Membrane-Bound Hsp70 an Activating Ligand for NK Cells

A Thesis Submitted for the Degree of Doctor of Natural Sciences
at the Faculty of Biology
at the Ludwig-Maximilians-University Munich

Catharina Christiane Groß
from Bonn-Bad Godesberg

Munich, July 2004

Completed at the chair of Molecular Oncology
Department of Hematology/Oncology
University Hospital of Regensburg

First Reviewer:	Prof. Dr. Elisabeth Weiß
Second Reviewer:	Prof. Dr. Heinrich Leonhardt
Supervisor:	Prof. Dr. Gabriele Multhoff

Submitted:	5th of July 2004
Oral examination:	1st of April 2005

EHRENWÖRTLICHE VERSICHERUNG

Hiermit versichere ich, Catharina Christiane Groß, ehrenwörtlich, daß die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Beihilfe angefertigt ist.

München, den 05.07.2004

_____

Catharina Groß

The important thing is not to stop questioning.
Curiosity has its own reason for existing.

Albert Einstein (1879-1955)

for my family and Thomas

APPENDIX

1 INTRODUCTION

1.1 Heat shock proteins (HSP) as activators of the immune system

Heat shock proteins (HSP) are highly conserved proteins constitutively expressed in pro- and eukaryotic cells. Their intracellular synthesis is induced by a variety of "stress stimuli" including heat shock, UV radiation, oxygen radicals, heavy metals, cytostatic drugs, and viral or bacterial infections. In the cytosol HSP are involved in folding/unfolding of proteins and nascent polypeptides, protection from protein-denaturation and -aggregation, in protein transport processes across membranes, and in antigen processing and presentation (DeNagel et al 1992, Hartl 1996). HSP are known to function as activators of the adaptive and innate immune system. Although the mechanism of release remains unclear, HSP have been found to be secreted by stressed tumor cells (Barreto et al 2003). Extracellular localized HSP either act as carrier molecules for the transport of immunogenic peptides to antigen presenting cells (APC) (Tamura et al 1997, Schild et al 1999, Udono et al 1993), or directly stimulate the innate immune system by the secretion of pro-inflammatory cytokines (Asea et al 2000a, Asea et al 2000b). Our laboratory identified membrane-bound Hsp70 as a recognition structure for the cytolytic attack mediated by natural killer (NK) cells (Multhoff et al 1997).

1.1.1 HSP as peptide carriers

Tumor-derived peptides, chaperoned by members of the HSP70 (Hsp70, Hsc70) and HSP90 (gp96) family (Tamura et al 1997, Schild et al 1999) can be taken up by APC via HSP-specific receptors including α-2 macroglobulin receptor CD91 (Binder et al 2000 a, Binder et al 2000b, Basu et al 2001), B-cell receptor CD40 (Becker et al 2002), or TLR2/4 (Asea et al 2002), in the presence or absence of CD14 (Asea et al 2000a (Figure 1). Representation of HSP-chaperoned immunogenic peptides on MHC class I molecules either through an endosomal or a TAP-proteasome-dependent ER-Golgi route (Castellino et al 2000) elicits a CD8+ cytotoxic T cell response (Udono et al 1993, Suto et al 1995) (Figure 1).

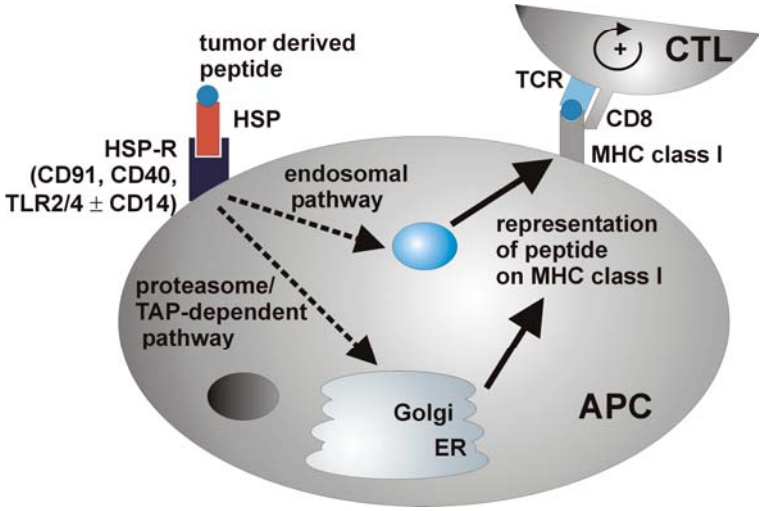


Figure 1: HSP as peptide carriers. HSP-peptide complexes can be uptaken by antigen presenting cells (APC) via HSP specific receptors including CD91, CD40, and TLR2/4 ± CD14. Tumor derived peptides are represented on MHC class I either through a proteasome/TAP-dependent pathway or an endosomal pathway. Presented peptides stimulate cytotoxic T cells.

The group of Srivastava developed a tumor vaccine approach based on this mechanism (Janetzki et al 2000, Belli et al 2002). In this approach, gp96 or Hsp70 peptide complexes were isolated from tumors and used as a vaccine for the stimulation of CD8+ T cells.

1.1.2 HSP as chaperokines

Beside their function as peptide-carriers extracellular localized HSP directly stimulate the innate immune system. It has been shown that both peptide loaded and free Hsp70 molecules are able to induce the secretion of proinflammatory cytokines including IL-1β, IL-6, and TNF-α by APC (Asea et al 2000a, Asea et al 2000b). The lipopolysaccharide (LPS) receptor CD14 together with members of the toll-like receptor family, namely TLR2 and TLR4, are involved in the signal transduction pathway resulting in cytokine release (Asea et al 2000a, Asea et al 2002). As shown in Figure 2, binding of Hsp70 to cell surface receptors TLR2/4 results in an activation of NF-κB and a rapid intracellular Ca^{2+} flux. Depending on the involvement of CD14 in binding of HSP either IL-1β, IL-6 and TNF-α, (Figure 2, left pathway) or TNF-α but not IL-1β or IL-6 are released (Figure 2, right pathway) (Asea et al 2000a).

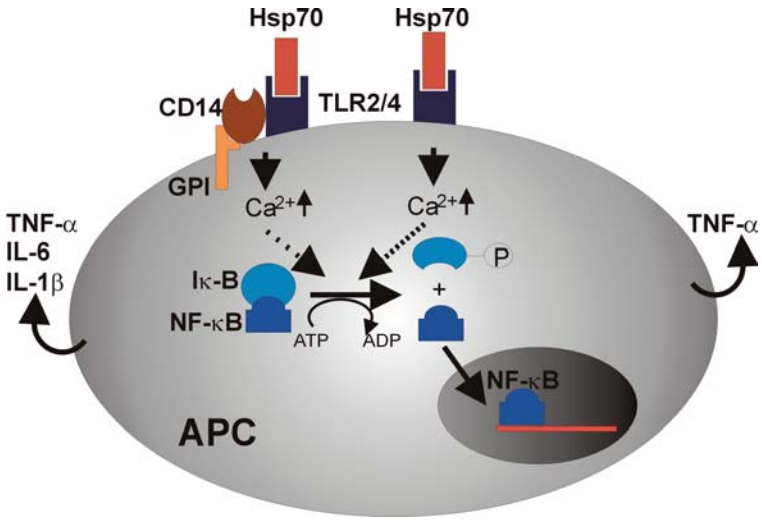


Figure 2: HSP as chaperokines. Binding of Hsp70 to TLR2/4 on the cell surface of APC results in an increase of intracellular calcium ions and activation of NF-κB. Depending whether CD14 is involved, APC secrete TNF-α, IL-6, and IL-1β (left pathway) or TNF-α alone (right pathway).

In addition to Hsp70 extracellular Hsp60 also has been found to activate APC through a CD14/TLR4 receptor pathway (Kol et al 2000, Ohashi et al 2000).

1.1.3 Hsp70 as an interacting partner for natural killer (NK) cells

In contrast to normal cells, tumor cells frequently express HSP on their plasma membranes (Ferrarini et al 1992, Piselli et al 1995, Tamura et al 1993, Multhoff et al 1995 a, Altmeyer et al 1995, Shin et al 2003). The expression of the inducible 72 kDa Hsp70 on the plasma membrane of tumor cells can be increased by physical (e.g. heat, γ-irradiation) and chemical (e.g. cytostatic drugs, alkyllysophospholipids) stress (Botzler et al 1996b, Botzler et al 1999, Rabinovich et al 2000, Feng et al 2001, Gehrmann et al 2002, Gehrmann et al unpublished data). Our group demonstrated that membrane-bound Hsp70 provides a tumor-selective target structure for the cytolytic attack mediated by NK cells (Multhoff et al 1995b, Multhoff et al 1997, Botzler et al 1996a). Furthermore, incubation of NK cells with recombinant Hsp70 and the C-terminal domain of Hsp70 (Hsp70-C) was found to stimulate proliferation and cytolytic activity against Hsp70 membrane-positive tumor cells (Multhoff et al 1999). Since CD94 membrane expression was up-regulated on NK cells following contact with Hsp70-protein (Multhoff et al 1999), an involvement of the C-type lectin receptor in the interaction of NK cells with Hsp70 was assumed.

1.2 Natural killer (NK) cells

Natural killer (NK) cells are large granular lymphocytes that comprise 5-20% of peripheral blood mononuclear cells (PBMNC) (Trincheri 1989). In contrast to T cells, NK cells do neither express the multimolecular CD3 complex nor α/β and γ/δ T cell receptors (TCR). Almost all NK cells are positive for the neuronal adhesion molecule CD56 mediating homo- and heterophilic cell adhesion (Lanier et al 1986, Lanier et al 1989). According to the cell surface density of CD56 two distinct NK cell subsets, the $CD56^{dim}$ and $CD56^{bright}$, could be separated (Figure 3) (Cooper et al 2001). The majority of NK cells (90%) express low levels of CD56 ($CD56^{dim}$) and high levels of the low affinity FcγRIII (CD16) (Figure 3A). Only 10% of the NK cells are $CD56^{bright}$ and $CD16^{dim/neg}$ (Figure 3B). The $CD56^{dim}$ cells contain more cytolytic granules (granzymes and perforin) than the $CD56^{bright}$ NK cells and are potent mediators of natural cytotoxicity, lymphokine activated killing (LAK) and antibody-dependent cytotoxicity (ADCC) (Lanier et al 1986). Following stimulation with IL-2 or IL-12 a drastically enhanced cytotoxicity is observed in the $CD56^{bright}$ NK cell population (Nagler et al 1989, Ellis et al 1989, Robertson et al 1992). $CD56^{dim}$ NK cells lack a strong cytokine response whereas the immunoregulatory $CD56^{bright}$ subsets secrete high amounts of IFN-γ, TNF-α, TNF-β, GM-CSF and IL-10 (see Figure 3A) following monokine activation (e.g. IL-15) (Fehniger et al 1999, Cooper et al 2002). Since $CD56^{bright}$ NK cells constitutively express the high (IL-2R$\alpha\beta\gamma$) and intermediate (IL-2R$\beta\gamma$) affinity IL-2 receptor (Figure 3A), these cells can be expanded *in vivo* and *in vitro* by stimulation with low doses (pM) of IL-2 (Caligiuri et al 1990 and Caligiuri et al 1993). In contrast, $CD56^{dim}$ cells expressing the intermediate (IL-2R$\beta\gamma$) IL-2 receptor (Figure 3B) show a weaker proliferative response against IL-2, even at high doses (1-10 nM) (Caligiuri et al 1990, Baume et al 1992). With respect to NK cell receptors (NKR), $CD56^{bright}$ NK cells show low or absent expression of killer cell immunoglobulin (Ig) receptors (KIR) and immunoglobulin-like transcript (ILT) and a high expression of CD94/NKG2 (Figure 3A). The $CD56^{dim}$ population could be subdivided into a $CD94/NKG2^{bright}$ and a $CD94/NKG2^{dim/-}$ expressing subpopulation (Nagler et al 1989, Voss et al 1998). $CD56^{bright}$ NK cells are believed to traffic to secondary lymphoid organs as they constitutively express high levels of the adhesion molecule L-selectin (CD62L) and the chemokine receptor CCR7 (Figure 3A). Both molecules are not expressed on $CD56^{dim}$ NK cells, but instead they show high

expression of the adhesion molecule PEN5/PSGL1 and the chemokine receptors CXCR1 and CX_3CR1 (Figure 3B) (Frey et al 1998, Campbell et al 2001).

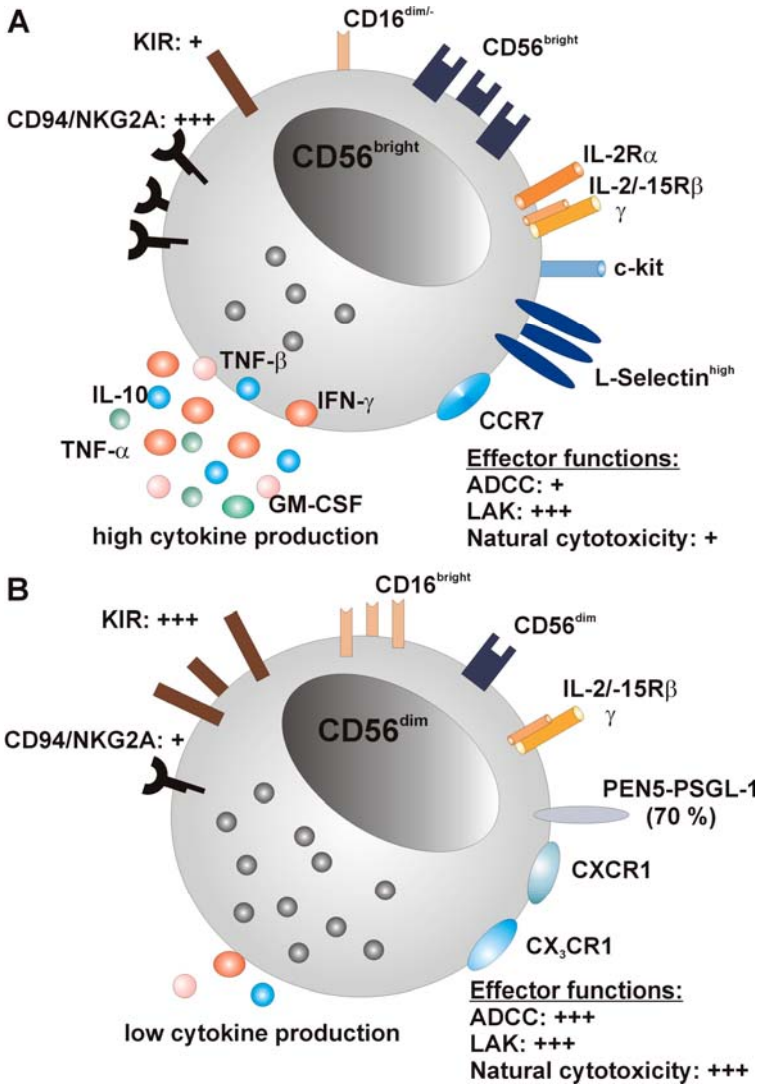


Figure 3: NK subpopulations. (A) $CD56^{bright}/CD16^{low/-}$ NK cells exhibit low antibody-dependent cellular cytotoxicty (ADCC) and natural cytotoxicity, but high lymphokine-activated killer (LAK) activity. They produce high levels of cytokines following stimulation with monokines. The expression of the C-type lectin-receptor CD94/NKG2A is bright, whereas Ig-like receptors (KIR) are weakly expressed. $CD56^{bright}$ NK cells express a number of cytokine and chemokine receptors including the high-affinity IL-2 receptor (IL-2Rαβγ), c-kit, and CC-chemokine receptor 7 (CCR7). The adhesion molecule L-selectin which is involved in trafficking to secondary lymph nodes (together with CCR7) is also highly expressed by these cells. (B) In contrast, $CD56^{dim}/CD16^{bright}$ NK cells are strong mediators of ADCC, LAK, and natural cytotoxicty, but secrete only low amounts of cytokines. In contrast to the $CD56^{bright}$ subpopulation these cells have high intracellular levels of cytotoxic granules. Also KIR are highly expressed by the $CD56^{dim}$ subpopulation. $CD56^{dim}$ cells express different cytokine (IL-2Rβγ) and chemokine (CXCR1 and CX_3CR1) receptors than $CD56^{bright}$ ones. Instead of L-selectin $CD56^{dim}$ cells highly express the adhesion molecule PEN5-P-selectin glycoprotein ligand-1 (PSGL-1). Figure 3 is adapted from Cooper et al 2001 and 2002.

1.2.1 NK cell receptors (NKR)

Function of NK cells is regulated by NK cell receptors (NKR). According to the missing-self theory, NK cells preferentially recognize and kill target cells with reduced, lost or altered self-MHC class I molecule expression (Ljunggren and Kärre 1990). Apart from MHC, tumor associated molecules present positive regulating ligands for NK cells. Our laboratory identified membrane-bound Hsp70 as a target recognition structure for NK cells (Multhoff et al 1997).

In humans the following major NKR-families have been identified (Table 1): killer cell immunoglobulin (Ig) receptors (KIR), immunoglobulin-like transcripts (ILT), C-type lectin receptors (Lanier 1998a, Borrego et al 2002), and natural cytotoxicity receptors (NCR) (Pessino et al 1998, Vitale et al 1998, Cantoni et al 1999, Pende et al 1999). A number of other cell surface molecules (e.g. 2B4) acting as co-receptors are also involved in triggering of NK cell function (Table 1) (Tanguye et al 2000, Valiante and Trinchieri 1993, Sivori et al 2000).

1.2.1.1 Killer cell Ig receptors (KIR)

The killer cell Ig receptors (KIR, CD158) are characterized by two or more extracellular localized immunoglobulin-like domains recognizing MHC class I allele groups including predominantly those of the HLA-B and HLA-C family (Moretta et al 1993, Wagtmann et al 1995, Pende et al 1996, Andre et al 2001). KIR genes are located on chromosome 19p13.4 and code for six activatory and six inhibitory members and allelic variants (Selvakumar et al 1997, Wilson et al 1997). They are single chain receptors with either two or three immunoglobulin-like domains (KIR**2D** or KIR**3D**) and a long (KIR2D**L** and KIR3D**L**) or a short (KIR2D**S** and KIR3D**S**) cytoplasmic tail. KIR with a long cytoplasmic domain contain one or two immunoreceptor tyrosine-based inhibition motifs (ITIM) and generate an inhibitory signal, whereas the short-tailed KIR have a positively charged amino acid in the transmembrane domain that interacts with adaptor molecules with immunoreceptor tyrosine-based activating motifs (ITAM) and generate an activatory signal (Biassoni et al 1996, Colonna and Samaridis 1995).

After binding of HLA molecules to inhibitory KIR, the tyrosine of the ITIM motif becomes phosphorylated by a member of the src kinase family (e.g. lck). Phosphorylated ITIM binds to the SH2 (Src homology 2) domains of protein tyrosine phosphatases SHP-1 and/or SHP-2, thereby releasing their catalytic site from

autoinhibition. (Olcese et al 1996, Burshtyn et al 1996, Barford and Neel 1998, Burshtyn and Long 1997). Two nonexclusive models are proposed for SHP-1 mediated inhibition: (i) SHP-1 inhibits NK cells by dephosphorylation of many proteins within the activatory pathway (Binstadt et al 1996, Binstadt et al 1998). (ii) A single key substrate that controls activation is targeted for dephosphorylation: e.g. dephosphorylation of the guanine nucleotide exchange factor Vav1 by SHP-1 is supposed to block activatory signals by inhibition of actin cytoskeleton rearrangements (Stebbins et al 2003).

When HLA binds to an activatory receptor type, DAP-12 an adaptor molecule becomes phosphorylated and binds to Syk/ZAP-70 (70 kDa, ζ-associated protein) which activates NK cells (Chu et al 1998, Brumbaugh et al 1997, Negishi et al 1995). Most inhibitory KIR have higher affinity for their HLA ligands than their activatory counterparts (Vales-Gomez 1998).

1.2.1.2 Immunoglobulin-like transcripts (ILT)

ILT that are predominantly expressed by myeloid cells, dendritic cells and B cells, also belong to the immunoglobulin-like superfamily (Colonna et al 1997, Cosman et al 1997). ILT-2 (Table 1), which is expressed on some NK cells interacts directly with a broad spectrum of classical and non-classical HLA molecules (Navarro et al 1999).

1.2.1.3 C-type lectin receptors

Most C-type lectin receptors are heterodimers consisting of CD94 (20-30 kDa) covalently bound to members of the NKG2 family (43 kDa) (Figure 4) (Lanier et al 1994, Lazetic et al 1996). These receptors interact with non-classical HLA-E molecules presenting peptides derived from signal sequences of HLA-A, HLA-B, HLA-C and HLA-G in a TAP-dependent manner (Braud et al 1998, Borrego et al 1998, Ulbrecht et al 1998, Lee et al 1998, Brooks et al 1999). The CD94 and NKG2 genes are closely linked on chromosome 12p12.3-p13.1 in the NK receptor complex (Sobanov et al 1999, Glienke et al 1998). CD94 is expressed by a single nonpolymorphic gene and has only a short cytoplasmic tail of 7 amino acids (Chang et al 1995). Therefore, CD94 alone is unable for signaling. Depending on its co-receptor (Figure 4) the heterodimeric CD94 complex fulfills inhibitory or activatory function: CD94/NKG2A and its splice variant NKG2B is an inhibitory receptor (Figure

4, left side), whereas CD94/NKG2C, CD94/NKG2E, its splice variant NKG2H, and CD94/NKG2F are activating receptors (Figure 4, middle) (Brooks et al 1997, Lanier 1998b). Similar to KIR with long cytoplasmic tails, the inhibitory receptors are signaling through SHP-1/2 that mediate inhibitory signals (Figure 4, left), whereas activatory receptors have a positively charged transmembrane domain that interacts with DAP-12 and thus mediates activating signals through Syk family members (Figure 4, middle). As shown for KIR, inhibitory receptors have a higher affinity to their ligands (Llano et al 1998). Only a minority of NK cell clones expresses both activating and inhibitory isoforms that recognize the same group of HLA alleles.

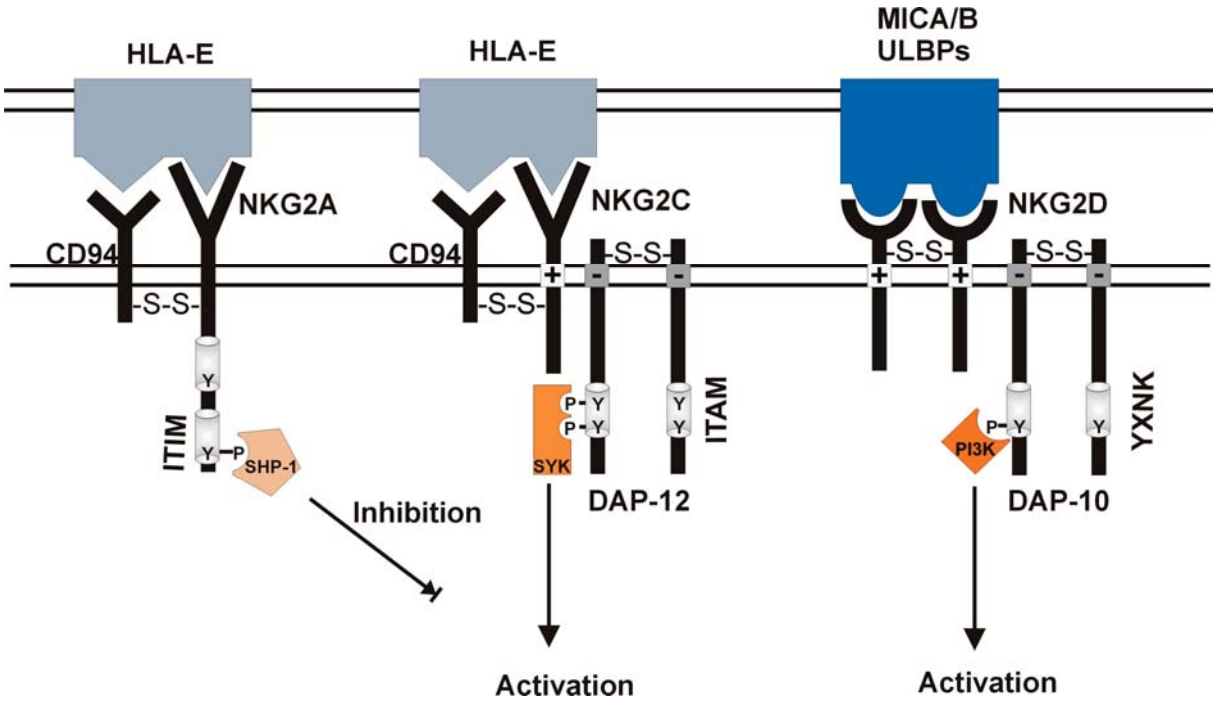


Figure 4: C-type lectin receptors. The C-type lectin receptors are disulfide-linked heterodimers of CD94 and members of the NKG2 family (either the inhibitory NKG2A or the activatory NKG2C). They recognize the nonclassical MHC class I molecule HLA-E. ITIM-containing NKG2A signals through SHP-1/2 that mediate inhibitory signals, whereas NKG2C has a positively charged amino acid in the transmembrane domain that interacts with DAP-12 and transduces activating signals through Syk family members. The activatory receptor NKG2D forms homodimers and binds to the MHC-like ligands MICA, MICB, and ULBP family. Through its positively charge in the transmembrane domain NKG2D associates with the adaptor molecule DAP-10 that contains an YXNK motif to bind PI-3kinase (PI3K) and sends activating signals through this alternative pathway. As the PI3K cascade is not inhibited by SHP-1/2, NKG2D may be able to induce a dominant activation signal.

The activating receptor NKG2D also belongs to the C-type lectin family, but in contrast to other NKG2 family members it forms only homodimers (Figure 4, right) (Wu et al 1999). NKG2D is constitutively expressed on NK cells, $\gamma\delta$ T cells and CD8+ T cells and interacts with the MHC class I chain-related (MIC) antigens, MICA and MICB, and the UL16 binding proteins (ULBP) that are stress-inducible or induced by

neoplastic transformation (Bauer et al 1999, Cosman et al 2001). The intracellular domain of NKG2D does not contain any signaling motif and therefore signaling is mediated through the adaptor molecule DAP-10 (Wu et al 1999). DAP-10 recruits phosphatidylinositol (PI)-3 kinase after phosphorylation. A selection of important NK cell receptors, their function and ligands are summarized in Table 1.

Receptor	Function	Ligand specificity	Chromosome
Ig-SF			
a) KIR			
CD158a (p58.1) KIR2DL1	inhibitory	HLA-Cw2, 4, 5, 6	19q13.4
CD158h (p50.1) KIR2DS1	activatory	HLA-Cw2, 4, 5, 6	19q13.4
CD158b1/2 (p58.2/3) KIR2DL2/3	inhibitory	HLA-Cw1, 3, 7, 8	19q13.4
CD158j/i (p50.2/3) KIR2DS2/4	activatory	HLA-Cw1, 3, 7, 8	19q13.4
CD158e1/2 (p70)		HLA-Bw4 alleles	19q13.4
KIR3DL1	inhibitory		
KIR3DS1	activatory		
CD158k (p140) KIR3DL2	inhibitory	HLA-A3, 11	19q13.4
CD158d (p49) KIR2DL4	inhibitory	HLA-G, others	19q13.4
b) ILT			
CD85j ILT-2/LIR-1	inhibitory	HLA-G, various	19q13.42
C-type lectin receptors			
CD94/NKG2A	inhibitory	HLA-E + leader peptides from HLA-A, -B, -C, -G	12p12.3-13.1
CD94/NKG2C	activatory	HLA-E + leader peptides from HLA-A, -B, -C, -G	12p12.3-13.1
NKG2D	activatory	MICA, MICB, H60, Rae1, ULBP 1, 2, 3	12p12.3-13.1
NCR			
NKp30	activatory	unknown	6
NKp44	activatory	unknown	6
NKp46	activatory	unknown	19q13.4
Coreceptors			
CD244 (2B4)	activatory	GPI-anchored CD48	1q23.3

Table 1: NK Receptors.

1.2.2 Mechanism of lysis mediated by NK cells

Cytotoxicity of NK cells is mediated by death receptor-mediated apoptosis including Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Eischen et al 1997, Arase et al 1995, Kayagaki et al 1999, Screpanti et al 2001), or by exocytosis of cytotoxic granules (Trapani et al 1994). The cytotoxic granules contain macromolecular complexes consisting of the proteoglycan serglycin (SG), the serine protease granzyme B (grB), and perforin (PFN). In addition, cytotoxic cells were also observed to secrete exclusively grB-SG complexes, PFN-SG complexes, and monomeric PFN (Metkar et al 2002). The mechanism by which granzyme B enters the target cells is poorly understood. Both, granzyme B uptake via a perforin pore and a "poreless" mechanism (Figure 5) are discussed.

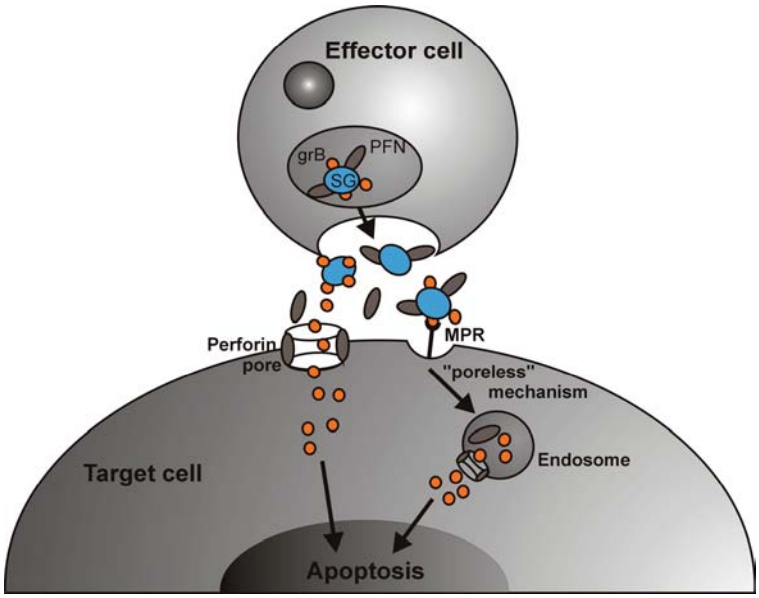


Figure 5: Mechanism by which granzyme B enters target cells. Effector cells including activated T cells and NK cells release serglycin (SG) – granzyme B (grB) – perforin (PFN), SG-grB, SG-PFN complexes. GrB enters the target cell via a perforin pore or a "poreless" mechanism mediated by the mannose 6-phosphate receptor. The release of grB from the endosome is perforin-dependent. Inside the target cell the serine protease grB induces apoptosis.

As illustrated in Figure 5 perforin polymerizes in the presence of Ca^{2+} and forms pores in the target cell membrane. These lesions can be formed in viable cells *in vitro* by addition of high concentrations of purified perforin. It was assumed that these pores enable granzyme B to be taken up into the target cell. However, *in vivo* there is no evidence for the stable transmembrane pore formation (Trapani and Smyth 2002). Moreover, it has been shown that the uptake of granzyme B into target cells does not necessarily require perforin. A receptor-mediated endocytosis for granzyme B was hypothesized (Shi et al 1997, Trapani et al 1996, Pinkoski et al 1998, Froelich et al

1996). In this "poreless" mechanism, the mannose 6-phosphate receptor (MPR) is controversely discussed as an entry port for granzyme B (Pinkoski et al 1998, Pinkoski et al 2002, Andrade et al 1998, Dressel et al 2003). However, the release of granzyme B from the endosomes and thereby the induction of apoptosis is perforin-dependent (Pinkoski et al 1998). Inside the target cell the serine protease granzyme B cleaves peptide bonds at Asp residues and thereby induces both, caspase-dependent and caspase-independent apoptosis (Figure 6) (Sarin et al 1997, Trapani et al 1998, Beresford et al 1999).

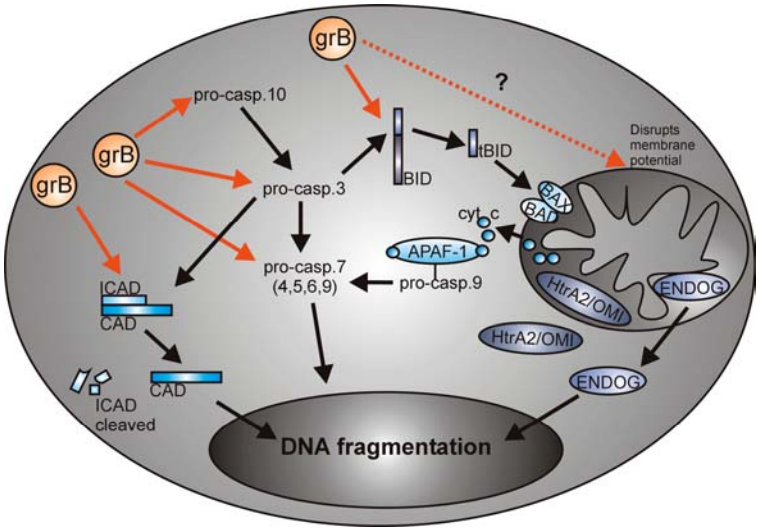


Figure 6: **Apoptotic pathways induced by granzyme B.** Cytosolic granzyme B (grB) activates pro-caspase 10 to its active form to initiate the caspase-dependent pathway. Caspase 10 then processes caspase 3, which, in turn, activates the further "downstream" caspases. In addition to caspase 10, granzyme B can directly process and activate caspase 3, 4, 5, 6, 7, and 9. In the caspase-independent pathway BH3-interacting domain death agonist (BID) is an early target of granzyme B. Truncated BID (tBID) disrupts the outer mitochondrial membrane which leads to the release of the pro-apoptotic factors cytochromce c (crucial for activating pro-caspase 9), HtrA2/OMI (blocks inhibitors of apoptosis), and endonuclease G (ENDOG, activates DNA damage). Released cytochrome c recruits pro-caspase 9 to form the apoptosome by binding to apoptotic protease-activating factor 1 (APAF1). In addition, granzyme B disrupts the mitochondrial transmembrane potential through an unknown mechanism. It can directly cleave inhibitor of CAD (ICAD) to free caspase-activated DNase (CAD) thus causing DNA fragmentation. Figure adapted from Froelich et al 1998 and Lieberman 2003.

In the caspase-dependent pathway (Figure 6), granzyme B is believed to initiate the proteolytic cascade by activating caspase 10 (Muzio et al 1996). Caspase 10, in turn, matures caspase 3 by cleaving the large and small subunits after Asp174. Activated caspase 3, which is a point of convergence for multiple induction pathways, initiates processing of caspase 7 and presumably other caspases. Additionally, granzyme B is believed to process and activate directly caspase 3, 4, 5, 6, 7, and 9 (Figure 6) (Froelich et al 1998). In contrast, the apoptotic pathways via Fas and TNF, which activate caspase 8 and/or caspase 2 respectively, are linear.

Another early target of granzyme B is BH3-interacting domain death agonist (BID) that destroys the integrity of the mitochondrial outer membrane when it is cleaved (Figure 6, Kuwana et al 2002). In addition to BID-mediated mitochondrial damage, which involves the BCL-2-associated protein X (BAX) and BCL-2 antagonist/killer (BAG) (Barry et al 2000, MacDonald et al 1999), granzyme B also directly disrupts the mitochondrial transmembrane potential in a caspase- and BID-independent manner (Alimonti et al 2001, MacDonald et al 1999). Disruption of the mitochondrial transmembrane potential results in the release of pro-apoptotic molecules including cytochrome c, HtrA2/OMI, and endonuclease G (ENDOG) from the intermembrane space into the cytosol (Figure 6) (Kuwana et al 2002, Barry et al 2000, Heibein et al 2000, Sutton et al 2000, Alimonti et al 2001). Released cytochrome c binds to apoptotic protease-activating factor (APAF) and thereby activates pro-caspase 9 (Figure 6). The homologue to the bacterial HSP Htr, HtrA2/OMI is a recently identified serine protease that inhibits the inhibitor of apoptosis protein (IAP) and is upregulated in response to heat shock. Endonuclease G, that is also released by the action of granzyme-B-cleaved BID, can induce oligonucleosomal DNA damage (Li et al 2001). Granzyme B can also activate the caspase-activated DNase (CAD) by cleaving its inhibitor (Thomas et al 2000, Sharif-Askari et al 2001). Therefore, granzyme B independently and directly activates at least two routes to DNA damage, even when caspase activation is blocked. In addition to targeting mitochondria and DNA degradation directly, granzyme B also directly cleaves several downstream caspase substrates.

1.3 Aims

1. Identification of the minimal NK cell stimulatory sequence of Hsp70
2. Characterization of the interaction of membrane-bound Hsp70 and NK cells
3. Definition of Hsp70-specific receptors on NK cells
4. Identification of the mechanism of lysis of Hsp70 membrane-positive tumor cells by NK cells

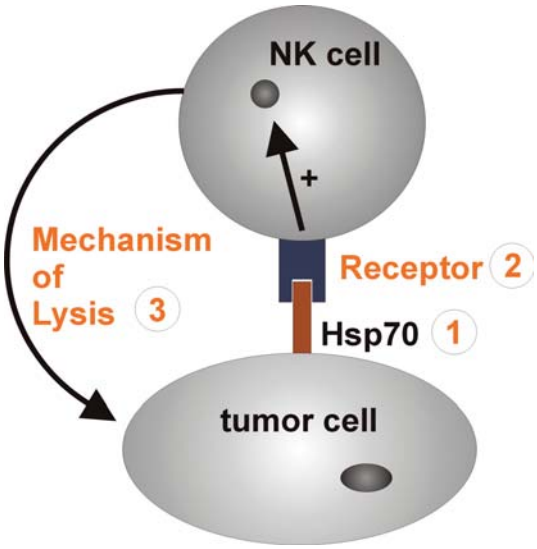


Figure 7: Schematic illustration of the aims of my work.
1. The sequence of Hsp70 presented on the cell surface of tumor cells will be elucidated and tested for its NK stimulatory capacity.
2. The interaction of NK cells with Hsp70 membrane-positive tumor cells will be characterized. Receptors involved in this interaction will be identified.
3. The mechanism of kill of Hsp70 membrane-positive tumor cells by NK cells will be identified.

2 MATERIAL AND METHODS

2.1 Cell lines

All cell lines were screened regularly for mycoplasma contamination by an enzyme immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) detecting *Mycoplasma arginini*, *Mycoplasma hyorhinis, Mycoplasma laidlawii*, and *Mycoplasma orale*. Only mycoplasma-free cell lines were used.

2.1.1 Tumor cell lines

The human tumor sublines CX+ and CX- were derived by fluorescence activated cell sorting (FACS) of CX-2 colon carcinoma cell line (TZB 61005, Tumorzentrum Heidelberg, Germany) with 60% Hsp70 membrane-positive cells using the Hsp70-specific monoclonal antibody cmHsp70.1 (Multhoff et al 1997) (multimmune GmbH, Regensburg, Germany). Hsp70 stable high expressing CX+ (90% Hsp70 membrane-positive cells) carcinoma cells differ significantly from Hsp70 stable low expressing CX- (20% Hsp70 membrane-positive cells) carcinoma cells. CX+ and CX- cells were kept in culture under exponential growth conditions by regular cell passages. Every three days cells were transferred after short term (maximum: 1 min) trypsination using trypsin/ethylene-diamine-tetra-acetic (EDTA) solution (PAN Biotech GmbH, Aidenbach, Germany) and 0.5 x 10^6 cells were seeded in 5 ml RPMI-1640 medium (Gibco, Eggenstein, Germany) supplemented with heat-inactivated 10% FCS (Gibco), 1 mM sodium-pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (all supplements were purchased from Life Technologies, Rockville, CA) in T25 culture flasks at 37°C and 5% CO_2.

The myelogenous cell line K562 was purchased from ATCC (CCL243, Rockville, MD) and cultured in RPMI-1640-medium supplemented with heat inactivated FCS, 1 mM sodium-pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

2.1.2 NK cell line YT

The IL-2 independent growing NK cell line YT (Yodoi et al 1985, ACC 434, DSMZ, Heidelberg, Germany) was cultivated in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 1 mM sodium-pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin ± 2 µg/ml of the 14-mer peptide TKD

(TKDNNLLGRFELSG, Bachem, Bubendorf, Switzerland); doubling time was 50 h to 60 h at a low cell density of 0.1 x 10^6 cells/ml.

2.2 Primary cells

2.2.1 Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Red Cross, Regensburg, Germany) or leukapheresis products (kindly provided by Clinical Chemistry, University Hospital of Regensburg) of healthy human volunteers by Ficoll density-gradient centrifugation. After two washing steps in RPMI-1640 medium depletion of monocytes was performed overnight. Monocyte-depleted PBL (5 x 10^6/ml) were incubated for 3 – 4 days in RPMI-1640 medium supplemented with 10% heat inactivated FCS, 1 mM sodium-pyruvate, 2 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, 100 IU/ml IL-2 $\pm$ 2 µg/ml TKD.

2.2.2 NK cells

Primary human NK cells were generated from PBL either by plastic adhesion following a modified protocol of Vujanovic (Vujanovic et al 1993) or by CD3-depletion or CD94-positive selection using a standard Miltenyi separation method.

To generate plastic adherent NK cells, PBL were separated into non-adherent CD3+ T cells and cytokine-dependent, transient (12 – 24 h) plastic adherent CD3-/CD16/56+ NK cell subpopulations after 12 h incubation in medium supplemented with 100 IU/ml IL-2.

For CD3-depletion, 100 x 10^6 PBL/ml were incubated with 200 µl MACS CD3 Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C. After washing with bovine serum albumin containing cell sorting buffer (MACS buffer, 10 – 20 x of labeling volume), CD3-negative NK cells were separated using a LS/VS column (Miltenyi Biotec, Bergisch Gladbach, Germany).

To obtain CD94-positive NK cells, PBL were separated by positive-selection using biotinylated CD94 monoclonal antibody (Ancell Immunology Research Products, Bayport, USA). Briefly 100 x 10^6 cells/ml were incubated with 5 µg biotin-conjugated CD94 mAb for 30 min at 4° C. After extensive washing (10 – 20 x of labeling volume) with MACS-buffer, cells (100 x 10^6/ml) were incubated with 200 µl MACS Anti-Biotin Microbeads (Miltenyi Biotec, Bergisch Gladbach Germany) for 15 min at 4°C under

gentle shaking. After washing, CD94-positive NK cells were separated using a LS/VS column.

Sorted cells (2 x 10^6/ml) were incubated for 3 – 4 days in RPMI-1640 medium with 10% heat inactivated FCS, 1 mM sodium-pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 IU/ml IL-2 ± 2 µg/ml TKD.

2.3 Flow cytometry

The following antibodies either conjugated or unconjugated were used for FACS analysis:

antibody (clone)	isotype	company
primary antibodies		
IgG1	mouse	Dianova, Hamburg, Germany
IgG1	rat	Dianova, Hamburg, Germany
IgG1-FITC	mouse	BD Biosciences, Heidelberg, Germany
IgG1-PE	mouse	BD Biosciences, Heidelberg, Germany
IgG2a	mouse	Dianova, Hamburg, Germany
IgG2a-PE	mouse	BD Biosciences, Heidelberg, Germany
IgG2b-FITC	mouse	BD Biosciences, Heidelberg, Germany
IgM-PE	mouse	Dianova, Hamburg, Germany
CD3-FITC/ CD16/56-PE	mouse IgG1/ mouse IgG2a	BD Biosciences, Heidelberg, Germany
CD3-PE	mouse IgG1	Dianova, Hamburg, Germany
CD6-PE	mouse IgG1	BD Biosciences, Heidelberg, Germany
CD7 (8H8.1)	mouse IgG2a	Coulter Immunotech, Marseille, France
CD8-PE (B9.11)	mouse IgG1	Dianova, Hamburg, Germany
CD11b	mouse IgG1	Coulter Immunotech, Marseille, France
CD14-FITC	mouse IgG2b	Coulter Immunotech, Marseille, France
CD16-FITC	mouse IgG1	BD Biosciences, Heidelberg, Germany
CD54-FITC	mouse IgG1	Coulter Immunotech, Marseille, France
CD56-FITC (NCAM16.2)	mouse IgG2b	BD Biosciences, Heidelberg, Germany
CD57-PE (NC1)	mouse IgM	Coulter Immunotech, Marseille, France
CD91	mouse IgG1	Progen Biotechnik, Heidelberg, Germany
CD94-PE (HP-3D9)	mouse IgG1	Ancell Immunology Research Products, Bayport, USA
CD158a-PE	mouse IgG1	Coulter Immunotech, Marseille, France
CD158b-PE	mouse IgG1	Coulter Immunotech, Marseille, France

granzyme B-PE (HC2)	mouse IgG1	Hölzel Diagnostics, Cologne, Germany
HLA-E (MEM-E/06)	mouse IgG1	EXIBO, Prague, Czech Republic
HLA-G (87G)	mouse IgG2a	kindly provided by Dr. D. Geraghty, Prague, Czech Republic
Hsp70-FITC (cmHsp70.1)	mouse IgG1	multimmune GmbH, Regensburg, Germany
Hsp70 (SPA810)	mouse IgG1	Stressgen, Victoria, CA
MHC class I-PE (W6/32)	mouse IgG2a	Cymbus Biotechnology, Dianova, Hamburg, Germany
MHC DR-PE (L243)	mouse IgG2b	Coulter Immunotech, Marseille, France
MICA (IMA310-0-C100)	mouse IgG1	Immatics Biotech, Tübingen, Germany
NKG2A (Z270)	mouse IgG1	kindly provided by Dr. L. Moretta, Milan, Italy
NKp30-PE (Z25)	mouse IgG1	Beckman Coulter, Krefeld, Germany
NKp46-PE (BAB281)	mouse IgG1	Beckman Coulter, Krefeld, Germany
p-glycoprotein mdr-1 gene-product (UIC2)	mouse IgG2a	Immunotech Coulter, Marseille, France
TLR2	mouse IgG2a	eBioscience, San Diego, USA
TLR4	mouse IgG2a	eBioscience, San Diego, USA
TLR9	mouse IgG1	Biocarta Europe, Hamburg, Germany
Secondary antibodies		
anti-mouse-FITC	goat IgG, IgM	DAKO, Glostrup, Denmark
anti-rat-FITC	rabbit IgG1	Dianova, Hamburg, Germany

Table 2: Antibodies for FACS analysis

Briefly, 0.5 x 10^6 cells were incubated either with unconjugated antibody or fluorescence-conjugated monoclonal antibody for 30 min at 4°C. After washing in PBS conditioned with 2% (cell lines) or 10% (primary material) heat-inactivated FCS, cells were analyzed directly by flow cytometry or incubated with a secondary antibody for another 30 min at 4°C. Then the cells were analyzed on a FACS Calibur instrument (BD Biosciences, Heidelberg, Germany). The percentage of positively-stained cells was calculated as the number of specifically-stained, propidium-iodide(PI)-negative, viable cells minus the number of cells stained with an isotype-matched control.

For intracellular staining, 0.5 x 10^6 cells were fixed in 0.25% paraformaldehyde (PFA) solution for 8 min and permeabilized in PBS containing 0.5% BSA and 0.1% saponin (permeabilisation solution). Permeabilized cells were incubated with a granzyme B-PE antibody or an isotype matched IgG1 control antibody at 4°C for 1 h in the dark. Following washing with permeabilisation solution and PBS, cells were fixed in 1%

paraformaldehyde solution and intracellular localized granzyme B was analyzed on a FACS Calibur instrument.

2.4 Light and immunofluorescence microscopy

Cells were analyzed using a Zeiss Axiovert 25 microscope (Zeiss, Jena, Germany) equipped with a 5 x objective. Pictures were taken with a Fuji FinePix S1 camera.
For fluorescence microscopy, labeled cells were fixed with 1% paraformaldehyde and embedded in "Fluorescent Mounting Medium" (Neo-Mount, Merck, Darmstadt, Germany) after cytospin. The samples were analyzed for transmission and fluorescence microscopy using a Zeiss Axioscop 2 Scanning microscope (Zeiss) equipped with a 40 x objective and standard filters (Filterset 10 for FITC- and Filterset 45 for PE-labeled cells). Multiplicative shading corrections were performed using the sofware Axiovision (Zeiss) and photographs were taken using the Axiocam camera (Zeiss).

2.5 Binding studies

2.5.1 FITC-labeling of Hsp70, BSA and TKD

1 mg/ml Hsp70 (Stressgen, Victoria, CA) and 1 mg/ml BSA (Sigma, St. Louis, USA) were incubated overnight with fluoresceine-isothiocyanate (FITC) (Sigma, St. Louis, USA) in 0.1 M sodium bicarbonate (pH 9.5) at 4°C. Unconjugated dye was removed by passing the reaction mixture over a PD-10 desalting column (Sephadex G-25, Amersham Bioscience, Uppsala, Sweden) equilibrated with PBS. Eluted fractions containing FITC-labeled protein were pooled and stored at 4°C in the dark. The number of FITC molecules bound per molecule protein was determined by measuring the OD at 280 nm and 495 nm. It was calculated as 4.1 FITC molecules for Hsp70 protein and 4.4 FITC molecules for BSA. Before use the solutions of the FITC-labeled proteins were centrifuged at 100,000 g in order to remove small particles.
FITC-labeled TKD was derived as a 94.3% pure, 2038.23 Da ($C_{92}H_{128}N_{22}O_{29}S$) FITC-γ-Abu-TKDNNLLGRFELSG-OH lyophilized material (Bachem, Bubendorf, Switzerland). The powder was diluted in sterile PBS at a stock concentration of 1 mg/ml. All experiments using FITC-labeled proteins and peptides were performed with identical stock solutions within three month.

2.5.2 Binding of BSA, Hsp70, and TKD to YT cells

Binding studies were performed with BSA-FITC, Hsp70-FITC and TKD-FITC. Therefore 5 x 10^6 YT cells per ml PBS supplemented with 2% FCS were incubated with 10, 25, 50 and 100 µg/ml BSA-FITC and Hsp70-FITC, and 0.2, 2.0, 4.0, and 8.0 µg/ml TKD-FITC for 45 min at 4°C. Binding was determined by flow cytometry. Directly before flow cytometric analysis the cells were co-stained with propidium iodide (PI). Only PI-negative viable cells were gated and analyzed on a FACS Calibur instrument.

2.6 Granzyme B ELISA

Granzyme B released by NK cells during the stimulation period either with IL-2 (100 IU/ml) alone or IL-2 plus TKD (2 µg/ml) was quantified by standard ELISA technique. Briefly, granzyme B antibody-coated (L-100-114, Hoelzel Diagnostics, Cologne, Germany) 96 well plates (Corning Costar, USA) were incubated with 100 µl samples or standard solutions at different concentrations in combination with the secondary capture antibody (Hoelzel Diagnostics), for 2 h at room temperature. After two washing steps, granzyme B was visualized by the addition of freshly prepared avidin—horseradish peroxidase (Hoelzel Diagnostics) for 1 h and substrate solution (Hoelzel Diagnostics) for another 25 min. Plates were counted on an ELISA reader at 450/650 nm.

2.7 ^{3}H thymidine uptake assay

The proliferation of NK and T cells after incubation with different Hsp70 derived peptides and Hsp70 protein was determined in a standard ^{3}H thymidine uptake assay (Strong et al 1973). 5 x 10^4 viable cells per 100 µl were seeded in 96-well flat-bottom microtiter plates in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 1 mM sodium-pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 100 IU/ml IL-2. Different amounts of Hsp70 (10 and 200 µg/ml) and TKD derived peptides (0.02 – 8 µg/ml) were added. Cells stimulated with IL-2 alone served as a negative control. After 48 h incubation at 37°C, the cells were pulsed with ^{3}H thymidine (0.5 µCi per well; Hartmann Analytic, Krefeld, Germany). Following another 12 h incubation period at 37°C cells were harvested onto a unifilter GF/96C well plate (Packard, GMI, Minnesota, USA) using the Harvester Filtermate 196 (Packard). Finally, 50 µl Micro Scint-20 (Perkin Elmer,

Boston, USA) were added per well and the total uptake was measured in a liquid scintillation counter (Packard).

2.8 Cytotoxicity Assays

2.8.1 ^{51}Cr release assay

The cytolytic activity of effector cells was measured in a standard ^{51}Cr radioisotope assay (MacDonald et al 1974). Dilutions of the effector cells were incubated with ^{51}Cr-labeled (100 µCi of $Na^{51}CrO_4$, Hartmann Analytic, Krefeld, Germany) tumor target cells (3 x 10^3 cells per well) in duplicates at a final volume of 200 µl of RPMI-1640 medium supplemented with 10% heat inactivated FCS at 37°C for 4 or 12 h in 96 well U-bottom plates. After the incubation period, supernatants (50 µl/well) were collected and the radioactivity was counted in a γ-counter (Packard, GMI, Minnesota USA). The percentage of specific lysis was determined according to the following equation: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] x 100. The percentage of spontaneous release (supernatant of tumor cells alone) was always less than 20% for each target cell.

The cytolytic activity was either blocked by incubation of tumor target cells with the Hsp70 specific monoclonal antibody cmHsp70.1 (5 µg/1 x 10^6 cells, multimmune GmbH, Regensburg, Germany) or by preincubation of NK cells with an antibody directed against the C-type lectin receptor CD94 (5 µg/ml, HP-3B1, mouse IgG2a, Coulter Immunotech, Marseille, France).

2.8.2 Granzyme B ELISPOT

In addition to the ^{51}Cr release assay, the cytolytic activity of NK cells was measured using a granzyme B ELISPOT assay (Rininsland et al 2000). Therefore, 96-well ELISPOT plates were coated overnight at 4°C with 100 µl/well granzyme B capture antibody (5 µg/ml, BD Biosciences, Heidelberg, Germany). Then, the plates were blocked with RPMI-1640 medium containing 10% heat-inactivated FCS for 2 h at room temperature. Tumor target cells (3 x 10^3 cells per well) and effector cells at different effector to target cell (E:T) ratios were seeded in duplicates at a final volume of 200 µl of RPMI-1640 medium supplemented with 10% FCS and incubated for 4 h at 37°C and 5% CO_2. After washing twice with deionized water and three times with PBS supplemented with 0.05% Tween-20, 100 µl/well biotinylated anti-human granzyme B antibody (2 µg/ml, BD Biosciences, Heidelberg, Germany) was added

and the plate was incubated for 2 h at room temperature. Then, the plates were washed three times with PBS supplemented with 0.05% Tween–20 and incubated with 100 µl/well avidin-HRP (1:100, BD Biosciences, Heidelberg, Germany) at room temperature for 1 h. After extensive washing with PBS supplemented with 0.05% Tween-20 (four times) and PBS (two times) 100 µl 3-amino-9-ethyl-carbazoele (AEC) substrate solution was added per well and plates were incubated for 25 min at room temperature in the dark. Substrate reaction was stopped with deionized water and after drying of the plates spots were counted automatically using the Bioreader-2000 (Biosys, Karpen, Germany).

2.9 Migration assay

Migration assays were performed in a transwell cell culture system (24 well plate, Costar, Corning Incorporated, NY, USA) in triplicates. The upper and the lower compartment were separated by a tissue culture polycarbonate membrane (polyvinyl-pyrrolidone-free, Nucleopore, Pleasanton, CA), 6.5 mm in diameter, with a pore size of 5 µm.

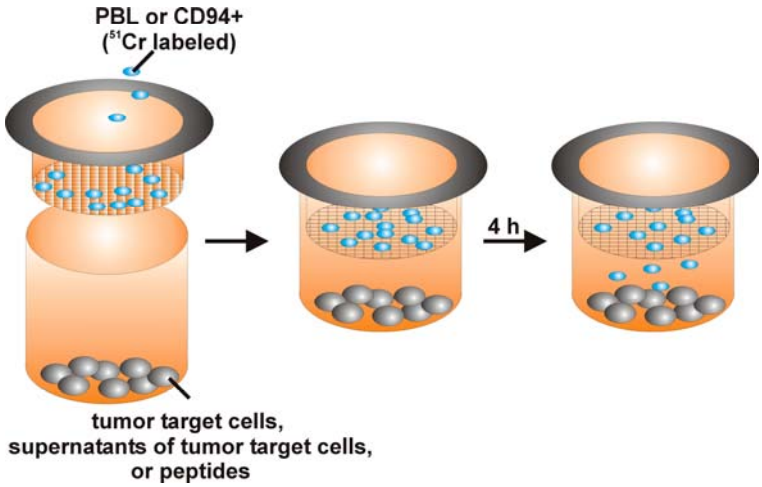


Figure 8: Principle of the migration assay. Target cells (for 48 h) or peptides are placed in the lower compartment. ^{51}Cr labeled PBL or CD94-positive NK cells are added in the upper compartment. After a 4 h incubation period, the cell suspension in the lower compartment is harvested and radioactivity is measured on a γ-counter.

0.1 x 10^6 tumor target cells (CX+/CX-), cell culture supernatants, and peptides were placed in a total volume of 600 µl of RPMI-1640 medium supplemented with 10% FCS in the lower compartment (Figure 8, left bottom). Then, 2 x 10^6 PBL or CD94-positively sorted cells stimulated with TKD were labeled with sodium [^{51}Cr]-chromate (100 µCi, Hartmann Analytic, Braunschweig, Germany) for 2 h. After extensive

washing 0.2 x 10^6 labeled cells were resuspendend in 100 µl RPMI-1640 medium supplemented with 10% FCS and added carefully to the upper compartment (Figure 8, left top). Recombinant IL-15 (10 ng/ml, Promocell GmbH, Heidelberg, Germany) served as a positive control for chemotaxis (Allavena et al 1997). After a 4 h incubation period in an humidified incubator at 37°C and 5% CO_2 (Figure 8, right), the cell suspension in the lower compartment was harvested (50 µl cell suspension) and radioactivity was measured on a γ-counter (Packard Instruments, GMI, Minnesota, USA). The percentage of migrated cells was determined according to the following equation: % specific migration = [(experimental value – spontaneous value) / (maximum value – spontaneous value)] x 100.

2.10 Apoptosis assays

2.10.1 Annexin-V staining

For annexin-V staining cells (0.5 x 10^6) were washed twice in annexin buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4) and incubated with 100 µl Annexin-V-Fluos labeling solution (Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at room temperature. After adding 400 µl annexin buffer, annexin positive cells were measured on a FACS Calibur flow cytometer.

2.10.2 DAPI staining

Methanol/acetone fixed cells (0.1 x 10^6 cells/100 µl) were incubated with 0.5 µg/µl 4,6-diamino-2-phenylindol (DAPI; Roche Diagnostics GmbH, Mannheim, Germany) in PBS/glycerol (3:1) for 15 min in the dark. Following washing in PBS the cells were mounted with "Fluorescent Mounting Medium" (Neo-Mount, Merck, Darmstadt, Germany) and analyzed for fluorescence using a Zeiss model Axioscop 2 scanning microscope (Zeiss, Jena, Germany) equipped with a 40 x objective and standard filters. Apoptosis was visualized with DAPI staining in 50 cells, each. Images were treated by multiplicative shading corrections using the software Axiovision (Zeiss).

2.10.3 Cytochrome c release

Cytochrome c was determined using a quantitative immunoassay (Quantikine®, R&D Systems, Wiesbaden, Germany). Briefly, untreated, camptothecin (4 mg/ml), or granzyme B (10 ng/ml)-treated cells (1.5 x 10^6/ml) were washed in PBS and treated

with lysis buffer (R&D Systems, Wiesbaden, Germany) for 1 h at room temperature. Following centrifugation at 1,000 x g for 15 min, supernatants were removed and 200 µl of a 1:100, 1:250, and a 1:500 dilution were used for a sandwich ELISA. Following incubation with substrate solution in the dark for 30 min the reaction was stopped. The optical density of each well was determined on an ELISA reader at 450 nm (MWG Biotech AG, Ebersberg, Germany). The amount of cytochrome c was determined according to a calibration curve.

2.11 Identification of human granzyme B

2.11.1 Membrane preparation

Membrane purification was performed by Dounce homogenization. Therefore, 1 x 10^9 cells were washed twice in 200 ml ice-cold PBS (500 g, 5 min, 4°C). After 10 min incubation in 7.5 ml Dounce buffer (10 mM TrisCl, 0.5 mM $MgCl_2$, pH 7.6) with protease inhibitors (Complete Mini, EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany) at 4°C, the cells were stroked 30 times in a chilled Dounce homogenizer on ice. Then 2.5 ml tonicity restoration buffer (10 mM TrisCl, 50 mM $MgCl_2$, pH 7.6) was added and the solution was centrifuged for 5 min at 500 g and 4°C. The supernatant was removed and centrifuged for 1 h at 100,000 g and 4°C. The pellet containing membranes was resuspended in 5 ml lysis buffer (300 mM NaCl, 50 mM TrisCl, pH 7.6) with protease inhibitors and solubilized 30-45 min by frequent gentle vortexing. Finally, insoluble material was spun down (10,000 g, 15 min, 4°C) and the supernatant (plasma membrane proteins) was stored at -20° C until used.

2.11.2 Affinity chromatography and immunoprecipitation

Bovine serum albumin (BSA, 1 mg/ml, Sigma, Munich, Germany), lyophilized, recombinant human Hsp70-protein (1 mg/ml, StressGen, British Columbia, CA), and Hsp70-peptide TKD (1 mg/ml, Bachem, Bubendorf, Switzerland) were incubated with equilibrated AminoLink-agarose beads (2 ml, Pierce, Biotechnology Inc., Rockford, USA) for 6 h, together with the reductant $NaCNBH_3$, followed by removal of uncoupled material and quenching non-reactive groups by extensive washing with 1 M Tris•HCl (pH 7.4). Binding capacity of BSA, Hsp70-protein, and Hsp70-peptide TKD was greater than 95%.

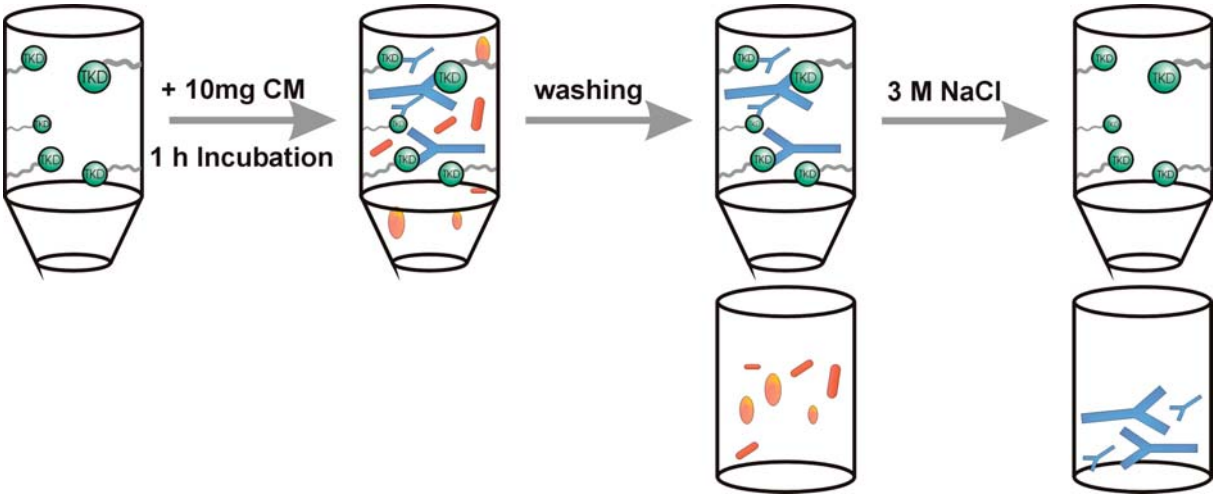


Figure 9: Affinity chromatography. TKD-conjugated columns are incubated with 10 mg cytoplasma membranes (CM) of NK cells or K562 cells. After 1 h incubation non-bound proteins are eluted by extensive washing. Bound proteins are eluted with 3 M sodium chloride.

As shown in Figure 9, 10 mg cytoplasma membranes of NK cells and the leukemic non-NK cell line K562 were loaded onto BSA, Hsp70-protein, and Hsp70-peptide TKD–conjugated columns for 1 h at room temperature. Columns were washed with 10 column volumes of 50 mM Tris buffer (pH 7.6). Bound proteins were eluted with 3 M sodium chloride in 50 mM Tris buffer in 5 fractions (1 ml). Each fraction was subjected to a SDS-PAGE using a 10% polyacrylamide slab gel and transferred onto polyvinylidene difluoride membranes.

2.11.3 Western-Blot analysis

Blots were blocked with 5% skim milk in PBS with 0.5% Tween 20 (Merck, Darmstadt, Germany) at room temperature for 1 h. Membranes were incubated with monoclonal antibody directed against granzyme B (2C5, IgG2a, BD Biosciences, Heidelberg, Germany), for 5 h at 4°C. Afterwards, blots were washed with PBS/0.5% Tween and incubated with a secondary mouse anti-IgG horseradish peroxidase antibody (Dianova, Hamburg, Germany), for 1 h at 4°C. Proteins were detected using the ECL detection kit (Amersham Biosciences Europe GmbH, Freiburg, Germany). Autoradiographs were recorded on X-omat films (Kodak, Stuttgart, Germany).

2.11.4 Protein identification by peptide mass fingerprinting (Collaboration with W. Koelch)

A 32 kDa protein band was isolated by affinity chromatography on immobilized Hsp70-protein or Hsp70-peptide TKD, which was excised from Coomassie Blue-stained gels, digested with trypsin and desalted using reversed phase ZIP tips (Millipore, Eschborn, Germany). The samples were embedded in 4-hydroxy-a-cyanocinnamic acid and the peptide masses were determined with a Perseptive Voyager DePro matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer in reflective mode. A peaklist was compiled with the m/z software (Proteometrics, Genomic Solutions, Ann Harbor, USA) and used for peak selection; the resulting peptide mass fingerprint was used to search the non-redundant NCBI protein data base using the Profound search engine (Proteometrics). Granzyme B was identified with 100% probability and $>$95% confidence.

3 RESULTS

Note: *Since part of my work was published (see below), only the representative results are shown in this chapter. For further results the publications comprised in the appendix are cited. The appendix contains reprints of the following publications:*

(i) **Gross C**, Hansch D, Gastpar R, Multhoff G. 2003a. Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. *Biol Chem* **384:** 267-279.

(ii) **Gross C**, Koelch W, DeMaio A, Arispe N, Multhoff G. 2003b. Cell surface-bound heat shock protein 70 (Hsp 70) mediates perforin-independent apoptosis by specific binding and uptake of granzyme B. *J Biol Chem* **278:** 41173-41181.

(iii) **Gross C**, Schmidt-Wolf IGH, Nagaraj S, Gastpar R, Ellwart J, Kunz-Schughart L, Multhoff G. 2003c. Hsp70-reactivity is associated with increased cell surface density of CD94/CD56 on primary NK cells. *Cell Stress Chaperones* **8:** 348-360.

(iv) Gastpar R, **Gross C**, Rossbacher L, Ellwart J, Riegger J, Multhoff G. 2004. The cell surface-localized Hsp70 epitope "TKD" induces migration and cytolytic activity selectively in human natural killer (NK) cells. *J Immunol* **172:** 972-980.

To examine the differences between Hsp70 membrane-positive and Hsp70 membrane-negative tumor cell lines, the colon carcinoma sublines CX+ (93% Hsp70 membrane-positive) and CX- (18% Hsp70 membrane-positive) and the pancreatic carcinoma sublines Colo+ (73% Hsp70 membrane-positive) and Colo- (34% Hsp70 membrane-positive) were used. Similar results were obtained with both systems. In this chapter only the results obtained in the CX+/CX- system are shown. Results obtained in the Colo+/Colo- system are also shown in the publications of the appendix and cited in the results part.

3.1 The Hsp70 peptide TKD is a target structure for NK cells

Previous work demonstrated that membrane-bound Hsp70 serves as a target structure for NK cells (Multhoff et al 1997). To identify the epitope of Hsp70 that interacts with NK cells, deletion mutants were produced lacking either the C- or N-terminal domain. It was shown, that similar to full-length Hsp70 C-terminal substrate binding domain of Hsp70 (Hsp70-C, aa$^{384-641}$, Figure 10) in combination with low dose IL-2 (100 IU/ml) stimulates the proliferative capacity and cytotoxic activity of NK cells (Botzler et al 1998, Multhoff et al 1999).

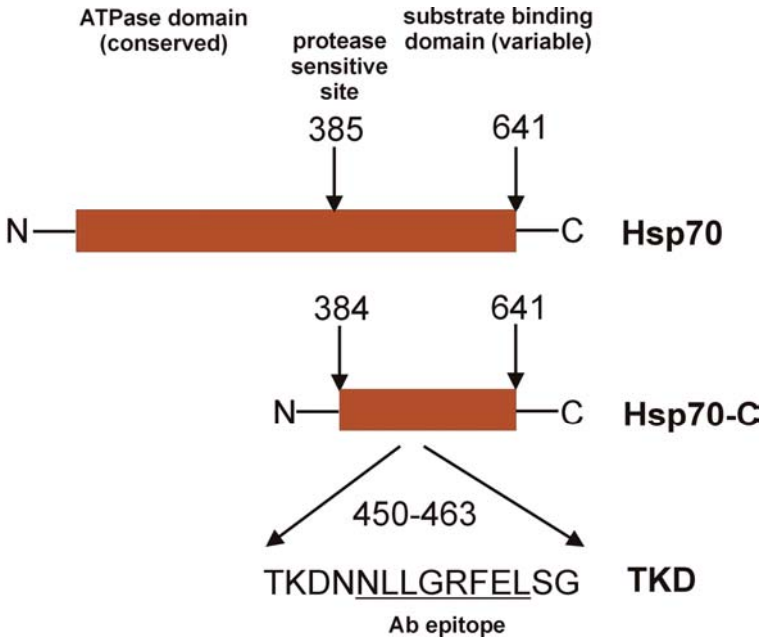


Figure 10: Hsp70, Hsp70-C, and TKD. Schematic illustration of Hsp70 protein, the C-terminal substrate binding domain of Hsp70 (Hsp70-C, aa$^{384-641}$), and the 14-mer peptide derived from the C-terminal domain of Hsp70 (TKD, aa$^{450-463}$). Underlined amino acids mark the 8-mer epitope that is recognized by the Hsp70 specific antibody cmHsp70.1.

3.1.1 The 14-mer peptide TKD stimulates the proliferative capacity of NK cells

An Hsp70-specific monoclonal antibody (cmHsp70.1) detecting membrane-bound Hsp70 on viable tumor cells was known to inhibit the immune response of NK cells against Hsp70 membrane-positive tumor cells (Multhoff et al 1999). By peptide scanning analyisis of the C-terminal domain of Hsp70 an 8-mer amino acid sequence (NLLGRFEL, aa$^{451-461}$, Figure 10) could be identified as the epitope of the Hsp70 specific antibody, detecting membrane-bound Hsp70 on the plasma membrane of viable tumor cells (Multhoff et al 2001). Based on the primary sequence of Hsp70 and

this 8-mer epitope the following peptides were synthesized and tested for their capacity to stimulate NK cells (Table 3): the 8-mer antibody binding epitope (NLL), a 13-mer C-terminal-extended peptide (GIPP), a 14-mer N-terminal-extended peptide (TKD), and a 12-mer N-terminal-extended peptide (TKD12). In addition, based on the primary sequence of Hsp70, different homologous peptides derived from Hsp70hom (88% sequence homology to Hsp70), the constitutive form Hsc70 (84% sequence homology to Hsp70), and the *Escherichia coli* derived DnaK (50% sequence homology to Hsp70), were synthesized (Table 3). One artificial 14-mer peptide termed LKD exhibiting one amino acid exchange at position one (T to L) was also tested for its stimulatory capacity on NK cells (Table 3).

Peptide	Origin of protein	aa	Sequence
NLL (8-mer)	Hsp70	454-461	NLLGRFEL
GIPP (13-mer)	Hsp70	454-466	NLLGRFELSGIPP
TKD (14-mer)	Hsp70	450-463	**TK**DN**NL**LG**R**FEL**SG**
TKD12 (12-mer)	Hsp70	450-461	TKDNNLLGRFEL
HOM (14-mer)	Hsp70hom	450-463	TKDNNLLGRFEL<u>T</u>G
HSC70 (14-mer)	Hsc70	450-463	TKDNNLLG<u>K</u>FEL<u>T</u>G
DNAK (14-mer)	DnaK	447-460	<u>AA</u>DN<u>KS</u>LG<u>Q</u>F<u>N</u>L<u>D</u>G
LKD (14-mer)	synthetic	450-463	<u>L</u>KDNNLLGRFELSG

Table 3: Amino acid (aa) sequences of peptides that were used for NK stimulation assays. Name of the peptide, origin of protein, and amino acid position and sequence are shown. Hsp70hom, the constitutive Hsc70, and the *Escherichia coli* derived DnaK are homologues of Hsp70. Bold positions mark amino acids that are presumably involved in the interaction with NK cells. Underlined positions mark amino acid exchanges compared to the primary sequence of Hsp70.

In a first set of experiments the 8-mer antibody binding epitope NLL, the 13-mer C-terminal-extended peptide GIPP, and the 14-mer N-terminal extended peptide TKD were tested for their capacity to stimulate the proliferation of NK cells in a ^{3}H thymidine assay.

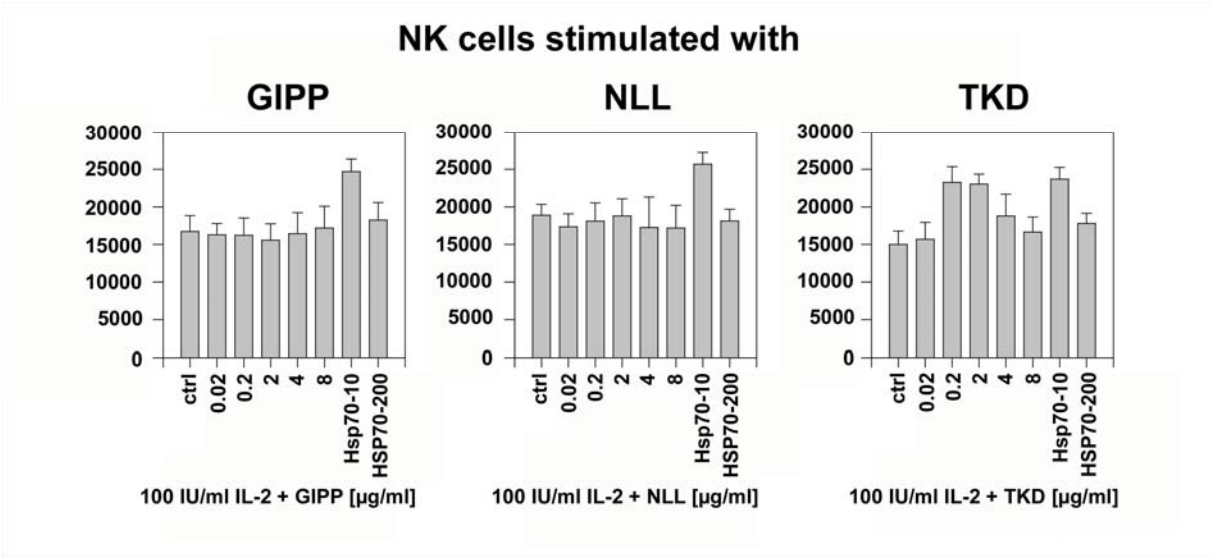


Figure 11: Comparison of the proliferative response of NK cells against Hsp70 derived peptides. Plastic adherent NK cells were stimulated either with IL-2 (100 IU/ml) alone (ctrl) or with IL-2 plus peptides GIPP (13-mer), NLL (8-mer), and TKD (14-mer) at concentrations of 0.02, 0.2, 2, 4, and 8 µg/ml. NK cells were also stimulated with Hsp70 protein at concentrations of 10 and 200 µg/ml. The proliferative response of NK cells was measured 66 h after peptide incubation and an 18 h incubation period with ^{3}H thymidine (0.5 µCi/ml). Values are given as the means of five independent experiments ± SD.

Therefore, plastic adherent NK cells were stimulated either with IL-2 (100 IU/ml) alone, or with IL-2 plus peptides at different concentrations. Stimulation of NK cells with low dose IL-2 plus rHsp70 served as a positive control. The concentration of 10 µg/ml has been defined as the optimal stimulatory dose for full length Hsp70 (Multhoff et al 1999). With respect to the molecular weight of the different peptides, the concentration that is equivalent to 10 µg of Hsp70 protein (72 kDa) was calculated as 0.2 µg/ml for all 13-mer and 14-mer peptides including GIPP (1452 Da) and TKD (1563 Da) and as 0.1 µg/ml for NLL (942 Da). Therefore, all peptides were tested in a concentration range of 0.02 to 8 µg/ml. As shown in Figure 11, neither the 8-mer peptide NLL nor the C-terminal-extended peptide GIPP were able to stimulate the proliferative capacity of NK cells at any of the tested concentrations. Only the N-terminal-extended 14-mer peptide TKD was able to stimulate the proliferative capacity of NK cells similar to full-length Hsp70 in a concentration range of 0.2-2 µg/ml.

It was shown that Hsc70 and DnaK failed to stimulate NK cells (Multhoff et al 1999). Furthermore, the homologue of Hsp70 (Hsp70hom) showed a weaker stimulatory capacity compared to Hsp70. Therefore, the stimulatory capacity of the 14-mer peptide TKD was compared to three 14-mer peptides based on the primary sequence of these proteins (HSC70, HOM, DNAK, Table 3). The stimulatory capacity of the N-

terminal extended 12-mer peptide (TKD12, Table 3), GIPP and NLL was also tested in this experiment.

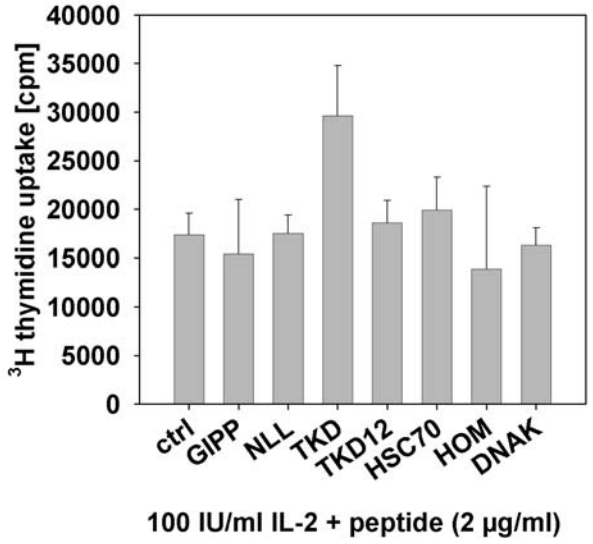


Figure 12: Comparison of the proliferative response of NK cells against different HSP70 derived peptides. Plastic adherent NK cells were stimulated either with IL-2 (100 IU/ml) alone or with IL-2 plus peptides GIPP (13-mer), NLL (8-mer), TKD (14-mer), TKD12 (12-mer), HSC70 (14-mer), HOM (14-mer), and DNAK (14-mer) at a concentration of 2 µg/ml. The proliferative response of NK cells was measured 48 h after peptide incubation and an 18 h incubation period with ^{3}H thymidine (0.5 µCi/ml). Values are given as the mean of two independent experiments ± SD.

As shown in Figure 12, only TKD plus low dose IL-2 was able to stimulate the proliferative capacity of NK cells at a concentration of 2 µg/ml. In the absence of IL-2, TKD exhibits no stimulatory activity towards NK cells.

3.1.2 The 14-mer peptide TKD stimulates the cytolytic activity of NK cells

Similar to full-length Hsp70, the 14-mer peptide TKD did not only stimulate the proliferative capacity of NK cells, but also the cytolytic activity against Hsp70 membrane-positive tumor cells. Therefore, NK cells were stimulated with low dose IL-2 (100 IU/ml) plus TKD (2 µg/ml) for 4 days and used as effector cells in a 4 h ^{51}Cr release assay. As target cells the Hsp70 high expressing (93% Hsp70 membrane-positive) CX+ cells and the Hsp70 low expressing (18% Hsp70 membrane-positive) CX- cells were used. These sublines were established from the colon carcinoma cell line CX-2 (66% Hsp70 membrane-positive cells) by flow cytometric cell sorting using the Hsp70-specific antibody cmHsp70.1. A phenotypic characterization of these sublines shown in Table 4 revealed that they only differ with respect to their capacity

to present Hsp70 on their cell membrane. All other cell surface markers tested including HLA class I, adhesion molecules ICAM (CD54) and NCAM (CD56), and MDR-1 were identically expressed by both sublines. HLA-E, the classical ligand for the C-type lectin receptor CD94, and the stress inducible MICA, the ligand for NKG2D, are weakly expressed by CX+/CX- cells.

specificity	antibody clone	positively stained cells [%]	
		CX+	CX-
HLA class I	W6/32	99 ± 1	99 ± 1
HLA DR	L243 B8.12.2	8 ± 3	6 ± 2
HLA-G	87G	6 ± 2	5 ± 2
HLA-E	MEM-E/06	11 ± 8	17 ± 3
MICA	AMO1	4 ± 1	5 ± 0
Hsp70	**cmHsp70.1**	**93 ± 3**	**18 ± 6**
CD54 (ICAM)	ICAM	69 ± 6	54 ± 5
CD106 (VCAM)	VCAM	9 ± 3	10 ± 3
CD57 (HNK1)	NC1	12 ± 4	14 ± 3
CD56 (NCAM)	NCAM 16.2	19 ± 7	17 ± 4
MDR-1 p-gp	UIC2	68 ± 8	64 ± 7

Table 4: Phenotypic characterization of Hsp70 membrane-positive CX+ and Hsp70 membrane-negative CX- tumor target cells. Cells were stained with FITC- or PE-conjugated antibodies. Mean values of at least three different experiments were shown.

As shown in Figure 13, TKD stimulated NK cells showed an enhanced lysis of Hsp70 membrane-positive CX+ cells compared to their Hsp70 membrane-negative counterparts at different effector to target cell ratios. When CD94-sorted NK cells were stimulated with the 14-mer peptide LKD exhibiting only one amino acid exchange at position 1 (T to L) no increase in the lysis of CX+ cells could be observed (right panel). Therefore, the 14-mer peptide TKD does not only enhance the proliferative activity of NK cells but also the cytolytic activity against Hsp70 membrane-positive tumor cells at a concentration of 2 µg/ml which is equivalent to 10 µg/ml full-length Hsp70-protein.

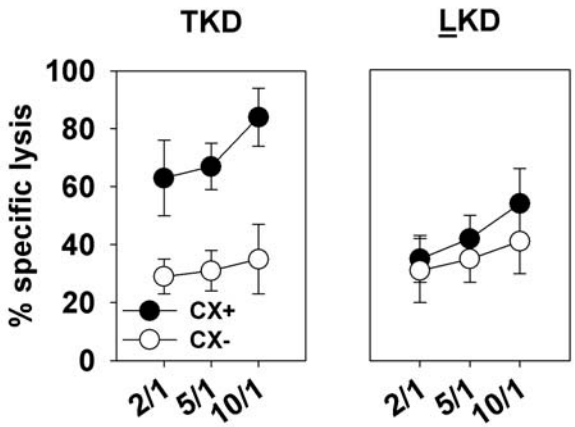


Figure 13: The 14-mer peptide TKD, but not the 14-mer peptide LKD, exhibiting one amino acid exchange at position 1 (T to L) stimulates Hsp70-reactivity of NK cells against Hsp70 membrane-positive target cells. CD94 sorted NK cells were stimulated for 4 days with low dose IL-2 (100 IU/ml) plus either TKD (2 µg/ml) (left panel) or LKD (2 µg/ml) (right panel) and used as effector cells at effector to target cell ratios ranging from 2/1 to 10/1 in a 4 h ^{51}Cr release assay. Hsp70 membrane-positive CX+ cells (solid circles) and Hsp70 membrane-negative CX- cells (open circles) were used as tumor target cells. The spontaneous release for each tumor target cell was always less than 20%.

In addition, the 14-mer peptide TKD enhances the secretion of the cytokines IFN-γ and TNF-α similar to Hsp70 (Multhoff et al 1999). Furthermore, the secretion of granzyme B was increased by stimulation of NK cells with low dose IL-2 plus TKD: NK cells (2 x 10^6/ml) incubated for 4 days with IL-2 (100 IU/ml) alone secreted 10 $\pm$ 7 ng/ml granzyme B, whereas NK cells stimulated with IL-2 plus TKD secreted 42 $\pm$ 11 ng/ml granzyme B (data from three independent experiments).

3.2 TKD stimulated NK cells migrate selectively towards Hsp70 membrane-positive tumor cells and supernatants derived thereof

3.2.1 Hsp70 membrane-positive tumor cells are infiltrated by TKD stimulated PBL

The 14-mer peptide TKD did not only enhance the cytotoxic activity of NK cells against Hsp70 membrane-positive tumor cells but also induces migration of NK cells towards them. A first hint that membrane-bound Hsp70 attracts NK cells was given by the following experiment: TKD stimulated PBL were co-incubated with exponentially growing Hsp70 membrane-positive CX+ and Hsp70 membrane-negative CX- cells. After 24 h the morphology of the cells in the co-cultures and controls (tumor cells alone) were analyzed by light microscopy (Figure 14).

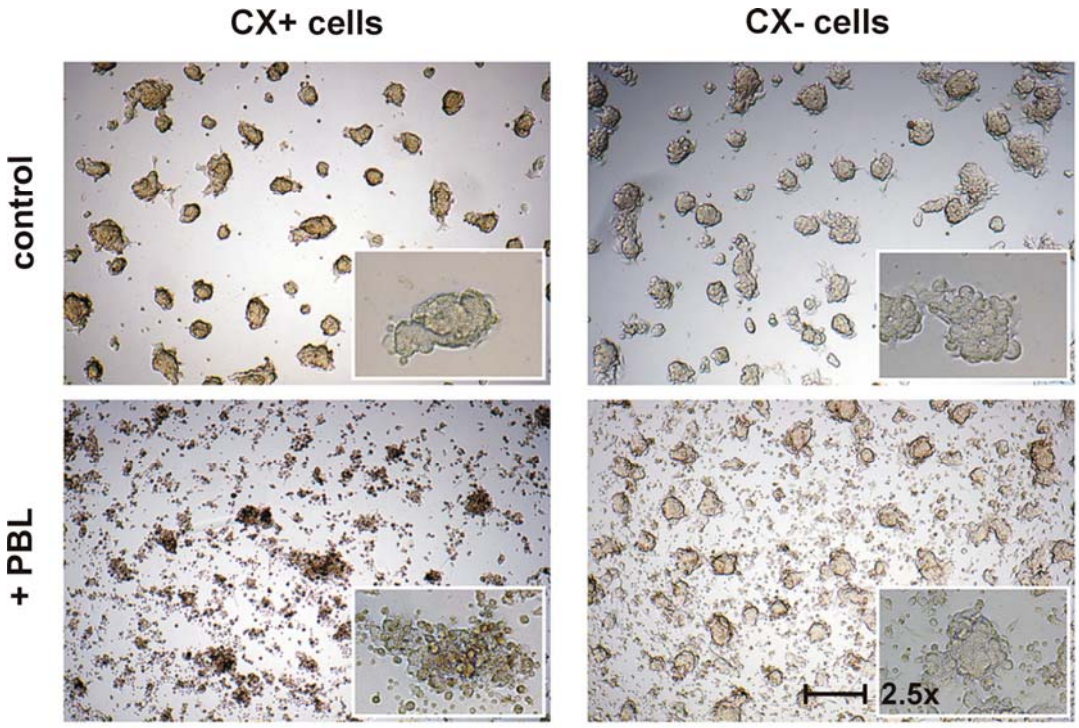


Figure 14: Infiltration of Hsp70 membrane-positive tumor cell clusters by TKD activated PBL. Hsp70 membrane-positive CX+ and Hsp70 membrane-negative CX- cells (0.1 x 10^6 cells/well) were seeded in 96 well flat-bottom plates and grown for 48 h. Tumor cells were co-incubated either with medium or with TKD stimulated PBL for another 24 h. Light microscopical analysis is shown for tumor cells only (control, upper row) or tumor cells co-incubated with TKD activated PBL (+ PBL. lower row) at a magnification of 50 x on a Zeiss Axiovert 25 microscope. Small inserts show a 2.5-fold magnification of one representative cell cluster. The scale bar in the lower right panel indicates 100 µm.

Under control conditions both cell lines showed a similar morphology forming adherent spheroidal cell clusters (Figure 14, control). If TKD activated effector cells

were added, the Hsp70 membrane-positive CX+ cell clusters were infiltrated by PBL, but not their Hsp70 membrane-negative counterparts (Figure 14, + PBL). Cell viability of CX+ cells also appeared to be reduced. This finding was confirmed by the fact that Hsp70 membrane-positive tumor cells are more susceptible to lysis mediated by TKD stimulated NK cells, than Hsp70 membrane-negative tumor cells (Figure 13).

3.2.2 TKD stimulated PBL selectively migrate towards Hsp70 membrane-positive tumor cells and supernatants derived thereof

Additional migration assays were performed using a transwell culture system. As tumor target cells the Hsp70 membrane-positive CX+ and Hsp70 membrane-negative CX- tumor cells were used. These cells were cultured in the lower compartment for 48 h. TKD activated PBL were added to the upper compartment and after a 4 h co-incubation period specific migration was measured. As shown in Figure 15A migration of TKD activated PBL towards Hsp70 plasma membrane-positive CX+ (11%) cells was higher than migration towards their negative counterparts (5%). As a positive control the cytokine IL-15 (10 ng/ml) was used as an attractant. Comparable to data published by Allavena et al (1997) migration towards IL-15 was 15% (data not shown). It has been shown that co-incubation of IL-2 activated NK cells and K562 target cells results in effector-target cell conjugates that trigger chemokine secretion and chemoattraction of other NK cells (Nieto et al 1998). To exclude chemoattraction induced by direct cell-cell contact, cell-free supernatants derived from CX+ and CX- tumor cells on day 2 were used. As demonstrated in Figure 15B left panel, TKD activated PBL showed 12% migration towards supernatants derived from CX+ cells, whereas only 3% of the PBL migrated towards supernatants derived from CX- cells. This suggests that migration of TKD activated PBL might be induced by a soluble factor that is produced selectively by Hsp70 membrane-positive tumor cells.

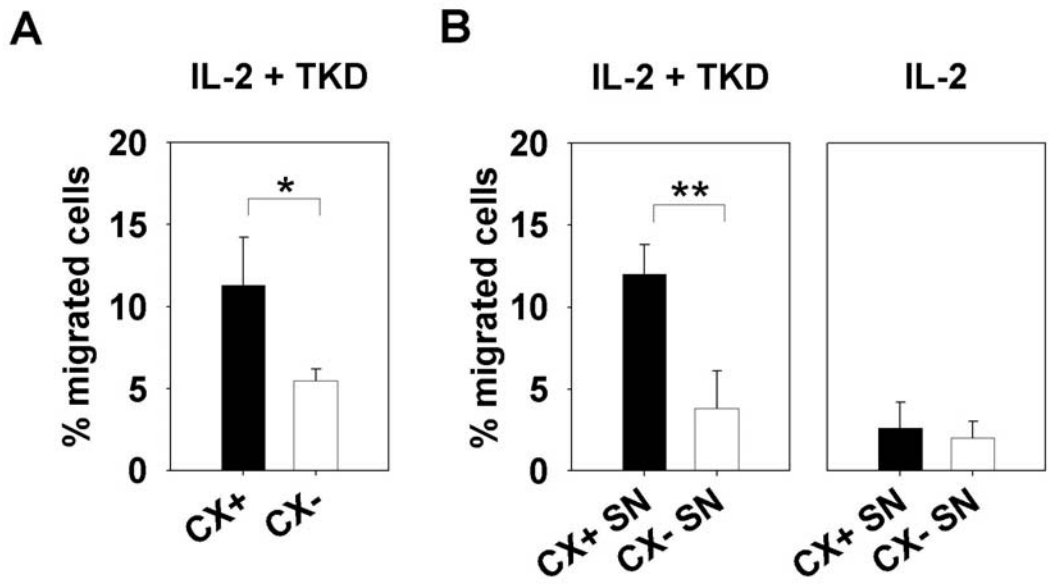


Figure 15: TKD activated PBL selectively migrate towards Hsp70 membrane-positive tumor cells and supernatants derived therof. Migration was analyzed in a transwell system with two compartments separated by a polycarbonate membrane with a pore size of 5 µm. (A) In the lower compartment CX+ and CX- tumor cells (0.2 x 10^6 cells/well) were grown for 48 h in 600 µl of RPMI-1640 medium supplemented with 10% heat inactivated FCS. PBL stimulated with low dose IL-2 (100 IU/ml) plus TKD (2 µg/ml) for 4 days, were labeled with sodium ^{51}chromate (100 µCi) and transferred into the upper chamber of the transwell system. Following a 4 h co-incubation period radioactivity was determined in PBL that migrated to the lower chamber. Specific migration was calculated as described in Materials and Methods. Results are shown as the mean ± SD of at least five independent experiments; statistically significant (student's $^{\tau}$-test) * $p < 0.05$. (B) In a next set of experiments 600 µl of cell-free culture supernatant (SN) of CX+ and CX- tumor cells cultured for 48 h were administered in the lower compartment. Following a 4 h co-incubation period with IL-2 and TKD stimulated PBL (left panel) or IL-2 stimulated PBL (right panel), radioactivity was determined in the migrated cells of the lower chamber. Specific migration was calculated as described above. Results are shown as the mean ± SD of at least five independent experiments; statistically significant (student's $^{\tau}$-test) * $p < 0.05$, ** $p < 0.005$.

Identical results were obtained with the pancreatic tumor system Colo+/Colo- (Gastpar, **Gross** et al 2004, appendix iv). Similar to the CX+/CX- sublines the Hsp70 membrane-positive Colo+ (73% Hsp70 membrane-positive cells) subline and the Hsp70 membrane-negative Colo- (34% Hsp70 membrane-positive cells) subline was generated from the pancreas carcinoma cell line Colo357 (50% Hsp70 membrane-positive cells) by flow cytometric cell sorting using the Hsp70 specific antibody cmHsp70.1. In this system TKD activated PBL migrated towards Colo+ cells (10%) and their supernatants (12%) whereas no migration against Colo- cells and their supernatants was observed (6% and 6% respectively). It has to be mentioned that stimulation of PBL with low dose IL-2 and TKD is a prerequisite for the induction of the migratory capacity since PBL stimulated with IL-2 alone did not migrate at all (Figure 15B, right panel).

3.2.3 NK cells migrate and exhibit lytic activity against Hsp70 membrane-positive tumor cells

A phenotypic analysis of the migrated cells revealed that selectively CD3-/CD16/56 NK cells but not CD3+ T cells were attracted by supernatants derived from Hsp70 membrane-positive tumor cells (Figure 16).

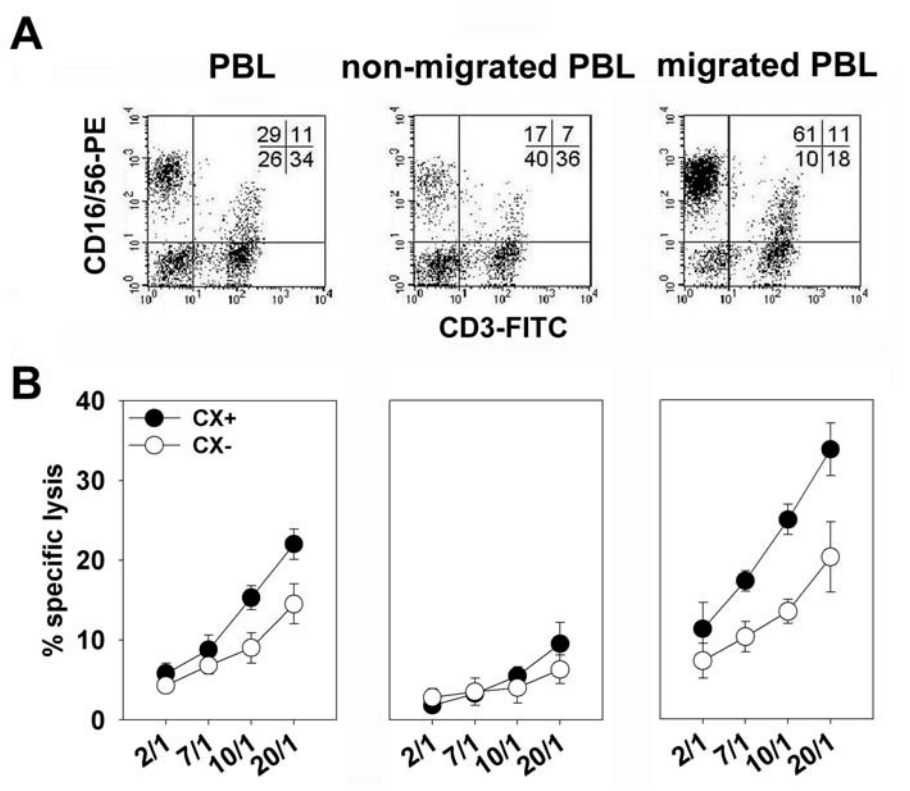


Figure 16: NK cells migrate towards and exhibit lytic activity against Hsp70 membrane-positive tumor target cells. A 4 h migration assay was performed using TKD stimulated PBL against supernatants of Hsp70 membrane-positive CX+ tumor cells to determine the phenotype and the lytic activity of non-migrated and migrated cells in comparison to the initial PBL population. (A) Non migrated PBL (middle panel), and migrated cells (right panel) were harvested and analyzed by multiparameter flow cytometry using CD3-FITC and CD16/56-PE antibodies. The phenotype of each cell population was compared to the initial PBL population (left panel) that had been stimulated with TKD for 4 days. Numbers in the dot blot diagrams indicate percentage of positively stained cells for each quadrant. Data of one representative result of three independent experiments is shown. (B) Concomitantly, the cytolytic activity of the three effector cell populations (initial PBL, non-migrated PBL, and migrated PBL) was tested in a standard 4 h ^{51}Cr release assay. CX+ (solid circles) and CX- (open circles) cells served as tumor target cells. Effector to target ratios ranged from 1/1 to 10/1. Mean values of three independent experiments ± SD is shown.

As shown in Figure 16A the cells of the original population contained 34% CD3 T cells, 11% CD3+/CD16/56+ NKT cells and 29% CD3-/CD16/56+ NK cells. The non-migrated cells showed no significant differences in the percentage of CD3+ T cells (36%) and CD3+/CD16/56+ NKT cells (7%), whereas a drastic decrease in CD3-/CD16/56+ NK cells from 29% to 17% could be observed. Concomitantly, the CD3-

/CD16/56+ increased significantly from 29% to 61% in the migrated cell population and the amount of CD3+/CD16/56- T cells was reduced from 34% to 18%. Taken together, these data indicate, that CD3-/CD16/56+ NK cells were specifically attracted by Hsp70 membrane-positive tumor cells and supernatants derived thereof. In addition, the migrated cell population showed a very strong lytic activity against Hsp70 membrane-positive CX+ target cells (solid circles) and a weaker activity against Hsp70 membrane-negative CX- target cells (open circles) (Figure 16B, right panel), whereas non-migrated cells showed a very weak lytic activity against all tumor target cells (Figure 16B, middle panel) indicating that CD3-/CD16/56+ NK cells were selectively attracted by Hsp70 membrane-positive tumor cells and specifically lyse them. Again, similar results were observed with the Colo+/Colo- system (Gastpar, **Gross** et al 2004, appendix iv).

Since NK cells not only migrated towards Hsp70 membrane-positive tumor cells, but also to supernatants derived thereof, a soluble factor was postulated that might be released by Hsp70 membrane-positive tumor cells. This was proven by the fact that both Hsp70 and TKD initiated migratory capacity in NK cells in a concentration-dependent (1 – 5 µg/ml), highly selective and chemokine-independent manner (Gastpar, **Gross** et al 2004, appendix iv). Taken together these results indicate that Hsp70 as well as TKD stimulate cytolysis, proliferation and chemotaxis in CD3-/CD16/56+ NK cells.

3.3 Binding studies of Hsp70 and TKD to NK cells

3.3.1 Hsp70 and TKD bind to the cell surface of the NK cell line YT

In addition to their immunostimulatory capacity, the interaction of full-length Hsp70 and the 14-mer peptide TKD with NK cells was investigated by immunofluorescence studies using the human NK cell line YT. Therefore, YT cells were treated with Hsp70-FITC (50 µg/ml) and TKD-FITC (8 µg/ml) for 45 min at 4°C. An unrelated protein, bovine serum albumin (BSA)-FITC (50 µg/ml), was used as a negative control. After extensive washing, cells were analyzed by fluorescence microscopy at a magnification of 500 x. As shown in Figure 17 both, Hsp70-FITC (Figure 17B) and TKD-FITC (Figure 17C), bound to the cell surface of YT cells at 4° C whereas no binding of BSA-FITC was observed at identical concentrations (Figure 17A).

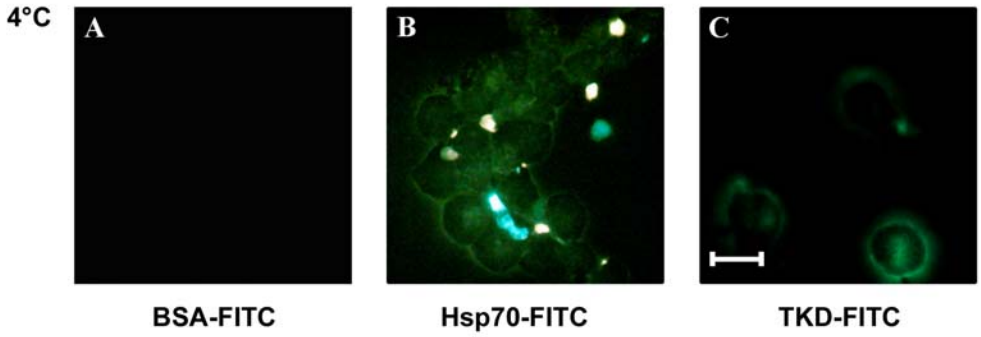


Figure 17: Specific cell surface binding of FITC-labeled Hsp70 and TKD to YT cells at 4°C. YT cells (5 x 10^6/ml) were treated with 50 µg/ml BSA-FITC (A), 50 µg/ml Hsp70-FITC (B), or 8 µg/ml TKD-FITC (C) at 4°C for 45 min. After extensive washing, cells were fixed with 1% paraformaldehyde and analyzed by fluorescence microscopy at a magnification of 500 x. The scale bar indicates 10 µm.

3.3.2 Binding of Hsp70 and TKD is concentration-dependent

To investigate whether cell surface binding of Hsp70-FITC is concentration-dependent, YT cells were incubated for 45 min with 10, 25, and 50 µg/ml Hsp70-FITC at 4°C. These concentrations have been defined as optimal for the stimulation of naive NK cells (Multhoff et al 1999). Identical concentrations of BSA-FITC were used as a control. Binding of Hsp70-FITC and BSA-FITC was analyzed by flow cytometry (Figure 18). The solid line represents binding of Hsp70-FITC (left panel) and BSA-FITC (right panel); a dashed line shows autofluorescence. Hsp70-FITC was bound specifically to YT cells at all concentrations (Figure 18, left panel): a

concentration of 10 µg/ml Hsp70-FITC resulted in 8% binding, 25 µg/ml in 29% binding, and 50 µg/ml in 71% binding. At 10 µg/ml and 25 µg/ml, the control protein BSA-FITC (Figure 18, right panel) was not bound by YT cells (2% and 4% binding, respectively); at a concentration of 50 µg/ml, binding of BSA-FITC was still significantly weaker (27% binding) as compared to Hsp70-FITC (71% binding). Concentrations above 50 µg/ml resulted in a non-specific binding of Hsp70-FITC and BSA-FITC (data not shown). Therefore, binding of full-length Hsp70 is dose dependent in a concentration range of 10 to 50 µg/ml.

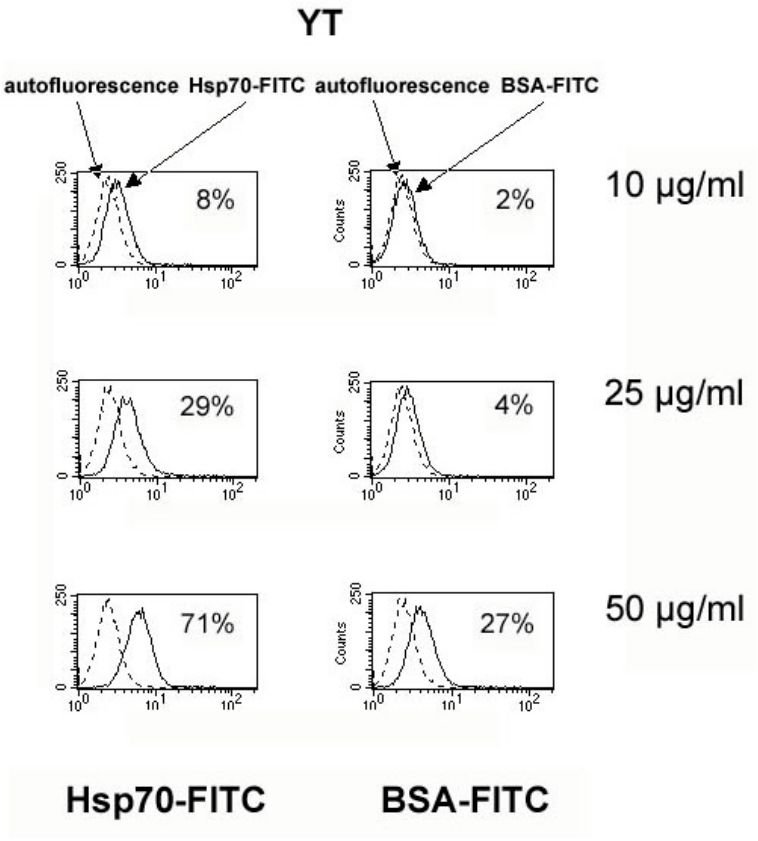


Figure 18: Hsp70 binding is concentration dependent. Concentration-dependent binding was determined by incubation of 5 x 10^6/ml YT cells with 10 µg/ml, 25 µg/ml, or 50 µg/ml of Hsp70-FITC (left panel), or BSA-FITC (right panel) at 4°C for 45 min. After extensive washing viable, PI-negative cells were analyzed by fluorescence analysis. Intensity of surface fluorescence (x-axis) was plotted against cell counts. The dashed line represents the autofluorescence; the solid line represents Hsp70 or BSA positively stained cells. Hsp70 or BSA binding is indicated in percent in the upper right corner of each graph, as the difference in the amount of positively stained cells minus autofluorescence-stained cells. Data of one representative result of at least three independent experiments is shown.

In addition to full-length Hsp70 protein concentration-dependent binding of TKD-FITC was investigated. Therefore, YT cells were incubated with TKD-FITC at concentrations ranging between 0.2 µg/ml and 8 µg/ml. The non-NK cell line K562 was used as a negative control. As shown in the left panel of Figure 19 specific

binding of TKD-FITC was observed at peptide concentrations of 2.0 µg/ml (12% binding), 4.0 µg/ml (19% binding), and 8.0 µg/ml (40% binding). An incubation of K562 cells with identical peptide concentrations revealed no significant binding of TKD-FITC. At a concentration of 8.0 µg/ml, the binding of TKD-FITC to YT cells as compared to K562 cells was still increased by 10-fold; 4% binding compared to 40% respectively. Concentrations above 8.0 µg/ml resulted in a non-specific interaction of TKD to both cell types.

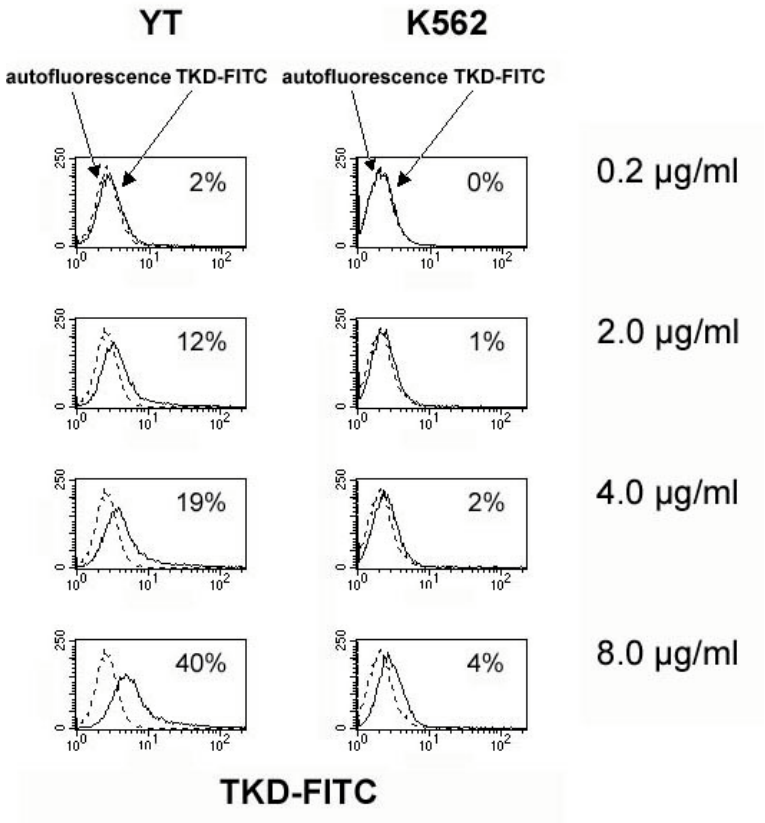


Figure 19: TKD binding is concentration dependent. TKD-FITC binding to YT (left panel) and the non-NK cell line K562 (right panel) is illustrated. Identical cell numbers of viable YT and K562 cells (5 x 10^6/ml each) were incubated with 0.2, 2.0, 4.0, or 8.0 µg/ml TKD-FITC as described in Figure 17. Data of one representative result of at least three independent experiments is shown.

In summary, a dose-dependent specific binding of both full-length Hsp70 (10 µg/ml – 50 µg/ml) and TKD (2 µg/ml – 8 µg/ml) but not of unrelated proteins was observed at 4°C. Interestingly, these concentration ranges also have been defined as optimal for stimulation of NK cell function including cytotoxicity, migration, and proliferation (Multhoff et al 1999, chapter 3.1).

3.3.3 Binding of Hsp70-FITC can be inhibited by free Hsp70

In order to show that Hsp70 binding is not only concentration-dependent but also highly specific, competition assays were performed using either unlabeled Hsp70-protein or an unrelated protein (glutathione-S-transferase, GST) in 20-fold excess (Figure 20). The binding of Hsp70-FITC (33% binding, Figure 20, left) was completely suppressed upon addition of a 20-fold excess of unlabeled Hsp70 (0% binding, Figure 20, middle), whereas an excess of unlabeled GST (20 x GST) only weakly competed for the Hsp70 binding site on YT cells (20% binding, Figure 20, right).

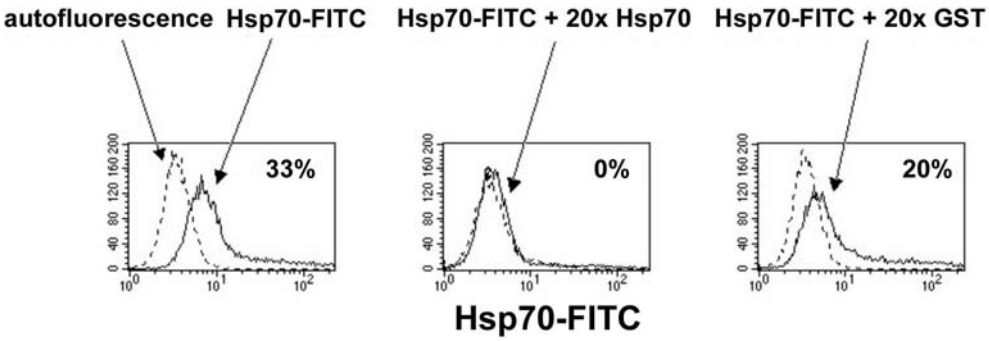


Figure 20: Hsp70 protein binding to YT cells is specific. Specificity of Hsp70 binding was measured by competition assays. YT cells (5 x 10^6/ml) were incubated with 20 µg/ml Hsp70-FITC, either alone or together with a 20-fold excess of unlabeled Hsp70 protein or unlabeled glutathione S-transferase (GST), at 4°C for 45 min. Data of one representative result of at least three independent experiments is shown.

These data indicate that binding of Hsp70/TKD to NK cells was concentration dependent and specific. These data led me to speculate about an Hsp70/TKD specific receptor.

3.4 The C-type lectin receptor CD94 is involved in Hsp70/TKD-NK cell interaction

3.4.1 CD94 is commonly expressed by YT cells and Hsp70-reactive NK cells

To characterize the molecular nature of the receptor responsible for Hsp70/TKD-NK cell interaction the NK cell line YT was used. A phenotypic characterization of YT cells revealed that none of the previously described HSP-receptors on APC including CD91 (Binder et al 2000a, Binder et al 200b, Basu et al 2001), TLR2, TLR4, and CD14 (Asea et al 2000a, Asea et al 2002) was present on the cell membrane of YT cells (Table5).

Antibody	Specificity	Positively stained cells [%]	+/-
	YT cell marker		
W6/32	**HLA class I**	**98.9 ± 0.2**	**+**
CD7	**gp40, FcμR, YT positive ctrl T cell marker**	**93.3 ± 0.7**	**+**
CD3	gp20-50, TcR associated complex	1.4 ± 0.2	-
CD6	gp100, thymocytes	0.0 ± 0.0	-
CD8	gp32, cytotoxic, suppressor T	0.0 ± 0.0	-
	NK cell marker		
CD11b	gp165, C3biR	0.4 ± 0.7	-
CD16	gp50-80, FcγR	2.8 ± 3.7	-
CD56	gp180, N-CAM	1.5 ± 0.9	-
CD57	p110, HNK1	0.0 ± 0.0	-
CD161	gp60, C-type lectin, KAR	0.0 ± 0.0	-
CD158a	p58.1, p50.1, Ig-SFR, KIR/KAR	0.0 ± 0.0	-
CD158b	p58.2, p50.2, IG-SFR, KIR, KAR	0.0 ± 0.0	-
CD94	**gp43, C-type lectin R, KIR/KAR**	**20-45.8 ± 6.0**	**+**
	HSP receptors on APC		
CD91	p600, α2-macroglobulin receptor	1.8 ± 0.7	-
CD14	gp55, LPS receptor	0.0 ± 0.0	-
TLR2	Toll-like receptor 2	1.0 ± 0.8	-
TLR4	Toll-like receptor 4	0.1 ± 0.1	-

Table 5: Phenotypic characterization of the NK cell line YT. IL-2 independent YT cells were cultured at cell densities ranging from 0.1 – 0.8 x 10^6 cells/ml (doubling time: 40-50 h). The data represent mean values of at least three independent experiments. Cell surface markers, commonly expressed by YT cells and Hsp70-reactive NK cells, are indicated in bold.

A comparison of YT cells and Hsp70-reactive primary NK cells demonstrated that only the C-type lectin receptor CD94 was commonly expressed by both cell types (Table 5).

3.4.2 The expression of CD94 is upregulated after stimulation of NK cells with Hsp70/TKD

As previously shown, CD94 was found to be upregulated after stimulation of naive NK cells with low dose IL-2 (100 IU/ml) plus full-length Hsp70 (Multhoff et al 1999). Similar to full-length Hsp70 the 14-mer peptide TKD revealed also an upregulated CD94 expression. As shown in Table 6, after stimulation with either low dose IL-2 or IL-2 plus TKD, peripheral blood mononuclear cells (PBMNC) derived from 25 different human volunteers revealed no significant differences in the amount of CD3 positive T cells. An incubation with IL-2 plus TKD resulted in a slight increase in the amount of CD56 and CD94 positive NK cells from 14.4 to 18.0 and from 13.5 to 17.4, respectively. With respect to the mean fluorescence intensity (mfi) significant changes were observed: after incubation with IL-2 plus TKD, the mfi of CD16 dropped from 873 to 583. In contrast, the mfi of the NK markers CD56 and CD94 increased from 534 to 981 and from 284 to 606, respectively.

	Percentage of positively stained cells ± SD [%] (mfi)		
Antibody	**Unstimulated (d0)**	**IL-2 (d4)**	**IL-2 + TKD (d4)**
CD3	71.9 ± 6.5 (200)	71.2 ± 4.6 (199)	70.0 ± 7.7 (205)
CD16	12.3 ± 7.8 (873)	13.2 ± 8.2 (821)	12.6 ± 7.1 (583)*
CD56	14.4 ± 7.8 (534)	12.8 ± 4.1 (715)*	18.0 ± 5.2 (981)*
CD94	13.5 ± 5.5 (284)	14.0 ± 5.3 (377)*	17.4 ± 4.3 (606)*

Table 6: Phenotypic characterization of unstimulated, IL-2 stimulated or IL-2 plus TKD stimulated PBMNC. Unstimulated PBMNC on d0 were compared by fluorescence analysis with PBMNC incubated for 4 days with either 100 IU/ml IL-2 alone, or IL-2 plus 2 µg/ml TKD. The cells were stained with the following mAb directed against T and NK cell-specific markers: CD3, CD16, CD56, and CD94. Flow cytometric analysis was performed using propidium iodide negative, viable cells of 25 different human volunteers. *significant difference in mean fluorescence intensity (mfi).

Similar results were obtained by stimulation of NK cells with low dose IL-2 plus TKD (**Gross** et al 2003c, appendix iii). Other cell surface receptors including the NCR NKp30 and NKp46 remained unaffected (**Gross** et al 2003c, appendix iii). Furthermore, a positive correlation of a high CD94 cell surface density and an

increased binding of Hsp70 was detected using the NK cell line YT (**Gross** et al 2003a, appendix i).

3.4.3 CD94 expression correlates with Hsp70-reactivity

With respect to the Hsp70-reactivity an increased cell surface density of CD94 on NK cells could be associated with an increased cytolytic activity against Hsp70 membrane-positive tumor cells. To confirm this finding, PBMNC were separated in a $CD94^{high}$ (CD94+++) and $CD94^{low}$ (CD94++) expressing NK cell subpopulation by flow cytometric cell sorting using a CD94 specific antibody (Figure 21). Both subpopulations were stimulated separately with low dose IL-2 and TKD for 4 days and used as effector cells in a ^{51}Cr cytotoxicity assay. As shown in Figure 21, lysis of Hsp70 membrane-positive tumor cells mediated by the $CD94^{high}$ cell population (solid diamonds) was stronger compared to the $CD94^{low}$ subpopulation (open diamonds).

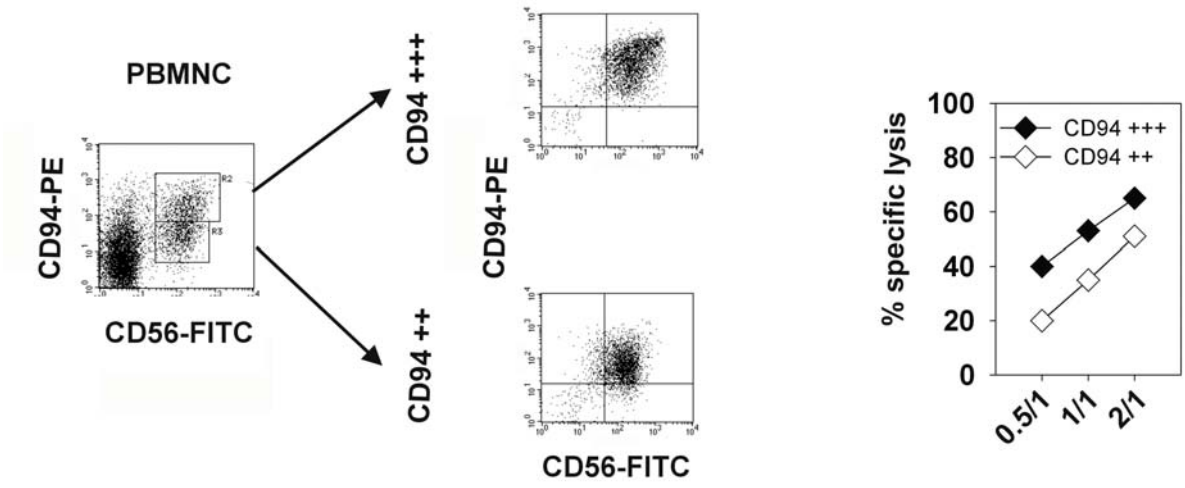


Figure 21: Comparative cytolytic activity of $CD94^{high}$ (+++) and $CD94^{low}$ (++) expressing NK cells. Peripheral blood mononuclear cells (PBMNC) were separated in $CD94^{high}$ (+++) and $CD94^{low}$ (++) expressing NK cells by flow cytometric cell sorting. Both subpopulations were stimulated with low dose IL-2 (100 IU/ml) and TKD (2 µg/ml) for 4 days and used as effector cells in a 4 h ^{51}Cr cytotoxicity assay. MHC class I- and Hsp70-positive CX-2 tumor cells were used as target cells. The spontaneous release for each target cell was less than 20%. Effector to target cell ratios were only 0.5/1, 1/1, and 2/1.

3.4.4 CD94 specific antibody blocks binding of Hsp70 and lysis of Hsp70 membrane-positive tumor cells

An involvement of CD94 in Hsp70 interaction was further confirmed by the fact, that both, binding of Hsp70 to YT cells (Figure 22) and lysis of Hsp70 membrane-positive tumor cells (Figure 23) could be abrogated by a CD94 specific antibody.

As shown in Figure 22, 51% of the YT cells were found to be CD94-positive (upper row). Following incubation with 20 µg/ml Hsp70-FITC 59% of the cells did bind Hsp70 (lower row, left panel). By preincubation of YT cells with 5 µg/ml CD94 antibody Hsp70-FITC binding could be abrogated (16% binding, lower row, middle panel), whereas preincubation with 5 µg/ml CD7 antibody only weakly competed for the Hsp70 binding site on YT cells (38% binding, lower row, right panel).

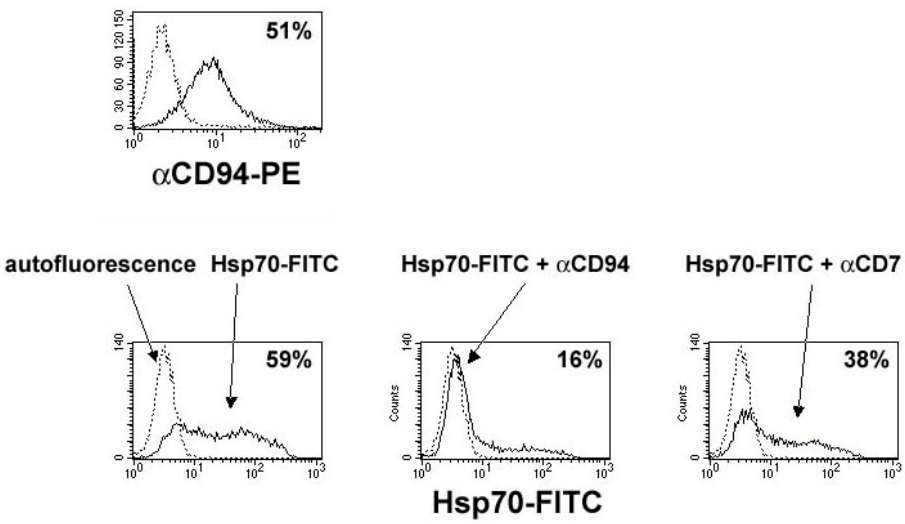


Figure 22: Blocking of Hsp70-FITC binding with a CD94 antibody. YT cells, either untreated (left panel) or after preincubation with CD94 mAb (HP-3B1, 5 µg/ml, middle panel) or CD7 mAb (5 µg/ml, right panel) for 30 min, were incubated with 20 µg/ml Hsp70-FITC at 4° C for 45 min. After extensive washing with PBS supplemented with 2% heat inactivated FCS viable, PI negative cells were analyzed by flow cytometry. The percentage of CD94-positive YT cells was 51%. The dashed line represents the autofluorescence; the solid line represents Hsp70 positively-stained cells. Specific Hsp70 protein binding is indicated in percent in the upper right corner of each graph. One representative data of three independent experiments is shown.

Furthermore, it could be shown that the CD94 specific antibody not only blocks binding of Hsp70 to NK cells but also inhibits the cytotoxicity of NK cells against Hsp70 membrane-positive tumor cells. This was demonstrated in a ^{51}Cr release assay (Figure 23). As target cells the Hsp70 membrane-positive CX+ and Hsp70 membrane-negative CX-cells were used. TKD stimulated CD3-depleted NK cells were used as effector cells.

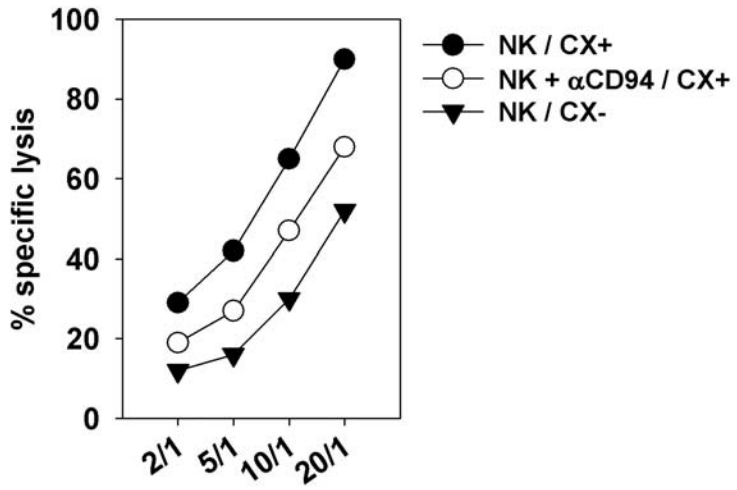


Figure 23: Blocking of lysis of Hsp70-positive tumor cells with a CD94 antibody. Hsp70 membrane-positive CX+ cells (circles) and Hsp70 membrane-negative CX- cells (triangles) were used as tumor target cells in a 4 h ^{51}Cr-release assay. The spontaneous release for each tumor target cell was always less than 20%. TKD stimulated CD3-depleted NK cells (4 days) either untreated (filled circles or triangles) or preincubated for 30 min with CD94 mAb (HP-3B1, 5 µg/ml) (open circles) were used as effector cells at different effector to target cell ratios ranging form 2/1 to 20/1. One representative data of three independent experiments is shown.

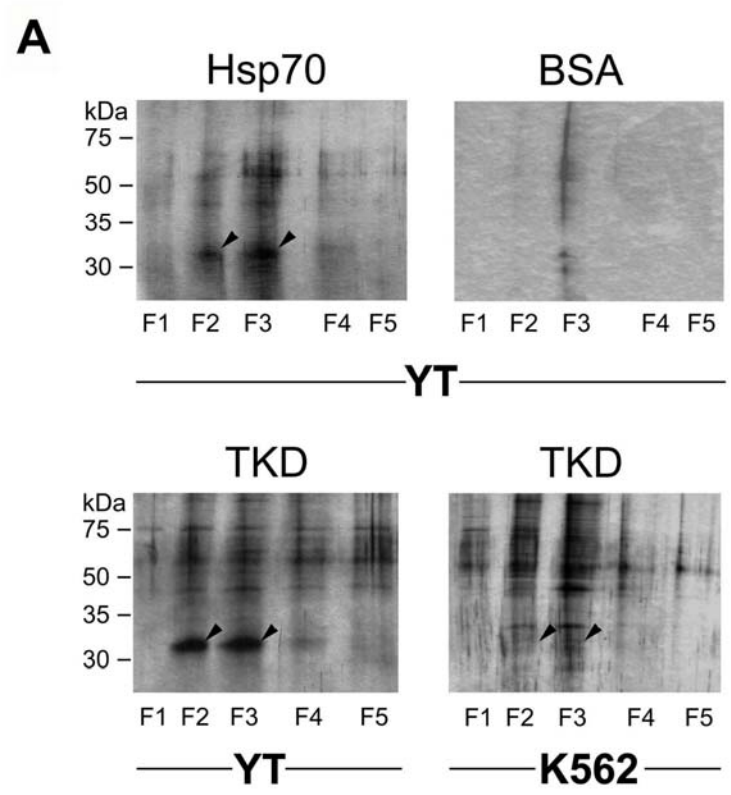
As illustrated in Figure 23, Hsp70 membrane-positive CX+ tumor cells (solid circles) were lysed significantly better as compared to their Hsp70 membrane-negative counterparts (solid triangles). Lysis of the Hsp70 membrane-positive CX+ cells could be decreased by preincubation of NK cells with an antibody directed against CD94 (open circles), whereas lysis of Hsp70 membrane-negative CX- cells was not affected by this antibody (data not shown). Multhoff et al have shown, that neither anti-CD57 antibody, nor HLA class I specific antibody have any inhibitory effect on the lysis of CX+ and CX- cells (Multhoff et al 1999).

In summary, it could be shown that (i) following incubation of NK cells with Hsp70/TKD, the expression of CD94 was upregulated, (ii) binding of Hsp70 to NK cells correlates with CD94 cell surface expression and could be abrogated by an CD94 specific antibody, and (iii) Hsp70 reactivity of NK cells correlated with CD94 cell surface expression and could be abrogated by an CD94 specific antibody. Taken together these data indicated that the C-type lectin receptor CD94 might be involved in the interaction of NK cells with Hsp70 membrane-positive tumor cells.

3.5 Mechanism of lysis of Hsp70 membrane-positive tumor cells

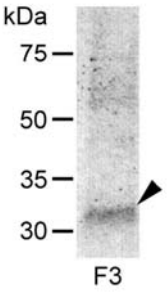
3.5.1 Human granzyme B interacts with Hsp70 and TKD

Although the preceding observations indicated that the 14-mer peptide TKD functions as a target recognition structure for NK cells, the mechanism how NK cells lyse Hsp70 membrane-positive tumor target cells remained to be elucidated. To answer this question, affinity chromatography was performed using sepharose columns coupled either with Hsp70 or TKD. Carrier or BSA-conjugated columns were used as controls. These columns were incubated with cell lysates of the NK cell line YT for 1 h. In addition cell lysates of the non-NK cell line K562 were used as a negative control. After extensive washing bound proteins were eluted in five fractions (F1 – F5) with 3 M sodium chloride. The eluted fractions were separated by SDS-PAGE and visualized by silver staining. A dominant 32 kDa protein band was observed in fractions two (F2) and three (F3) of YT cell eluates obtained from Hsp70 and TKD columns (Figure 24A). This band was not detected in eluates of carrier (data not shown) or BSA-conjugated columns nor in material eluted from the TKD affinity columns loaded with K562 cell lysates (Figure 24A). Additionally the 32 kDa protein band derived from F3 of the TKD-conjugated column was excised from a SDS-PAGE stained with Coomassie Blue and digested with trypsin (Figure 24B). The resulting peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass peptide fingerprinting (MALDI-TOF) in collaboration with the laboratory of W. Koelch. Sequences of the tryptic peptides exhibited 100% homology with granzyme B (Figure 24B). The identity of the 32 kDa protein as granzyme B was further confirmed by Western blot analysis using a specific antibody (2C5) against granzyme B. Fractions of YT cell lysates on Hsp70 and TKD columns revealed the presence of a granzyme B protein band by Western blotting (Figure 24C). However, no granzyme B was detected in the eluted fraction of Hsp70 and TKD affinity columns loaded with K562 cell lysates (Figure 24). Intracellular flow cytometry using a PE-conjugated granzyme B antibody (HC2-PE) showed positive staining for cytoplasmic granzyme B in YT cells, but not in K562 cells (**Gross** et al 2003b, appendix ii). In summary, these data indicate that granzyme B interacts with Hsp70 and TKD.



B

Identification of human granzyme B by MALDI-TOF analysis



Mr observed	Mr expected	peptide start	peptide end	sequence
877.364	877.396	145	151	MTVQEDR
893.369	893.391	145	151	MTVQEDR
1048.572	1048.566	192	201	VAGIVSYGR
1055.549	1077.556	136	144	HSHTLQEVKV
1288.702	1288.703	73	83	RPIPHPAYNPK
1442.722	1442.740	61	72	EQEPTQQFIPVK

Figure 24A, B: Identification of granzyme B as an interacting partner for Hsp70 and TKD. (A) Hsp70, BSA, and TKD columns were incubated either with cell lysates of the NK cell line YT or the non-NK cell line K562. Bound proteins were eluted from the columns in 5 fractions (F1 - F5), and resolved on SDS-PAGE. Following silver staining, eluates of YT cells derived from Hsp70 and TKD columns revealed a dominant 32 kDa protein band in fractions two (F2) and three (F3). No 32 kDa protein band was detectable in YT eluates derived from BSA columns and in K562 eluates derived from TKD columns. The position of the 32 kDa band is indicated with an arrowhead. (B) The tryptic peptides of the Coomassie Blue-stained 32 kDa band of fraction 3 (F3), derived from the TKD column, correspond to human granzyme B. The probability of identification was 100% and the estimated Z-score was 1.89 corresponding to >95% confidence.

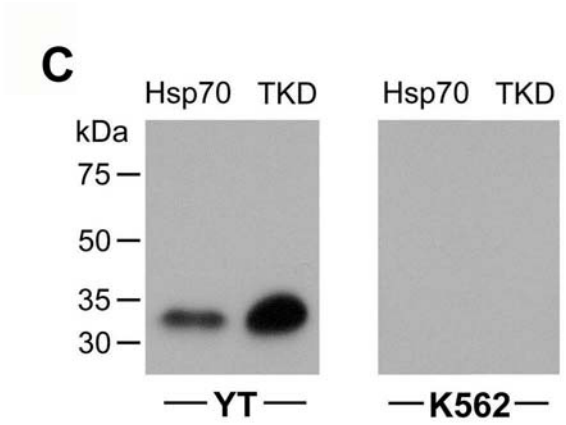


Figure 24C: Identification of granzyme B as an interacting partner for Hsp70 and TKD. (C) Corresponding Western blot analysis of YT and K562 cell eluates (F3) following incubation with Hsp70 and TKD columns. The blot was autoradiographed and the localization of granzyme B was visualized by immunostaining with the granzyme B specific monoclonal antibody 2C5. Eluates of YT cells (left), but not of K562 cells (right) revealed a 32 kDa granzyme B protein band.

3.5.2 Granzyme B binds specific to the cell surface of Hsp70 membrane-positive tumor cells and is taken up at 37°C

The preceding findings resulted in the following working hypothesis: if a tumor cell expresses Hsp70 on the cell surface, granzyme B might bind to it and might be taken up. In the case of an Hsp70-negative tumor cell no binding and uptake of granzyme B should be detected. This hypothesis was proven using the CX+ and CX- tumor system. Both cell types were incubated with perforin-free purified granzyme B at 4°C and at 37°C. Binding and uptake was shown by fluorescence microscopy (Figure 25A) and FACS analysis (Figure 25B), using an antibody directed against granzyme B (HC2-PE). Compared to untreated tumor cells (Figure 25A, control), granzyme B was bound to the cell surface of Hsp70 membrane-positive CX+ cells at 4°C (Figure 25A, grB 4°C). After a temperature shift to 37°C granzyme B was taken up by the Hsp70 membrane-positive tumor cells (Figure 25A, grB 37°C). In contrast, Hsp70 membrane-negative CX- cells neither exhibited any granzyme B cell surface binding at 4°C nor uptake at 37°C (data not shown). Furthermore, it could be shown that granzyme B was selectively taken up by Hsp70 membrane-positive tumor cells in a concentration-dependent manner, but not by their Hsp70 membrane-negative counterparts (Figure 25B). Therefore, Hsp70 membrane-positive CX+ cells and Hsp70 membrane-negative CX- cells were treated with different amounts of perforin-free, purified granzyme B (1 µg/ml, 2 µg/ml, and 4 µg/ml). A concentration-dependent uptake of granzyme B was detected in Hsp70 membrane-positive CX+ cells after

incubation with 2 and 4 µg/ml granzyme B, but not in their Hsp70 membrane-negative counterparts.

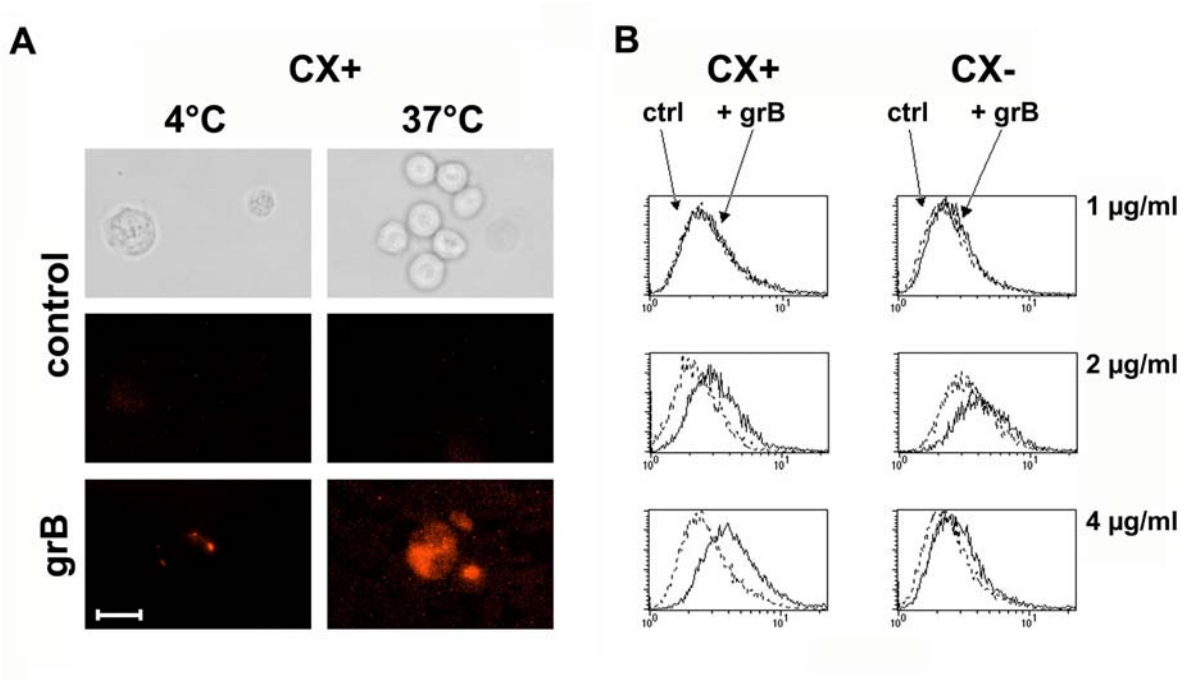


Figure 25: Specific cell surface binding and uptake of granzyme B (grB) by Hsp70 membrane-positive tumor cells. (A) Comparative binding of granzyme B (2 µg/ml) to the cell surface of CX+ tumor cells at 4°C, and uptake into the cytosol after a temperature shift to 37°C for 30 min, using the PE-conjugated granzyme B-specific monoclonal antibody HC2-PE. Light microscopy (first row); immunofluorescence of cells without granzyme B (control, second row); immunofluorescence of cells after addition of granzyme B (grB, third row). One representative fluorescence microscopy of three showing identical results is shown; magnification 400 x. (B) Intracellular flow cytometry of permeabilized CX+/CX- (n = 2) tumor cells using granzyme B-specific monoclonal antibody HC2-PE before (dashed line) and after (solid line) incubation of the tumor cells with 1, 2, and 4 µg/ml granzyme B at 37°C for 30 min. Only CX+ but not CX- cells showed a dose-dependent shift of the granzyme B peak to the right, indicating uptake of extracellularly offered granzyme B. Scale bar represents 10 µm.

Identical results were obtained using the pancreatic carcinoma sublines Colo+ and Colo- (**Gross** et al 2003b, appendix ii). In summary, these data indicated specific binding and internalization of granzyme B in Hsp70 membrane-positive tumor cells.

3.5.3 Granzyme B causes apoptosis selectively in Hsp70 membrane-positive tumor cells

After internalization the serine protease granzyme B cleaves procaspases into their activated form, and thereby induces programmed cell death (apoptosis) by promoting DNA fragmentation. Furthermore, granzyme B is involved in the release of cytochrome c from the mitochondria into the cytosol (Berke 1995, Froelich et al 1999, Shresta et al 1999). Since granzyme B was specifically taken up by Hsp70 membrane-positive tumor cells, only these cells should become apoptotic after treatment with granzyme B. Using three independent apoptosis assays (annexin V-FITC staining, nuclear DNA fragmentation and mitochondria cytochrome c release), it could be demonstrated that granzyme B caused apoptosis specifically in Hsp70 membrane-positive tumor cells, but not in their Hsp70 membrane-negative counterparts.

In a first set of experiments Hsp70 membrane-positive CX+/Colo+ and Hsp70 membrane-negative CX-/Colo- cells were incubated with 10 ng/ml enzymatically active granzyme B for 4, 12 and 24 h. The topoisomerase inhibitor camptothecin (cam, 4 µg/ml) was used as a positive control reagent for the induction of apoptosis. After a 4 h incubation period, neither camptothecin nor granzyme B initiated apoptosis in any of the tested tumor cells.

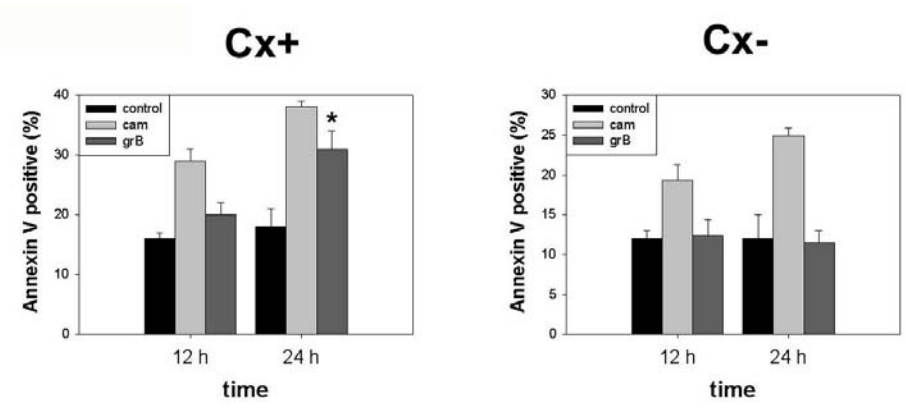


Figure 26: Isolated granzyme B (grB) induces apoptosis selectively in Hsp70 membrane-positive tumor cells (annexin-V staining). Percentage of annexin-V positive and propidium iodide-negative CX+ (left) and CX- (right) tumor cells, either untreated (black bars), or following incubation either with camptothecin (4 µg/ml; light gray bars) or granzyme B (10 ng/ml; dark gray bars) for 12 and 24 h. The data represent the mean of four independent experiments ± SD. * marks granzyme B values significantly different from control ($p < 0.005$).

After a 12 and 24 h incubation period with camptothecin significant apoptosis was observed in all tumor sublines (Figure 26). If the tumor sublines were incubated with

physiological relevant concentrations of granzyme B, the amount of annexin-V positive cells was increased in Hsp70 membrane-positive CX+/Colo+ cells, but not in their Hsp70 membrane-negative counterparts after 12 h and even more pronounced after 24 h. Figure 26 shows the results of the CX+/CX- system. For the Colo+/Colo- system see **Gross** et al 2003b, appendix ii.

In line with the annexin-V staining, both CX+ and CX- cells exhibited a typical positive DAPI staining pattern, as a sign for DNA fragmentation, following treatment with camptothecin, as compared with untreated controls (Figure 27).

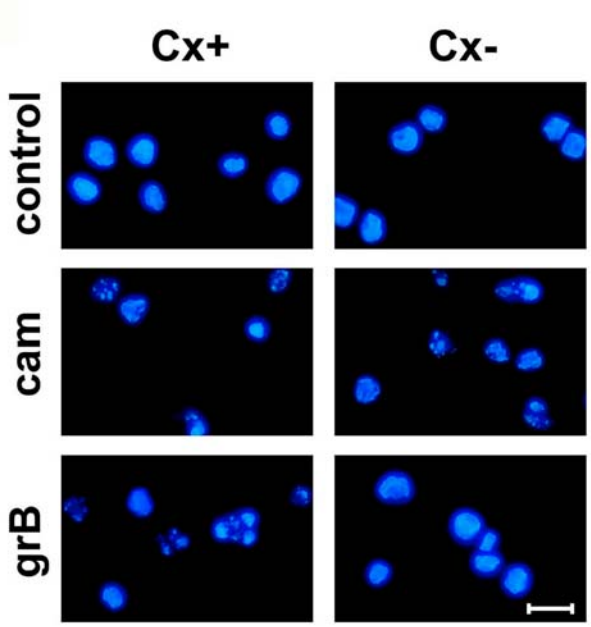


Figure 27: Isolated granzyme B (grB) induces apoptosis selectively in Hsp70 membrane-positive tumor cells (DAPI staining). Untreated (control), camptothecin (4 µg/ml; cam), or granzyme B (10 ng/ml; grB) treated CX+ and CX- tumor cells (24 h) were stained with DAPI. Considerable nuclear DNA fragmentation was observed in all tumor sublines following incubation with camptothecin (middle panel). After incubation with granzyme B only CX+ tumor cells exhibited nuclear DNA fragmentation (lower panel, left). No signs of apoptosis were observed in CX- tumor cells following incubation with granzyme B (lower panel, right). Scale bar represents 10 µm.

DNA fragmentation was observed selectively in Hsp70 membrane-positive CX+ tumor cells, 24 h post-treatment with granzyme B, whereas no DNA fragmentation was detected in their Hsp70 membrane-negative counterparts (Figure 27). Identical results were observed in the Colo+/Colo- system (**Gross** et al 2003b, appendix ii).

Cytochrome c release was measured following incubation of CX+ and CX- cells with granzyme B and camptothecin (Table 7). After 24 h incubation with granzyme B, cytochrome c concentration was increased 1.8-fold (0.382 mg/ml *versus* 0.690 mg/ml) in CX+ cells, whereas no increase after treatment with granzyme B was

observed in CX- cells (0.452 *versus* 0.425 mg/ml). An incubation with camptothecin (4 µg/ml) for 24 h resulted in a comparable, 1.5-fold increase in cytochrome c concentrations in both CX+ and CX- tumor cells.

Cells	Cytochrome c [mg/ml]		
	Control	**Camptothecin**	**Granzyme B**
		-fold increase	
CX+	0.382 ± 0.02	0.555 ± 0.04*	0.690 ± 0.08*
	1.0	*1.5*	*1.8*
CX-	0.452 ± 0.02	0.672 ± 0.02*	0.425 ± 0.075
	1.0	*1.5*	*0.9*

Table 7: Isolated granzyme B (grB) induces apoptosis selectively in Hsp70 membrane-positive tumor cells (cytochrome c release). Quantitative determination of human cytochrome c in CX+ and CX- tumor cells either untreated (control), or following incubation with camptothecin (4 µg/ml) or granzyme B (10 ng/ml), for 24 h. The data represent the mean of four independent experiments ± SD; * values significantly different from control ($p < 0.05$).

Taken together, these results indicate that following binding and selective uptake, via membrane-bound Hsp70, granzyme B initiates apoptosis in a perforin-independent manner.

3.5.4 Granzyme B released by TKD activated NK cells mediates apoptosis in Hsp70 membrane-positive tumor cells

To test the physiological relevance of these findings the intracellular levels of granzyme B in NK cells were measured before (d0) and after treatment with TKD (d3). It was shown, that intracellular levels of granzyme B were upregulated in NK cells 1.4-fold following stimulation with TKD plus low dose IL-2. Concomitantly, lysis of Hsp70 membrane-positive CX+ cells was elevated 1.5-fold in TKD stimulated NK cells compared to naive NK cells (Figure 28, left panel). Lysis of Hsp70 membrane-negative CX- cells remained unaffected (Figure 28, right panel). Furthermore, the increased cytolytic activity of TKD-stimulated NK cells against Hsp70 membrane-positive CX+ cells could be inhibited by Hsp70-specific antibody (cmHsp70.1) down to the level of lysis of Hsp70 membrane-negative tumor cells (1.7-fold inhibition, Figure 28, left), indicating that Hsp70 serves as an entry port for granzyme B. Identical results could be obtained in the Colo+/Colo- system (**Gross** et al 2003b, appendix ii).

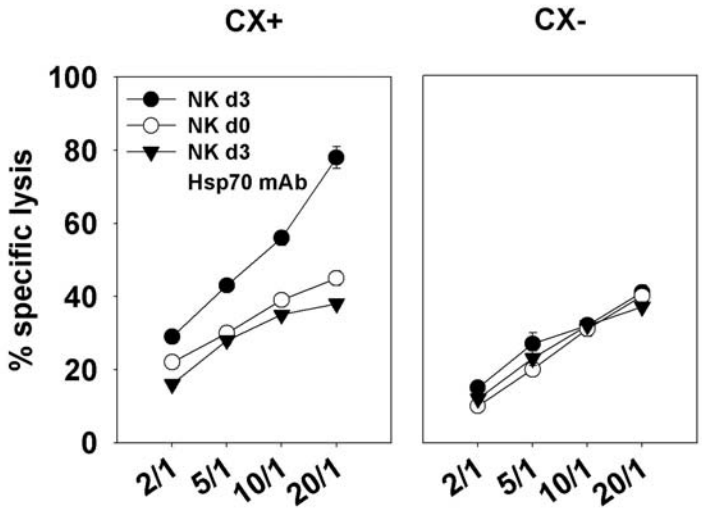


Figure 28: Selective kill of Hsp70 membrane-positive tumor cells, mediated by NK cells, is blockable by Hsp70-specific monoclonal antibody and correlates with granzyme B secretion. Killing of CX+ (left panel) and CX- (right panel) tumor target cells by naive, unstimulated (NK d0, open circles), and TKD stimulated NK cells (NK d3, filled circles and filled triangles) was quantified in 12 h ^{51}Cr release assays. Intracellular granzyme B levels in naive NK cells (NK d0) versus TKD-stimulated NK cells (NK d3) increased 1.4-fold; concomitantly lysis of CX+ cells was elevated 1.5-fold at different effector to target ratios. Lysis of CX- tumor cells remained unaffected. Furthermore, the increased cytolytic activity of TKD-stimulated NK cells (NK d3) against CX- tumor cells was inhibited by the Hsp70 specific antibody cmHsp70.1 (Hsp70 mAb,) down to the level of lysis of Hsp70 membrane-negative tumor cells (1.7-fold inhibition, filled triangles). Cytotoxicity was determined at E:T ratios ranging form 2/1 to 20/1. Spontaneous release of each target cell was below 10%. The data represent the mean of three independent experiments ± SD.

Apart from this observation, Hsp70 membrane-positive tumor target cells stimulated the production and release of granzyme B in primary NK cells. This was shown by an ELISPOT assay measuring the release of granzyme B in a co-incubation of either unstimulated (NK d0) or TKD-stimulated NK cells with CX+/CX- and Colo+/Colo- tumor target cells. Co-incubation of tumor cells with unstimulated NK cells (NK d0) always resulted in very low granzyme B release, independent of the tumor target cell line and the effector to target cell (E:T) ratio. In controls, the number of spots was always less than 20. After a 3 day stimulation period with TKD (NK d3) followed by a 4 h co-incubation time with tumor cells, granzyme B release was significantly up-regulated. At an E:T ratio of 5:1, the following numbers of granzyme B spots were determined: CX+: 260 ± 20; CX-: 165 ± 6; Colo+: 137 ± 55, Colo-: 66 ± 8. Concomitantly, Hsp70 membrane-positive tumor target cells (CX+/Colo+) were lysed significantly better as compared to their negative counterparts (Figure 28). In summary these data suggested a dual role for Hsp70 and TKD in the immune response against cancer: On the one hand it stimulates the cytolytic activity of NK cells by an increased production of the pro-apoptotic enzyme granzyme B, on the

other hand it facilitated uptake of granzyme B selectively into Hsp70 membrane-positive tumor target cells.

4 DISCUSSION

4.1 The 14-mer peptide TKD serves as a target structure for NK cells

Tumor cells frequently express Hsp70, the major stress-inducible member of the HSP70 family, on their plasma membranes (Tamura et al 1993, Multhoff et al 1995 a, Farkas et al 2003, Kleinjung et al 2003). These tumor cells are more susceptible for the lysis mediated by NK cells. Furthermore, it could be shown that 3 to 4 days stimulation of NK cells with Hsp70 (10 µg/ml) and low dose IL-2 (100 IU/ml) enhanced the proliferative and cytolytic activity of NK cells (Multhoff et al 1999). It could be demonstrated that similar to full-length Hsp70 and the C-terminal domain of Hsp70 (Hsp70-C, $aa^{384-641}$, Botzler et al 1998), the 14-mer peptide TKD (TKDNNLLGRFELSG, $aa^{450-463}$) stimulated the proliferative capacity and cytolytic activity of NK cells. TKD is an N-terminal extended peptide of the 8-mer binding epitope of the Hsp70 specific antibody cmHsp70.1. This antibody detects cell surface-bound Hsp70 on viable tumor cells (Multhoff et al 1995) and inhibits NK cell mediated lysis of Hsp70 membrane-positive tumor cells (chapter 3.5.4, Figure 28). Other Hsp70 specific antibodies directed against the N-terminal ATPase domain or epitopes in the C-terminal domain different from NLLGRFEL ($aa^{454-461}$) neither detect membrane-bound Hsp70 on viable tumor cells nor block the cytolytic function of NK cells (Botzler et al 1998). Therefore, one might speculate that the 8-mer is exposed to the extracellular milieu or that other Hsp70 epitopes are hidden and thus not accessible to antibodies.

Since the 8-mer peptide NLL, the C-terminal extended 13-mer peptide GIPP and the 12-mer N-terminal extended peptide TKD12 failed to stimulate NK cells the 14-mer peptide TKD represents the minimal stimulatory sequence for NK cells. Comparison of TKD with other 14-mer peptides for example the sequence of Hsp70hom (88% homology to Hsp70, HOM, TKDNNLLGRFELTG, 1 aa exchange at postion 462), the constitutively expressed highly homologous Hsc70 (84% homology, HSC70, TKDNNLLGKFELTG, 2 aa exchanges at position 458 and 462), and the E. coli derived Hsp70 homologue DnaK (50% homology, DNAK, AADNKSLGQFNLDG, 7 aa exchanges at position 450, 451, 454, 455, 458, 460, and 462) revealed that the TKD region is quite variable (Altschul et al 1999). Functionally already one aa exchange at position 450 (T to L, LKD, LKDNNLLGRFELTG) or at position 462 (S to T, HOM) is sufficient to inhibit the immunostimulatory activity of this peptide.

Stimulation of NK cells with TKD is concentration dependent. The optimal stimulatory dose of TKD is 0.2 – 2.0 µg/ml. This concentration is equivalent to 10 – 100 µg/ml full-length Hsp70 that is also known to stimulate NK cells. Concentrations above the optimal dose 2 µg/ml TKD or 200 µg/ml Hsp70 protein, respectively, failed to stimulate NK cells. These findings lead to the hypothesis that Hsp70/TKD NK cell interaction is saturable and thus might be receptor mediated.

Stimulation of NK cells with low dose IL-2 plus TKD does not only enhance the proliferative and cytolytic activity of NK cells, but also its migratory capacity. Only Hsp70 membrane-positve tumor cells showed chemotactic properties. In coincubation and migration assays, TKD stimulated PBL selectively infiltrated Hsp70 membrane-positive tumor cells, but not their Hsp70 membrane-negative counterparts. Since supernatants derived from Hsp70 membrane-positive tumor cells did also enhance migration a soluble factor was assumed. It was shown that TKD stimulated PBL only migrated towards supernatants derived from Hsp70 membrane-positive tumor cells but not to that of Hsp70 membrane-negative counterparts.

Phenotypical analysis of the migrated and non-migrated cell population showed that mainly CD3-/CD16/56+ NK cells, but not CD3+ T cells migrated towards these supernatants. In addition, the migrated cell population also showed an enhanced cytotoxicity against Hsp70 membrane-positive tumor cells. Recent data demonstrated that Hsp70 can be released by tumor cells (Barreto et al 2003, Broquet et al 2003). Here, it could be shown that TKD stimulated NK cells migrated towards full-length Hsp70 and TKD in a concentration (1 – 5 µg/ml) dependent manner. Chemokinesis or random cell mobility was excluded, since NK cells only migrated in a positive gradient.

Migration towards TKD is highly selective, because TKD related peptides with 2 (HSC70) and 7 aa exchanges (DNAK) failed to stimulate migration (Gastpar, **Gross** et al 2004). It is important to note that TKD stimulation is a prerequisite for induction of migration, since NK cells stimulated with IL-2 alone did not show any migratory capacity towards Hsp70 membrane-positive tumor cells.

It has been shown that IL-2 activated NK cells can induce regression of established lung and liver tumors (Schwarz et al 1989, Vujanovic et al 1995, Whiteside et al 1989, Yasmura et al 1994). However, the antitumor effect is often limited, because primary tumors and metastases are not efficiently infiltrated by effector cells. Stimulation of NK cells with the 14-mer peptide TKD plus low dose IL-2 might

enhance both, the migratory capacity and cytolytic activity of NK cells and thus might provide an innovative basis for the development of a cellular immunotherapy.

4.2 The C-type lectin receptor CD94 is involved in NK-Hsp70 interaction

The 14-mer peptide TKD was identified as the minimal NK stimulatory sequence of Hsp70. Activation of NK cells *via* TKD is concentration dependent (0.2 – 2.0 µg/ml) and specific. The same could be shown for interaction of Hsp70/TKD with the NK cell line YT. Using FITC labeled full-length Hsp70 protein and TKD it could be demonstrated that cell surface binding is specific and saturable and therefore might be receptor mediated: (i) binding of Hsp70-FITC could be abrogated by 20 x excess of free Hsp70 but not of an unrelated protein (GST) and (ii) Hsp70-FITC and TKD-FITC bound specifically to the cell surface of YT cells in a concentration dependent manner (10 – 50 µg/ml and 2 – 8 µg/ml respectively). These concentrations also have been found to stimulate NK cell function (chapter 4.1, Multhoff et al 1999, Hantschel et al 2000). Concentrations above 50 µg/ml (Hsp70) or 8 µg/ml (TKD) resulted in non-specific binding. These observations differed from published data (Basu et al 2001), demonstrating a receptor-mediated, specific binding of Hsp90, gp96, Hsc70 and Hsp70 to CD11b-positive macrophages at concentrations of 200 µg/ml. Therefore, it was assumed that interaction of NK cells with Hsp70 is mediated by receptors different from that expressed by APC. This hypothesis was further confirmed by the fact that NK cells interact specifically with Hsp70/TKD, whereas Hsp70hom/HOM, Hsc70/HSC70, and DnaK/DNAK failed to stimulate NK cell activity (Multhoff et al 1999). In contrast APC have been found to interact with several HSP, including Hsc70, Hsp70, Hsp90, gp96, and Hsp60 (Kol et al 2000, Ohashi et al 2000, Habich et al 2002). Furthermore, a phenotypical analysis of the Hsp70 specific NK cell line YT revealed that none of the recently described HSP-receptors including CD91 (Binder et al 2000 a, Basu et al 2001), TLR2, TLR4, and CD14 (Asea et al 2000 a, Asea et al 2002) were expressed on the cell surface of YT cells. A comparison of YT cells with Hsp70 reactive NK cells, showed that only C-type lectin receptor CD94 (Lanier et al 1994, Lazetic et al 1996), which was described to interact with HLA-E molecules, presenting leader peptides of MHC class I molecules including HLA-A, HLA-B, HLA-C, and HLA-G (Braud et al 1998, Borrego et al 1998, Ulbrecht et al 1998, Lee et al 1998, Brooks et al 1999), was commonly expressed by both cell types. An increased CD94 expression was observed when NK cells were stimulated either with full-length Hsp70 protein (10 µg/ml) or the 14-mer peptide TKD (2 µg/ml) in combination with low dose IL-2. Furthermore, it could be shown that binding of Hsp70 and Hsp70-reactivity correlates with CD94 membrane-expression

on NK cells. In addition, a CD94 specific antibody suppressed both, binding of Hsp70 to YT cells and cytolytic activity of NK cells against Hsp70 membrane-positive tumor cells. However, CD94 alone is unlikely to be responsible for the interaction with Hsp70/TKD for several reasons: (i) CD94 as a homodimer is unable to mediate cell signaling and (ii) transiently CD94 transfected COS cells did not exhibit significant Hsp70 or TKD binding (data not shown). Although these data support the idea that CD94 is involved in the interaction of NK cells with Hsp70/TKD, the co-receptor of CD94 that mediates signaling has not been defined. CD94 is known to form heterodimers with members of the NKG2 family. Depending if its partner molecule contains an ITIM or ITAM motif the heterodimeric CD94 complex fullfills inhibitory or activatory function. Since binding of Hsp70/TKD activates NK cells it is likely that the co-receptor of CD94 is a member of the NKG2 family that contains an ITAM motif. This includes NKG2C, NKG2E and its splice variant NKG2H, and NKG2F (Brooks et al 1997, Lanier 1998b). The activating receptor NKG2D can be excluded, since NKG2D forms homodimers.

4.3 Membrane-bound Hsp70 mediates perforin-independent apoptosis by specific binding an uptake of granzme B

Although it was shown that the 14-mer peptide TKD functions as a target recognition structure for NK cells, the mechanism how NK cells lyse Hsp70 membrane-positive tumor cells remained to be elucidated. In the cytoplasm, Hsp70 binds hydrophobic residues of denaturated polypeptides and co-chaperones *via* the substrate binding pocket, localized in the C-terminus in an ATP-dependent manner (Hartl et al 2002, Hartl et al 1996, Rüdiger et al 1997, Demand et al 1998). To identify molecules that are involved in the interaction of NK cells with Hsp70 membrane-positive tumor cells affinity chromatography was performed with cell lysates from the NK cell line YT. To characterize interacting partners specific for membrane-bound Hsp70, an affinity column coupled with recombinant Hsp70 or TKD. TKD was used since this sequence is believed to be exposed on the cytoplasma membrane. A dominant 32 kDa protein was eluted from Hsp70 and TKD columns incubated with NK cell lysates. This protein was neither detected using control columns (uncoupled, BSA-conjugated, or GST-conjugated) or cell lysates of a non-NK cell line. The serine protease granzyme B was identified as the interacting partner for Hsp70 and TKD by matrix-assisted laser desorption ionization time-of-flight mass peptide fingerprinting (MALDI-TOF) and by Western Blot analysis. This observation is confirmed by the finding that Hsp70 and Hsp27 binds to granzyme-immobilized sepharose columns (Beresfold et al 1998).

Granzyme B is an important pro-apoptotic enzyme produced by activated T cells and also by NK cells. One mechanism of NK cell-mediated killing involves exocytosis of cytotoxic granules containing serglycin, serine proteases and perforin (Trapani et al 1994, Metkar et al 2002). Granzyme B is believed to enter target cells by endocytosis via the mannose 6-phosphat receptor. However, the release of grB from the endosome into the cytosol is perforin-dependent (Pinkoski et al 1998, Pinkoski et al 2002, Andrade et al 1998). The group of A. De Maio has shown that Hsc70 forms a functionally stable ATP-dependent cation channel in acidic phospholipid membranes (Arispe and De Maio 2000, Arispe et al 2002). In collaboration with A. De Maio, it was shown that Hsp70 also forms ion channels in the cell surface of Hsp70 membrane-positive CX+ tumor cells; no channel formation was observed in Hsp70 membrane-negative CX- tumor cells. Together with the fact, that granzyme B binds specifically to Hsp70/TKD the following working hypothesis could be postulated: if a tumor cell expresses Hsp70 on its cell surface granzyme B might bind to it and is possibly taken

up by this tumor cell. In the case of an Hsp70-negative tumor cell no binding and uptake of granzyme B should be observed. This hypothesis was proven using the CX+/CX- and Colo+/Colo- system. It was shown that granzyme B selectively bound to the cell surface of Hsp70 membrane-positive tumor cells (CX+/Colo+) and was internalized by them after a temperature shift from 4°C to 37°C (Figure 29).

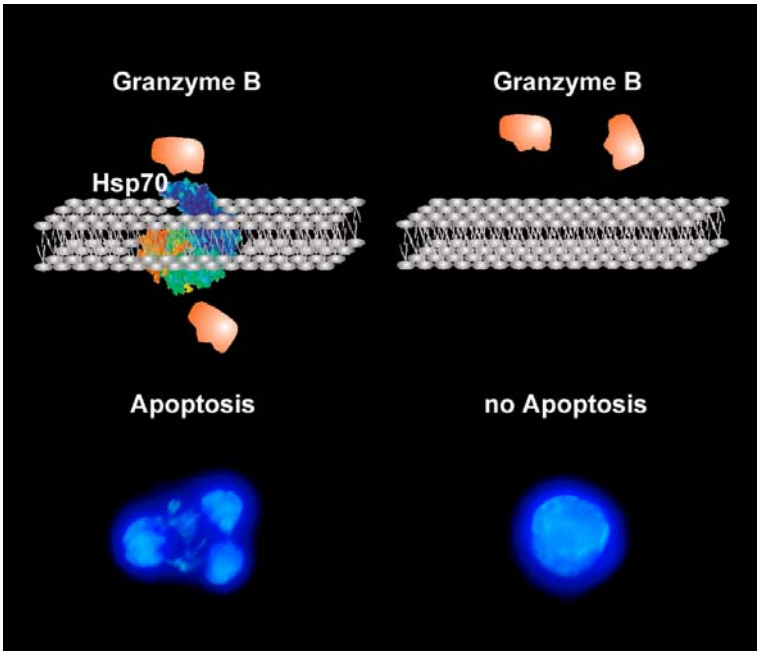


Figure 29: Granzyme B causes apoptosis selectively in Hsp70 membrane-positive tumor cells. Granzyme B binds to the cell surface of Hsp70 membrane-positive tumor cells via the extracellularly exposed 14-mer peptide TKD. It is internalized in Hsp70 membrane-positive tumor cells which undergo apoptosis. In the case of Hsp70 membrane-negative tumor cells, granzyme B remains outside of the cell and cannot induce apoptosis.

After internalization the serine protease granzyme B induces both caspase-dependent and –independent apoptosis by promoting DNA fragmentation (Froelich et al 1998, Beresfold et al 1999, Thomas et al 2000, Sharif-Askari et al 2001, Figure 6 Introduction). Moreover granzyme B causes the release of pro-apoptotic molecules (e.g. cytochrome c) by disruption of the mitochondrial transmembrane potential (Alimonti et al 2001, MacDonald et al 1999, Kuwana et al 2002, Figure 6 Introduction). Since granzyme B was specifically taken up by Hsp70 membrane-positive tumor cells, only these cells should selectively undergo apoptosis after treatment with granzyme B. Using three idependent apoptosis assays detecting membrane changes (Annexin V staining), nuclear DNA fragmentation (DAPI staining), and mitochondrial disintegrity (cytochrome c release), it could be demonstrated that physiological relevant concentrations of granzyme B (Spaneny-Dekking et al 1998) caused apoptosis perforin-independent and specifically in Hsp70

membrane-positive CX+/Colo+ cells, but not in their Hsp70 membrane-negative counterparts (CX-/Colo-) (Figure 29). These differences were not obtained with the alkaloid camptothecin, which inhibits DNA topoisomerase by stabilizing the cleavable complex and thereby causing apoptosis. This indicates that initiation of apoptosis by granzyme B and camptothecin involves different routes. Variable intracellular Hsp70 levels could be excluded since tumor sublines with differential Hsp70 membrane-expression express similar amounts of intracellular Hsp70 even under stress (Multhoff et al 1997). Moreover, binding and uptake of granzyme B *via* extracellular exposed TKD might be a critical step for induction of apoptosis. This hypothesis is supported by the fact that cytolytic activity of NK cells against Hsp70 membrane-positive tumor cells could be reduced by the Hsp70 specific antibody cmHsp70.1 that binds to TKD. It is possible that this antibody prevents tumor cells from apoptosis by blocking specific binding and uptake of granzyme B. Taken together these data showed a new perforin-independent pathway for the induction of apoptosis by granzyme B (Figure 30).

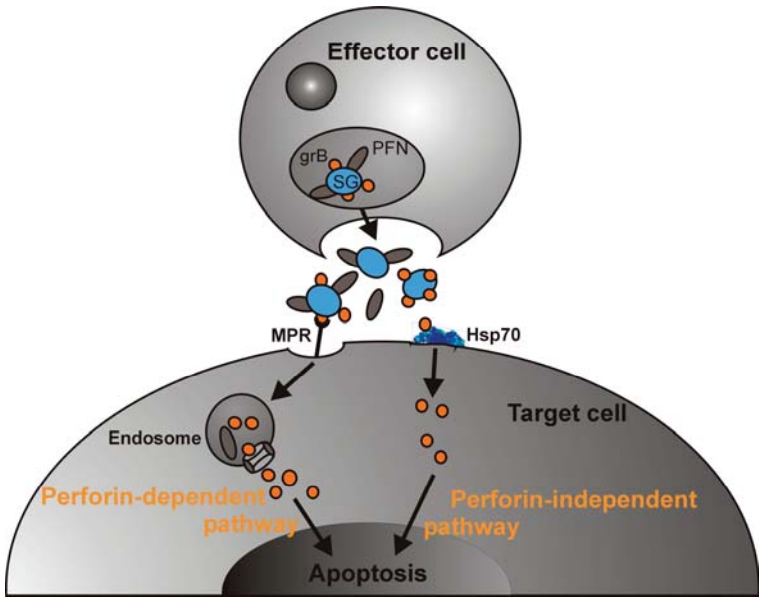


Figure 30: Perforin-dependent/-independent pathway. Effector cells including activated T cells and NK cells release serglycin (SG) – granzyme B (grB) –perforin (PFN), SG-grB, and SG-PFN complexes. In the perforin-dependent pathway grB is endocytosed by the target cell via the mannose 6-phosphat receptor. The release of grB into the cytosol is perforin-dependent. In the perforin-independent pathway grB enters the target cell via membrane-bound Hsp70. Inside the target cell the serine protease granzyme B induces apoptosis

Apart from the observation that Hsp70 serves as an entry port for granzyme B into Hsp70 membrane-positive tumor target cells, Hsp70 and TKD stimulate the

production and release of granzyme B in primary NK cells. Therefore, cell surface expression of Hsp70 does not only enhance sensitivity of tumor target cells to apoptotic cell death but also induces NK cell activation. In contrast to *in vitro* applied granzyme B, activated NK cells also kill Hsp70 membrane-negative tumor cells to a certain extent. Since this weak lysis was not blockable with the Hsp70 specific antibody cmHsp70.1, another pathway for killing of Hsp70 membrane-negative tumor cells is hypothesized. Furthermore, NK cells lysed Hsp70 membrane-positive tumor target cells more efficiently as compared to *in vitro* provided granzyme B. This could be explained by the fact that killing mediated by NK cells include physical cell-to-cell contact which is likely to result in high local granzyme B concentrations at the tumor cells. Since these concentrations cannot be quantified experimentally, the *in vitro* apoptosis assays were performed with granzyme B concentrations similar to those found in human serum (Spaeny-Dekking et al 1998). These concentrations are propably different from those secreted by NK cells and therefore have different efficacy. However, the relative release of granzyme B from NK cells after tumor target cell contact could be determined by ELISPOT analysis. A correlation between the cytotoxic response of activated NK cells against Hsp70 membrane-positive tumor cells with the relative release of granzyme B by these cells could be observed. These findings were supported by the fact that increasing amounts of *in vitro* provided granzyme B are still selectively taken up by Hsp70 membrane-positive tumor cells.

In summary these data suggested a dual role for Hsp70 and TKD in the immune response against cancer: On the one hand NK cells are stimulated to produce and deliver granzyme B on the other hand it facilitated uptake of granzyme B selectively into Hsp70 membrane-positive tumor target cells.

5 SUMMARY

A broad variety of tumor cells express Hsp70 on their cell surface. These tumor cells are more sensitive to the lysis mediated by NK cells. Here, it was shown that the 14mer peptide TKD (TKDNNLLGRFELSG, $aa^{450-463}$ derived from the C-terminus of Hsp70 is the minimal sequence of Hsp70 that has the capacity to activate NK cells. NK cells incubated with low dose IL-2 (100 IU/ml) plus TKD (2 µg/ml) showed an enhanced proliferative capacity and an enhanced cytolytic activity and migratory capacity against Hsp70 membrane-positive tumor cells. Furthermore, secretion of the cytokines IFN-γ and TNF-α was enhanced. TKD stimulated NK cells also showed enhanced intracellular levels of the serine protease granzyme B. Since binding of full-length Hsp70 protein and TKD to NK cells was specific and concentration-dependent, an involvement of a Hsp70 specific receptor was hypothesized. It was shown that the C-type lectine receptor CD94 is involved in Hsp70/TKD-NK cell interaction: (i) CD94 was upregulated in NK cells after incubation with Hsp70/TKD; (ii) binding of Hsp70 correlated with CD94 expression on NK cells; (ii) binding of Hsp70 could be inhibited by a CD94 specific antibody; (iii) Hsp70 reactivity correlated with CD94 expression on NK cells; and (iv) Hsp70 reactivity of NK cells could be inhibited by a CD94 specific antibody. Finally the mechanism how NK cell lyse Hsp70 membrane-positive tumor cells could be elucidated. It was shown that the serine protease granzyme B binds to Hsp70/TKD on the cell surface of Hsp70 membrane-positive tumor cells and is specifically taken up by these cells. As demonstrated by different apoptosis assays (annexin V staining, cytochrome c release, and DAPI staining) granzyme B causes apoptosis specifically in Hsp70 membrane-positive tumor cells, but not in their Hsp70 membrane-negative counterparts.

6 REFERENCES

Allavena PC, Giardiana G, Bianchi G, Mantovani A. 1997. IL-15 is chemotactic for natural killer cells and stimulates their adhesion to vascular endothelium. *J Leukocyte Biol* **61:** 729-735.

Alimonti JB, Shi L, Baijal PK, Greenberg AH. 2001. Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. *J Biol Chem* **276:** 6974-6982.

Altmeyer A, Maki RG, Feldweg AM, Heike M, Protopopov VO, Masur SK, Srivastava PK. 1996. Tumor-specific cell surface expression of the –KDEL containing, endoplasmic reticular heat shock protein gp96. *Int J Cancer* **61:** 272-279.

Altschul S, Madden TL, Schaeffer AA, Zhang J, Zhang Z, Miller W, Schild H. 1999. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389-3402.

Andrade F, Poy S, Nicholson D, Thornberry N, Rosen A, Casciola-Rosen L. 1998. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implication for CTL induced apoptosis. *Immunity* **8:** 451-460.

Andre P, Biassoni R, Colonna M, Cosman D, Lanier LL, Long EO, Lopez-Botet M, Moretta A, Moretta L, Praham P, Trowsdale J, Vivio E, Wagtmann N, Wilson MJ. 2001. New nomenclature for MHC receptors. *Nat Immunol* **2:** 661.

Arase H, Arase N, Saito T. 1995. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J Exp Med* **181:** 1235-1238.

Arispe N, De Maio A. 2000. Lipid interaction differentiates the constitutive and stress-induced heat shock proteins Hsc70 and Hsp70. *J Biol Chem* **275:** 30839-30843.

Arispe N, Doh M, De Maio A. 2002. ATP and ADP modulate a cation channel formed by Hsc70 in acidic phospholipid membranes. *Cell Stress Chaperones* **7:** 330-338.

Asea A, Kraeft S-K, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GX, Calderwood SK. 2000a. Hsp70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* **6:** 435-442.

Asea A, Kabingu E, Stevenson MA, Calderwood SK. 2000b. Hsp70 peptide-bearing and peptide-negative preparations act as chaperokines. *Cell stress and Chaperones* **5:** 425-431.

Asea A, Rehli M, Kabinfu E, Boch JA, Baré O, Auron PE, Stevenson MA, Calderwood SK. 2002. Novel Signal Transduction pathway utilized by extracellular Hsp70: Role of TLR2 and TLR4. *J Biol Chem* **227:** 15028-15034.

Barford D, Neel BG. 1998. Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2. *Structure* **6:** 249-254.

Barreto A, Gonzalez JM, Kabingu E, Asea A, Fiorentino S. 2003. Stress-induced release of Hsc70 from human tumors. *Cellular Immunology* **222:** 97-104.

Barry M, Heibein JA, Pinkoski MJ, Lee SF, Moyer RW, Green DR, Bleackley RC. 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol* **20:** 3781-3794.

Basu S, Binder RJ, Ramalingam T, Srivastava PK. 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* **14:** 303-313.

Bauer S, Groh V, Wu J, Steinle A, Philips JH, Lanier LL, Spies T. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285:** 727-729.

Baume DM, Robertson MJ, Levine H, Manley TJ, Schow PW, Ritz J. 1992. Differential response to interleukin 2 define functionally distinct subsets of human natural killer cells. *Eur J Immuno.* **22:** 1-6.

Becker T, Hartl F-U, Wieland F. 2002. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. *J Cell Biol* **158:** 1277-1285.

Belli F, Testori A, Rivoltini L, Maio M, Andreola G, Sertoli MR, Gallino G, Piris A, Catterlan A, Lazzari I, Carrabba M, Scita G, Santantonio C, Pilla L, Tragni G, Lombrardo C, Arienti F, Marchiano A, Queirolo P, Bertolini F, Cova A, Lamaj E, Ascani L, Camerini R, Corsi M, Cascinelli N, Lewis JJ, Srivastava P, Parmiani G. 2002. Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96-peptide complexes: clinical and immunologic findings. *J Clin Oncol* **20:** 4169-4180.

Beresford PJ, Jaju M, Friedman RS, Yoon MJ, Lieberman J. 1998. A role for heat shock protein 27 in CTL-mediated cell death. *J Immunol* **161:** 161-167.

Beresford PJ, Xia Z, Greenberg AH, Lieberman J. 1999. Granzyme A loading induces rapis cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity* **10:** 585-594.

Berke G. 1995. The CTL' s kiss of death. *Cell* **81:** 9-16.

Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, Conte R, Poggi A, Moretta A, Moretta L. 1996. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med* **183:** 645-650.

Binder RJ, Han DK, Srivastava PK. 2000a. CD91: a receptor for heat shock protein gp96. *Nature* **1:** 151-155.

Binder RJ, Harris ML, Ménoret A, Srivastava PK. 2000b. Saturation, competition, and specificity in interaction of heat shock proteins (hsp) gp96, hsp90, and hsp70 with CD11b+ cells. *J Immunol* **165:** 2582-2587.

Binstadt BA, Brumbaugh KM, Dick CJ, Scharenberg AM, Williams BL, Colonna M, Lanier LL, Kinet J-P, Abraham RT, Leibson PJ. 1996. Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation. *Immunity* **5:** 629-638.

Binstadt BA, Billadeau DD, Jevremovic D, Williams BL, Fang N, Yi TL, Koretzky GA, Abraham RT, Leibson PJ. 1996. SLP-76 is a direct substrate of SHp-1 recruited to killer cell inhibitory receptros. *J Biol Chem* **273:** 27518-27523.

Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG confers protection from natural killer cell-mediated lysis. *J Exp Med* **187:** 813-818.

Borrego F, Kabat J, Kim DK, Kieto L, Maasho K, Penta J, Solana R, Coligan JE. 2002. Structure and function of major histocompatibility complex (MHC) class I specific receptors expressed on human natural killer (NK) cells. *Mol Immunol.* **38:** 637-660.

Botzler C, Issels R, Multhoff G. 1996a. Heat-shock protein 72 cell-surface expression on human lung carcinoma cells is associated with an increased sensitivity to lysis mediated by adherent natural killer cells. *Cancer Immunol Immunother* **43:** 226-230.

Botzler C, Kolb H-J, Issels RD, Multhoff G. 1996b. Noncytotoxic alkyl-lysophospholipid treatment increases sensitivity of leukemic K562 cells to lysis by natural killer cells (NK). *Int J Cancer* **65:** 633-638.

Botzler C, Gloria L, Issels RD, Multhoff G. 1998. Definition of extracellular localized epitopes of Hsp70 involved in an NK immune response. Cell Stress Chaperones **3:** 6-11.

Botzler C, Ellwart J, Eissner G, Issels R, Multhoff G. 1999. Synergistic effects of heat and ET-18-OCH3 on membrane expression of hsp70 and lysis of leukemic K562 cell. *Exp. Hematol.* **27:** 470-478.

Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D' Andrea A, Ogg GS, Lazetic S, Young NT, Bell JS, Phillips JH, Lanier LL, McMichael AJ. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391:** 795-799.

Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE. 1997. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J Exp Med* **185:** 795-800.

Brooks AG, Borrego F, Posch PE, Patamawenu A, Scorzelli CJ, Ulbrecht M, Weiss EH, Coligan JE. 1999. Specific recognition of HLA-E, but not classical HLA class I molecules by soluble CD94/NKG2A and NK cells. *J Immunol* **162:** 305-313.

Broquet AH, Thomas G, Masliah J, Trugnan G, Bachelet M. 2003. Expression of the molecular chaperone Hsp70 in detergent resistant microdomains correlates with its membrane delivery and release. *J Biol Chem* 278: 21601-21606.

Brumbaugh KM, Binstadt BA, Billadeau DD, Schoon RA, Dick CJ, Ten RM, Leibson PJ. 1997. Functional role for Syk tyrosine kinase in natural killer cell-mediated natural cytotoxicity. *J Exp Med* **186:** 1965-1974.

Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, Yi T, Kinet J-P, Long EO. 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitory receptor. *Immunity* **4:** 77-85.

Burshtyn DN, Long EO. 1997. Regulation through inhibitory receptros: lessons from natural killer cells. *Trends Cell Biol* **7:** 473-479.

Cantoni C, Bottino C, Vitale M, Pessino A, Augugliaro R, Malaspina A, Parolini S, Moretta L, Moretta A, Biassoni R. 1999. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J Exp Med* **189:** 787-796.

Caligiuri MA, Zmuidzina A Manley FJ, Levine H, Smith KA, Ritz J. 1990. Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. *J Exp Med* **171:** 1509-1526.

Caligiuri MA, Murray C, Robertson MJ, Wang E, Cochran K, Cameron C, Schow P, Ross ME; Klumpp TR, Soitter RJ et al. 1993. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. J *Clin Invest* **91:** 123-132.

Campbell JJ, Qin S, Unutmaz D, Soler D, Murphy KE, Hodge MR, Wu L, Batcher EC. 2001. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol* **166:** 6477-6482.

Castellino F, Boucher PE, Eichelberg K, Mayhew M, Rothman JE, Houghton AN, Germain RN. 2000. Receptor mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. *J Exp Med* **191:** 1957-1964.

Chang C, Rodriguez A, Carretero M Lopez-Botet M, Pillips JH. Lanier LL. 1995. Molecular characterization of human CD94: a type II membrane glycoprotein related to the C-type lectin superfamily. *Eur J Immunol* **22 (9):** 2433-2437.

Chu DH, Morita CT, Weiss A. 1998. The Syk family of protein tyrosine kinase in T-cell activation and development. *Immunol Rev* **165:** 167-180.

Colonna M, Samaridis J. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* **268:** 405-408.

Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J, Augman L, Cella M, Lopez-Botet M. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med* **186:** 1809-1818.

Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Gayur T, Carson WE, Caligiuri MA. 2001. Human natural killer cells: a unique innate immunoregulatory role for the $CD56^{bright}$ subset. *Blood* **97:** 3146-3151.

Cooper MA, Bush JE, Fehniger TA, VanDeusen JB, Waite RE, Liu Y, Aguila HL. Caligiuri MA. 2002. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood* **100:** 3633-3638.

Cosman D, Fanger N, Borges L, Kubin M, Chin W, Pereson L, Hsu ML. 1997. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity* **7:** 273-282.

Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* **14:** 123-133.

Demand J, Lüders J, Höhfeld J. 1998. The carboxy-terminal domain of Hsc70 provides binding sites for a distinct set of chaperone cofactors. *Mol Cell Biol* **18:** 2023-2028.

DeNagel CD, Pierce SK. 1992. A case for chaperones in antigen processing. *Immunol Today* **3:** 86-89.

Dressel R, Raja SM, Häning S, Seidler T, Froelich CJ, von Figura K, Günther E. 2003. Granzyme-mediated cytotoxicity does not involve the mannose 6-phophate receptors on target cells. *J Biol Chem* **279:** 202000-20210.

Eischen CM and Leibson PJ. 1997. Role for NK-cell-associated Fas ligand in cell-mediated cytotoxictiy and apoptosis. *Res Immunol* **148:** 164-169.

Ellis TM, Fischer RI. 1989. Functional heterogeneity of Leu 19"bright"+ and Leu 19"dim"+ lymphokine-activated killer cells. *J Immunol* **142:** 2949-2954.

Fehniger TA, Shah MH, Turner MJ, VanDeusen JB, Whitman SP, Cooper MA, Suzuki K. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response: *J Immunol* **162:** 4511-4520.

Feng H, Zeng Y, Whitesell L, Katsanis E. 2001. Stressed apoptotic tumor cells express heat shock proteins and elicit tumor-specific immunity. *Blood* **97:** 3503-3512.

Ferrarini M, Helatai S, Zocchi MR, Rugarli C. 1992. Unusual expression and localization of heat-shock proteins in human tumor cells. *Int J Cancer* **51:** 613-619.

Frey M, Pachianathan NB, Gehniger TA, Ross ME, Wang WC, Stewart CC, Caligiuri MA, Evans SS. 1998. Differential expression and function of L-selectin on CD56bright and CD56dim natural-killer-cell subsets. *J Immunol* **31:** 792-801.

Froelich CJ, Orth K, Turbov J, Seth P, Gottlieb R, Babior B, Shah GM, Bleackley RC, Dixit VM, Hanna W. 1996. New paradigm for lymphocyte granule-mediated cytotoxicity. target cells bind and internalize granzyme B, but and endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. *J Biol Chem* **271:** 29073-29079.

Froelich CJ, Dixit VM, Yang X. 1998. Lymphocyte granule-mediated apoptosis. matters of viral mimicry and deadly proteases. Immunol Today **19:** 30-36.

Gehrmann M, Pfister K, Utzler P, Gastpar R, Margulis B, Multhoff G. 2002. Effects of antineoplastic agents on cytoplasmic and membrane-bound heat shock protein 70 (Hsp70) levels. *Biol Chem* **383:** 1715-1725.

Glienke J, Sobanov Y, Brostjan C, Steffens C, Nyugen C, Lehrach H, Hofer E, Francis F. 1998. The genomic organization of NKG2C, E, F, and D receptor genes in the human natural killer gene complex. *Immunogenetics* **48:** 163-173.

Habich C, Baumgart K, Kolb H, Burkart V. 2002. The receptor for HSP60 on macrophages is saturable, specific and distinct from receptors for other HSPs. *J Immunol* **168:** 569-576.

Hantschel M, Pfister K, Jordan A, Scholz R, Andreesen R, Schmitz G, Schmetzer H, Hiddemann W, Multhoff G. 2000. Hsp70 plasma-membrane expression on primary tumor biopsy material and bone marrow of leukemic patients. *Cell Stress Chaperones* **5:** 438-442.

Hartl FU. 1996. Molecular chaperones in protein folding. *Nature* **381:** 571-580.

Hartl FU, Hayer-Hartl M. 2002. Molecular chaperones in the cytosol from nascent chain to folded protein. *Science* **295:** 1825-1828.

Heibein JA, Goping IS, Barry M, Pinkoski MJ, Shore GC, Green DR, Bleackley RC. 2000. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members Bid and Bax. *J Exp Med* **192:** 1391-1402.

Janetzki S, Palla D, Rosenhauer V, Lochs H, Lewis JJ, Srivastava PK. 2000. Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study. *Int J Cancer* **88:** 232-238.

Kayagaki N, Yamaguchi N, Nakayama M, Takeda K, Akiba H, Tsutsui H, Okamura H, Nakanishi K, Okumura K, Yagita H. 1999. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J Immunol* **163:** 1906-1913.

Kleinjung T, Arndt O, Feldmann HJ, Bockmühl U, Gehrmann M, Zilch T, Pfister K, Schönberger J, Marienhagen J, Eilles C, Rossbacher L, Multhoff G. 2003. Heat shock protein 70 (Hsp70) membrane expression on head-and neck cancer biopsy – a target for natural killer (NK) cells. *Int J Radiation Oncology Biol Phys* **57:** 820-826.

Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA. 2000. Cutting Edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for Hsp60 activation of mononuclear cells. *J Immunol* **164:** 13-17.

Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD. 2002. Bid, Bax and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111:** 331-342.

Lanier LL, Le AM, Civin CI, Loken MR, Philips JH. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T Lymphocytes. *J Immunol* **136:** 4480-4486.

Lanier LL, Testi R, Bindl J, Phillips JH. 1989. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med* **169:** 2233-3238.

Lanier LL, Chang C, Philips JH. 1994. Human NKR-P1a. a disulfide linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *J Immunol* **153:** 2317-2323.

Lanier LL. 1998a. NK cell receptors. *Annu Rev Immunol* **16:** 359-393.

Lanier LL. 1998b. Activating and inhibitory NK cell receptors. *Adv Exp Med Biol* **452:** 13-18.

Lazetic S, Chang C, Houchins JÜ, Lanier LL, Philips JH.1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J Immunol* **157:** 4741-4745.

Lee N, Llano M, Carretero A, Ishitani A, Navarro F, Lopez-Botet M, Geraghty DE. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci USA* **95:** 5199-5204.

Lieberman J. 2003. The ABCS of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* **3:** 361-370.

Li LY, Luo X, Wang X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412:** 95-99.

Ljunggren HG, Kärre K. 1990. In search of the missing self: MHC molecules and NK recognition. *Immunol Today*. **11:** 237-244.

Llano M, Lee N, Navarro F, Garcia P, Albar JP, Geraghty DE, Lopez-Botet M. 1998. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur J Immunol* **28:** 2854-2863.

MacDonald HR, Engers HD, Cerottini JC, Brunner KT. 1974. Generation of cytotoxic T lymphocytes in vitro. *J Exp Med* **140:** 718-722.

MacDonald G, Shi L, Vande Velde C, Lieberman J, Greenberg AH. 1999. Mitochondria-dependent and-independent regulation of granzyme B-induced apoptosis. *J Exp Med* **189:** 131-144.

Metkar SS, Wang B, Aguillar-Santelises M, Raja SM, Uhlin-Hansen L, Podack E, Trapani JA, Foelich CJ. Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore-formation. *Immunity* 16: 417-428.

Moretta A, Vitale M, Bottino C, Orrengo AM, Morelli L, Angugliaro R, Barbaresi M, Ciccone E, Moretta L. 1993. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med* **178**: 597-604.

Multhoff G, Botzler C, Wiesent M, Müller E, Meier T, Wilmanns W, Issels RD. 1995a. A stressinducible 72-kDa heat-shock protein (Hsp72) is expressed on the surface of human tumor cells, but not on normal cells. *Int J Cancer* **61:** 272-279.

Multhoff G, Botzler C, Wiesnet M, Eissner G, Issels RD. 1995b. CD3- large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72-kD heat shock protein on human sarcoma cells. *Blood* **86:** 1374-1382.

Multhoff G, Botzler C, Jennen L, Schmidt J, Ellwart J, Issels RD. 1997. Heat shock protein 72 on tumor cells: A recognition structure for Natural Killer cells. *J Immunol* **158:** 4341-4350.

Multhoff G, Mizzen L, Winchester CC, Milner CM, Wenk S, Kampinga HH, Laumbacher B, Johnson J. 1999. Heat shock protein 70 (Hsp70) stimulates proliferation and cytolytic activity of NK cells. *Exp Hematol* **27:** 1627-1636.

Multhoff G, Pfister K, Gehrmann M, Hantschel M, Gross C, Hafner M, Hiddemann W. 2001. A 14-mer Hsp70 peptide stimulates natural killer (NK) cell activity. *Cell Stress Chaperones* **6:** 337-344.

Nagler A, Lanier LL, Cwirla S, Phillips JH. 1989. Comparative studies of human FcRIII-positive and negative natural killer cells. *J Immunol* **143:** 3183-3191.

Navarro F, Liano M, Bellon T, Colonna M, Geraghty DE, Lopez- Botet M. 1999. The ILT2 (LIR2) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cell. *Eur J Immunol* **29:** 277-283.

Negishi I, Motoyama N, Nakayama K, Senju S, Hatakeyama S, Zhang Q, Chan AC, Loh DY. 1995. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature* **376:** 435-438.

Nelson A, De Maio A. 2000. ATP and ADP modulate a cation channel formed by Hsc70 in acidic phospholipid membranes. *J Biol Chem* **275:** 30839-30843.

Nieto MF, Navarro F, Perez-Villar JJ, del Pozzo MA, Gonzalez-Amaro R, Mellado M, Frade JMR, Martinez C. Lopez-Botet M, Sanchez-Madrid F. 1998. Roles of chemokines and receptor polarization in NK-target cell interaction. *J Immunol*. **161:** 3330-3339.

Ohashi K, Burkhart V, Flohé S, Kolb H. 2000. Cutting Edge: heat shock protein 60 is a putative endogenous ligand of the Toll-Like Receptor-4 complex. *J Immunol* **164:** 558-561.

Olcese L, Lang P, Vély F, Cambiaggi A, Mrguet D, Blery M, Hippen KL, Biassoni R, Moretta A, Moretta L, Cambier JC, Vivier E. 1996. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphates. *J Immunol* **156:** 4531-4534.

Pende D; Biassoni R, Cantoni C, Verdiani S, Falco M, diDonato C, Accame L, Bottino C, Moretta A, Moretta L. 1996. The natural killer cell receptor specific for HLA-A allotypes; a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kDa disulphide-linked dimer. *J Exp Med* **184:** 505-518.

Pende D, Parolini S, Pessino A, Sivori S, Augugliaro R, Morelli L, Marcenaro E, Accame L, Malaspina A, Biassoni R. 1999. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human NK cells. *J Exp Med* **190:** 1505-1516.

Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, Biassoni R, Moretta A. 1998. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med* **188:** 953-960.

Pinkoski M, Hobmann M, Heibbein JA, Tomasselli K, Li F, Seth P, Froelich C, Bleakley CR. 1998. Entry and trafficking of granzyme B in target cells during granzyme B-perforin-mediated apoptosis. *Blood* **92:** 1044-1054.

Pinkoski M, Green J, Douglas R. 2002. Lymphocyte apoptosis: refining the paths to perdition. *Curr Opin Hematol* **9:** 43-49.

Piselli P, Vendetti S, Poccia F, Cicconi R, Mattei M, Bolognesi A, Stipe F, Colizzi V, 1995. *In vitro* and *in vivo* efficacy of heat shock protein specific immunotoxins on human tumor cells. *J Biol Regul Homeost Agents* **9:** 55-62.

Rabinovich BA, Shannon J, Su R-C, Miller RG. 2000. Stress renders T cell blasts sensitive to killing by activated syngenic NK cells. *J Immunol* **165:** 2390-2397.

Rininsland FH, Helms T, Asaad R, Boehm BO, Tary-Lehmann M. 2000. Granzyme B ELISPOT assay for ex vivo measurements of T cell immunity. *J Immunol Methods* **240:** 143-155.

Robertson MJ, Soiffer RJ, Wolf SF, Manley TJ, Donahne C, Young D, Hermann SH, Ritz J. 1992. Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *J Exp Med* **175:** 779-788.

Robertson MJ, Cochran KJ, Cameron C, Le JM, Tantravahi R, Titz J. 1996. Characterization of a cell line, NKL, derived from an aggressive human natural killer leukemia. *Exp Hematol* **24:** 410-415.

Rüdiger S, Buchberger A, Bukau B. 1997. Interaction of Hsp70 chaperones with substrates. *Nat Struct Biol* **4:** 342-349.

Sarin A, Williams MS, Alexander-Miller MA, Berzofsky JA, Zacharchuk CM, Henkart PA. 1997. Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases. *Immunity* **6:** 209-215.

Schild H-J, Arnold-Schild A, Lammert E, Rammensee H-G. 1999. Stress proteins and immunity mediated by cytotoxic T lymphocytes. *Curr Opin Immunol* **11:** 109-114.

Schwarz RE, Vujanovic NL, Hiserodt JC. 1989. Enhanced anti-metastatic activity of lymphokine-activated killer cells purified and expanded by their adherence to plastic. *Cancer Res* **49:** 1441-1446.

Screpanti V, Wallin RP, Ljunggren HG, Grandien A. 2001. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. *J Immunol* **167:** 2068-2073.

Selvakumar A, Steffens U, Palanisamy N, Chaganti RS, Dupont B. 1997. Genomic organization and allelic polymorphism of the human killer cell inhibitory receptor gene KIR 103. *Tissue Antigens* **49:** 564-573.

Sharif-Askari E, Alam A, Rheaume E, Beresford PJ, Scotto C, Sharma K. Lee D, DeWolf WE, Nuttall ME, Lieberman J, Sekaly RP. 2001. Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. *EMBO J* **20:** 3101-3113.

Shi L, Mai S, Israels S, Browne KA, Tapani JA, Greenberg AH. 1997. Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. *J Exp Med* **185:** 855-866.

Shin BK, Wang H, Yim AM. Le Naour F, Brichory G, Jang JH, Zhao R, Puravs E, Tra J, Michael CW, Misek DE, Hannash SM. 2003. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. *J Biol Chem* **278:** 7607-7616.

Shresta S, Graubert TA, Thomas DA, Raptis SZ, Ley TJ. 1999. Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity* **10:** 595-605.

Sivori S, Parolini S, Falco M, Marcenaro E, Biassoni R, Bottino C, Moretta L, Moretta A. 2000. 2B4 functions as a co-receptor in human NK cell activation. *Eur J Immunol* **30:** 787-793.

Spaeny-Dekking EH, Hanna WL, Wolbink AM, Wever PC, Kiummer AJ, Swaak AJ, Middeldorp J, Huisman HG, Froelich C, Hack CA. 1998. Extracellular granzymes A and B in humans detection of native species during CTL responses in vitrao and in vivo. *J Immunol* **160:** 3610-3616.

Stebbins CC, Watzl C, Billadeau DD, Leibson PJ, Burshtyn DN, Long EO. 2003. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol Cell Biol* **23:** 6291-6299.

Strong DM, Ahmed AA, Thurman GB, Sell KW. 1973. In vitro stimulation of murine spleen cells using a microculture system and a multiple automated sample harvester. *J Immunol Methods* **2:** 279-284.

Sutton VR, Davis JE, Cancilla M, Johnstone RW, Ruefli AA, Sedelies K, Browne KA, Trapani JA. 2000. Initiation of apoptosis by granzyme B requires direct cleavage of Bid, but not direct granzyme B-mediated caspase activation. *J Exp Med* **192:** 1403-1414.

Sobanov Y, Glienke J, Brostjan C, Lehrach H, Francis F, Hofer E. 1999. Linkage of the NKG2 and CD94 receptor genes to D12S77 in the human natural killer gene complex. *Immunogenetics* **49:** 99-105.

Suto R, Srivastava PK. 1995. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* **269:** 1585-1587.

Tamura Y, Tsuboi N, Sato N, Kikuchi K. 1993. 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. *J Immunol* **151:** 5516-5524.

Tamura Y, Peng P, Liu K, Daou M, Srivastava PK. 1997. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* **278:** 117-120.

Tangye SG, Cherwinski H, Lanier LL, Philips JH. 2000. 2B4-mediated activation of human natural killer cells. *Mol Immunol* **37:** 493-501.

Thomas DA, Du C, Xu M, Wang X, Ley TJ. 2000. DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. *Immunity* **12:** 621-632.

Trapani JA, Smyth MJ, Apostolidis VA, Dawson M, Bowne KA. 1994. Granule serine proteases are normal nuclear constituents of natural killer cells. *J. Biol Chem.* **269:** 18359-18365.

Trapani JA, Browne KA, Smyth MJ, Jans DA. 1996. Localization of granzyme B in the nucleus. A putative role in the mechanism of cytotoxic lymhocyte-mediated apoptosis. *J Biol Chem* **271:** 4127-4133.

Trapani JA, Jans DA, Jans PJ Smyth MJ, Browne KA, Sutton VR. 1998. Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. *J Biol Chem* **273:** 27934-27938.

Trapani JA, Smyth MJ. 2002. Functional significance of the perforin/granzyme cell death pathway. Nature Reviews **2:** 735-747.

Trinchieri G. 1989. Biology of natural killer cells. *Adv Immunol* **47:** 176-187.

Udono H, Srivastava PK. 1993. Heat shock protein 70 associated peptides elicit cancer immunity. *J Exp Med* **178:** 1391-1396.

Ulbrecht M, Modrow S, Srivastava R, Person PA, Weiss EH. 1998. Interaction of HLA-E with peptides and the peptide transporter in vitro: implications for its function in antigen presentation. *J Immunol* **160:** 4375-4385.

Valiante NM, Tinchieri G. 1993. Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes. *J Exp Med* **178:** 1397-1406.

Vales-Gomez M, Reyburn HAT, Mandelboim M, Strominger JL. 1998. Kinetics of interaction of HLA-C ligands with natural killer cell inhibitory receptors. *Immunity* **9:** 337-344.

Vitale M, Bottino C, Sivori S, Sanseverino L, Casticoni R, Marceuro A, Augugliaro R, Moretta L, Moretta A. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med* **187 (12):** 2065-2072.

Voss SD, Daley J, Ritz J, Robertson MJ. 1998. Participation of the CD94 receptor complex in costimulation of human natural killer cells. *J Immunol* **160:** 1618-1626.

Vujanovic NL, Rabinovich H, Lee YJ, Herbermann RB, Whiteside TL. 1993. Distinct phenotypic and functional characteristics of human natural killer cells obtained by rapid IL-2 adherence to plastic . *Cell Immunol* **151:** 133-13.

Vujanovic NL, Yasumura S, Hirabayashi H, Lin WC, Watkins S, Herberman RB, Whiteside TL. 1995. Antitumor activities of subsets of human IL-2 activated natural killer cells in solid tissues. *J Immunol* **154:** 281-289.

Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. 1995. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and functional transfer. *Immunity* **3:** 801-809.

Whiteside TL, Vujanovic NL, Herberman RB. 1989. Natural killer cells in tumor therapy. *Curr Top Micobiol Immunol* **230:** 221-244.

Wilson MJ, Torka M, Trowsdale J. 1997. Genomic organization of a human killer cell inhibitory receptor gene. *Tissue Antigens* **49:** 574-579.

Wu J, Song Y, Bakker AB, Bauer S, Spies T, Lanier LL, Philips JH. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* **285:** 730-732.

Yasumura S, Lin WC, Hirabayshi H, Vujanovic NL, Herberman RB, Whiteside TL. 1994. Immunotherapy of liver metastases of human gastric carcinoma with IL-2 activated natural killer cells. *Cancer Res* **54:** 3808-3816.

Yodoi J, Teshigawara K, Nikaido T, Fukui K, Noma T, Honjo T, Takigawa M, Sasaki M, Minato N, Tsudo M, et al. 1985. TGF (IL-2)-receptor inducing factor(s). I. Regulation of IL-2 receptor on natural killer-like cell line (YT cells): *J Immunol* **143:** 1623-1630.

7 ACKNOWLEDGEMENTS

At this point I want to thank all the people who have contributed to this work:

- First of all, Gabriele Multhoff, for her support and stimulating discussions during experiments and data analysis, for her never-ending enthusiasm which was very helpful during the critical period of this work, and for giving me the opportunity to publish my work and to present parts of it at the Cell Stress and Chaperone congress in Québec.
- My first supervisor Prof. Dr. Elisabeth Weiß for her support, her comments, and her constructive suggestions concerning this work.
- My second supervisor Prof. Dr. Heinrich Leonhardt for his assessment and his interest in this work.
- Prof. Dr. Reinhard Andreesen for giving me the opportunity to complete my work at the department of Hematology/Oncology.
- The members of the group of Gabriele Multhoff for supporting me in my project and thus making me feel comfortable in the lab: Claus Botzler for his support at the beginning of my work, Robert Gastpar for his good co-operation during the migration project, Andreas Wortmann for helping me with the CliniMACS separation, Mathias Gehrmann for his introduction to the fluorescence microscopy, Lydia Roßbacher for her excellent technical assistance, and Gerald Thonigs for helping me to cultivate the huge amounts of YT cells needed for membrane preparation.
- The members of the group of Andreas Mackensen, namely Karin Fischer, Sandra Vogl, Monika Laumer, Annegret Rehm, and Jana Heyman for their support and friendly atmosphere.
- The red cross in Regensburg for providing us buffy coats and the Department for Clinical Chemistry of the University Hospital of Regensburg, namely Prof. Dr. Gerd Schmitz and Prof. Dr. Miriam Klouche for providing us leukapheresis products.
- The group of Prof. Dr. Hans Wolf namely Tanja Bauer and Birgit Bauer for giving me the oppotunity to use the ELISPOT reader.

- All the people who contributed to identify the mechanism of how NK cells kill Hsp70 membrane-positive tumor cells: Prof. Dr. Walther Koelch for performing the MALDI-TOF analysis which was a milestone of my work, Dr. Antonio deMaio and Dr. Nelson Arispe for cation channel activity measurements of the CX+/CX- cells, and Prof. Dr. Marja Jäättelä and Dr. Christopher Froelich for constructive suggestions.
- My parents for their believe in me and for their support during my studies.
- My sister Michaela for her listening and her good suggestions.
- Last but not least, Thomas for his love and support.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft SFB-455: Viral function and immunomodulation, by European Union Grant TRANSEUROPE QLRT 2001 01936, and by multimmune GmbH Regensburg

8 CURRICULUM VITAE

Personal data

Name:	Catharina Christiane Groß
Date and place of birth:	29th of March 1973, Bonn-Bad Godesberg, Germany

PhD thesis

Since 11/1999	PhD thesis at the Ludwig-Maximilians-University, Munich, Germany; Prof. Dr. E. Weiß Completed at the group of Molecular Oncology, Department of Hematology/Oncology, University Hospital of Regensburg, Germany; Prof. Dr. G. Multhoff Subject: Membrane-Bound Hsp70 an Activating Ligand for NK Cells

Scientific education

09/1998 – 06/1999	Diploma thesis at the Institute of Biotechnology II, Forschungszentrum Jülich, Germany; Prof. Dr. C. Wandrey Subject: Stroma-Free Cultivation and Expansion of Human Hematopoetic Stem Cells
11/1993 – 06/1999	Studies of biology at the Friedrich-Wilhelms-University, Bonn, Germany Major subjects: microbiology, genetics and cell biology Minor subjects: biochemistry, chemistry and physics Grade: diploma biology
11/1992 – 10/1993	Studies of chemistry at the Friedrich-Wilhelms-University, Bonn, Germany

School

08/1983 – 05/1992	Gustav-Stresemann-Gymnasium, Bad Wildungen, Germany Grade: academic standard required for university entrance
18/1979 – 07/1983	Primary school Helenental, Bad Wildungen, Germany

Visiting student

09/1996 – 06/1997	Visiting student (ERASMUS scholarship) at the University of East Anglia, Norwich, Great Britain Subjects: genetics, microbiology, English and Spanish

Practical training

06/1997 – 08/1997	Practical training at the Institute for Biotechnology II, Forschungszentrum Jülich, Germany; Prof. Dr. C. Wandrey Subject: Cultivation of Animal Cells
01/1997 – 03/1997	Research Project at the Institute of Food Research, Norwich, Great Britain; Prof. Dr. M. Schweitzer Subject: Testing for DNA Instability and Mismatch Repair in *Saccharomyces cerevisiae*

Scholarship

07/1996 – 06/1999	Studienstiftung des deutschen Volkes

Membership

Since 08/2003	Cell Stress Society International

Participation at conferences

09/2003	GvHD – State of the Art. 1st Regensburg Postgraduate Course on GvHD, Regensburg, Germany
09/2003	1st International Congress on Stress Responses in Biology and Medicine, Québec, Canada Poster presentation
03/2003	2nd International Symposium on the Clinical Use of Cellular Products: Cellular Therapy 2003, Regensburg, Germany Poster presentation
10/2002	Conference of the German and Austrian Society of Hematology and Oncology, Munich, Germany Poster presentation
03/2001	1st International Symposium on the Clinical Use of Cellular Products: Cellular Therapy 2001, Regensburg, Germany Poster presentation

Publications

Krause SW, Gastpar R, Andreesen R, **Gross C**, Ullrich H, Thonigs G, Pfister K, Multhoff G. 2004. Treatment of Colon and Lung Cancer Patients with *ex Vivo* Heat Shock Protein 70-Peptide-Activated, Autologous Natural Killer Cells: A Clinical Phase I Trial. *Clin Cancer Res* **10:** 3699-3707.

Gastpar R, **Gross C**, Rossbacher L, Ellwart J, Riegger J, Multhoff G. 2004. The cell surface-localized Hsp70 epitope "TKD" induces migration and cytolytic activity selectively in human natural killer (NK) cells. *J Immunol* **172:** 972-980.

Farkas B, Hantschel M, Magyarlaki M, Becker B, Scherer K, Landthaler M, Pister K, Gehrmann M, **Gross C**, Mackensen A, Multhoff G. 2003. Heat shock protein 70 membrane expression and melanoma-associated marker phenotype in primary and metastatic melanoma. *Melanoma Res* **13:** 147-152.

Gross C, Schmidt-Wolf IGH, Nagaraj S, Gastpar R, Ellwart J, Kunz-Schughart L, Multhoff G. 2003. Hsp70-reactivity is associated with increased cell surface density of CD94/CD56 on primary NK cells. *Cell Stress Chaperones* **8:** 348-360:

Gross C, Koelch W, DeMaio A, Arispe N, Multhoff G. 2003. Cell surface-bound heat shock protein 70 (Hsp 70) mediates perforin-independent apoptosis by specific binding and uptake of granzyme B. *J Biol Chem* **278:** 41173-41181.

Gross C, Hansch D, Gastpar R, Multhoff G. 2003. Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. *Biol Chem* **384:** 267-279.

Moser C, Schmidbauer C, Gürtler U, **Gross C**, Gehrmann M, Thonigs G, Pfister K, Multhoff G. 2002. Inhibition of tumor growth in mice with severe combined immunodeficiency is mediated by heat shock protein 70 (Hsp70)-peptide activated, CD94 positive natural killer cells. *Cell Stress Chaperones* **7:** 365-373.

Multhoff G, Pfister K, Gehrmann M, Hantschel M, **Gross C**, Hafner M, Hiddemann W. 2001. A 14-mer Hsp70 peptide stimulates natural killer (NK) cell activity. *Cell Stress Chaperones* **6:** 337-344.

Linguistic proficiency:

English

French (knowledge aquired at school)

Spanish (basics)

APPENDIX

(i) **Gross C**, Hansch D, Gastpar R, Multhoff G. 2003a. Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. *Biol Chem* **384:** 267-279.

(ii) **Gross C**, Koelch W, DeMaio A, Arispe N, Multhoff G. 2003b. Cell surface-bound heat shock protein 70 (Hsp 70) mediates perforin-independent apoptosis by specific binding and uptake of granzyme B. *J Biol Chem* **278:** 41173-41181.

(iii) **Gross C**, Schmidt-Wolf IGH, Nagaraj S, Gastpar R, Ellwart J, Kunz-Schughart L, Multhoff G. 2003c. Hsp70-reactivity is associated with increased cell surface density of CD94/CD56 on primary NK cells. *Cell Stress Chaperones* **8:** 348-360.

(iv) Gastpar R, **Gross C**, Rossbacher L, Ellwart J, Riegger J, Multhoff G. 2004. The cell surface-localized Hsp70 epitope "TKD" induces migration and cytolytic activity selectively in human natural killer (NK) cells. *J Immunol* **172:** 972-980.

Biol. Chem., Vol. 384, pp. 267–279, February 2003 · Copyright © by Walter de Gruyter · Berlin · New York

Interaction of Heat Shock Protein 70 Peptide with NK Cells Involves the NK Receptor CD94

Catharina Gross, Daniel Hansch, Robert Gastpar and Gabriele Multhoff*

Department of Hematology and Oncology, University Hospital Regensburg, Franz-Josef Strauss Allee 11, D-93053 Regensburg, Germany

*Corresponding author

Full-length Hsp70 protein (Hsp70) and the C-terminal domain of Hsp70 (Hsp70C) both stimulate the cytolytic activity of naive natural killer (NK) cells against Hsp70-positive tumor target cells. Here, we describe the characterization of Hsp70-NK cell interaction with binding studies using the human NK cell line YT. Binding of recombinant Hsp70 protein (Hsp70) and the C-terminal domain of Hsp70 (Hsp70C) to YT cells is demonstrated by immunofluorescence studies. A phenotypic characterization revealed that none of the recently described HSP-receptors (α2-macroglobulin receptor CD91, Toll-like receptors 2, 4, 9, CD14) are expressed on YT cells. Only the C-type lectin receptor CD94 is commonly expressed by YT cells and Hsp70 reactive NK cells. A correlation of the cell density-dependent, variable CD94 expression and the binding capacity of Hsp70 was detected. Furthermore, Hsp70 binding could be completely abrogated by preincubation of YT cells with a CD94-specific antibody. Competition assays using either unlabeled Hsp70 protein or an unrelated protein (GST) in 20-fold excess and binding studies with escalating doses of Hsp70 protein provide evidence for a specific and concentration-dependent interaction of Hsp70 with YT cells.

In addition to Hsp70 and Hsp70C, a 14-mer Hsp70 peptide termed TKD is known to exhibit comparable stimulatory properties on NK cells. Similar to full-length Hsp70 protein (10 μg/ml–50 μg/ml), a specific binding of this peptide to YT cells was observed at 4 °C, at equivalent concentrations (2.0 μg/ml–8.0 μg/ml). Following a 30 min incubation period at 37 °C, membrane-bound Hsp70 protein and Hsp70 peptide TKD were completely taken up into the cytoplasm.

Key words: Binding studies / Hsp70 protein / Hsp70 peptide / NK cell receptor CD94.

Introduction

Heat shock proteins (HSP) are highly conserved proteins that inhabit nearly all subcellular compartments. Environmental stress as well as differentiation, proliferation and maturation result in increased HSP levels (Lindquist and Craig, 1988; DeNagel and Pierce, 1992). The major role of intracellular-residing HSP is protecting cells against lethal damage induced by stress, and support of the folding and transport processes of newly produced polypeptides and abberant proteins (Hartl, 1996). Recently, extracellular-localized and membrane-bound HSPs have been found to be important in eliciting immune responses. Uptake of HSP (gp96, Hsp90, Hsp70, and calreticulin) peptide complexes by antigen-presenting cells (APC) *via* the α2-macroglobulin receptor CD91 and presentation by MHC class I molecules (Arnold-Schild *et al.,* 1999; Binder *et al.,* 2000a,b; Sondermann *et al.,* 2000; Basu *et al.,* 2001) elicits an antigen-specific $CD8^+$ T cell response (Schild *et al.,* 1999; Wells and Malkovsky, 2000; Binder *et al.,* 2001). Binding of extracellular HSP70 to monocytes stimulates the secretion of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, through a CD14-associated pathway that is dependent on intracellular calcium and on the activation of NF-κB (Asea *et al.,* 2000a). For this 'chaperokine effect', HSP70-chaperoned peptides are not required (Asea *et al.,* 2000b). Our group demonstrated that membrane-bound Hsp70 renders tumor cells more sensitive to the cytolytic activity of transiently plastic adherent natural killer (NK) cells (Vujanovic *et al.,* 1995; Multhoff *et al.,* 1995b, 1997; Hantschel *et al.,* 2000). By sequencing of the Hsp70 antibody epitope of clone C92F3B1 (Reineke *et al.,* 1996), which is known to inhibit the cytolytic NK activity against Hsp70-positive tumor cells, a 14-mer peptide TKDNNLLGRFELSG (TKD) could be identified as the minimal Hsp70-NK cell interacting peptide (Multhoff *et al.,* 2001). Incubation of purified NK cells, either with full-length Hsp70 protein, with the C-terminal domain of Hsp70 (Hsp70C), or with the Hsp70 peptide at a concentration of 10 μg/ml Hsp70 protein or 2 μg/ml Hsp70 peptide, resulted in a comparable stimulation of the cytolytic function of NK cells against Hsp70-positive tumor target cells (Multhoff *et al.,* 1999, 2001). In contrast, related HSP70 members, including the constitutively-expressed Hsc70 (84% homology to Hsp70) and DnaK, the *E. coli*-derived Hsp70 (50% homology to Hsp70), did not affect NK cell activity (Botzler *et al.,* 1998). Since saturation and specificity are typical attributes for a receptor-ligand interaction we speculate about the existence of Hsp70-specific receptors on NK cells. Functionally, experimental evidence exists that the C-type lectin KIR/KAR receptor CD94 is involved in the interaction of NK cells with Hsp70 protein for several reasons: (i) following incubation of purified NK cells with Hsp70 protein and the Hsp70 peptide, CD94 expression is up-regulated; (ii) the cytolytic activity of NK cells

against Hsp70-positive tumor target cells is selectively reduced by the addition of anti-CD94 antibody; other NK-specific antibodies did not influence NK-mediated killing (Multhoff *et al.,* 1999).

Here the involvement of CD94 as a putative receptor for Hsp70 is analyzed using the IL-2-independent, human NK cell line YT (Yodoi *et al.,* 1985; Drexler and Matsuo, 2000). The Hsp70-YT cell interaction is investigated in binding studies using either unconjugated or FITC-labeled full-length Hsp70 protein, the C-terminal domain of Hsp70 (Hsp70C), and Hsp70 peptide TKD.

Results

Binding of Full-Length Hsp70 Protein (Hsp70) and the C-Terminal Domain of Hsp70 (Hsp70C) to YT Cells

Previously, we observed that incubation of naive human NK cells with full-length Hsp70 protein enhances the cytolytic activity against Hsp70-positive tumor cells. This stimulatory activity was concentration dependent (10 µg/ml– 100 µg/ml) and restricted to Hsp70, the major stress-inducible member of the HSP70 group. Other related members of this family, including Hsc70 (84% homology to Hsp70) or DnaK (50% homology to Hsp70), did not stimulate NK cell activity (Multhoff *et al.,* 1999). In a first experiment, we wanted to know whether full-length Hsp70 protein binds to the human NK cell line YT. Following incubation of YT cells with 50 µg/ml unlabeled Hsp70 protein at 4 °C, binding was measured by indirect flow cytometry, using the Hsp70-specific monoclonal antibody (mAb) SPA810. The epitope of SPA810 is localized within the C-terminal domain of Hsp70 (Botzler *et al.,* 1998), but differs from that of the Hsp70-specific Ab clone C92F3B1 (Multhoff *et al.,* 2001). Only the latter mAb inhibits the cytolytic activity of NK cells against Hsp70-positive tumor target cells in antibody blocking studies (Multhoff *et al.,* 1995a,b). We assumed that the epitope of clone C92F3B1 might be involved in the Hsp70-NK cell interaction and therefore not be detectable in binding assays. As shown in Figure 1, a significantly increased mean fluorescence value of the Hsp70 peak was observed with the mAb SPA810, but not with the mAb clone C92F3B1 (data not shown). As negative controls, YT cells that had not been incubated with Hsp70 protein were stained with the Hsp70-specific mAb SPA810 and secondary Ab (mAb ctrl, dashed line). In addition, Hsp70 protein-incubated YT cells were stained with an isotype-matched control Ab and a secondary Ab (isotype ctrl, dotted line).

Our previous data indicate that part of the C-terminal substrate binding domain, and not the N-terminal ATPase domain of Hsp70, is exposed to the extracellular milieu if expressed on the cell surface of Hsp70-positive tumor cells (Botzler *et al.,* 1998). Therefore, we asked the question whether the C-terminal domain Hsp70C also

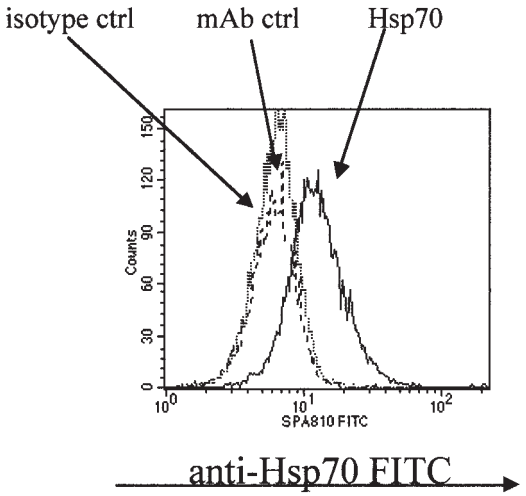


Fig. 1 Hsp70 Protein Binding to YT Cells as Determined by Indirect Immuno-Flow Cytometry.
YT cells (5×10^6/ml) were incubated with 50 µg/ml Hsp70 protein at 4 °C for 45 min. Binding of Hsp70 was measured by indirect flow cytometry using Hsp70-specific mAb SPA810. This antibody detects an epitope within the C-terminal substrate binding domain of Hsp70 that differs from that of the blocking Hsp70 antibody, clone C92F3B1. Intensity of surface fluorescence (x-axis) was plotted against cell counts. The dotted line represents the isotype-matched control (isotype ctrl) of YT cells incubated with Hsp70 protein; the dashed line represents the SPA810 mAb control (mAb ctrl) of YT cells that had not been incubated with Hsp70 protein; the solid line represents Hsp70 positively-stained cells. Only viable PI-negative cells were used for the analysis. The data show one representative experiment out of five.

binds to YT cells. For these binding studies the fusion protein Hsp70C-GST, consisting of the C-terminal domain of human Hsp70 (Hsp70C, aa 384 – 563) and glutathione S-transferase (GST), was used. As shown in Figure 2A, the mean fluorescence value of the indirect Hsp70-staining using the mAb SPA810, as compared to the mAb SPA810 control of untreated YT cells (mAb ctrl, dashed line), or the isotype-matched control of Hsp70C-GST-treated YT cells (isotype control, dotted line), shifted to the right. A similar staining pattern was obtained with a GST-specific mAb (Figure 2B). As controls, either the GST mAb (mAb ctrl) was incubated with untreated YT cells, or Hsp70C-GST treated YT cells were incubated with an isotype-matched control antibody (isotype ctrl). As shown in Figure 2C, GST alone at an equivalent concentration did not bind to YT cells non-specifically. In summary, these data indicate by indirect immunofluorescence studies that the NK cell line YT binds full-length Hsp70 protein and the C-terminal domain Hsp70C fused to GST; no binding of GST alone was observed.

Phenotypic Characterization of YT Cells

Since naive NK cells have been found to interact with Hsp70 proteins in a concentration-dependent manner, we hypothesize that the NK cell line YT, similar to antigen-presenting cells (APC), might express Hsp70-spe-

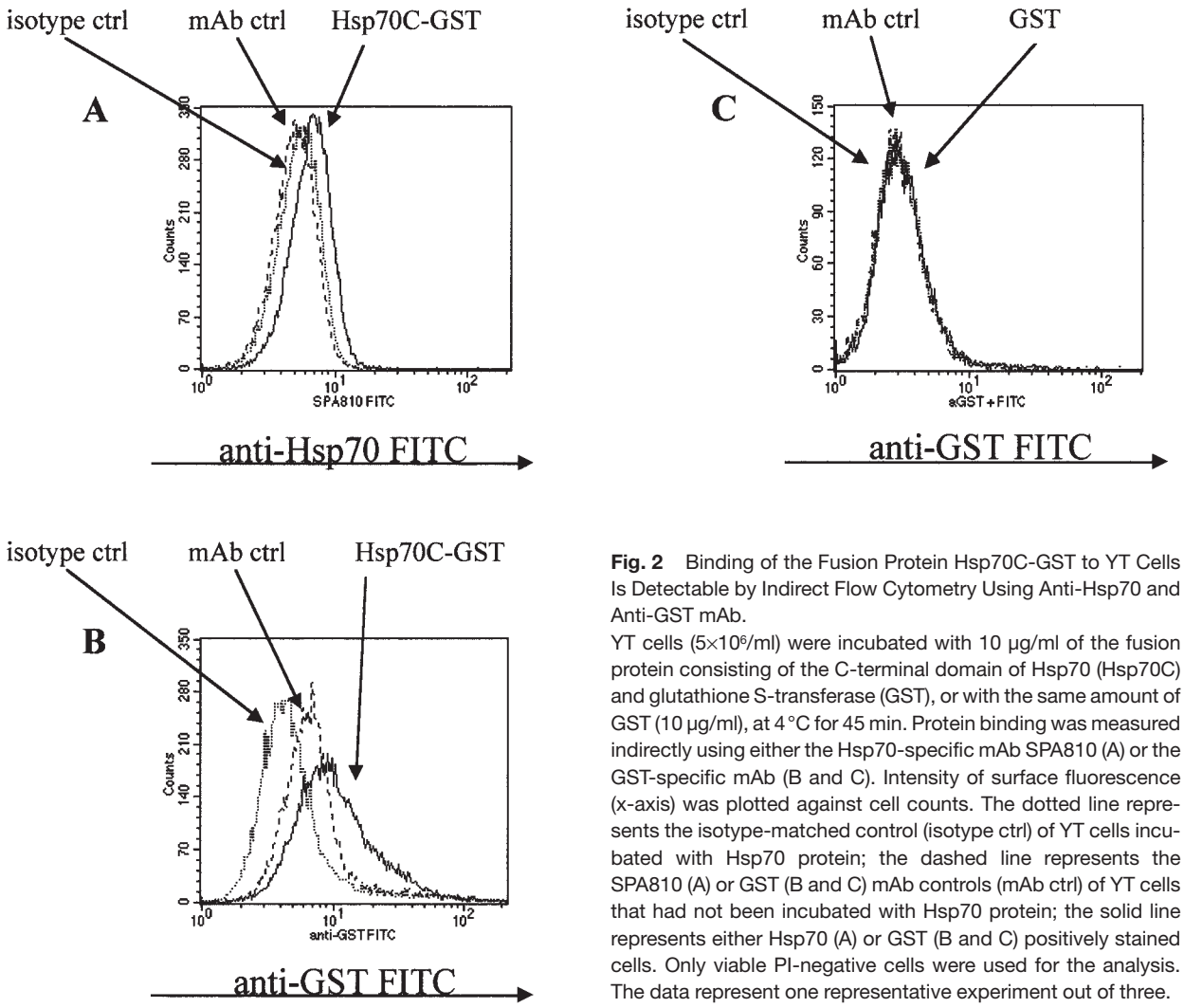


Fig. 2 Binding of the Fusion Protein Hsp70C-GST to YT Cells Is Detectable by Indirect Flow Cytometry Using Anti-Hsp70 and Anti-GST mAb.
YT cells (5×10^6/ml) were incubated with 10 µg/ml of the fusion protein consisting of the C-terminal domain of Hsp70 (Hsp70C) and glutathione S-transferase (GST), or with the same amount of GST (10 µg/ml), at 4 °C for 45 min. Protein binding was measured indirectly using either the Hsp70-specific mAb SPA810 (A) or the GST-specific mAb (B and C). Intensity of surface fluorescence (x-axis) was plotted against cell counts. The dotted line represents the isotype-matched control (isotype ctrl) of YT cells incubated with Hsp70 protein; the dashed line represents the SPA810 (A) or GST (B and C) mAb controls (mAb ctrl) of YT cells that had not been incubated with Hsp70 protein; the solid line represents either Hsp70 (A) or GST (B and C) positively stained cells. Only viable PI-negative cells were used for the analysis. The data represent one representative experiment out of three.

cific receptors on the cell surface. In an effort to identify this putative Hsp70 receptor, a phenotypic characterization was performed (Table 1). In addition to common YT cell markers, T and newly defined NK cell-specific Killer cell inhibitory/activating, Ig-like, superfamily receptors (KIR) and C-type lectin receptors were tested (Braud *et al.,* 1998; Lanier *et al.,* 1998; Lanier, 2000; Vales-Gomez *et al.,* 2000), as well as HSP receptors that had been identified on APC (Binder *et al.,* 2000a; Asea *et al.,* 2000a; Ohashi *et al.,* 2000). As a positive control the MHC class I expression was determined using the antibody W6/32. In line with published data (Drexler and Matsuo, 2000), YT cells were found to be positive for the pluripotent stem cell and leukemic marker CD7 (FcµR). As an NK cell line, they lack expression of classical T cell markers including CD3, CD6 and CD8. Several NK cell-specific markers, including CD11b, CD16 (Lanier *et al.,* 1988), CD56, CD57, the immunoglobulins KIR p 58.1, p 58.2 (Moretta *et al.,* 1995; Long, 1999) and the activating C-type lectin receptor CD161 (Lanier *et al.,* 1994) were also not present on YT cells. Only CD94 is commonly expressed by YT cells and Hsp70-reactive NK cells. However, the expression levels of CD94 varied; a negative correlation was found with an increased cell density of YT cells.

As mentioned earlier, several receptors, including the α2-macroglobulin receptor CD91, the LPS receptor CD14, and the Toll-like receptors TLR 2, 4, and 9, have been discussed as potential HSP receptors on APC that mediate HSP binding and/or signal transduction (Asea *et al.,* 2000a,b; Binder *et al.,* 2000a; Ohashi *et al.,* 2000; Habich *et al.,* 2002). To understand their involvement in the interaction of NK cells with Hsp70, their membrane expression was measured on YT cells. As summarized in Table 1, neither the α2-macroglobulin receptor CD91, which was identified as a common receptor for gp96, Hsp90 and Hsp70 on APC (Binder *et al.,* 2000b; Basu *et al.,* 2001), nor one of the the Toll-like receptors (TLR 2, 4, 9) alone, or in combination with CD14 (Asea *et al.,* 2000a; Kol *et al.,* 2000), were found to be expressed on YT cells.

CD94 Is Involved in the Binding of Hsp70-FITC to YT Cells

Based on previous results with naive human NK cells, experimental evidence exists that the C-type lectin receptor

Table 1 Phenotypical Analysis of the NK Cell Line YT.

Antibody	Specificity	Positively stained cells (%)	+/–
	YT cell marker		
W6/32	**MHC class I**	98.9 ± 0.2	+
CD7	gp40, FcμR, YT positive ctrl T cell marker	93.3 ± 0.7	+
CD3	gp20 – 50, TcR associated complex	1.4 ± 0.2	–
CD6	gp100, thymocytes	0.0 ± 0.0	–
CD8	gp32, cytotoxic, suppressor T	0.0 ± 0.0	–
	NK cell marker		
CD11b	gp165, C3biR	0.4 ± 0.7	–
CD16	gp50 – 80, FcγR	2.8 ± 3.7	–
CD56	gp180, N-CAM	1.5 ± 0.9	–
CD57	p110, HNK1	0.0 ± 0.0	–
CD161	gp60, C-type lectin, KAR	0.0 ± 0.0	–
CD158a	p58.1, p50.1, Ig-SFR, KIR/KAR	0.0 ± 0.0	–
CD158b	p58.2, p50.2, Ig-SFR, KIR/KAR	0.0 ± 0.0	–
CD94	**gp43, C-type lectin R, KIR/KAR**	20 – 45.8 ± 6.0	+
	HSP receptors on APC		
CD91	p600, α2-macroglobulin receptor	1.8 ± 0.7	–
CD14	gp55, LPS receptor	0.0 ± 0.0	–
TLR 2	Toll-like receptor 2	1.0 ± 0.8	–
TLR 4	Toll-like receptor 4	0.1 ± 0.2	–
TLR 9	Toll-like receptor 9	2.9 ± 0.2	–

IL-2 independent YT cells were cultured at cell densities ranging from 0.1 – 0.8×10^6 cells/ml (doubling time: 40 – 50 h). The data represent mean values of at least three independent experiments. Cell surface markers, commonly expressed by YT cells and Hsp70-reactive NK cells, are indicated in bold. A negative correlation of the CD94 expression and increased cell densities was observed. Abbreviations: APC, antigen presenting cells; ctrl, control; gp, glycoprotein; LPS, lipopolysaccharide; MHC, major histocompatibility complex; p, protein; R, receptor; TcR, T cell receptor.

CD94 might be involved in the interaction with Hsp70: (i) following incubation of isolated, pure NK cells with Hsp70 protein or Hsp70C, the expression of CD94 was up-regulated (Multhoff *et al.,* 1999). (ii) The cytolytic activity of NK cells could be up-regulated by a 4-day incubation period with Hsp70 protein (Multhoff *et al.,* 1999; Hantschel *et al.,* 2000). (iii) Antibody blocking studies using a CD94-specific antibody on the effector cells reduced the cytolytic response of NK cells against Hsp70-positive tumor target cells specifically (Multhoff *et al.,* 1999). (iv) CD94 is commonly expressed on Hsp70-reactive NK cells and the NK cell line YT (Table 1). Therefore, there was a special interest in studying the role of CD94 as a potential receptor for the interaction with Hsp70.

By flow cytometry we have shown a negative correlation of cell density and CD94 expression on YT cells (Figure 3A). At elevated cell densities (0.8×10^6 cells/ml) the CD94 expression was much weaker as compared to low cell densities (0.1×10^6 cells/ml). If the cells were diluted in fresh medium on day 0, day 2, day 4 and day 6, the percentage of CD94-positive cells increased from 13% to 23%. Concomitantly, the percentage of Hsp70C-GST binding as determined by indirect immunofluorescence studies increased from 12% to 42% (Figure 3A). These data provide evidence that CD94 might be involved in the interaction of YT cells with Hsp70.

To further support this hypothesis, antibody blocking studies were performed. In these experiments Hsp70 binding was not only demonstrated by indirect flow cytometry but also by direct means using FITC-labeled Hsp70 protein. As shown in Figure 3B, 41% of the YT cells were positively stained with the CD94 antibody. Following incubation with 20 μg/ml Hsp70-FITC for 45 min, binding was found on 33% of the cells. This Hsp70-FITC binding could be abrogated by preincubation of YT cells with 5 μg/ml CD94 antibody; following antibody incubation only 6% Hsp70-FITC binding was detected (Figure 3B). As a control, a CD7-specific antibody was used for blocking; this antibody did not affect Hsp70-FITC binding (data not shown).

Binding of Full-Length Hsp70 Protein to YT Cells Is Specific and Concentration-Dependent

Specificity of Hsp70-FITC binding to CD94-positive YT cells was confirmed by competition assays. Hsp70-FITC binding (20 μg/ml) at a CD94 expression level of 41% was 33%. Upon addition of a 20-fold excess of unlabeled Hsp70 (20x Hsp70), binding of Hsp70-FITC was completely suppressed (0% binding), whereas the identical amount of unlabeled GST (20x GST) only weakly competed for the Hsp70 binding site on YT cells. As shown in

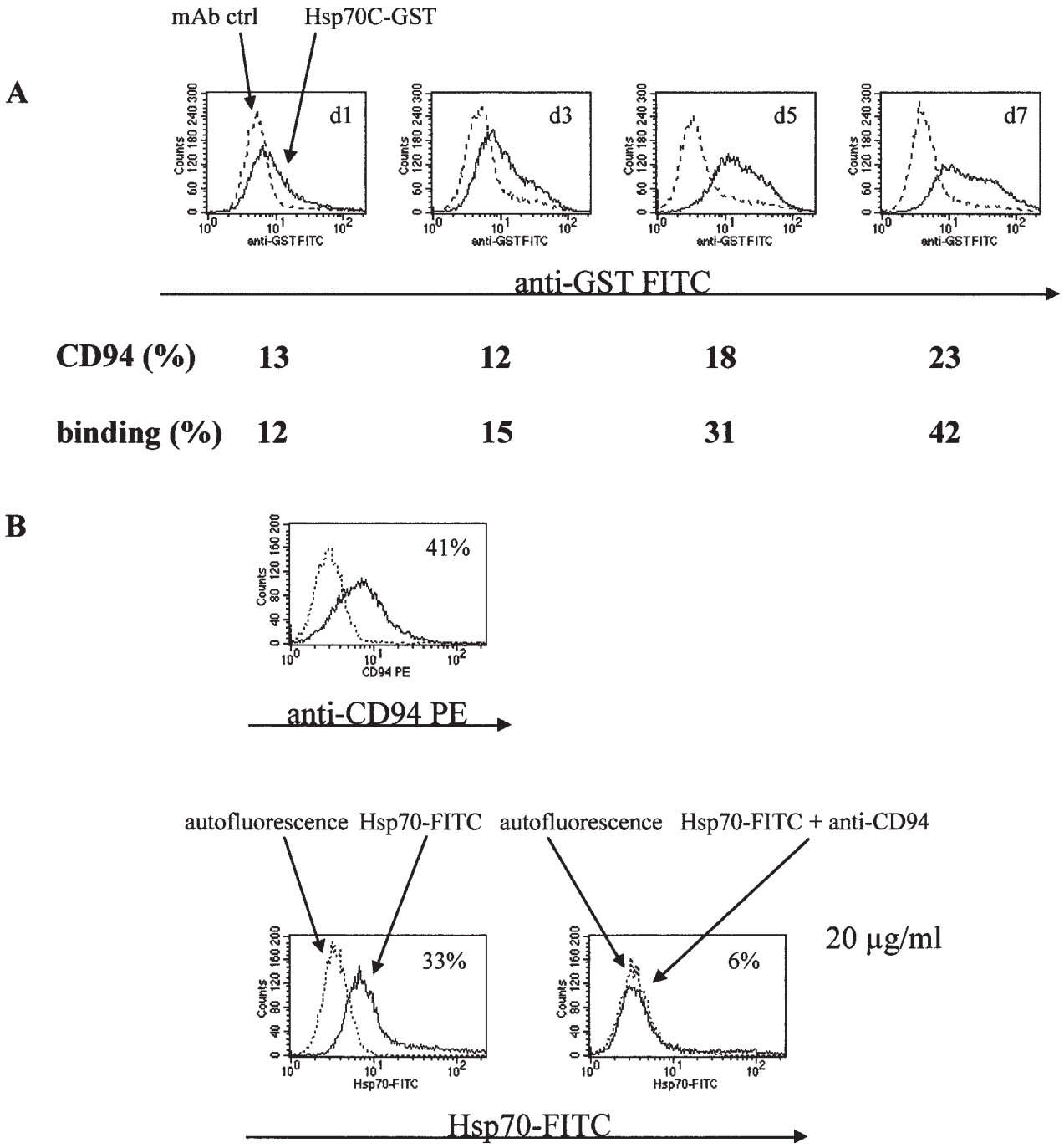
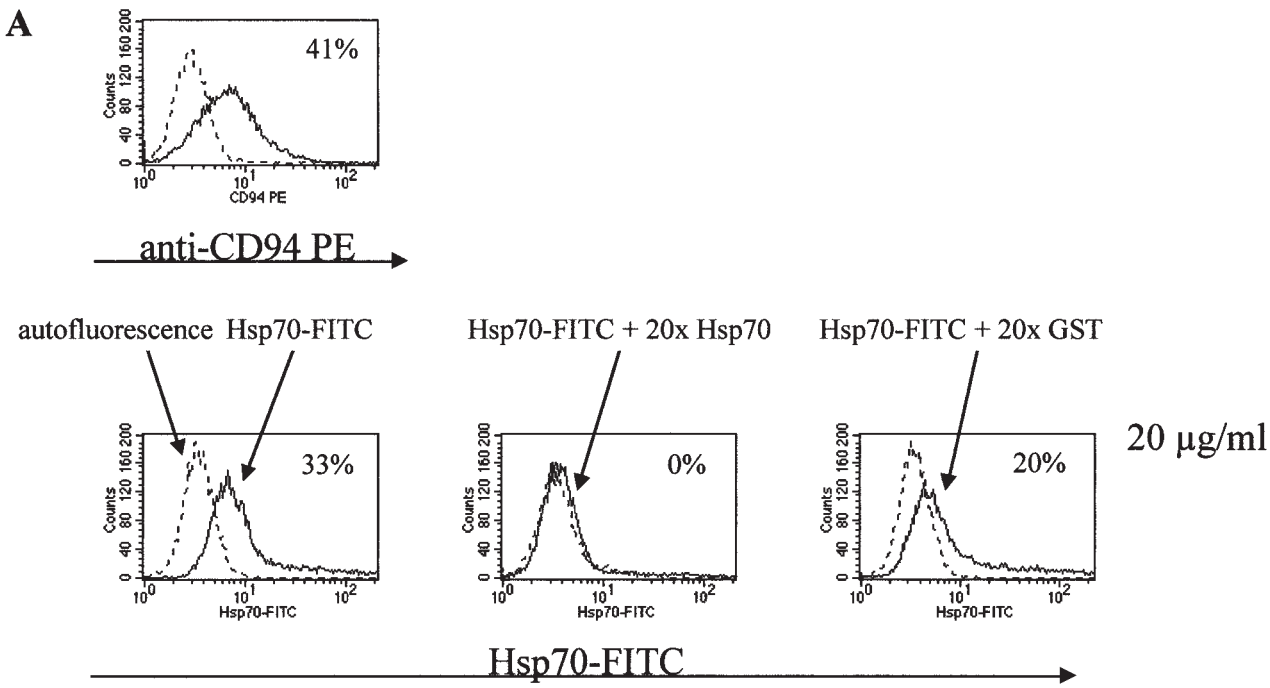
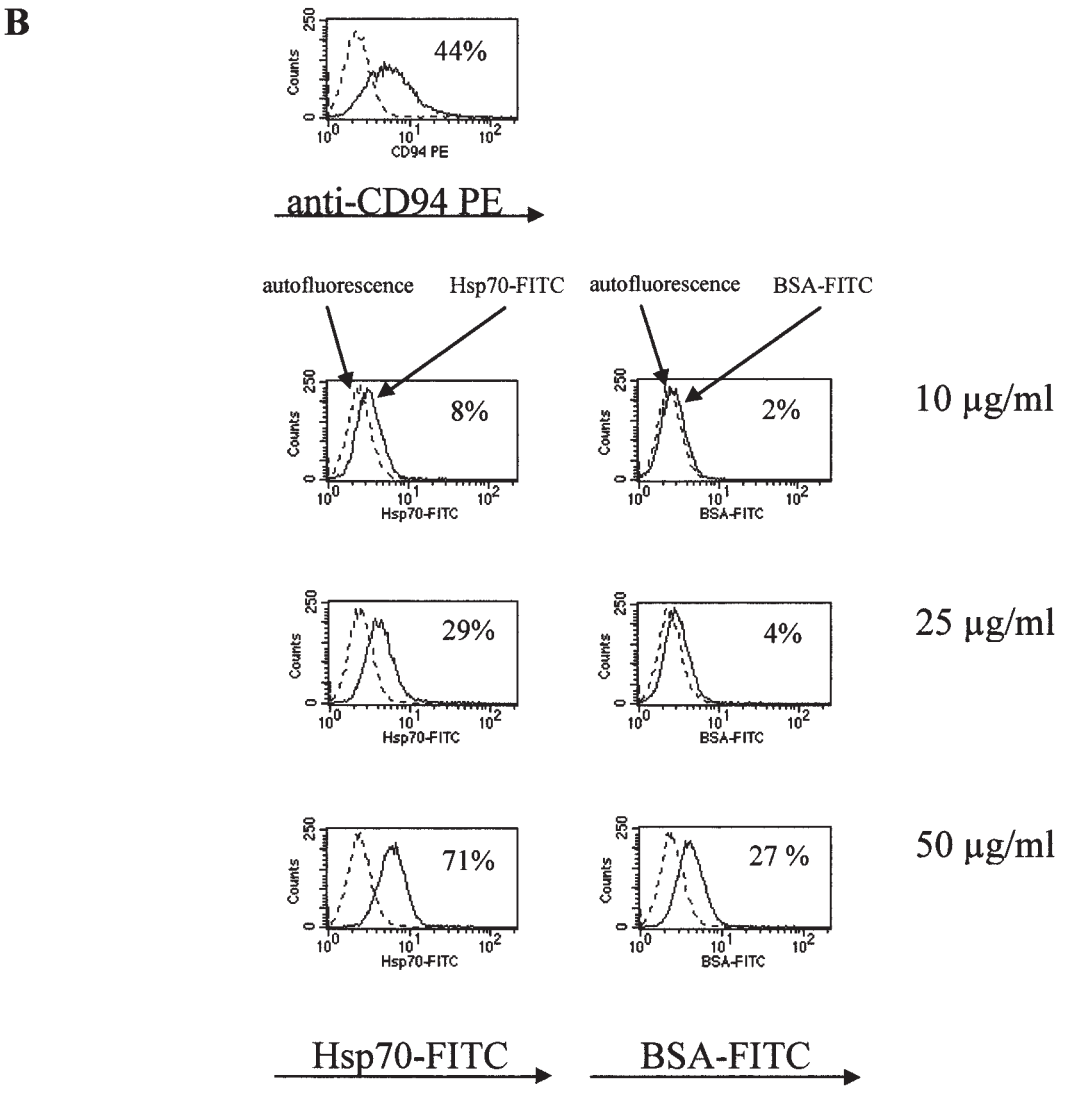


Fig. 3 Hsp70 Protein Binding Is Associated with CD94 Cell Surface Expression on YT Cells.
CD94 expression was measured concomitant with Hsp70C-GST binding using a mAb directed against GST (A). YT cells were cultivated for seven days at decreasing cell densities (0.8, 0.4, 0.2, 0.1×10^6/ml). The CD94 expression increased with decreasing cell densities. On days 1, 3, 5, and 7 YT cells were incubated with Hsp70C-GST fusion protein as described in Figure 2.
Hsp70 binding was also determined directly using FITC-labeled Hsp70 protein (Hsp70-FITC; B). YT cells, either untreated (left panel) or after preincubation with CD94 mAb (5 µg/ml) for 30 min (right panel), were incubated with 20 µg/ml Hsp70-FITC (B) at 4 °C for 45 min. Following incubation cells were extensively washed with PBS and fixed in 1% PFA solution. The percentage of CD94-positive YT cells was 41%. The fluorescence pattern was analyzed by flow cytometry. The dashed line represents the autofluorescence; the solid line represents Hsp70 positively-stained cells. Specific Hsp70 protein binding is indicated in percent in the upper right corner of each graph.

Figure 4A, Hsp70-FITC binding was still 20% if a 20-fold excess of unlabeled GST was applied together with Hsp70-FITC. The data represent one experiment out of three demonstrating identical results. To determine the concentration range of specific Hsp70 binding, YT cells were incubated with Hsp70-FITC at concentrations ranging from 10 µg/ml to 50 µg/ml (Figure 4B). These concentrations have been defined as optimal for the stimulation


A
41%
Counts
CD94 PE
anti-CD94 PE
autofluorescence
Hsp70-FITC
Hsp70-FITC + 20x Hsp70
Hsp70-FITC + 20x GST
33%
0%
20%
20 µg/ml
Hsp70-FITC
Hsp70-FITC


B
44%
CD94 PE
anti-CD94 PE
autofluorescence
Hsp70-FITC
autofluorescence
BSA-FITC
8%
2%
10 µg/ml
29%
4%
25 µg/ml
71%
27 %
50 µg/ml
Hsp70-FITC
BSA-FITC

of naive NK cells (Multhoff *et al.,* 1999). As negative controls, bovine serum albumin (BSA-FITC) and glutathione S-transferase (GST-FITC) were chosen and used at identical concentration ranges. All proteins had been conjugated to FITC with a comparable coupling efficiency: for Hsp70-FITC it was calculated as 4.1, for BSA-FITC as 4.4 and for GST-FITC as 4.5. YT cells were incubated with 10, 25, and 50 µg/ml Hsp70-FITC, BSA-FITC,

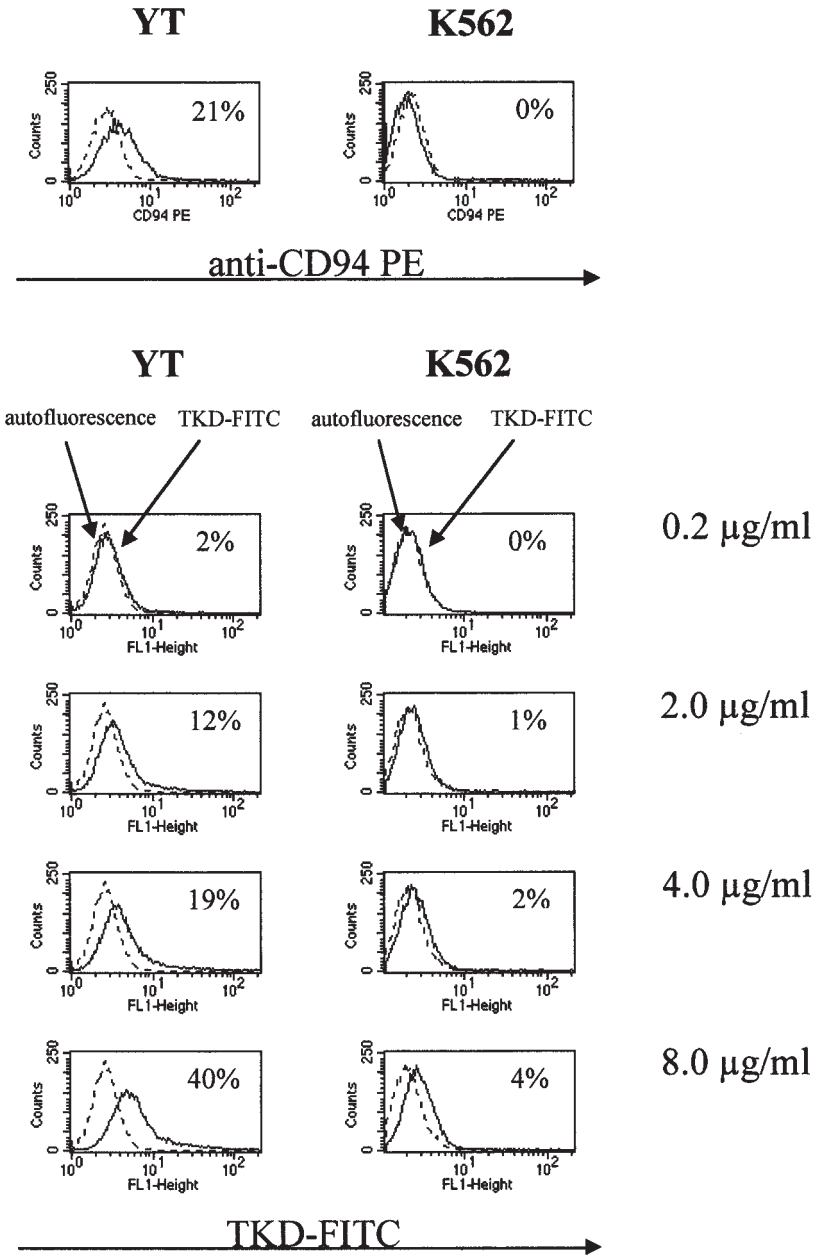


Fig. 5 Specific and Concentration-Dependent Binding of Hsp70 Peptide TKD to YT Cells Is Associated with CD94 Expression.
The upper row shows flow cytometric analysis of the CD94 expression on the cell lines YT (21%) and K562 (0%). Below, TKD-FITC binding to YT (left) and to K562 cells (right) is illustrated. Identical cell numbers of viable YT and K562 cells (5×10^6/ml each) were incubated with the following concentrations of TKD-FITC: 0.2, 2.0, 4.0, and 8.0 µg/ml for 45 min at 4 °C. Only viable, PI-negative YT and K562 cells were analyzed. Intensity of surface fluorescence (x-axis) was plotted against cell counts. The dashed line represents the autofluorescence; the solid line represents Hsp70 peptide positively stained cells. Specific peptide binding is indicated in percent in the upper right corner of each graph, as the difference in the amount of positively stained cells minus autofluorescence-stained cells.

Fig. 4 Hsp70 Protein Binding to YT Cells Is Specific and Concentration-Dependent.
Specificity of Hsp70 binding was measured by competition assays (A). YT cells (5×10^6/ml) were incubated with 20 µg/ml Hsp70-FITC, either alone or together with a 20-fold excess of unlabeled Hsp70 protein or unlabeled glutathione S-transferase (GST), at 4 °C for 45 min. 41% of the YT cells were positively stained with CD94.
Concentration-dependent binding was determined by incubation of YT cells with different amounts of Hsp70-FITC or BSA-FITC (B). YT cells were incubated with 10, 25, or 50 µg/ml Hsp70-FITC (left), or BSA-FITC (right) as described in Figure 3.

and GST-FITC for 45 min. In order to avoid endocytosis the whole incubation procedure was performed at 4 °C. As shown in Figure 4A, YT cells used in this experiment exhibit CD94 expression on 44% of the cells. Specific binding to YT cells was observed at all protein concentrations, a concentration of 10 µg/ml Hsp70-FITC resulted in 8% binding, 25 µg/ml in 29% binding, and 50 µg/ml in 71% binding. At 10 µg/ml and 25 µg/ml, the negative control protein BSA-FITC was not bound to YT cells (2% and 4% binding, respectively); at a concentration of 50 µg/ml, binding of BSA-FITC was still significantly weaker (27% binding) as compared to Hsp70-FITC (71% binding). The solid line represents binding of Hsp70-FITC in the left panel and of BSA-FITC in the right panel; autofluorescence is shown by a dashed line. GST-FITC, as an additional negative control protein, did not bind to YT cells at a concentration of 50 µg/ml (data not shown). However, concentrations above 50 µg/ml result in a non-specific binding of all FITC-labeled proteins with YT cells (data not shown). Regarding these results, we conclude that a specific binding of Hsp70-FITC is detectable within a concentration range of 10 µg/ml to 50 µg/ml.

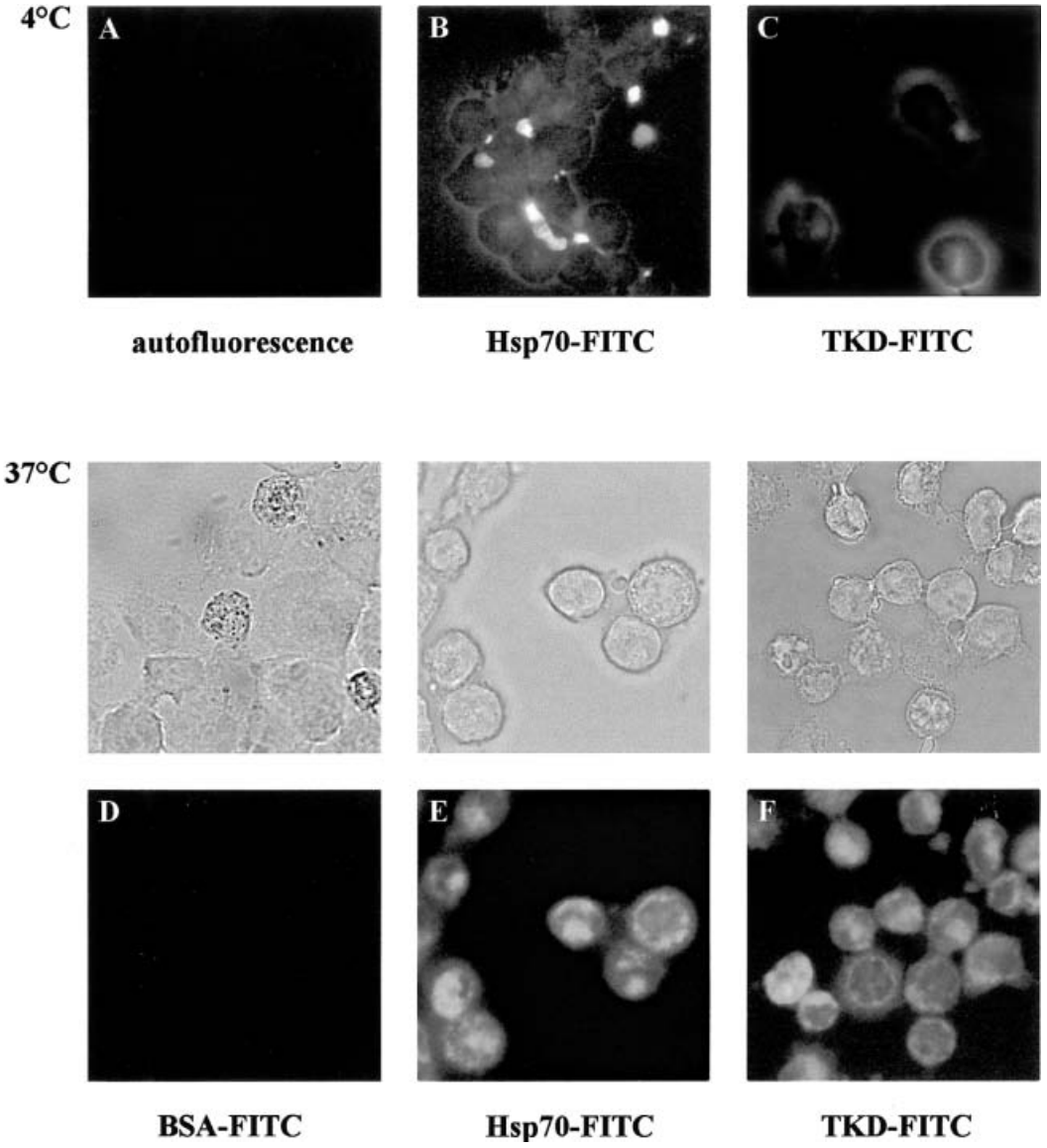


Fig. 6 Specific Cell Surface Binding of FITC-Labeled Hsp70 Protein and Hsp70 Peptide TKD at 4 °C (A, B, C), and Uptake Following Incubation at 37 °C (D, E, F) by YT Cells.
Binding at 4 °C: autofluorescence of untreated YT cells (A) incubated at 4 °C for 45 min is shown in (A). YT cells were incubated either with 50 µg/ml Hsp70-FITC (B) or 8.0 µg/ml TKD-FITC (C) at 4 °C for 45 min, as described in the legend to Figure 5, fixed with 1% paraformaldehyde and then analyzed for fluorescence microscopy at a magnification of 50×.
Uptake at 37 °C: YT cells were treated with BSA-FITC (50 µg/ml), Hsp70-FITC (50 µg/ml) and TKD-FITC (8 µg/ml), as described above and then incubated at 37 °C for 30 min, following extensive washing. After fixation in 1% paraformaldehyde, cells were analyzed for transmission microscopy (middle row). The corresponding fluorescence microscopy (lower row) of YT cells incubated with BSA-FITC (background fluorescence) is shown in (D), with Hsp70-FITC in (E), and with TKD-FITC in (F); magnification 50×.

Specific Binding of Hsp70 Peptide TKD to YT Cells

In addition to full-length Hsp70, a 14-mer Hsp70 peptide localized in the C-terminal substrate binding domain of Hsp70 (aa 450 – 463; TKDNNLLGRFELSG) has been found to possess stimulatory properties on NK cells (Multhoff *et al.,* 2001). Therefore, we were interested in examining whether the FITC-conjugated Hsp70 peptide TKD also binds to YT cells in a concentration-dependent manner. According to previous data the concentration range was chosen between 0.2 µg/ml and 8.0 µg/ml Hsp70 peptide. The CD94 expression on YT cells that had been used for this binding study was 21% (Figure 5, upper graph). As shown in the lower graph of Figure 5, specific binding of TKD-FITC to the NK cell line YT was observed at peptide concentrations of 2.0 µg/ml (12% binding), 4.0 µg/ml (19% binding) and 8.0 µg/ml (40% binding). An incubation of the CD94 negative control cell line, K562, with identical peptide concentrations revealed no significant binding of TKD-FITC. At a concentration of 8.0 µg/ml, the binding of TKD-FITC to YT cells as compared to K562 cells was still increased by 10-fold; 4% binding compared to 40%, respectively. Concentrations above 8.0 µg/ml result in a non-specific interaction of the Hsp70 peptide TKD to both cell types. Similar results were seen with other CD94-negative cells (Küppner *et al.,* 2001). Binding studies using an irrelevant FITC-labeled 14-mer control peptide (PAASIDRSTKPPLL) revealed no specific binding to YT cells (data not shown).

With respect to our Hsp70 protein and Hsp70 peptide binding studies, we propose that specific binding of Hsp70 protein to YT cells is found at concentrations of 10 µg/ml to 50 µg/ml and of Hsp70 peptide at equivalent concentrations of 2.0 µg/ml to 8.0 µg/ml. These data support previous work, which indicated that within these concentration ranges both Hsp70 protein and Hsp70 peptide increase the cytolytic activity of naive NK cells (Multhoff *et al.,* 1999, 2001).

Binding and Uptake of Hsp70-FITC as Compared to Hsp70 Peptide (TKD) by YT Cells

The binding of FITC-labeled Hsp70 protein (Hsp70-FITC) and Hsp70 peptide TKD (TKD-FITC) was demonstrated by indirect and direct flow cytometry, and could be confirmed by fluorescence microscopy. As compared to autofluorescence (Figure 6A), YT cells incubated either with 50 µg/ml FITC-labled Hsp70 protein (B, Hsp70-FITC) or with 8.0 µg/ml Hsp70 peptide (C, TKD-FITC) at 4 °C for 45 min, exhibit a ring-shaped fluorescence (Figure 6, upper row). These data clearly indicate a typical cell surface binding of Hsp70 protein and Hsp70 peptide TKD to the NK cell line YT. In a second set of experiments, YT cells were incubated with BSA-FITC (Figure 6D, 50 µg/ml), Hsp70-FITC (E, 50 µg/ml) and Hsp70 peptide TKD-FITC (F, 8 µg/ml), as described above. Following binding of Hsp70 protein and peptide to YT cells at 4 °C, the cells were washed in order to remove non-specifically bound FITC-labeled material. Then, viable cells were incubated at the physiological temperature of 37 °C for another 30 min. Following fixation, fluorescence microscopy was performed. In the middle row of Figure 6, light microscopy of the protein- and peptide-treated, fixed YT cells is illustrated. As shown in the corresponding fluorescence microscopy (Figure 6, lower row), the ring-shaped cell surface staining profoundly changes. As compared to transmission microscopy, all cells that had been pre-incubated either with Hsp70-FITC (E) or TKD-FITC (F) exhibited a cytoplasmic staining pattern. As a control, YT cells were incubated with BSA-FITC (50 µg/ml) under identical conditions. Neither at 4 °C nor following incubation at 37 °C was any staining observed (D). These data provide evidence that Hsp70-FITC (E) and TKD-FITC (F) have been selectively internalized from the cell surface into the cytoplasm within 30 min.

Kinetic studies revealed that under physiological conditions, already after 10 min 50% of the YT cells internalized FITC-labeled Hsp70 from the cell surface (data not shown). Regarding these results, we speculate that Hsp70 protein and Hsp70 peptide uptake is a temperature-dependent, rapid process.

Discussion

Previously, we have shown that membrane-bound Hsp70 renders tumor cells more sensitive to the cytolytic activity of transiently plastic adherent, CD94-positive human NK cells. This finding has been verified in several tumor cell types including carcinomas, sarcomas and leukemia (Multhoff *et al.,* 1995a; Botzler *et al.,* 1996; Multhoff, 1997; Hantschel *et al.,* 2000). Within an autologous human colon carcinoma cell system, CX+ and CX-, which differ only with respect to the Hsp70 cell surface expression pattern, the role of Hsp70 as the recognition site for NK cells was determined (Multhoff *et al.,* 1997). The efficiency of human NK cells to eradicate Hsp70-positive tumors could be confirmed *in vivo* in a xenograft SCID/beige tumor mouse model (Multhoff *et al.,* 2000). A 14-mer sequence, TKDNNLLGRFELSG (aa 450 – 463), in the C-terminal substrate binding domain of Hsp70 could be identified as a target recognition site for NK cells by sequencing the epitope of the Hsp70-specific antibody clone C92F3B1 (Welch and Suhan, 1986) that abrogates NK-mediated lysis against Hsp70-positive tumor target cells (Multhoff *et al.,* 2001). This sequence has been found to be unique for human Hsp70. Furthermore, it has been demonstrated that incubation of purified, naive NK cells with either full-length Hsp70 protein, the C-terminal domain of Hsp70 (aa 384 – 563), or with the 14-mer Hsp70 peptide TKD, induces comparative immunostimulatory effects (Multhoff *et al.,* 1999).

Although the functional role of Hsp70 protein and Hsp70 peptide on NK cells is clear, the molecular basis for the interaction of Hsp70 protein, Hsp70C-terminus or Hsp70 peptide remains to be elucidated. Since the availability of purified naive NK cells is limited, the leukemic

NK cell line YT was used for binding studies. Our data provide evidence for a specific and saturable interaction of either unlabeled or FITC-conjugated Hsp70, Hsp70C and Hsp70 peptide TKD with YT cells. For Hsp70 and Hsp70C-GST fusion protein a specific binding to YT cells was seen within a concentration range of 10 µg/ml to 50 µg/ml and for Hsp70 peptide TKD within a concentration range of 2.0 µg/ml to 8.0 µg/ml. These concentrations have been found to stimulate NK cell function (Multhoff *et al.,* 1999, 2001; Hantschel *et al.,* 2000). Therefore, we propose that YT cells provide a reliable model system to study Hsp70-NK interaction. Hsp70 protein concentrations above 50 µg/ml and Hsp70 peptide concentrations above 8.0 µg/ml result in a non-specific interaction with different cell types. At these high concentrations, Hsp70 is bound non-specifically also by non-NK cells. These findings differ from published data (Basu *et al.,* 2001), which demonstrated a receptor-mediated, specific binding of Hsp90, gp96, Hsc70 and Hsp70 to CD11b-positive macrophages at concentrations up to 200 µg/ml. With respect to these observations and due to the fact that, compared to APC, NK cells fulfill different immunological functions, we speculate that NK cells might use other Hsp70 receptors that require different concentrations. Several receptors, including CD91 (Binder *et al.,* 2000b; Basu *et al.,* 2001) and members of the Toll-like receptor family, either alone or in a receptor-complex with CD14 (Kol *et al.,* 2000; Pfeiffer *et al.,* 2001), are presently discussed as putative HSP60, HSP70 and HSP90 receptors on APC. To support our hypothesis that NK cells use other Hsp70-specific receptors, the expression pattern of APC receptors was tested on YT cells. None of these receptors was found on YT cells. Apart from this observation, several other results support the idea that the Hsp70 receptor on NK cells differs from that on APC: NK cells selectively interact with Hsp70, the major stress-inducible member of the HSP70 family. Neither the *E. coli*-derived Hsp70 homologue DnaK (50% homologous to Hsp70) nor the constitutively expressed, highly homologous human Hsc70 (84% homology to Hsp70) were able to stimulate NK cell activity (Multhoff *et al.,* 1999). In contrast, APCs have been found to interact with several HSPs, including Hsc70, Hsp70, Hsp90, gp96, and Hsp60 (Kol *et al.,* 2000; Ohashi *et al.,* 2000; Habich *et al.,* 2002). However, it is still a matter of debate as to which receptors interact with a distinct HSP. For NK cells, a 14-mer Hsp70 peptide derived from the C-terminal domain of Hsp70 could be determined as the minimal stimulatory sequence (Multhoff *et al.,* 2001). This peptide sequence has been found to be unique for the human stress-inducible Hsp70 as determined by protein database blast search (Altschul *et al.*, 1997). Already two conservative amino acid exchanges within this peptide at positions 458 and 462 abrogate the stimulatory activity of the peptide. To our knowledge, no HSP70 or HSP90 peptide sequence has been identified that exhibits such selectivity for APCs.

Although saturability and specificity, as two attributes of a receptor-ligand interaction, have been demonstrated for the interaction of Hsp70 protein and Hsp70 peptide with NK cells, and although it became obvious that APCs use different receptors, the molecular nature of the putative Hsp70 receptor on NK cells remains elusive. Our data provide evidence that the C-type lectin receptor CD94, known to interact with HLA-E molecules, presenting leader peptides of MHC class I molecules (Braud *et al.,* 1998; Lanier *et al.,* 1998; Long, 1999), is involved in Hsp70 interaction. An increased CD94 expression is observed if purified NK cells are incubated either with Hsp70 protein or the Hsp70 peptide TKD in combination with low dose IL-2. A CD94 antibody specifically reduces the cytolytic activity of NK cells against Hsp70-positive tumor target cells (Multhoff *et al.,* 1999; Multhoff *et al.,* 2001) and also suppresses binding of Hsp70 to YT cells. Finally, Hsp70 binding correlates with the expression levels of CD94, and CD94 is the only receptor commonly expressed on the cell surface of NK cells and the NK cell line YT. However, CD94 alone is unlikely to be responsible for the interaction with Hsp70 for several reasons: (i) CD94 as a homodimer is unable to mediate cell signalling and (ii) transiently CD94 transfected COS cells did not exhibit significant Hsp70 protein or Hsp70 peptide binding (data not shown). Although our data support the idea that CD94 is involved in the interaction of NK cells with Hsp70 protein and Hsp70 peptide TKD, the co-receptor of CD94 that mediates signalling following contact with Hsp70 has not yet been defined.

Materials and Methods

Cell Lines

The IL-2 independent, CD7-positive, leukemic NK cell line YT (ACC 434, DSMZ, Heidelberg, Germany) was cultivated in RPMI-1640 medium (Gibco-BRL, Life Technologies, Rockville, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, BioWhittaker, Walkersville, USA), 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin (all supplements were purchased from Life Technologies); doubling time was between 40 and 50 h at constant cell densities ranging from $0.1 - 0.8 \times 10^6$ cells/ml. Increasing cell densities correlate with decreased CD94 expression; cell densities above 0.8×10^6 cells/ml, result in a significantly decreased cell viability (Yodoi *et al.,* 1985; Drexler and Matsuo, 2000).

The myelogenous cell line K562 was purchased from ATCC (CCL 243, Rockville, USA) and grown in YT cell medium. The non-NK cell line K562 was used as a negative control in binding studies.

Monoclonal Antibodies and Flow Cytometry

The following mouse or rat-anti-human monoclonal antibodies (mAb), either unconjugated* or fluorescein-conjugated, were used for the phenotypic characterization of YT cells and for binding studies: isotype-matched control antibodies (Becton Dickinson, Heidelberg, Germany; Coulter Immunotech, Marseille, France), CD3 (IgG1, Dianova, Hamburg, Germany), CD6 (IgG1, Pharmingen, Becton Dickinson, Heidelberg, Germany), CD7 (IgG2a, Coulter, Immunotech), CD8 (IgG1, Dianova), CD11b* (IgG1, Coulter Immunotech), CD16 (IgG1, Becton Dick-

inson), CD56 (IgG2b, Becton Dickinson), CD57 (IgM, Coulter Immunotech), CD91* (IgG1, Progen Biotechnik, Heidelberg, Germany), CD94 (IgG1, HP-3D9, Ancell Immunology Research Products, Bayport, USA), CD158a (IgG1, p58.1, Coulter Immunotech), CD158b (IgG1, p58.2, Coulter Immunotech), CD161 (IgG2a, Coulter Immunotech), NKG2A (IgG1, Z270, kindly provided by L. Moretta), W6/32* (IgG2a, Cymbus Biotechnology, Dianova), TLR 2 (IgG2a, eBioscience, San Diego, USA), TLR 4 (IgG2a, eBioscience), TLR 9* (IgG1, Biocarta Europe, Hamburg, Germany), Hsp70* mAbs (IgG1, SPA810, StressGen, British Columbia, Canada), and clone C92F3B1 (IgG1, multimmune GmbH, Regensburg, Germany) (Welch and Suhan, 1986), glutathione S-transferase (GST) rat-anti-human mAb (kindly provided by Dr. E. Kremmer, GSF – Institute for Molecular Immunology, Munich, Germany). All antibody preparations contained 0.1% sodium azide.

For flow cytometry, 0.5 – 1×10^6 cells were incubated for 30 min at 4 °C, either with unconjugated* or fluorescence-conjugated primary mAbs. After washing in PBS conditioned with 2% FCS, the cells were either analyzed directly by flow cytometry or following incubation with a secondary FITC-rabbit anti-mouse/rat Ig Ab (Dianova, Hamburg, Germany) for another 30 min at 4 °C. Then, the cells were analyzed on a FACSCalibur instrument (Becton Dickinson). The percentage of positively-stained cells was calculated as the number of specifically-stained, propidium-iodide (PI)-negative, viable cells minus the number of cells stained with an isotype-matched control antibody.

FITC-Labeling of Hsp70 Protein, Bovine Serum Albumin (BSA), Glutathione S-Transferase (GST) and Hsp70 Peptide TKD

Hsp70 protein (1 mg/ml; StressGen), BSA (1 mg/ml; fraction V; Sigma, St. Louis, USA), and GST (1 mg/ml; Sigma) were incubated with fluoresceine-isothiocyanate (FITC, isomer I, Sigma) in 0.1 M sodium bicarbonate (pH 9.5) overnight at 4 °C. Unconjugated dye was removed by passing the reaction mixture over a PD-10 desalting column (Sephadex G-25, Amersham Bioscience, Uppsala, Sweden) equilibrated in PBS. Eluted fractions containing FITC-labeled protein were pooled and stored at 4 °C in the dark.

The number of dye molecules bound per molecule protein was determined by measuring the OD at 280 nm and 495 nm. It was calculated as 4.1 for Hsp70 protein, 4.4 FITC molecules for BSA, and 4.5 FITC molecules for GST, respectively. Before use the solutions of the FITC-labeled proteins were centrifuged at 100 000 *g* in order to remove small particles.

FITC-labeled Hsp70 peptide TKD (Multhoff *et al.,* 2001) was derived as a 94.3% pure, 2038.23 Da ($C_{92}H_{128}N_{22}O_{29}S$) FITC-γ-Abu-TKDNNLLGRFELSG-OH lyophylized material (Bachem, Bubendorf, Switzerland). The powder was diluted in sterile PBS at a stock concentration of 1 mg/ml. All experiments using FITC-labeled proteins and peptides were performed with identical stock solution, within three months.

Binding of BSA, GST, Hsp70 Protein, Hsp70C and Hsp70 Peptide TKD to YT Cells

For binding studies, the following proteins and peptides, either unlabeled or FITC-labeled, were used: bovine serum albumin (BSA), glutathione S-transferase (GST), full-length recombinant Hsp70 protein (Hsp70; SPP755, StressGen), a fusion protein, consisting of the C-terminal substrate binding domain (aa 384 – 563) of Hsp70 and glutathione S-transferase, (Hsp70C-GST), and Hsp70 peptide TKD. Briefly, YT cells (5×10^6 cells/ml), harvested at a cell density of 0.1 – 0.8×10^6, were incubated for 45 min at 4 °C with proteins and peptides at different concentrations in a total volume of 1 ml PBS containing 2% FCS. For BSA and Hsp70 protein the concentrations 10, 25, 50, and 100 µg/ml, for Hsp70C-GST 10 µg/ml and for Hsp70 peptide TKD 0.2, 2.0, 4.0, and 8.0 µg/ml, were used. Binding of non-FITC-labeled proteins was measured indirectly by flow cytometry, using mAbs against Hsp70 (SPA810, StressGen) and against GST. Binding of FITC-labeled BSA, GST, Hsp70 protein, and Hsp70 peptide TKD was determined by direct flow cytometry, after incubation of viable, non-fixed YT or K562 cells with the relevant labeled or unlabeled proteins and peptides, at 4 °C. Directly before flow cytometric analysis the cells were co-stained with propidium iodide (PI). Only PI-negative viable cells were gated and analyzed on a FACSCalibur instrument. As negative controls, either the fluorescence of untreated cells was determined (autofluorescence), or untreated cells were stained with primary and secondary mAbs (mAb ctrl), or cells treated with protein were incubated with the relevant isotype-matched control Ab and secondary Ab (isotype ctrl). Binding was determined in percent: the amount of untreated cells (autofluorescence) or cells incubated with the adequate negative control Abs was subtracted from the amount of positively-stained cells that had been incubated with the different proteins and peptides.

Fluorescence Microscopy

In order to visualize binding, 5×10^6 YT cells were incubated for 45 min at 4 °C, with 50 µg/ml Hsp70-FITC or with 8.0 µg/ml TKD-FITC, as described above. Protein and peptide uptake were studied on non-fixed cells that were allowed to bind TKD-FITC and Hsp70-FITC at 4 °C. After two washing steps in PBS, the cells were incubated at 37 °C for 30 min.

Following cytospin, the paraformaldehyde-fixed cells were embedded in Fluorescent Mounting Medium (Neo-Mount, Merck, Darmstadt, Germany). The samples were analyzed for transmission and fluorescence using a Zeiss Axioscop 2 scanning microscope (Zeiss Jena, Germany) equipped with a 63× (apochromat) objective.

Acknowledgments

The work was supported by the Wilhelm-Sander Stiftung (2000.054.1), BMBF (BioChance, 0312338) and by multimmune GmbH. The authors want to thank Gerald Thonigs and Lydia Roßbacher for excellent technical assistance, Dr. Michael Rehli for transfection experiments and Prof. Dr. Walter Koelch for help with protein database BLAST SEARCH.

References

Altschul, S., Madden, T.L., Schaeffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389 – 3409.

Arnold-Schild, D., Hanau, D., Spehner, D., Schmid, C., Rammensee, H.-J., Salle, E.H., and Schild, H.-J. (1999). Receptor-mediated endocytosis of hsp by professional antigen-presenting cells. J. Immunol. *162,* 3757 – 3760.

Asea, A., Kraeft, S.K., Kurt-Jones, E.A., Stevenson, M-A., Chen, L.B., Finberg, R.W., Koo, G.C., and Calderwood, S.K. (2000a). Hsp70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperone and cytokine. Nature Med. *6,* 435 – 442.

Asea, A., Kabingu, E., Stevenson, M.A., and Calderwood, S.K. (2000b). Hsp70 peptide-bearing and peptide-negative prepa-

rations act as chaperokines. Cell Stress Chaperones *5*, 425 – 431.

Basu, S., Binder, R.J., Ramalingam, T., and Srivastava, P.K. (2001). CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70 and calreticulin. Immunity *14,* 303 – 313.

Binder, R.J., Harris, M.L., Menoret, A., and Srivastava, P.K. (2000a). Saturation, competition, and specificity of heat shock proteins (hsp) gp96, hsp90, and hsp70 with CD11b$^+$ cells. J. Immunol. *165,* 2582 – 2586.

Binder, R.J., Han, D.K., and Srivastava, P.K. (2000b). CD91: a receptor for heat shock protein gp96. Nature Immunol. *1,* 151 – 155.

Binder, R.J., Blachere, N.E., and Srivastava, P.K. (2001). Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. J. Biol. Chem. *276*, 17163 – 17171.

Botzler, C., Issels, R., and Multhoff, G. (1996). Heat-shock protein 72 cell-surface expression on human lung carcinoma cells in associated with an increased sensitivity to lysis mediated by adherent natural killer cells. Cancer Immunol. Immunother. *43,* 226 – 230.

Botzler, C., Li, G., Issels, R., and Multhoff, G. (1998). Definition of extracellular localized epitopes of Hsp70 involved in an NK immune response. Cell Stress Chaperones *3*, 6 – 11.

Braud, V.M., Allan, D.S.J., O`Callaghan, C.A., Soderstrom, K., D`Andrea, A., Ogg, G.S., Lazetic, S., Young, N.T., Bell, J.I., Phillips, J.A., Lanier, L.L., and McMichael, J.A. (1998). HLA-E binds to natural killer cell receptors CD94/NKG2A, B, and C. Nature *391,* 795 – 798.

DeNagel, D.C., and Pierce, S.K. (1992). A case for chaperones in antigen processing. Immunol. Today *3*, 86 – 89.

Drexler, H.G., and Matsuo, Y. (2000). Malignant hematopoietic CLL lines: *in vitro* models for the study of natural killer cell leukemia-lymphoma. Leukemia *14,* 777 – 782.

Hantschel, M., Pfister, K., Jordan, A., Scholz, R., Andreesen, R., Schmitz, G., Schmetzer, H., Hiddemann, W., and Multhoff, G. (2000). Hsp70 plasma membrane expression on primary tumor biopsy material and bone marrow of leukemic patients. Cell Stress Chaperones *5,* 438 – 442.

Habich, C., Baumgart, K., Kolb, H., and Burkart, V. (2002). The receptor for HSP60 on macrophages is saturable, specific and distinct from receptors for other HSPs. J. Immunol. *168,* 569 – 576.

Hartl, F.U. (1996). Molecular chaperones in protein folding. Nature *381,* 571 – 580.

Kol, A., Lichtman, A.H., Finberg, R.W., Libby, P., and Kurt-Jones, E.A. (2000). Hsp60 activates the innate immune response: CD14 is an essential receptor for Hsp60 activation of mononuclear cells. J. Immunol. *164,* 13 – 21.

Küppner, M., Gastpar, R., Gelwer, G., Nössner, E., Ochmann, O., Scharner, A., and Issels, R. (2001). The role of Hsp70 in dendritic cell maturation: Hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. Eur. J. Immunol. *31,* 1602 – 1609.

Lanier, L.L. (2000). Turning on natural killer cells. J. Exp. Med. *191,* 1259 – 1262.

Lanier, L.L., Ruitenberg, J.J., and Phillips, J.H. (1988). Functional and biochemical analysis of CD16 antigen on NK cells and granulocytes. J. Immunol. *141,* 3478 – 3485.

Lanier, L., Chang, C., and Phillips, J.H. (1994). Human NKR-P1A – a disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. J. Immunol. *153,* 2417 – 2419.

Lanier, L.L, Corliss, B., Wu, J., and Phillips, J.H. (1998). Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity *8,* 693 – 701.

Lindquist, S., and Craig, E.A. (1988). The heat shock proteins. Annu. Rev. Genet. *22,* 631 – 677.

Long, E.O. (1999). Regulation of immune responses through inhibitory receptors. Annu. Rev. Immunol. *17,* 875 – 904.

Moretta, A., Sivori, S., Vitale, M., Pende, D., Morelli, L., Augugliaro, R., Bottino, C., and Moretta, L. (1995). Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J. Exp. Med. *182*, 875 – 879.

Multhoff, G. (1997). Hsp72, a hyperthermia-inducible immunogenic determinant on leukemic K562 and Ewing`s sarcoma cells. Int. J. Hyperthermia *13,* 39 – 48.

Multhoff, G., Botzler, C., Wiesnet, M., Müller, E., Meier, T., Wilmanns, W., and Issels, R. (1995a). A stress-inducible 72 kDa heat-shock protein (Hsp72) is expressed on the surface of human tumor cells, but not on normal cells. Int. J. Cancer *61*, 272 – 275.

Multhoff, G., Botzler, C., Wiesnet, M., Eissner, G., and Issels, R. (1995b). CD3$^-$ large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72 kDa heat shock protein on human sarcoma cells. Blood *86,* 1374 – 1382.

Multhoff, G., Botzler, C., Jennen, L., Schmidt, J., Ellwart, J., and Issels, R. (1997). Heat shock protein 72 on tumor cells. A recognition structure for natural killer cells. J. Immunol. *158,* 4341 – 4350.

Multhoff, G., Mizzen, L., Winchester, C.C., Milner, C.M., Wenk, S., Kampinga, H.H., Laumbacher, B., and Johnson, J. (1999). Heat shock protein 70 (Hsp70) stimulates proliferation and cytolytic activity of NK cells. Exp. Hematology *27,* 1627 – 1636.

Multhoff, G., Pfister, K., Botzler, C., Jordan, A., Scholz, R., Burgstahler, R., and Hiddemann, W. (2000). Adoptive transfer of human NK cells in mice with severe combined immunodeficiency inhibits growth of Hsp70 expressing tumors. Int. J. Cancer *88,* 791 – 797.

Multhoff, G., Pfister, K., Gehrmann, M., Hantschel, M., Gross, C., Hafner, H., and Hiddemann, W. (2001). A 14-mer Hsp70-peptide stimulates NK cell activity. Cell Stress Chaperones *6,* 337 – 374.

Ohashi, K., Burkart, V., Flohe, S., and Kolb, H. (2000). Hsp60 is a putative endogeneous ligand of the Toll-like receptor 4 complex. J. Immunol. *164,* 558 – 563.

Perez-Villar, J.J., Melero, I., Rodriguez, A., Carretero, M., Aramburu, J., Sivori, S., Orengo, A.M., Moretta, A., and Lopez-Botet, M. (1995). Functional ambivalence of the Kp43 (CD94) NK cell associated surface antigen. J. Immunol. *154,* 5779 – 5782.

Pfeiffer, A., Böttcher, A., Orso, E., Kapinsky, M., Nagy, P., Bodnar, A., Spreitzer, I., Liebisch, G., Drobnik, W., Gempel, K. *et al.* (2001). Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. Eur. J. Immunol. *31,* 3153 – 3164.

Reineke, U., Ehrhard, B., Sabat, R., Volk, H.D., and Schneider-Mergener, J. (1996). Mapping discontinuous epitopes using-cellulose-bound peptide libraries. Immunobiol. *196,* 96 – 104.

Schild, H.-J., Arnold-Schild, A., Lammert, E., and Rammensee, H.-G. (1999). Stress proteins and immunity mediated by cytotoxic T lymphocytes. Curr. Opin. Immunol. *11,* 109 – 114.

Sondermann, H., Becker, T., Mayhew, M., Wieland, F., and Hartl, F.-U. (2000). Characterization of a receptor for heat shock protein 70 on macrophages and monocytes. Biol. Chem. *381,* 1165 – 1174.

Vales-Gomez, M., Reyburn, H., and Strominger, J. (2000). Mole-

cular analysis of the interactions between human NK receptors and their HLA ligands. Human Immunol. *61,* 28–38.

Vujanovic, N.L., Yasumura, S., Hirabayashi, H., Lin, W.C., Watkins, S., Herberman, R.B., and Whiteside, T.L. (1995). Antitumor activities of subsets of human IL-2 activated natural killer cells in solid tissues. J. Immunol. *154,* 281–289.

Welch, W.J., and Suhan, J.P. (1996). Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell. Biol. *103,* 2035–2052.

Wells, A.D., and Malkovsky, D. (2000). Heat shock proteins, tumor immunogenicity, and antigen presentation: an integrated view. Immunol. Today *21,* 129–132.

Yodoi, J., Teshigawara, K., Nikaido, T., Fukui, K., Noma, T., Honjo, M., Takigawa, M. Sasaki, M., Minato N., Tsudo, M., Uchiyama, T., and Maeda, M. (1985). TCGF (IL-2)-receptor inducing factors. I. Regulation of IL-2 receptor on a NK-like cell line (YT cells). J. Immunol. *134,* 1623–1630.

Received May 22, 2002; accepted September 2, 2002

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 42, Issue of October 17, pp. 41173–41181, 2003
Printed in U.S.A.

Cell Surface-bound Heat Shock Protein 70 (Hsp70) Mediates Perforin-independent Apoptosis by Specific Binding and Uptake of Granzyme B*

Received for publication, March 14, 2003, and in revised form, June 19, 2003
Published, JBC Papers in Press, July 21, 2003, DOI 10.1074/jbc.M302644200

Catharina Gross‡, Walter Koelch§, Antonio DeMaio¶, Nelson Arispe‖, and Gabriele Multhoff‡**

From the ‡University Hospital Regensburg, Department of Hematology, 93053 Regensburg, Germany, the §University of Glasgow CRC Beatson Laboratories, Institute of Biomedical and Life Sciences, Glasgow G61 1BD, Scotland, United Kingdom, the ¶Division of Pediatric Surgery and Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the ‖Department of Anatomy and Cell Biology, Uniformed Services of Health Sciences, Bethesda, Maryland 20814

Cell surface-bound heat shock protein 70 (Hsp70) renders tumor cells more sensitive to the cytolytic attack mediated by natural killer (NK) cells. A 14-amino acid Hsp70 sequence, termed TKD (TKDNNLLGRFELSG, $aa^{450-463}$) could be identified as the extracellular localized recognition site for NK cells. Here, we show by affinity chromatography that both, full-length Hsp70-protein and Hsp70-peptide TKD, specifically bind a 32-kDa protein derived from NK cell lysates. The serine protease granzyme B was uncovered as the 32-kDa Hsp70-interacting protein using matrix-assisted laser desorption ionization time-of-flight mass peptide fingerprinting. Incubation of tumor cells with increasing concentrations of perforin-free, isolated granzyme B shows specific binding and uptake in a dose-dependent manner and results in initiation of apoptosis selectively in tumor cells presenting Hsp70 on the cell surface. Remarkably, Hsp70 cation channel activity was also determined selectively in purified phospholipid membranes of Hsp70 membrane-positive but not in membrane-negative tumor cells. The physiological role of our findings was demonstrated in primary NK cells showing elevated cytoplasmic granzyme B levels following contact with TKD. Furthermore, an increased lytic activity of Hsp70 membrane-positive tumor cells could be associated with granzyme B release by NK cells. Taken together we propose a novel perforin-independent, granzyme B-mediated apoptosis pathway for Hsp70 membrane-positive tumor cells.

Elevated cytoplasmic Hsp70[1] levels have been found to protect tumor cells against programmed cell death (1). However, evidence has accumulated indicating that the presence of Hsp70 on the plasma membrane or in the extracellular milieu is highly immunogenic and exposes target cells to immunological attack (2). Following receptor-mediated uptake (3) and re-presentation by antigen presenting cells, HSP-chaperoned peptides elicit a cytotoxic, CD8+ T cell response (4). Recently, several receptors, including CD91, Toll-like receptors 2/4 (TLR2/4), and CD40 (5) have been identified to mediate the interaction of HSP90- (gp96), HSP70- (Hsp70, Hsc70), and HSP60-peptide complexes with antigen presenting cells (6–9). A peptide-independent "chaperokine effect" has been described for members of the HSP70 family. Binding of exogeneous HSP70 to monocytes via TLR2/4 induces receptor clustering in a CD14-dependent pathway (10) and the secretion of proinflammatory cytokines via the MyD88/IRAK/NFκ-B signal transduction pathway (11–13). We detected Hsp70, the major-stress inducible member of the HSP70 family, selectively in the plasma membrane of tumor cells, but not in normal cells by cell surface biotinylation and immunofluorescence (14). This finding was confirmed most recently by proteomic profiling of tumor cell membranes (15).

The amount of membrane-bound Hsp70 on tumor cells positively correlates with their sensitivity to lysis mediated by natural killer (NK) cells. Physical (*i.e.* heat, irradiation) and chemical (*i.e.* cytostatic drugs, alkyllysophospholipids) stress has been found to increase Hsp70 surface expression on tumor cells and thereby renders them better targets for NK cells (16–18). Incubation of purified NK cells with recombinant Hsp70 increases the cytolytic activity against Hsp70 membrane-positive tumor cells (19). NK cells have been found to interact specifically with a 14-amino acid Hsp70 sequence, termed TKD (TKDNNLLGRFELSG, $aa^{450-463}$), on the C-terminal of this protein. This region (TKD) is present in the ectoplasmic domain of viable tumor cells (20). Therefore, it was not surprising that similar to full-length Hsp70 (19), Hsp70-peptide TKD exhibits a comparable immunostimulatory capacity to NK cells (20). Although the preceding observations indicate that Hsp70-peptide functions as a tumor-selective target recognition structure for NK cells (21), the mechanism by which NK cells lyse Hsp70 membrane-positive tumor target cells remained to be elucidated.

EXPERIMENTAL PROCEDURES

Cells

The NK cell line YT was cultured at low cell densities ranging between 0.1 and 0.5 × 10^6 cells/ml in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) supplemented with 6 mM L-glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; Invitrogen).

The tumor sublines CX+/CX− and Colo+/Colo− were derived by cell

* This work was supported by a grant of the Wilhelm-Sander Stiftung, European Union Grant TRANSEUROPE QLRT 2001 01936, and by multimmune GmbH. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Prof. for Molecular Oncology, University Hospital Regensburg, Dept. of Hematology, Franz-Josef Strauss Allee 11, 93053 Regensburg, Germany. Tel.: 49-941-944-5575; Fax: 49-941-944-5572; E-mail: gabriele.multhoff@klinik.uni-regensburg.de.

[1] The abbreviations used are: Hsp, heat shock protein; NK, natural killer; BSA, bovine serum albumin; PE, phycoerythrin; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; grB, granzyme B.

sorting of CX-2 colon (Hsp70 positive: 60%) and Colo357 pancreas (Hsp70 positive: 60%) carcinoma cell lines using the Hsp70-specific monoclonal antibody C92F3B1 (Multimmune GmbH, Regensburg, Germany), according to a previously described protocol (21). Hsp70 stably high expressing CX+ (Hsp70 positive: 80%) and Colo+ (Hsp70 positive: 85%) carcinoma sublines differ significantly from Hsp70 stably low expressing CX− (Hsp70 positive: 25%) and Colo− (Hsp70 positive: 35%) carcinoma cells. Carcinoma sublines and the non-NK leukemic cell line K562 were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 6 mM L-glutamine and antibiotics. Exponentially growing tumor cells (day 1 after feeding of the cells) were used for granzyme B and camptothecin treatment.

All cell lines were screened regularly for mycoplasma contaminations by an enzyme immunoassay detecting *Mycoplasma arginini*, *Mycoplasma hyorhinis, Achdeoplasma laidlawii*, and *Mycoplasma orale* (Roche Diagnostics). Only mycoplasma-free cell lines were used.

Primary NK cells were generated by CD3-depletion of peripheral blood mononuclear cells using a standard Miltenyi biotin separation method (Miltenyi, Bergisch Gladbach, Germany). Sorted cells were activated by incubation of the NK cells for 3 days in supplemented RPMI 1640 medium containing 100 IU/ml interleukin-2 (Chiron, Ratingen, Germany) and 2 μg/ml Hsp70-peptide TKD (TKDNNLLGRFELSG, $aa^{450-463}$, Bachem Bubendorf, Switzerland).

Affinity Chromatography and Immunoprecipitation

Bovine serum albumin (BSA, 1 mg/ml, Sigma), lyophilized, recombinant human Hsp70-protein (1 mg/ml, StressGen, British Columbia, Canada), and Hsp70-peptide TKD (1 mg/ml, Bachem, Bubendorf, Switzerland) were incubated with equilibrated AminoLink-agarose beads (2 ml, Pierce) for 6 h, together with the reductant $NaCNBH_3$, followed by removal of uncoupled material by extensive washes with Tris buffer and quenching non-reactive groups. Binding capacity of BSA, Hsp70-protein, and Hsp70-peptide TKD was greater than 95%. Cell lysates of NK cells and the leukemic non-NK cell line K562 were loaded onto BSA, Hsp70-protein, and Hsp70-peptide TKD-conjugated columns for 1 h at room temperature.

Columns were washed with 10 column volumes of 20 mM Tris buffer, bound proteins were eluted with 3 M sodium chloride in 20 mM Tris buffer, in 5 fractions (1 ml). Each fraction was subjected to a SDS-PAGE using a 10% polyacrylamide slab gel and transferred onto polyvinylidene difluoride membranes.

Western Blot Analysis

Blots were blocked with skim milk (0.1%) and incubation with monoclonal antibody directed against granzyme B (2C5, IgG2a, BD Biosciences, Heidelberg, Germany), for 5 h at 4 °C. Blots were washed and incubated with a secondary mouse anti-IgG horseradish peroxidase antibody (Dianova, Hamburg, Germany), for 1 h at 4 °C. Proteins were detected using the ECL kit (Amersham Biosciences) for 5 s.

Protein Identification by Peptide Mass Fingerprinting

A 32-kDa protein band was isolated by affinity chromatography on immobilized Hsp70-protein or Hsp70-peptide TKD, which was excised from Coomassie Blue-stained gels, digested with trypsin, and desalted using reversed phase ZIP tips (Millipore, Eschborn, Germany). The samples were embedded in 4-hydroxy-α-cyanocinnamic acid and the peptide masses were determined with a Perseptive Voyager DePro matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer in reflective mode. A peaklist was compiled with the *m/z* software (Proteometrics) and used for peak selection; the resulting peptide mass fingerprint was used to search the non-redundant NCBI protein data base using the Profound search engine (Proteometrics). Granzyme B was identified with 100% probability and >95% confidence.

Membrane Preparation

Membrane purification was performed by Dounce homogenization (100×10^6 tumor cells) in hypotonic, EDTA-free buffer containing the protease inhibitor phenylmethylsulfonyl fluoride followed by sequential centrifugation at $1{,}000 \times g$ for 5 min and at $100{,}000 \times g$, at 4 °C, for 60 min. The pellet containing membranes was resuspended in 2 ml of 0.3 M NaCl in 50 mM Tris buffer, 0.5% Nonidet P-40, pH 7.6.

Cation Channel Activity Measurements

Large unilamellar liposomes were prepared from plasma membranes derived of Hsp70 membrane-positive and membrane-negative tumors and applied to an orifice of about 100–120 μm in diameter with a Teflon film separating two compartments. The ionic solutions containing either symmetrical concentrations of KCl ($200_{cis}/200_{trans}$ mM) or asymmetric concentrations ($200_{cis}/50_{trans}$ mM) and 5 mM K-Hepes, 7.1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and ATP (2 mM). The two ionic compartments were electrically connected via agar bridges and Ag/AgCl pellet electrodes to the input of a voltage clamp amplifier. Current was recorded using patch clamp amplifier (Axopatch-1D; Axon Instruments, CA) and data were stored on a PCM/VCR digital system (Toshiba) with a frequency response in the range from direct current to 25,000 Hz. Off-line analysis of the channel activity was carried out using the software package Pclamp 6 and Axosope (Axon Instruments).

Flow Cytometry

Cells (0.5×10^6) were fixed in paraformaldehyde (1% paraformaldehyde in PBS) for 10 min, and permeabilized in PBS containing BSA (0.5%), NaN_3 (0.1%), and saponin (0.1%). Permeabilized cells were incubated either with the granzyme B-phycoerythrin (PE)-conjugated monoclonal antibody HC2-PE (IgG1; Hölzel Diagnostika, Cologne, Germany) or with an isotype-matched IgG1 control antibody, at 4 °C for 1 h, in the dark. Following washing intracellular localized granzyme B was analyzed on a FACSCalibur instrument (BD Biosciences).

Treatment

Stock solutions of camptothecin (4 mg/ml, Sigma, Munich, Germany) were diluted in dimethyl sulfoxide and stored at 4 °C in the dark. Granzyme B (Hölzel Diagnostics, Cologne, Germany) solutions were freshly prepared directly before usage. Exponentially growing cells ($0.5–1.5 \times 10^6$/ml) were incubated either with camptothecin at a final concentration of 4 μg/ml or with purified, enzymatically active granzyme B (10 ng/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml) (22) for 10 min, and 30 min either at 4 or 37 °C. After washing in RPMI 1640 medium binding and uptake was determined in non-permeabilized and permeabilized tumor cells by flow cytometry and fluorescence microscopy on a Axioscop 25 scanning microscope (Zeiss, Jena, Germany) equipped with a ×40 objective and standard filters. Images were treated by multiplicative shading correction using the software Axiovison (Zeiss Vison, Jena, Germany). Granzyme B was visualized in red by using the HC2-PE antibody.

Apoptotic cell death was measured after incubation of tumor cells with 10 ng/ml granzyme B for 4, 12, and 24 h by different apoptosis assays, as described below.

Apoptosis Assays

Annexin V-FITC Staining—Briefly, cells were washed twice in Hepes buffer containing 5 mM $CaCl_2$ and incubated with annexin V-FITC (Roche Diagnostics) for 10 min at room temperature. Annexin V-FITC positively stained cells were measured on a FACSCalibur flow cytometer (BD Biosciences).

DAPI Staining—Methanol/acetone fixed cells (0.1×10^6 cells/100 μl) were incubated with 0.5 μg/μl 4,6-diamino-2-phenylindole (DAPI) in PBS/glycerol (3:1) for 15 min in the dark. Following washing in PBS the cells were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) and analyzed for fluorescence using a Zeiss model Axioscop 2 scanning microscope (Zeiss, Jena, Germany) equipped with a ×40 objective and standard filters. Apoptosis was visualized with DAPI staining in 50 cells, each. Images were treated by multiplicative shading correction using the software Axiovision (Zeiss).

Cytochrome c—Cytochrome *c* was determined using a quantitative immunoassay (DCDCO, R&D Systems, Wiesbaden, Germany). Briefly, untreated, camptothecin (4 μg/ml), or granzyme B (10 ng/ml)-treated cells (1.5×10^6/ml) were washed in PBS and treated with lysis buffer for 1 h at room temperature. Following centrifugation at $1{,}000 \times g$ for 15 min, supernatants were removed and 200 μl of a 1:100, 1:250, and a 1:500 dilution was used for a sandwich enzyme-linked immunosorbent assay. Following incubation with substrate solution in the dark for 30 min the reaction was stopped. The optical density of each well was determined on an enzyme-linked immunosorbent assay reader at 450 nm. The amount of cytochrome *c* was determined according to a calibration curve.

^{51}Cr Release Assay and Inhibition Assay

NK cell-mediated cytotoxicity was measured using a 12-h ^{51}Cr radioisotope assay. As target cells the colon carcinoma sublines CX+ and CX− were used. For blocking studies the monoclonal antibody C92F3B1 and an isotype matched control antibody (IgG1) were used at a final concentration of 5 μg/1×10^6 cells. Following incubation of CX+ and CX− target cells with the antibodies for 30 min at 4 °C, the cells were labeled with ^{51}Cr and the cytotoxicity assay was performed as described

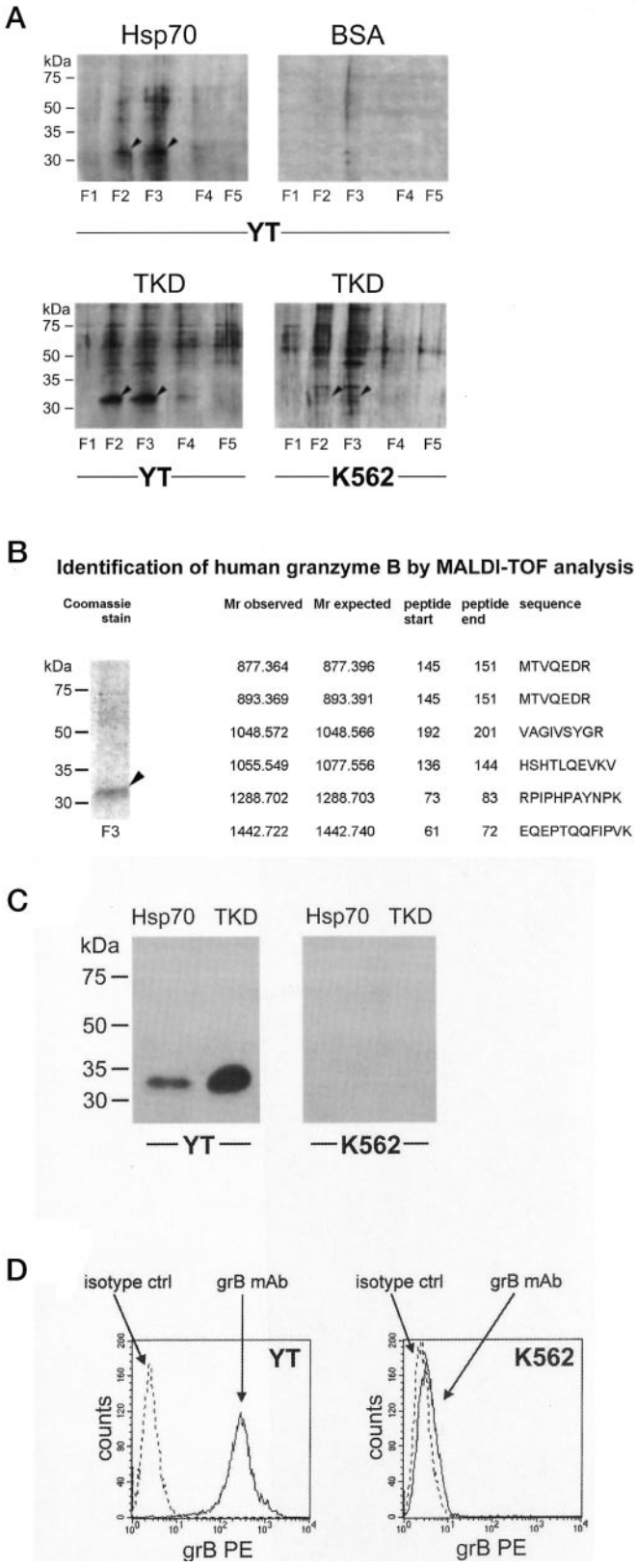


FIG. 1. **Identification of granzyme B as the interacting partner for Hsp70-protein and Hsp70-peptide TKD.** *A*, Hsp70-protein (Hsp70), BSA, and Hsp70-peptide (TKD) columns were incubated either with cell lysates of the NK cell line YT or the non-NK cell line K562. Bound proteins were eluted from the columns in 5 fractions (*F1–F5*), resolved on SDS-PAGE. Following silver stain, eluates of YT cells derived from Hsp70 and TKD columns revealed a dominant 32-kDa protein band in fractions two (*F2*) and three (*F3*). No 32-kDa protein band was detectable in YT eluates derived from BSA columns and in K562 eluates derived from TKD columns. The position of the 32-kDa band is indicated with an *arrowhead*. *B*, the tryptic peptides of the Coomassie Blue-stained 32-kDa band of fraction 3 (*F3*), derived from the TKD column, correspond to human granzyme B. The probability of identification was 100% and the estimated Z-score was 1.89 corresponding to >95% confidence. *C*, corresponding Western blot analysis of YT and K562 cell eluates (*F3*) following incubation with Hsp70-protein (Hsp70) and Hsp70-peptide (TKD) columns. The blot was autoradiographed and the localization of granzyme B was visualized by immunostaining with the granzyme B specific monoclonal antibody 2C5. Eluates of YT cells (*left*), but not of K562 cells (*right*) revealed a 32-kDa granzyme B protein band. *D*, intracellular flow cytometry of permeabilized YT cells (*left*) and K562 cells (*right*) using the PE-conjugated granzyme B-specific monoclonal antibody HC2-PE (*solid line*), as compared with an isotype-matched negative control antibody (*dashed line*). Only YT cells, but not K562 cells, contain cytoplasmic granzyme B.

by MacDonald (23). The percentage of specific lysis was calculated as: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100.

Granzyme B ELISPOT

Granzyme B release was compared in unstimulated NK cells (NK d0) and TKD-stimulated NK cells (NK d3) after a 4-h co-incubation period with tumor cell lines CX+/CX− and Colo+/Colo−, at different effector to target cell ratios (E:T) ranging from 20:1 to 2:1. For detection a granzyme B ELISPOT kit (number 552572, BD Biosciences) was used. Briefly, 96-well ELISPOT plates (MAIPN45, Millipore) were coated overnight at 4 °C with capture antibody, blocked with RPMI 1640 culture medium containing 10% fetal calf serum and incubated with tumor and effector cells for 4 h at 37 °C, as specified before. After washing in deionized water and wash buffers A and B, biotinylated anti-granzyme B antibody was added (2 μg/ml) for 2 h. After another two washing steps granzyme B was visualized by the addition of freshly prepared avidin-horseradish peroxidase (1 h) and substrate solution (25 min incubation period). Spots were counted automatically using Immuno Spot Series I analyzer.

RESULTS

Granzyme B Is a Partner of Hsp70 and Hsp70-Peptide TKD—Proteins that interact with Hsp70 were identified by affinity chromatography on immobilized Sepharose columns coupled either with human Hsp70-protein or a peptide derived from the C-terminal region of Hsp70, termed TKD (TKDNNLLGRFELSG, aa$^{450-463}$). TKD corresponds to 14-amino acids localized in the extracellular domain of Hsp70, present on Hsp70 membrane-positive tumor cells, which mediate recognition by NK cells (20). TKD was used in the affinity column to exclude the recognition of proteins by the substrate binding domain of Hsp70. Recently, we showed that Hsp70-protein and TKD, specifically bind to the NK cell line YT (24). Therefore, we assumed that YT cells provide an ideal tool for identifying Hsp70-interacting partners. YT cell lysates were fractionated on immobilized Hsp70 and TKD columns. The material bound to the columns was eluted with 3 M sodium chloride in five fractions. As controls, YT cell lysates were administered to carrier or BSA-conjugated columns. Moreover, lysate of a non-NK cell line (K562) was fractionated on TKD-conjugated affinity column. The eluted fractions were separated by SDS-PAGE and visualized by silver staining. A dominant protein band of apparent molecular weight of 32,000 was observed in fractions two (F2) and three (F3) of YT cell eluates derived from Hsp70-protein (Hsp70) or Hsp70-peptide (TKD) columns (Fig. 1*A*). This band was not detectable in eluates of carrier (data not shown) or BSA-conjugated columns nor in material eluted from the TKD affinity columns loaded with K562 cell lysates (Fig. 1*A*). Additionally, the 32-kDa protein band derived from F3 was excised from a SDS-PAGE stained with Coomassie Blue and digested with trypsin (Fig. 1*B*). The resulting peptides were analyzed by MALDI-TOF peptide mass fingerprinting. Sequences of the tryptic peptides exhibited 100% homology with granzyme B (Fig. 1*B*). The identity of the 32-kDa protein

band as granzyme B was further confirmed by Western blot analysis using a specific antibody (2C5) against granzyme B. Fractionation of YT cell lysates on Hsp70-protein (Hsp70) and Hsp70-peptide (TKD) columns revealed the presence of granzyme B protein band by Western blotting (Fig. 1*C*). However, no granzyme B was detected in the eluted fraction of Hsp70 and TKD affinity columns loaded with K562 cell lysates (Fig. 1*C*). Also flow cytometry using a phycoerythrin-conjugated granzyme B antibody HC2-PE showed positive staining for cytoplasmic granzyme B in YT cells, but not in K562 cells (Fig. 1*D*). In summary, these data indicate that granzyme B interacts with full-length Hsp70-protein as well as Hsp70-peptide TKD.

Specific Binding and Internalization of Granzyme B in Hsp70 Membrane-positive Tumor Cells—The preceding findings posed the question whether membrane-bound Hsp70 might enable specific binding and entry of granzyme B into the cytosol. Therefore, perforin-free, purified granzyme B was co-incubated with tumor cell sublines CX+/CX− and Colo+/Colo− that exhibit differential Hsp70 membrane expression. A light microscopical analysis of untreated CX+ and Colo+ cells (control) at 4 *versus* 37 °C is shown in the *upper row* of each panel (Fig. 2*A*). The corresponding immunofluorescence microscopy of the cells at 4 and 37 °C is illustrated below (control). Initially, none of the cells showed any granzyme B staining, neither on the cell surface nor in the cytoplasm. However, after a 15-min incubation period of the cells with purified granzyme B (grB) at 4 °C, a ring-shaped fluorescence, indicating a typical cell surface staining, was detected on Hsp70 membrane-positive CX+ and Colo+ tumor sublines (Fig. 2*A*, *left panel*). A temperature shift from 4 to 37 °C during the 30-min incubation period resulted in uptake of granzyme B, as determined by a cytoplasmic staining pattern in CX+ and Colo+ tumor sublines (Fig. 2*A*, *right panel*). In contrast, the Hsp70 membrane-negative counterparts CX− and Colo− neither exhibited any granzyme B cell surface binding at 4 °C nor uptake at 37 °C (data not shown). Flow cytometry analysis of permeabilized cells revealed a faint shift of the granzyme B peak to the right selectively in Hsp70 membrane-positive CX+ and Colo−, but not in CX− and Colo− tumor sublines, if the cells were co-incubated with 1 μg/ml granzyme B for 30 min at 37 °C (Fig. 2*B*, *upper graph*). A dose-dependent increase in granzyme B uptake, in Hsp70 membrane-positive tumor cells (CX+/Colo+), was detected after co-incubation with 2 and 4 μg/ml granzyme B (Fig. 2*B*, *lower graph*). However, even at the highest concentration of 4 μg/ml, granzyme B was internalized much more pronounced by Hsp70 membrane-positive as compared with Hsp70-negative tumor cells (CX−/Colo−).

Potential ion channels formed by Hsp70 may play a role in the mechanism of selective granzyme B uptake in Hsp70 membrane-positive tumor cells. Indeed, a particular ion conductance pathway was observed after incorporation of vesicles derived from purified phospholipids of Hsp70 membrane-positive (CX+) tumor sublines. This was not seen in vesicles obtained from Hsp70 membrane-negative (CX−) tumor cells (data not shown). Based on these results one might speculate about an ion channel activity facilitating uptake of granzyme B selectively into Hsp70 membrane-positive tumor cells.

In Vitro Provided Granzyme B Induces Apoptosis Selectively in Hsp70 Membrane-positive Tumor Cells—Differences in the inducibility of apoptosis were studied by co-incubation of Hsp70 high- (CX+/Colo+) and low- (CX−/Colo−) expressing carcinoma cells (21) with 10 ng/ml enzymatically active granzyme B (22) for 4, 12, and 24 h. The topoisomerase inhibitor camptothecin at a final concentration of 4 μg/ml served as a positive control for apoptosis. Programmed cell death was determined by using three different apoptosis assays including annexin V-FITC, DAPI staining, and mitochondrial cytochrome *c* release. After a 4-h incubation period, neither camptothecin nor granzyme B initiated apoptosis in any of the tested tumor cells (data not shown), indicating that our tumor carcinoma cell lines are more resistant to apoptotic cell death, as compared with the acute T cell leukemia cell line Jurkat. After a 12- and 24-h incubation period with camptothecin significant apoptosis was observed in all tumor sublines (Fig. 3*A*). It appeared that the colon carcinoma sublines CX+/CX− are better protected toward a camptothecin-mediated cell death as compared with the pancreas carcinoma sublines Colo+/Colo−. However, no significant differences in the inducibility of apoptosis by using camptothecin was observed between Hsp70 membrane-positive and Hsp70 membrane-negative tumor cells. Interestingly, this was not the case if the tumor sublines were incubated with granzyme B at a concentration that is found in the serum under physiological conditions (10 ng/ml): 12 h post-treatment the amount of annexin V-FITC positive cells was equally up-regulated in Hsp70 membrane-positive CX+/Colo+ tumor cells (1.3-fold); after 24 h the increase in apoptotic cells was significant in Hsp70 membrane-positive CX+ (1.8-fold) and in Colo+ (2.4-fold) tumor cells (Fig. 3*A*). In line with these results the amount of Hsp70 membrane-positive leukemic K562 cells (25) was similarly up-regulated (1.8-fold) following contact with granzyme B (data not shown). In contrast, the amount of apoptotic CX− and Colo− tumor cells with stably low Hsp70 membrane expression levels remained unaltered before and after a 12- and 24-h co-incubation period with granzyme B (Fig. 3*A*).

To exclude apoptosis initiated by anoikes light microscopical analysis of untreated (control), camptothecin- (*cam*), and granzyme B (grB)-treated CX+/CX− and Colo+/Colo− tumor cells were performed. As shown in Fig. 3*B*, 24 h post-treatment with granzyme B, neither Hsp70 membrane-positive nor -negative tumor cell lines exhibited any signs of loss in plastic adherence. Regarding these findings we ruled out the possibility that anoikes might be a possible mechanism for the induction of apoptotic cell death in Hsp70 membrane-positive tumor sublines. It is important to note that all apoptosis assays were determined within the adherent cell population following a short term (<1 min) trypsinization.

Consistent with the results derived by annexin V-FITC staining all cell types, CX+/CX-, Colo+/Colo−, exhibited a positive DAPI nuclear fragmentation staining, as a typical sign of apoptosis at a later stage, following treatment with camptothecin, as compared with untreated control cells (Fig. 3*C*). Again, DNA fragmentation was detected only in Hsp70 membrane-positive CX+ and Colo+ tumor cells, 24 h post-treatment with granzyme B (*grB*). In line with the annexin V-FITC staining results, no signs of DNA fragmentation were observed in Hsp70 membrane-negative CX− and Colo− cells (Fig. 3*C*). As an additional test for apoptotic cell death, cytochrome *c* release was measured following incubation of CX+ and CX− cells with granzyme B and camptothecin. As summarized in Table I, following incubation with granzyme B for 24 h, cytochrome *c* concentration was elevated 1.8-fold (0.382 mg/ml *versus* 0.690 mg/ml) in CX+ cells. However, no increase in cytoplasmic cytochrome *c* was observed in CX− cells following treatment with granzyme B (0.452 *versus* 0.425 mg/ml). An incubation with camptothecin (4 μg/ml) for 24 h results in a comparable, 1.5-fold increase in cytochrome *c* concentrations in CX+ and CX− tumor cells. In summary these results indicate that following binding and selective uptake, via membrane-bound Hsp70, granzyme B initiates apoptosis in a perforin-independent manner.

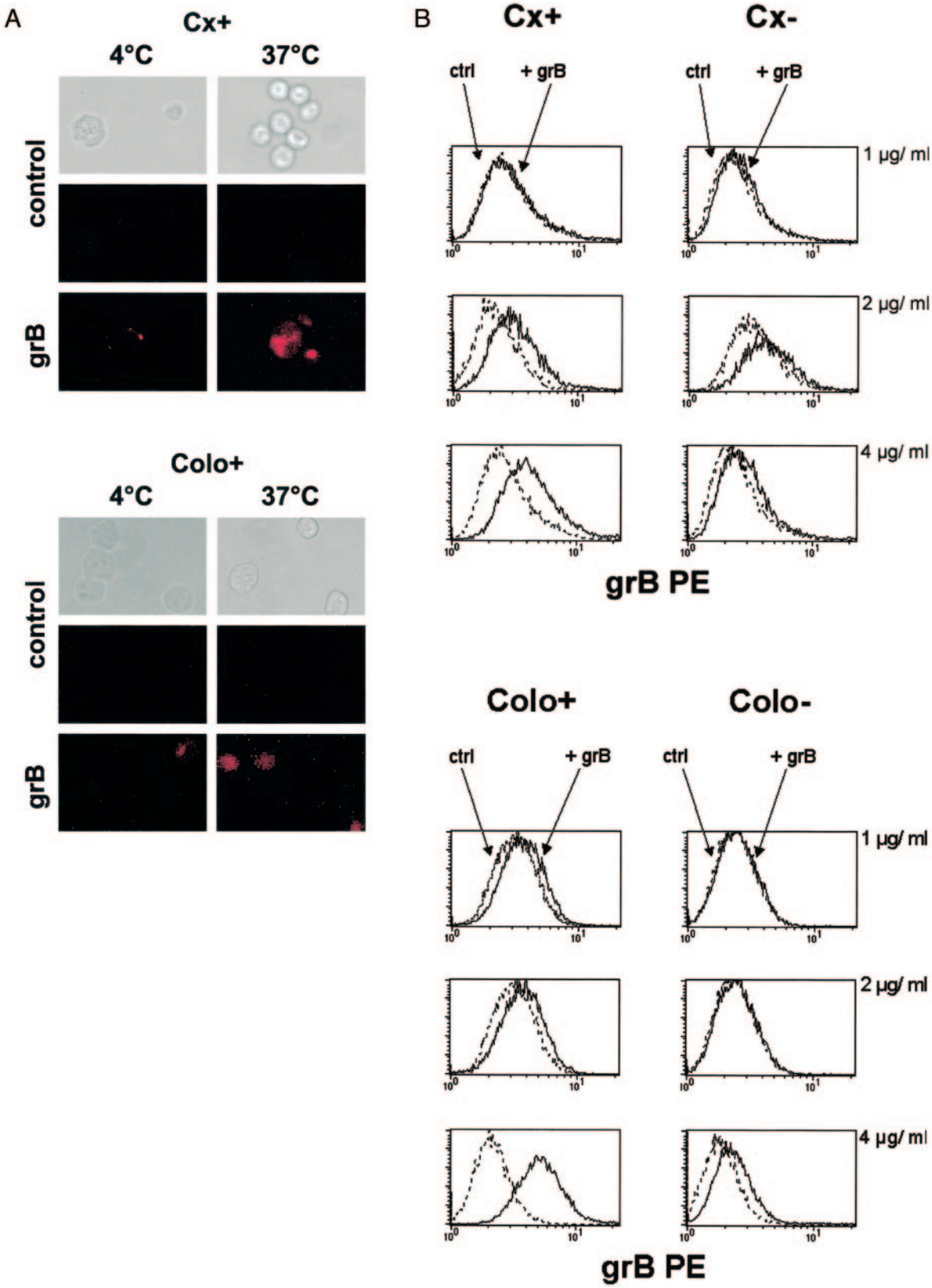


FIG. 2. **Specific cell surface binding and uptake of granzyme B (*grB*) by Hsp70 membrane-positive tumor cells.** *A*, comparative binding of granzyme B (2 µg/ml) to the cell surface of CX+/CX− and Colo+/Colo− tumor cells at 4 °C, and uptake into the cytosol after a temperature shift to 37 °C for 30 min, using the PE-conjugated granzyme B-specific monoclonal antibody HC2-PE. *First row*, light microscopy; *second row*, immunofluorescence of cells without granzyme B (control); *third row*, immunofluorescence of cells after addition of granzyme B, as specified (*grB*). One representative fluorescence microscopy of three showing identical results is illustrated; magnification ×40. *B*, intracellular flow cytometry of permeabilized CX+/CX− ($n = 2$) and Colo+/Colo− ($n = 4$) tumor cells using granzyme B-specific monoclonal antibody HC2-PE before (*dashed line*) and after (*solid line*) incubation of the tumor cells with 1, 2, and 4 µg/ml granzyme B at 37 °C for 30 min. Only CX+ and Colo+, but not CX− and Colo− cells showed a dose-dependent shift of the granzyme B peak to the *right*, indicating uptake of extracellular offered granzyme B. *ctrl*, control.

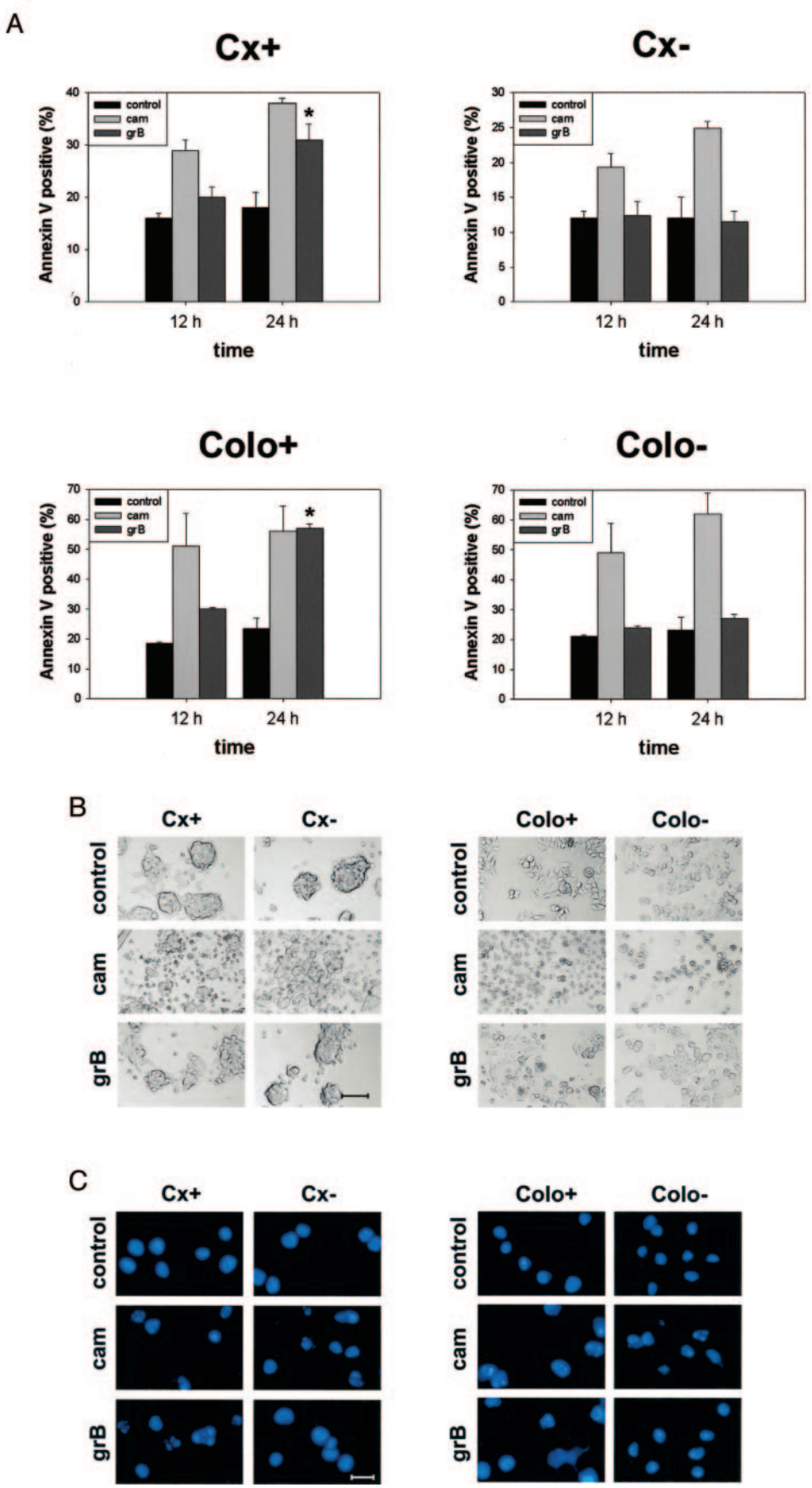


FIG. 3. **Isolated granzyme B (*grB*) induces apoptosis selectively in Hsp70 membrane-positive tumor cells.** *A*, percentage of annexin V-FITC positive and propidium iodide-negative CX+/Colo+ (*left*) and CX−/Colo− (*right*) cells, either untreated (*black bars*), or following incubation either with camptothecin (4 μg/ml; *light gray bars*) or granzyme B (10 ng/ml; *dark gray bars*) for 12 and 24 h. The data represent the mean of three to four independent experiments ± S.D.; * marks granzyme B values significantly different from control ($p < 0.05$). *B*, light microscopical analysis

TABLE I

Quantitative determination of human cytochrome c in CX+ and CX− tumor cells either untreated (control), or following incubation with camptothecin (4 μg/ml) or granzyme B (10 ng/ml), for 24 h

The data represent the mean of four independent experiments ± S.E.

Cells	Cytochrome *c* (mg/ml)		
	Control	Camptothecin	Granzyme B
		-fold increase	
CX+	0.382 ± 0.02	0.555 ± 0.04[a]	0.690 ± 0.08[a]
	1.0	1.5	1.8
CX−	0.452 ± 0.02	0.672 ± 0.02[a]	0.425 ± 0.075
	1.0	1.5	0.9

[a] Values significantly different from control ($p < 0.05$).

Granzyme B Released by TKD-activated NK Cells Mediates Apoptosis in Hsp70 Membrane-positive Tumor Cells—The physiological role of our findings was tested in functional assays using naive and Hsp70-peptide (TKD)-stimulated human NK cells. Previously, we have shown that incubation of NK cells with Hsp70-protein at concentrations between 10 and 50 μg/ml or with equivalent TKD concentrations between 0.2 and 2.0 μg/ml resulted in increased cytolytic activity against Hsp70 membrane-positive tumor target cells. Concomitantly, the expression of CD94, the killer cell inhibitory/activatory C-type lectin receptor, was up-regulated (24). Although it was known that Hsp70 acts as a tumor-selective recognition structure for NK cells (21, 25), it remained unclear whether tumor cells die by necrosis or apoptosis. To elucidate this question, NK cells were incubated with Hsp70-peptide TKD (2 μg/ml) for 3 days. A significant increase (1.4-fold) in cytoplasmic granzyme B levels was observed within 3 days of stimulation in CD3-negative NK cells. In contrast, granzyme B expression was not up-regulated in CD3-positive T cells following identical treatment conditions.

Killing of CX+/CX− and Colo+/Colo− tumor cells following contact with freshly isolated, unstimulated (NK d0), or TKD-stimulated NK cells (NK d3) was compared in a standard 12-h ^{51}Cr release assay (Fig. 4). Because of experimental limitations, the co-incubation period of NK and tumor cells in the cytotoxicity assay could not be extended to 24 h, like in our *in vitro* apoptosis assays.

Concomitant with the increase in cytoplasmic granzyme B, the cytolytic activity of TKD-stimulated NK cells (NK d3) against CX+ target cells was enhanced 1.5-fold and that of Colo+ target cells 2.0-fold at effector to target (E:T) ratios ranging between 20:1 and 2:1. In contrast, the cytolytic activity against Hsp70 membrane-negative CX− and Colo− cells was not elevated. The increased lysis of Hsp70 membrane-positive tumor cells was decreased in both cell systems down to the degree of lysis of Hsp70 membrane-negative tumor cells, by Hsp70-specific monoclonal antibody, that is known to recognize membrane-bound Hsp70-peptide TKD on viable tumor cells (25). In contrast, the lower lysis of Hsp70 membrane-negative tumor cells remained unaffected after incubation with Hsp70 antibody. Technically, it is not possible to quantify the absolute amount of granzyme B that is transferred from NK cells into tumor cells by cell-to-cell contact. However, relative values of granzyme B release could be determined by ELISPOT analysis. Therefore, a comparison of the cytolytic response of freshly isolated, unstimulated (NK d0) and TKD-stimulated NK cells

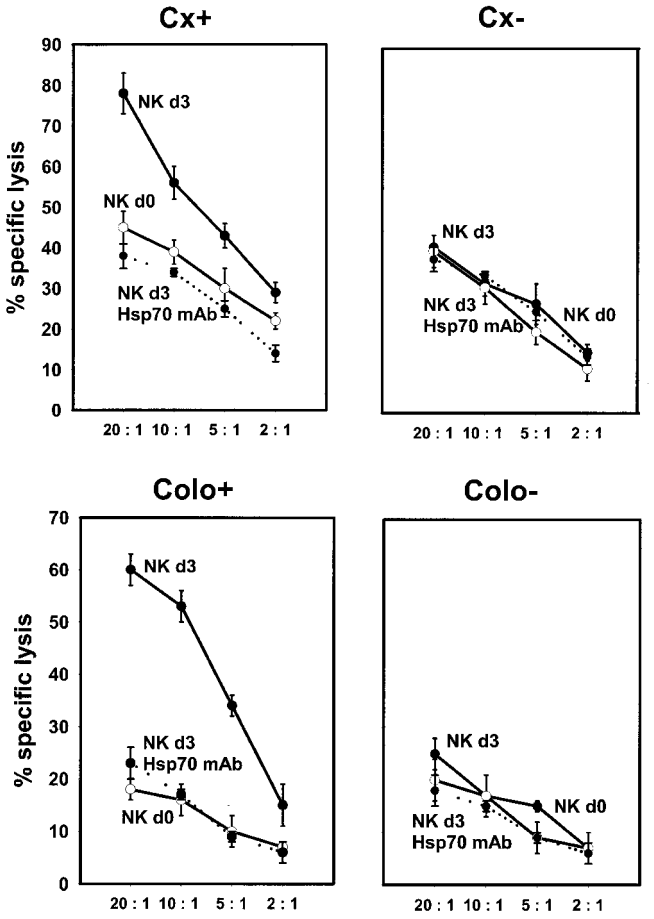


FIG. 4. **Selective kill of Hsp70 membrane-positive tumor cells, mediated by NK cells, is blockable by Hsp70-specific monoclonal antibody and correlates with granzyme B secretion.** Killing of CX+/Colo+ (*left panel*) and CX−/Colo− (*right panel*) tumor target cells by naive, unstimulated (NK d0), and Hsp70-peptide TKD-stimulated NK cells (NK d3) was quantified in 12-h ^{51}Cr release assays. Intracellular granzyme B levels in naive NK cells (NK d0) *versus* TKD-stimulated NK cells (NK d3) increased 1.4-fold; concomitantly, lysis of CX+ cells was elevated 1.5-fold, that of Colo+ cells 2.0-fold at different effector to target ratios. Lysis of CX− and Colo− tumor cells remained unaffected. Furthermore, the increased cytolytic activity of TKD-stimulated NK cells (NK d3 Hsp70 monoclonal antibody) against CX+ and Colo+ cells was inhibited by Hsp70-specific antibody down to the level of lysis of Hsp70 membrane-negative tumor cells (1.7-fold inhibition, *dotted line*). Cytotoxicity was determined at E:T ratios ranging from 20:1 to 2:1; spontaneous release of each target cell was below 10%. The data represent the mean of three independent experiments ± S.D.

(NK d3) against CX+/CX− and Colo+/Colo− tumor cells was performed concomitantly with the definition of the granzyme B release. Irrespectively of the tumor cell line and the E:T cell ratio, co-incubation of tumor cells with unstimulated NK cells (NK d0) always results in very low granzyme B release; the number of spots was always less than 20. After a 3-day stimulation period with TKD (NK d3) followed by a 4-h co-incubation time with tumor cells, granzyme B release was significantly up-regulated. At an E:T ratio of 5:1, the number of granzyme B spots, as determined in three independent experiments, was as follows: CX+, 260 ± 20; CX−, 165 ± 6; Colo+, 137 ± 55; Colo−, 66 ± 8. Concomitantly, Hsp70 membrane-

of adherent growing CX+/CX and Colo+/Colo− tumor cell clusters either untreated (control) or following treatment with camptothecin (*cam*, 4 μg/ml) or granzyme B (*grB*, 10 ng/ml), for 24 h. *Scale bar* indicates 100 μm. *C*, in parallel, either untreated (*control*), camptothecin (*cam*), or granzyme B (*grB*)-treated CX+/CX− and Colo+/Colo− tumor cells (24 h) were stained with DAPI. Considerable nuclear DNA fragmentation was observed in all tumor sublines following incubation with camptothecin (*middle panel*). After incubation with granzyme B only CX+ and Colo+ cells exhibited nuclear DNA fragmentation (*lower panel*, *left*). No signs of apoptosis were observed in CX- and Colo− tumor cells following incubation with granzyme B (*lower panel*, *right*). *Scale bar* represents 10 μm.

positive tumor target cells (CX+/Colo+) were lysed significantly better as compared with their negative counterparts (CX−/Colo−). These data strongly suggest that lysis of Hsp70 membrane-positive tumor cells by TKD-activated NK cells is associated with granzyme B release. Proteases, like granzyme B, initiate apoptosis by a intracellular mechanism (35). In Fig. 2, *A* and *B*, specific binding and a dose-dependent internalization of *in vitro* provided granzyme B was detected selectively in Hsp70 membrane-positive tumor cells. With respect to these findings, we assumed that differences in lysis of CX+/CX− and Colo+/Colo− tumor cells are because of a different capacity of the tumor cells to internalize granzyme B.

DISCUSSION

Previously we have demonstrated that tumor cells, but not normal cells, present Hsp70 on their plasma membrane (14). Antibody mapping revealed that part of the C-terminal substrate binding domain is exposed to the extracellular milieu (26). The epitope of this antibody corresponds to a 14-amino acid sequence, termed TKD (TKDNNLLGRFELS, $aa^{450-463}$). Thus, exposure of TKD on the cell surface sensitizes tumor cells to the cytolytic activity of NK cells (20). Furthermore, Hsp70-peptide TKD exhibits activatory properties on NK cells, which is comparable with the activity observed by equivalent amounts of full-length Hsp70 (19). Although it is obvious that Hsp70-protein and Hsp70-peptide TKD efficiently trigger NK cell activity, the mechanism how NK cells kill Hsp70 membrane-positive tumor target cells remained unclear.

To identify molecules that are involved in the interaction of NK cells with Hsp70-positive tumor cells, Hsp70 conjugated to Sepharose was used as affinity bait to isolate Hsp70-binding proteins from lysates of the human NK cell line YT (27). In the cytoplasm, Hsp70 binds hydrophobic residues of denatured polypeptides and co-chaperones via the substrate binding pocket, localized in the C-terminal region of the protein, in an ATP-dependent manner (28–31). To characterize interacting partners specific for membrane-bound Hsp70, an affinity column with Hsp70-peptide TKD, was also used (20). A dominant 32-kDa protein band was eluted from Hsp70-protein and Hsp70-peptide columns with lysates of NK cells. This protein band was not detected with lysates of non-NK cells. The intensity of the 32-kDa protein band was more pronounced in eluates of TKD columns because at the molar level a 50–100-fold excess Hsp70-peptide was coupled to the column, as compared with full-length Hsp70-protein. By MALDI-TOF and Western blot analysis the Hsp70-interacting partner could be identified as granzyme B (M_r expected 32,000). This observation is consistent with a prior report indicating binding of Hsp70 and Hsp27 to granzyme-immobilized Sepharose columns (32).

One mechanism of NK cell-mediated killing involves the exocytosis of cytotoxic granules containing perforin and serine proteases (33). After internalization the protease granzyme B cleaves procaspases into their activated form, and thereby induces programmed cell death by promoting DNA fragmentation (34–36). Also gene deletion studies demonstrated that granzyme B is critical for the induction of apoptosis (37). It is assumed that granzyme B enters target cells either in a perforin-dependent or perforin-independent way. Presently, the mannose 6-phosphate receptor is discussed to be involved in the process of endocytosis/pinocytosis of granzyme B (38–40). Here we provide evidence for a perforin-independent induction of apoptosis mediated by the interaction of granzyme B with cell surface-bound TKD. Incubation of human colon and pancreas carcinoma sublines that differ in Hsp70 membrane expression, with isolated, enzymatic active granzyme B, at concentrations found in human serum under physiological conditions (41), induces apoptosis selectively in Hsp70 membrane-positive tumor cells, as determined by annexin V-FITC staining, nuclear DNA fragmentation, and cytochrome *c* release. Cell viability of Hsp70 membrane-negative tumor sublines was not affected by granzyme B. These differences in the induction of apoptosis were not observed with the topoisomerase inhibitor camptothecin. Therefore, we speculate that initiation of apoptosis by granzyme B and camptothecin involves different routes. Our results that binding of granzyme B to the extracellular exposed region of Hsp70, defined by the Hsp70-peptide TKD, may be critical for uptake and for the induction of apoptosis. This observation is supported by the finding that the enhanced cytolytic activity of NK cells against Hsp70 membrane-positive tumor cells could be blocked by an Hsp70-specific monoclonal antibody, which binds to TKD. It is conceivable that cell surface-bound Hsp70 antibody prevents binding and internalization of granzyme B to tumor cells and thus protects them from apoptosis.

Earlier observations have shown that only an Hsp70 antibody directed against the C-terminal part TKD was able to recognize cell surface-localized Hsp70 on viable tumor cells (26). This suggests that the protein may be incorporated into the lipid bilayer. This option has been supported by recent evidence indicating interaction of HSP70s with phospholipids of the plasma membrane (42). Moreover, an ion conductance pathway was observed in artificial lipid bilayers after incorporation of Hsc70 (43). More recently, these proteins were found to interact with lipids in a liposome aggregation assay (44). Therefore, it is possible that Hsp70 is also embedded in the plasma membrane of tumor cells. Here we report on a selective cation channel activity in plasma membrane of Hsp70 positive tumor cells. Based on these results, one might speculate about channel formation, which facilitate binding and uptake of granzyme B into Hsp70 membrane-positive tumor cells. In the absence of Hsp70 in the plasma membrane, these channels cannot be created and thus uptake of granzyme B is prohibited. In the cytosol Hsp70 frequently cooperates with other proteins. Therefore, we were interested to identify molecules that may be tightly associated with Hsp70 in the plasma membrane. Unpublished data [2] of our group suggest that the silencer of death domain, also termed Bag-4, is co-localized with Hsp70 on the cell surface. Ongoing studies investigate the role of Hsp70 in concert with Bag-4 in the formation of ion channels.

Keeping in mind that tumor sublines with differential Hsp70 membrane expression did not differ with respect to their intracellular Hsp70 levels, neither under physiological conditions nor following stress (21), differences in the sensitivity to granzyme B-mediated apoptosis cannot be explained by differential cleavage of Hsp70 inside the cell. Also modulations in the expression of major histocompatibility complex class I molecules, known to differentially induce killer cell inhibitory/activating receptors on NK cells, could be ruled out, because the major histocompatibility complex class I cell surface pattern was identical in Hsp70 membrane-positive and -negative tumor sublines (21).

Apart from the observation that Hsp70 serves as an entry port for granzyme B into Hsp70 membrane-positive tumor target cells, Hsp70-protein and TKD stimulate the production and release of granzyme B in primary NK cells. This finding can explain why high Hsp70 membrane expression not only predisposes tumor target cells to apoptotic cell death but also favors NK cell activation. In contrast to *in vitro* applied granzyme B, activated NK cells also kill Hsp70 membrane-negative tumor cells to a certain extent. However, this weak lysis was not

[2] M. Gehrmann, J. Marienhagen, H. Eichholtz-Wirth, E. Fritz, J. Ellwart, M. Jäätellä, T. Zilch, and G. Multhoff, unpublished observations.

blockable with Hsp70 antibody. Together with the result that lysis of Hsp70 membrane-positive tumor cells was reduced to the degree of lysis observed with Hsp70 membrane-negative tumor cells by Hsp70 antibody, we assumed that Hsp70-mediated granzyme B internalization and apoptosis induction is only one mode how activated NK cells kill tumor cells. Furthermore, in comparison to *in vitro* provided granzyme B, killing mediated by NK cells engage a physical cell-to-cell contact that is likely to result in high local granzyme B concentrations at the tumor cells that cannot be quantified experimentally. Therefore, our *in vitro* apoptosis assays were conducted with granzyme B concentrations similar to those found in human serum (41). These concentrations are undoubtedly different to that secreted by NK cells and hence it would be rather surprising if they had the same efficacy. Although the absolute amount of granzyme B transferred from NK cells into tumor cells could not be defined, the cytotoxic response of activated NK cells against Hsp70 membrane-positive tumor cells correlated with the relative release of granzyme B into the medium, as determined by ELISPOT analysis. Kinetical studies indicating that increasing amounts of *in vitro* provided granzyme B are still selectively taken up by Hsp70 membrane-positive tumor cells further support these findings.

In summary our data suggest a dual role for Hsp70-protein and Hsp70-peptide TKD in the immune response against cancer. On the one hand it stimulates the production and delivery of granzyme B by NK cells, on the other hand it facilitates uptake of granzyme B selectively into Hsp70 membrane-positive tumor target cells. We propose the Hsp70 epitope TKD is a naturally occurring interacting partner for granzyme B that elucidates one part of the puzzling role of membrane-bound Hsp70 in the natural defense mechanisms against tumor cells.

Acknowledgments—We thank Marja Jäättelä and Christopher Froelich for constructive suggestions. We also thank Gerald Thonigs and Lydia Rossbacher for excellent technical assistance.

REFERENCES

1. Nylandsted, J., Brand, K., and Jäättelä, M. (2000) *Ann. N. Y. Acad. Sci.* **926,** 122–125
2. Schild, H.-J., Arnold-Schild, A., Lammert, E., and Rammensee, H.-G. (1999) *Curr. Opin. Immunol.* **11,** 109–114
3. Arnold-Schild, D., Hanau, D., Spehner, D., Rammensee, H.-J., Salle, E. A., and Schild, H. J. (1999) *J. Immunol.* **162,** 3757–3760
4. Suto, R., and Srivastava, P. K. (1995) *Science* **269,** 1585–1588
5. Becker, T., Hartl, F. U., and Wieland, F. (2002) *J. Cell Biol.* **158,** 1277–1285
6. Basu, S., Binder, R. J., Ramalingam, T., and Srivastava, P. K. (2001) *Immunity* **14,** 303–313
7. Binder, R. J., Han, D. K., and Srivastava, P. K. (2000) *Nat. Immunol.* **1,** 151–155
8. Sondermann, H., Becker, T., Mayhew, M., Wieland, F., and Hartl, F. U. (2000) *Biol. Chem.* **381,** 1165–1174
9. Ohashi, K., Burkart, V., Flohe, S., and Kolb, H. (2000) *J. Immunol.* **164,** 558–561
10. Pfeiffer, A., Böttcher, A., Orso, E., Kapinsky, M., Nagy, P., Bodnar, A., Spreitzer, I., Liebisch, G., Drobnik, W., Gempel, K., Horn, M., Holmer, S., Hartung, T., Multhoff, G., and Schmitz, G. (2001) *Eur. J. Immunol.* **31,** 3153–3164
11. Asea, A., Kraeft, S. K., Kurt-Jones, E. A., Stevenson, M. A., Chen, L. B., Finberg, R. W., Coo, G. C., and Claderwood, S. K. (2000) *Nat. Med.* **6,** 435–442
12. Asea, A., Kabingu, E., Stevenson, M. A., and Calderwood, S. K. (2000) *Cell Stress Chaperones* **5,** 425–431
13. Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Bare, O., Auron, P. E., Stevenson, M. A., and Calderwood, S. K. (2002) *J. Biol. Chem.* **277,** 15028–15034
14. Multhoff, G., Botzler, C., Wisenet, M., Müller, E., Meier, T., Wilmanns, W., and Issels, R. (1995) *Int. J. Cancer* **61,** 272–279
15. Shin, B. K., Wang, H., Yim, A. M., LeNaour, F., Brichory, F., Jang, J. H., Zhao, R., Puravs, E., Tra, J., Michael, C. W., Misek, D. E., and Hanash, S. M. (2002) *J. Biol. Chem.* **278,** 7607–7616
16. Botzler, C., Kis, K., Eissner, G., Issels, R., and Multhoff, G. (1999) *Exp. Hematol.* **27,** 470–478
17. Rabinovich, B. A., Shannon, J., Su, R.-C., and Miller, R. G. (2000) *J. Immunol.* **165,** 2390–2397
18. Feng, H., Zeng, Y., Whitesell, L., and Katsanis, E. (2001) *Blood* **97,** 3505–3512
19. Multhoff, G., Mizzen, L., Winchester, C. C., Milner, C. M., Wenk, S., Kampinga, H., Laumbacher, B., and Johnson, J. (1999) *Exp. Hematol.* **27,** 1627–1636
20. Multhoff, G., Pfister, K., Gehrmann, M., Hantschel, M., Gross, C., Hafner, M., and Hiddemann, W. (2001) *Cell Stress Chaperones* **6,** 337–374
21. Multhoff, G., Botzler, C., Wiesnet, M., Eissner, G., and Issels, R. (1997) *J. Immunol.* **158,** 4341–4350
22. Shi, L., Yang, X., Froelich, C., and Greenberg, A. H. (2000) *Methods Enzymol.* **322,** 125–143
23. MacDonald, H. R., Engers, H. D., Cerottini, J. C., and Brunner, K. T. (1974) *J. Exp. Med.* **140,** 718–722
24. Gross, C., Hansch, D., Gastpar, R., and Multhoff, G. (2003) *Biol. Chem.* **384,** 267–279
25. Multhoff, G., Botzler, C., Wiesnet, M., Eissner, G., and Issels, R. (1995) *Blood* **86,** 1374–1382
26. Botzler, C., Li, G., Issels, R., and Multhoff, G. (1998) *Cell Stress Chaperones* **3,** 6–11
27. Drexler, H. G., and Matsuo, Y. (2000) *Leukemia* **17,** 777–782
28. Hartl, F. U., and Hayer-Hartl, M. H. (2002) *Science* **295,** 1825–1828
29. Hartl, F. U. (1996) *Nature* **381,** 571–580
30. Rüdiger, S., Buchberger, A., and Bukau, B. (1997) *Nat. Struct. Biol.* **4,** 342–349
31. Demand, J. Lüders, J., and Höhfeld, J. (1998) *Mol. Cell Biol.* **18,** 2023–2028
32. Beresfold, P. J., Jaju, M., Friedmann R. S., Yoon, M. J., and Liebermann, J. (1998) *J. Immunol.* **161,** 161–167
33. Trapani, J. A., Smyth, M. J., Apostolidis, V. A., Dawson, M., and Bowne, K. A. (1994) *J. Biol. Chem.* **269,** 18359–18365
34. Berke, G. (1995) *Cell* **81,** 9–16
35. Froelich, C. J., Dixit, V. M., and Yang, X. (1998) *Immunol. Today* **19,** 30–36
36. Shresta, S., Graubert T. A., Thomas D. A., Raptis, S. Z., and Ley, T. J. (1999) *Immunity* **10,** 595–605
37. Tschopp, J., and Nabholz, M. (1990) *Annu. Rev. Immunol.* **8,** 279–302
38. Pinkoski, M., Hobmann, M., Heibein, J. A., Tomasselli, K., Li, F., Seth, P., Froelich, C., and Bleakley, C. R. (1998) *Blood* **92,** 1044–1054
39. Pinkosi, M., Green, J., and Douglas, R. (2002) *Curr. Opin. Hematol.* **9,** 43–49
40. Andrade, F., Poy, S., Nicholson, D., Thornberry, N., Rosen, A., and Casciola-Rosen, L. (1998) *Immunity* **8,** 451–460
41. Spaeny-Dekking, E. H., Hanna, W. L., Wolbink, A. M., Wever, P. C., Kiummer, A. J., Swaak, A. J., Middeldorp, J., Huisman, H. G., Froelich, C., and Hack, C. A. (1998) *J. Immunol.* **160,** 3610–3616
42. Mamelak, D., and Lingwood, C. (1997) *Glycoconj. J.* **14,** 715–722
43. Arispe, N., and De Maio, A. (2000) *J. Biol. Chem.* **275,** 30839–30843
44. Arispe, N., Doh, M., and De Maio, A. (2002) *Cell Stress Chaperones* **7,** 330–338

Cell Stress & Chaperones (2003) **8** (4), 348–360

Article no. csac. 2003.474

Heat shock protein 70-reactivity is associated with increased cell surface density of CD94/CD56 on primary natural killer cells

Catharina Gross,[1] Ingo G.H. Schmidt-Wolf,[2] Srinivas Nagaraj,[2] Robert Gastpar,[1] Joachim Ellwart,[3] Leoni A. Kunz-Schughart,[4] and Gabriele Multhoff[1]

[1]Department of Hematology and Oncology, Molecular Oncology, University Hospital Regensburg, 93053 Regensburg, Germany
[2]Division of Hematology and Oncology, Department of Medicine, University Hospital Bonn, Bonn, Germany
[3]GSF—Institute of Molecular Immunology, Munich, Germany
[4]Institute of Pathology, University of Regensburg, 93053 Regensburg, Germany

Abstract Previously we described an involvement of the C-type lectin receptor CD94 and the neuronal adhesion molecule CD56 in the interaction of natural killer (NK) cells with Hsp70-protein and Hsp70-peptide TKD. Therefore, differences in the cell surface density of these NK cell–specific markers were investigated comparatively in CD94-sorted, primary NK cells and in established NK cell lines NK-92, NKL, and YT after TKD stimulation. Initially, all NK cell types were positive for CD94; the CD56 expression varied. After stimulation with TKD, the mean fluorescence intensity (mfi) of CD94 and CD56 was upregulated selectively in primary NK cells but not in NK cell lines. Other cell surface markers including natural cytotoxicity receptors remained unaffected in all cell types. CD3-enriched T cells neither expressing CD94 nor CD56 served as a negative control. High receptor densities of CD94/CD56 were associated with an increased cytolytic response against Hsp70 membrane–positive tumor target cells. The major histocompatibility complex (MHC) class I–negative, Hsp70-positive target cell line K562 was efficiently lysed by primary NK cells and to a lower extent by NK lines NK-92 and NKL. YT and CD3-positive T cells were unable to kill K562 cells. MHC class-I and Hsp70-positive, Cx+ tumor target cells were efficiently lysed only by CD94-sorted, TKD-stimulated NK cells with high CD94/CD56 mfi values. Hsp70-specificity was demonstrated by antibody blocking assays, comparative phenotyping of the tumor target cells, and by correlating the amount of membrane-bound Hsp70 with the sensitivity to lysis. Remarkably, a 14-mer peptide (*L*KD), exhibiting only 1 amino acid exchange at position 1 (T to L), neither stimulated Hsp70-reactivity nor resulted in an upregulated CD94 expression on primary NK cells. Taken together our findings indicate that an MHC class I–independent, Hsp70 reactivity could be associated with elevated cell surface densities of CD94 and CD56 after TKD stimulation.

INTRODUCTION

Natural killer (NK) cells are large granular lymphocytes comprising 5% to 20% of the peripheral blood mononuclear cells (PBMNCs) (Trinchieri 1989). In contrast to T cells, NK cells do neither express the multimolecular CD3 complex nor α/β and γ/δ T cell receptors. Almost all NK cells are positive for the neuronal adhesion molecule CD56, mediating homo- and heterophilic cell adhesion (Lanier et al 1986, 1989). With respect to the cell-surface density of CD56, 2 distinct NK cell subsets, the $CD56^{dim}$ and $CD56^{bright}$ could be distinguished (Cooper et al 2001). In the early host defense, NK cells secrete immunoregulatory cytokines including interferon-γ, interleukin (IL)–2, IL-12, tumor necrosis factor–α and granulocyte-macrophage colony–stimulating factor. NK cells also mediate natural cytotoxicity against virus-infected cells and tumor cells by release of apoptotic enzymes including granzymes and perforin or by antibody-dependent cellular cytotoxicity through the low affinity FcγRIII receptor CD16 (Lanier et al 1988). The cytolytic activity of NK cells is

Received 9 July 2003; Accepted 23 July 2003.
Correspondence to: Gabriele Multhoff, Tel 0049 941 944 5575; Fax 0049 941 944 5572; E-mail: gabriele.multhoff@klinik.uni-regensburg.de.

regulated by killer cell receptors, which are known to interact with self–major histocompatibility complex (MHC) class-I or class I–like molecules (Lanier 1998a, Borrego et al 2002). According to the missing-self theory, NK cells preferentially recognize and kill virus-infected or transformed cells with reduced, lost, or altered self-MHC class-I molecule expression (Ljunggren and Kärre 1990). In humans, 3 distinct killer cell receptor families have been identified (Lanier 1998a): killer cell immunoglobulin (Ig) receptors (KIRs), Ig-like transcripts (ILTs), and C-type lectin receptors. The KIRs are characterized by 2 or 3 extracellular localized Ig-like domains recognizing MHC class-I alleles including HLA-A, HLA-B, and HLA-C (Moretta et al 1993; Wagtmann et al 1995; Pende et al 1996). Depending on their cytoplasmic domain containing an immunoreceptor tyrosine–based inhibition motif or an immunoreceptor tyrosine–based activating motif, NK cells mediate inhibitory and activating signals (Colonna and Samaridis 1995; Biassoni et al 1996). ILTs also belong to the Ig-like superfamily (Colonna et al 1997; Cosman et al 1997). They are inhibitory receptors expressed predominantly on myeloid cells, dendritic cells, and B cells (Colonna et al 1997). ILT-2, which is expressed on some NK cells interacts directly with a broad spectrum of classical and nonclassical HLA molecules including HLA-G (Navarro et al 1999). Most C-type lectin receptors are heterodimers consisting of CD94 covalently bound to members of the NKG2 family (Lanier et al 1994; Lazetic et al 1996). These receptors interact with nonclassical HLA-E molecules, presenting leader peptides derived from HLA-A, HLA-B, HLA-C, and HLA-G (Borrego et al 1998; Braud et al 1998; Lee et al 1998). CD94/NKG2A is an inhibitory receptor, whereas CD94/NKG2C, CD94/NKG2E, and CD94/NKG2F are activating receptors (Brooks et al 1997; Lanier 1998b). The activating receptor NKG2D also belongs to the C-type lectin family, but in contrast to other members it forms homodimers (Wu et al 1999). NKG2D is constitutively expressed on NK cells, γ/δ T cells, and CD8+ T cells and interacts with the MHC class I chain–related (MIC) antigens, MICA and MICB, and the UL16-binding proteins, which are induced by stress or neoplastic transformation (Bauer et al 1999; Cosman et al 2001). Recently, a new group of activatory receptors, termed natural cytotoxicity receptors (NCRs), consisting of NKp30 (Pende et al 1999), NKp44 (Vitale et al 1998; Cantoni et al 1999), and NKp46 (Pessino et al 1998) have been identified. Although NKp30 and NKp46 are constitutively expressed by almost all peripheral blood NK cells, the ligands regulating their expression and activatory function have not been defined yet.

By binding assays, an involvement of the C-type lectin receptor CD94 in the interaction of NK cells with Hsp70, the major stress-inducible member of the Hsp70 group could be demonstrated (Gross et al 2003). Tumor cells expressing the C terminal–localized Hsp70 sequence ''TKDNNLLGRFELSG'' (TKD, $aa_{450–463}$) on their cell surface are preferentially recognized and lysed by NK cells (Multhoff et al 1999). Hsp70-protein and Hsp70-peptide TKD, both induce proliferative and cytolytic activity in NK cells (Multhoff et al 1999; 2001). Concomitantly, the expression of the C-type lectin receptor CD94 and the neuronal adhesion-molecule CD56 was found to be upregulated on CD3-negative NK cells (Multhoff et al 1999; Moser et al 2002). Other NK cell–specific receptors of the KIR family remained unaffected (Multhoff et al 1999). Because it is known that the cell surface density of CD56 (Cooper et al 2001) and CD94 (Voss et al 1998; Jacobs et al 2001; Lian et al 2002; Gunturi et al 2003) are associated with the cytolytic function, we aimed to study changes in the mean fluorescence intensity (mfi) of these 2 markers in CD94-positive NK cell lines in response to Hsp70-peptide TKD. These data were compared with the cytolytic capacity against classical MHC class I–negative NK target cells (K562) and MHC class I–positive but Hsp70-positive (Cx+) or Hsp70-negative (Cx−) tumor target cells.

MATERIALS AND METHODS

Tumor cell lines

The myelogenous cell line K562 was purchased from American Type Culture Collection (CCL 243, Rockville, MD, USA) and cultured in Roswell Park Memorial Institute-1640 medium (Gibco, Eggenstein, Germany) supplemented with heat-inactivated 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin (all supplements purchased from Life Technologies, Rockville, MD, USA) at 37°C and 5% CO_2.

The colon carcinoma sublines Cx+ (Hsp70 membrane–positive) and Cx− (Hsp70 membrane–negative) derived by cell sorting (Multhoff et al 1997) from Cx2 colon carcinoma cells (Tumorzentrum Heidelberg, Heidelberg, Germany) differ with respect to their capacity to express Hsp70 on the plasma membrane. Cells were kept in culture under exponential growth conditions by regular cell passages. Every 3 days, cells were transferred after short-term (maximum, 1 minute) trypsinization using trypsin and ethylenediamine-tetraacetic acid solution (Gibco), and 0.5×10^6 cells were seeded in 5 mL fresh medium in T25 culture flasks.

In the cytotoxicity assay, K562 cells were used as a classical NK target, whereas Cx+ and Cx− cells were applied as MHC class I–positive tumor target cells.

NK cell lines

The human NK lymphoma cell line, NK-92 (Gong et al 1994) (ACC 488, DSMZ, Heidelberg, Germany), was

grown in RPMI-1640 medium with 20% heat-inactivated FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin, and 100 IU/mL IL-2; doubling time was 40 to 50 hours at cell densities ranging from 0.2 to 0.6×10^6 cells/mL.

NKL cell line (Robertson et al 1996) was cultured in RPMI-1640 medium with 10% heat-inactivated FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin, and 100 IU/mL IL-2; doubling time was 40 hours at cell densities ranging from 0.5 to 1×10^6 cells/mL.

The IL-2 independent growing NK cell line YT (Yodoi et al 1985) (ACC 434, DSMZ) was cultivated in RPMI-1640 medium with 10% heat-inactivated FCS, 1 mM sodium-pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin; doubling time was 50 to 60 hours at a constant low cell density of 0.1×10^6 cells/mL.

Cytokine-induced killer cells

PBMNCs were isolated from buffy coats of healthy human volunteers by Ficoll separation followed by monocyte depletion. Peripheral blood lymphocytes were grown in RPMI-1640 medium with 10% heat-inactivated FCS, 25 mM Hepes (Invitrogen GmbH, Karlsruhe, Germany), 2 mM L-glutamine, 1% penicillin-streptomycin, and 50 μM 2-mercaptoethanol. A quantity of 1,000 IU/mL of human recombinant interferon-γ (Imukin, Roche Diagnostics GmbH, Mannheim, Germany) was added on day 0. After a 24-hours incubation period, CD3 monoclonal antibody (mAb, Ortho Biotech, Neuss, Germany), 50 ng/mL (38.1; P. Martin, University of Washington, Seattle, WA, USA), 300 IU/mL IL-2 (Proleukin, Chiron Behring GmbH, Marburg, Germany), and 100 IU/mL rIL-1β (Roche Diagnostics GmbH) were added. Cells were incubated at 37°C and 5% CO_2 and subcultured every 3 days in fresh medium at a cell density of 0.4×10^6 cells/mL (Schmidt-Wolf et al 1991, 1997).

CD94-positive NK cells

PBMNCs were separated by positive selection using biotinylated CD94 mAb (HP3-D9, Ancell Immunology Research Products, Bayport, MN, USA) and a standard Miltenyi biotin separation method (Miltenyi, Bergisch Gladbach, Germany). Briefly, 100×10^6 cells/mL were incubated with 5 μg biotin-conjugated CD94 mAb for 30 minutes at 4°C. After extensive washing with bovine serum albumin containing magnetic cell-sorting buffer (MACS-buffer, Miltenyi), cells were incubated with anti-biotin beads for 15 minutes at 4°C under gentle shaking. After washing, CD94-positive cells were separated using a LS/VS column (Miltenyi).

Sorted cells were incubated for 4 days in RPMI-1640 medium with 10% heat-inactivated FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin, 100 IU/mL IL-2, and 2 μg/mL Hsp70-peptide TKD (TKDNNLLGRFELSG, purity >96%; Bachem, Bubendorf, Switzerland) at 37°C. As a control, CD94-sorted NK cells were also incubated with the mutated peptide *L*KD (LKDNNLLGRFELSG, purity >96%; Bachem) under identical culture conditions. In long-term incubation experiments CD94-sorted NK cells were cultivated in fresh medium containing TKD (2 μg/mL) plus low-dose IL-2 (100 IU/mL) weekly for 5 weeks.

CD3-positive T cells (CD3)

CD3-positive T cells were separated by positive selection from PBMNC using the CD3-separation kit (Miltenyi) according to a protocol described above.

Sorted cells were incubated for 4 days in RPMI-1640 medium with 10% heat-inactivated FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin, 100 IU/mL IL-2, and 2 μg/mL Hsp70-peptide TKD (Bachem) at 37°C.

Treatment

Exponentially growing tumor cells were either heat shocked at a temperature of 42°C for 1 hour, followed by a recovery period of 12 hours or treated with 1 μM paclitaxel (Taxol-100, Bristol Arzneimittel, Munich, Germany) for 2 hours also followed by a recovery period of 12 hours. Previously published viability assays revealed that both treatment conditions were nonlethal for the tumor cells (Multhoff et al 1997; Gehrmann et al 2002). After treatment, cells were analyzed with respect to their MHC class-I, class-II, and Hsp70 membrane expression by flow cytometry.

Flow cytometry

The following mouse fluorescein-conjugated mAb, were used for phenotypic characterization of effector and target cells: isotype-matched controls (Becton Dickinson, Heidelberg, Germany), CD54 fluorescein isothiocyanate (FITC) (Immunotech Coulter, Marseille, France), CD56 FITC (IgG2b, Becton Dickinson), CD57 phycoerythrin (PE) (IgM, Immunotech Coulter), CD94 PE (IgG1, HP-3D9, Ancell Immunology Research Products), CD3 FITC and CD16/CD56 PE (Simultest, IgG1, Becton Dickinson), NKp30 PE (IgG1, clone Z25, Beckmann Coulter, Krefeld, Germany), NKp46 (IgG1, clone BAB281, Beckmann Coulter), W6/32 PE (anti-MHC class-I, IgG2a, Cymbus Biotech, Eagle Close, UK), L243 PE (MHC DR, IgG2b, Immunotech Coulter), 87G (HLA-G, IgG2a, kindly provided by Dr D. Geraghty), TP25.99 (HLA-E, IgG1, kindly pro-

vided by Dr S. Ferrone), UIC2 (MDR-1, p glycoprotein, IgG2a, Immunotech Coulter), IMA310–0-C100 (MICA, IgG1, Immatics Biotech, Tübingen, Germany), and cm-Hsp70.1 FITC (Hsp70, IgG1, Multimmune GmbH, Regensburg, Germany).

Briefly, 0.5×10^6 cells were incubated with fluorescein-conjugated mAb at 4°C, for 30 minutes. After washing in phosphate-buffered saline supplemented either with 2% (NK-92, NKL, YT, K562, Cx+, Cx− cell lines) or 10% (CD94-positive, CD3-positive, cytokine-induced killer (CIK) primary cells) heat-inactivated FCS, the cells (200, 000) were analyzed on a FACS Calibur instrument (Becton Dickinson). The percentage of positively stained cells was calculated as the number of specifically stained cells minus the number of cells stained with an irrelevant isotype-matched control antibody.

Cell-mediated lympholysis assay

The cytolytic activity of primary effector cells and NK cell lines was monitored in a standard ^{51}Cr cytotoxicity assay (MacDonald et al 1974). Different titrations (2:1, 1:1, 0.5:1 or 10:1, 5:1, 2:1 or 40:1, 20:1, 10:1) of effector and ^{51}Cr-labeled (5 mCi of $Na^{51}CrO_4$, NEN-Dupont, Boston, MA, USA) tumor target cells (3×10^3 cells per well) were incubated in duplicate at a final volume of 200 μL (RPMI-1640 medium supplemented with 10% FCS) at 37°C in 96-well U-bottom plates (Greiner, Melsungen, Germany). For antibody blocking, tumor cells (0.5×10^6 cells) were incubated for 15 minutes with 5 μg/mL Hsp70-specific mAb (cmHsp70.1) after labeling and then used in the cytotoxicity assay without further washing. After a 4-hours incubation period, supernatants were collected and the radioactivity was quantified on a γ-counter (Canberra Packard Instruments, Dreieich, Germany). Spontaneous release was determined using the supernatants of target cells that had not been coincubated with effector cells. Maximum release was determined in the suspensions of ^{51}Cr-labeled target cells. Specific lysis (%) was calculated according to the following equation: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. The percentage of spontaneous release in all experiments was always less than 20% for each target cell line.

RESULTS AND DISCUSSION

Separation of CD94-positive NK cells and CD3-positive T cells

We have shown previously that the C-type lectin receptor CD94 is involved in the interaction of NK cells with Hsp70, the major stress-inducible member of the Hsp70 family (Gross et al 2003). Tumor cells expressing the Hsp70 epitope ''TKDNNLLGRFELSG'' on their cell surface are recognized and lysed by CD3-negative, CD94-positive NK cells (Multhoff et al 1999). Incubation of primary human NK cells with Hsp70-peptide TKD plus low-dose IL-2 for 4 days results in an enhanced cytolytic activity against Hsp70-positive tumor cells (Multhoff et al 2001). Concomitantly, the percentage of CD94- and CD56-positive cells increased. Other NK cell markers including CD16 and KIRs (CD158a/b; CD158e1/e2; CD158k) remained unaffected. IL-2 alone increases the expression of CD94 and CD56 marginally, indicating that TKD might have a synergistic effect on the upregulation of these NK cell–specific markers (Multhoff et al 1999). In the present study, modulations in the protein density of CD56 and CD94 were compared in CD94-sorted, primary NK cells, and NK cell lines before and after stimulation with TKD. Concomitantly, the cytolytic capacity against the classical MHC class I–negative NK target cells (K562) and the MHC class I–positive, Hsp70 membrane–positive (Cx+) and membrane–negative (Cx−) tumor cells was tested.

In the first set of experiments, CD94-positive NK cells were separated from PBMNCs of 8 healthy human volunteers by magnetic activated cell sorting (MACS). As a negative control, CD3-positive T cells were selected from 3 healthy human donors. Both positively enriched cell populations were cultured separately in the presence of low-dose IL-2 (100 IU/mL) and Hsp70-peptide TKD (2 μg/mL). The concentration of the supplements was identified as the optimal activating dose for human NK cells in kinetical studies (Multhoff et al 1999). The cell surface expression pattern of CD56, CD94, CD3, and CD16/56 in unstimulated PBMNC is illustrated in Fig. 1A. Freshly isolated cells were not phenotyped for CD94 and CD3 because antibody-coupled beads were still bound to the cell surface (data not shown). On day 4, after separation and stimulation a flow-cytometric analysis was performed. One representative result of the multiparameter flow cytometric analysis is illustrated in Fig. 1B, C. The mean percentages of positively stained cells ± standard deviation of all experiments are summarized and given on the right hand side of each individual graph. After positive selection for CD94 and stimulation with TKD, 63 ± 12% of the cells were found to be double positive for CD94 and CD56; 22 ± 9% were positive for CD94, only (upper panel). A minor part of cells (2 to 3 ± 3%) was found to be CD94- and CD3-negative. Because CD94 separation was not complete (purity >85%), on day 4 after stimulation, between 12% and 29% contaminating CD3-positive T cells were detected. With respect to the NK cell marker CD16/56 and the T cell marker CD3 this cell population contained 54% NK cells and 16% T cells (lower panel). A positive selection using a CD3-specific antibody followed by identical stimulation with TKD re-

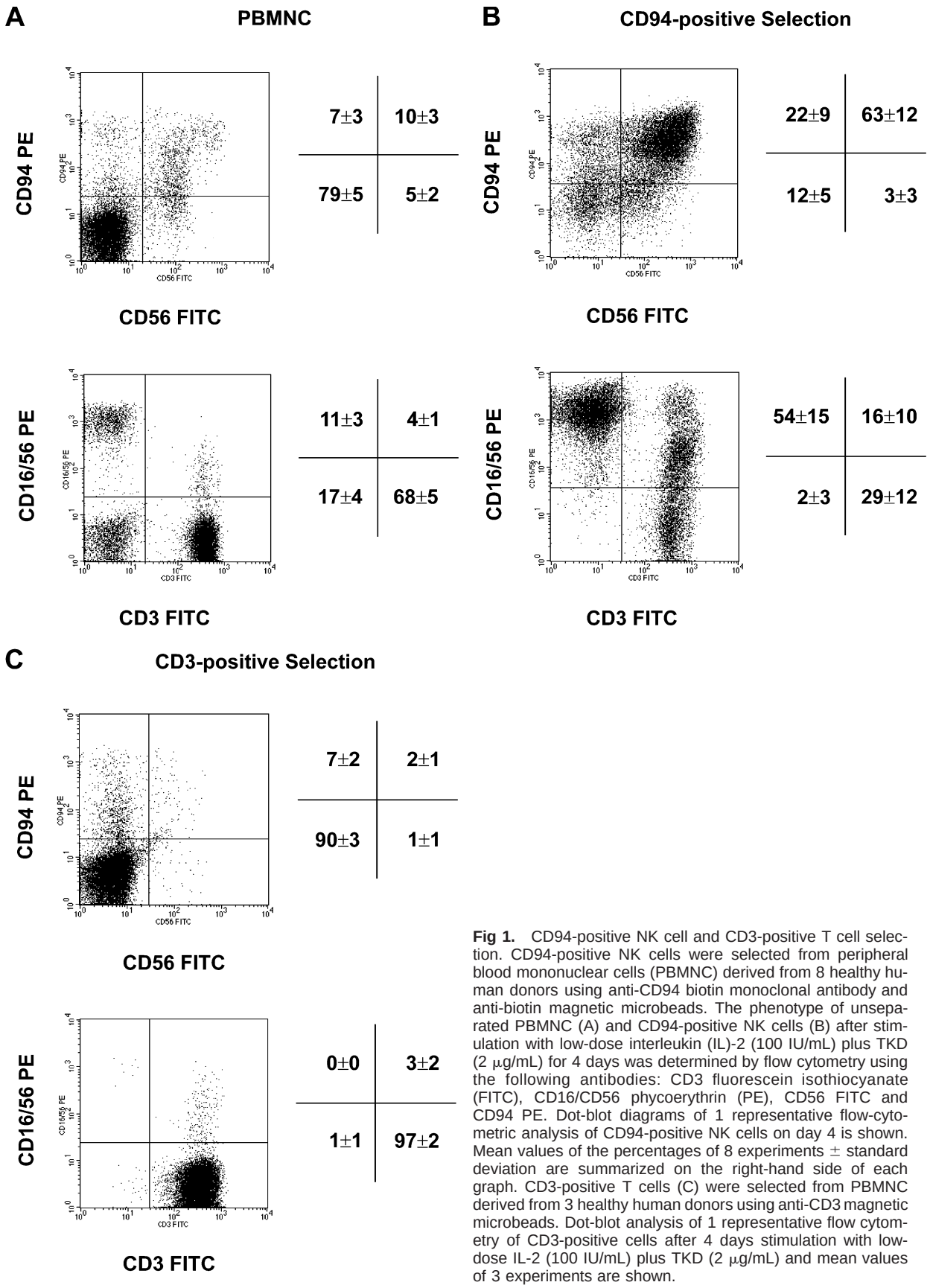


Fig 1. CD94-positive NK cell and CD3-positive T cell selection. CD94-positive NK cells were selected from peripheral blood mononuclear cells (PBMNC) derived from 8 healthy human donors using anti-CD94 biotin monoclonal antibody and anti-biotin magnetic microbeads. The phenotype of unseparated PBMNC (A) and CD94-positive NK cells (B) after stimulation with low-dose interleukin (IL)-2 (100 IU/mL) plus TKD (2 μg/mL) for 4 days was determined by flow cytometry using the following antibodies: CD3 fluorescein isothiocyanate (FITC), CD16/CD56 phycoerythrin (PE), CD56 FITC and CD94 PE. Dot-blot diagrams of 1 representative flow-cytometric analysis of CD94-positive NK cells on day 4 is shown. Mean values of the percentages of 8 experiments ± standard deviation are summarized on the right-hand side of each graph. CD3-positive T cells (C) were selected from PBMNC derived from 3 healthy human donors using anti-CD3 magnetic microbeads. Dot-blot analysis of 1 representative flow cytometry of CD3-positive cells after 4 days stimulation with low-dose IL-2 (100 IU/mL) plus TKD (2 μg/mL) and mean values of 3 experiments are shown.

vealed that almost all cells (97%) were CD3-positive T cells; no CD16/56-positive NK cells (3%) were found. The percentage of CD94/CD56–positive cells was only 7%.

After long-term stimulation, the CD94/CD56–positive phenotype remained stable. Cells were incubated in fresh medium supplemented with TKD plus low-dose IL-2 once a week. Figure 2 shows representative dot-blot profiles of the CD94/CD56 staining of CD94-enriched NK cells on days 14, 21, 28, and 35. Although the percentages of CD94/CD56–positive cells remained constant, the mfi decreased during long-term culture (data not shown). The number of CD3-positive T cells did not increase; indicating that even repeated stimulation rounds with IL-2 plus TKD does not favor T cell growth. From these results, we conclude that it is possible to keep CD94/CD56–positive NK cells in culture for several weeks.

A comparison of the protein density, as determined by the mfi, of unstimulated and TKD-stimulated CD94-sorted NK cells and NK cell lines NK-92, NKL, and YT revealed that a significant increase in CD94 and CD56 expression was detected only with primary NK cells, within 4 days of stimulation (Table 1).

Comparative phenotypic characterization of the cell-surface markers CD94 and CD56 on primary NK, CIK, and T cells and on NK cell lines NK-92, NKL, and YT

In addition to the CD94 and CD56 expression, differences in the expression pattern of newly defined NCRs, NKp30 and NKp46 were tested in CD94-enriched NK cells, CD3-enriched T cells, and in NK cell lines NK-92, NKL, and YT after treatment with IL-2 plus TKD. In contrast to primary human NK cells, neither NK cell lines nor T cells showed any difference in their CD94, CD56, or NCR expression pattern after TKD stimulation. The percentages of positively stained cells and mfi values of all markers derived from 3 to 8 independent experiments after stimulation are summarized in Table 2. After cell sorting and TKD stimulation, 85% of the primary NK cells were positive for the C-type lectin receptor CD94; 63% of these cells also expressed the neuronal adhesion molecule CD56. NKp30 and NKp46 was found on 58% and 75% of the CD94-sorted cells, respectively, and on the NK lines NK-92 (29% and 62%) and NKL (78% and 77%) at variable percentages. These markers did not differ before and after stimulation with TKD. YT cells were negative for NKp30 (0%) but strongly positive for NKp46 (98%). CD3-sorted T cells were negative for all tested markers. Both NK cell lines, NK-92 and NKL, were found to express CD94 (99%). However, coexpression of CD56 was determined only on NK-92 cells (99%). In contrast, only a small subpopulation of 15% of the NKL cells did express CD56. As shown previously, the percentage of CD94-positive cells varied on YT cells between 13% and 52%, depending on the number of cells in culture; a negative correlation was consistently found with increasing cell densities (Gross et al 2003). With the culture conditions tested here, 37% to 54% of the YT cells were positive for CD94; CD56 was almost not detectable. CIK cells were also analyzed with respect to their CD94 and CD56 expression pattern. Because CIK cells are a heterogeneous cell population consisting of a mixture of T and NK cells, the percentages for CD94 and CD56 varied from 18% to 54% and from 29% to 67%, respectively. Because of this high variability, standard deviations were not provided for CIK cells.

With respect to the mfi, again striking differences were found between primary NK cells and NK cell lines. Only primary NK cells reacted on TKD stimulation with a significantly upregulated mfi of CD94 from 156 to 416, and of CD56 from 114 to 393, between day 0 and day 4 (Table 1). The expression of NCRs remained unaffected by TKD stimulation (data not shown). In all other cell types, the mfi values of the tested markers remained unaltered. In comparison with NK cell lines, primary NK cells showed the highest expression levels of the cell surface markers CD94 and CD56 on day 4. The CD94 mfi values on CIK cells, NK-92, and NKL cells were 57, 170, and 193, respectively. Very low CD94 mfi levels were detectable in YT cells. With respect to CD56, the mfi values of CIK and NK-92 cells was 181 and 72, respectively; NKL cells showed low mfi values of 4. Positive mfi values for CD56 were not found in YT cells and CD3-positive T cells, lacking CD56 molecules on their cell surface. Significant mfi values of NKp30 and NKp46 were only found in CD94-sorted NK cells, after stimulation with TKD.

In summary, although the percentages of CD94-positive cells were comparably high in primary NK cells and NK cell lines NK-92 and NKL, striking differences were recorded with respect to the cell surface density of this receptor. Furthermore, only primary NK cells reacted with a significant upregulation in the cell surface density of CD94 after stimulation with TKD. All other tested markers remained unaltered. Similar results were seen for the neuronal adhesion molecule CD56. To distinguish the different NK cell types on the basis of their CD94/CD56 receptor density, the mfi values were marked in Figure 3 with + and − symbols as follows: mfi > 300, +++; mfi 150–300, ++; mfi 10–150, +; mfi < 10, −.

Association of the cell surface intensity of CD94/CD56 with Hsp70 reactivity

The cytolytic response of the different effector cells, prestimulated with TKD, was tested in a 4-hours cytotoxicity assay using the myeloid cell line K562 and the colon carcinoma sublines Cx+ and Cx− as target cells. A phenotypic characterization of the tumor cell lines is summarized in Table 3. Only Cx+ and Cx− colon carcinoma

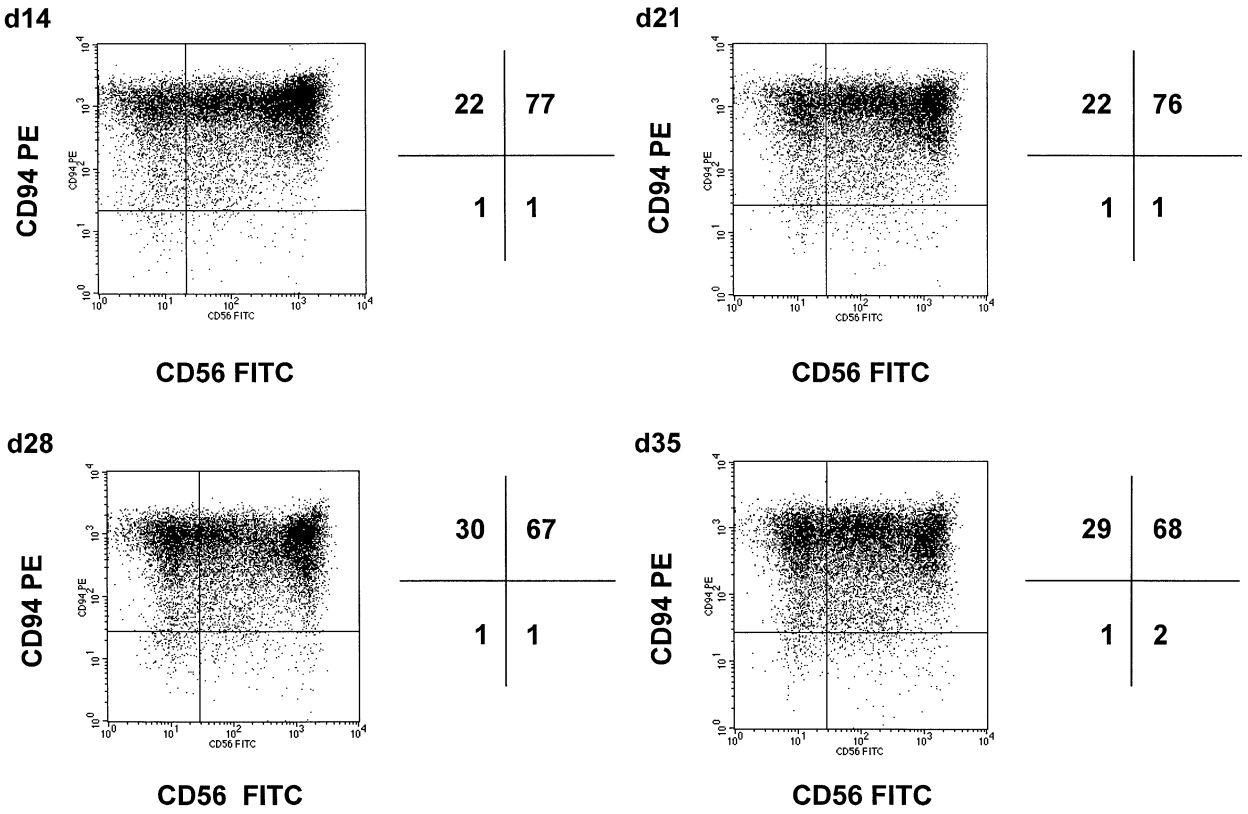


Fig 2. CD94 and CD56 expression pattern on CD94-sorted, primary natural (NK) cells after long-term (5 weeks) stimulation with TKD. CD94-positive NK cells were cultured in medium containing low-dose interleukin (IL)-2 (100 IU/mL) plus TKD (2 μg/mL). Medium was completely renewed once a week. Cell density was kept at 2×10^6 cells/mL. Phenotypic characterization was performed by flow cytometry on days 14, 21, 28, and 35 using fluorescence-conjugated CD56 fluorescein isothiocyanate and CD94 phycoerythrin antibodies. The data represent 1 representative profile of 3 independent experiments.

Table 1 Comparative analysis of the mean fluorescence intensity (mfi) of unstimulated and interleukin (IL)-2 (100 IU/mL) plus TKD (2 μg/mL) stimulated CD94-positive, primary natural killer (NK) cells and NK cell lines NK-92, NKL, and YT

	Mean fluorescence intensity (mfi)	
	Unstimulated	**IL-2 plus TKD**
CD94-positive NK cells		
CD94	156 ± 42	416 ± 176**
CD56	114 ± 62	393 ± 264**
NK-92		
CD94	175 ± 13	173 ± 12
CD56	76 ± 19	72 ± 22
NKL		
CD94	148 ± 10	152 ± 10
CD56	0 ± 0	0 ± 0
YT		
CD94	8 ± 1	7 ± 2
CD56	0 ± 0	0 ± 0

Cells were stained with the following antibodies: CD94 phycoerythrin, CD56 fluorescein isothiocyanate. Mean values from 8 independent experiments are summarized ± standard deviation.

** Significantly different from control ($P < 0.05$).

cells were found to be positive for MHC class-I molecules; the classical NK target cell line K562 was MHC class I–negative. With respect to the missing-self theory (Ljunggren and Kärre 1990), K562 cells are lysed because of a deficient MHC class-I expression associated with a lack of inhibitory signals for KIRs. On the other hand about 50% of the K562 cells do express Hsp70 on their cell surface and thus provide a recognition signal for the activating form of the C-type lectin receptor CD94. Therefore, we assumed that recognition of K562 cells is mediated by at least 2 receptor mechanisms: inactivating of inhibitory KIRs due to the missing MHC class-I expression and activating of the C-type lectin receptor due to the Hsp70 membrane expression. Because it is known that Cx+ and Cx− exhibit an identical MHC class-I and class-II expression pattern but differ significantly with respect to their Hsp70 membrane expression (Multhoff et al 1997), an involvement of KIR could be ruled out for the Hsp70 reactivity. In addition to MHC class-I, class-II, and Hsp70, the expression of several adhesion molecules including CD54 (intracellular adhesion molecule (ICAM)), CD106 (vascular adhesion molecule (VCAM)), CD57 and CD56 (neuronal adhesion molecule (NCAM))

Table 2 Comparative phenotypic characterization of CD94-positive, primary natural killer (NK) cells, CD3-positive T cells, cytokine-induced killer cells, and NK cell lines NK-92, NKL, YT stimulated with low dose interleukin-2 plus TKD for 4 days

	Positively stained cells (%)					
	CD94 (n = 8)	**CIK (n = 3)**	**NK-92 (n = 6)**	**NKL (n = 3)**	**YT (n = 6)**	**CD3 (n = 3)**
CD94	85 ± 7[a]	18–54[b]	99 ± 0	99 ± 0	37–54[b]	7 ± 2
CD56	63 ± 12	29–63[b]	99 ± 1	15 ± 3	3 ± 1	3 ± 1
NKp30	58 ± 14	not tested	29 ± 8	78 ± 9	0 ± 0	0 ± 0
NKp46	75 ± 8	not tested	62 ± 16	77 ± 2	98 ± 1	0 ± 0
	Mean fluorescence intensity (mfi)					
	CD94[a]	**CIK**	**NK-92**	**NKL**	**YT**	**CD3**
CD94	416 ± 176[a]	57 ± 25	173 ± 12	154 ± 10	7 ± 1	0 ± 0
CD56	393 ± 264	181 ± 48	72 ± 22	4 ± 1	0 ± 0	0 ± 0
NKp30	182 ± 75	not tested	8 ± 1	12 ± 3	0 ± 0	0 ± 0
NKp46	502 ± 223	not tested	8 ± 1	10 ± 1	44 ± 7	0 ± 0

Cells were stained with the following fluorescein-conjugated antibodies: CD3 fluorescein isothiocyanate, CD16/56 phycoerythris (PE), CD56 FITC, CD94 PE, NKp30 PE, and NKp46 PE.

[a] mfi of CD94/CD56-positive cells.

[b] Heterogeneous cell population, a range or a median value is provided.

were determined on the target cell lines. K562, Cx+ and Cx− tumor cells were strongly positive for CD54. With respect to the adhesion molecules CD106, CD57, and CD56, only Cx+ and Cx− tumor cells showed a comparable but weak expression; K562 cells were negative for these markers. Also, the multidrug resistance gene product MDR-1 was comparably expressed on Cx+ and Cx− tumor cells but not on K562 cells. With respect to the expression of the stress-inducible MHC-related molecule MICA, only K562 cells were found to be positive. HLA-E

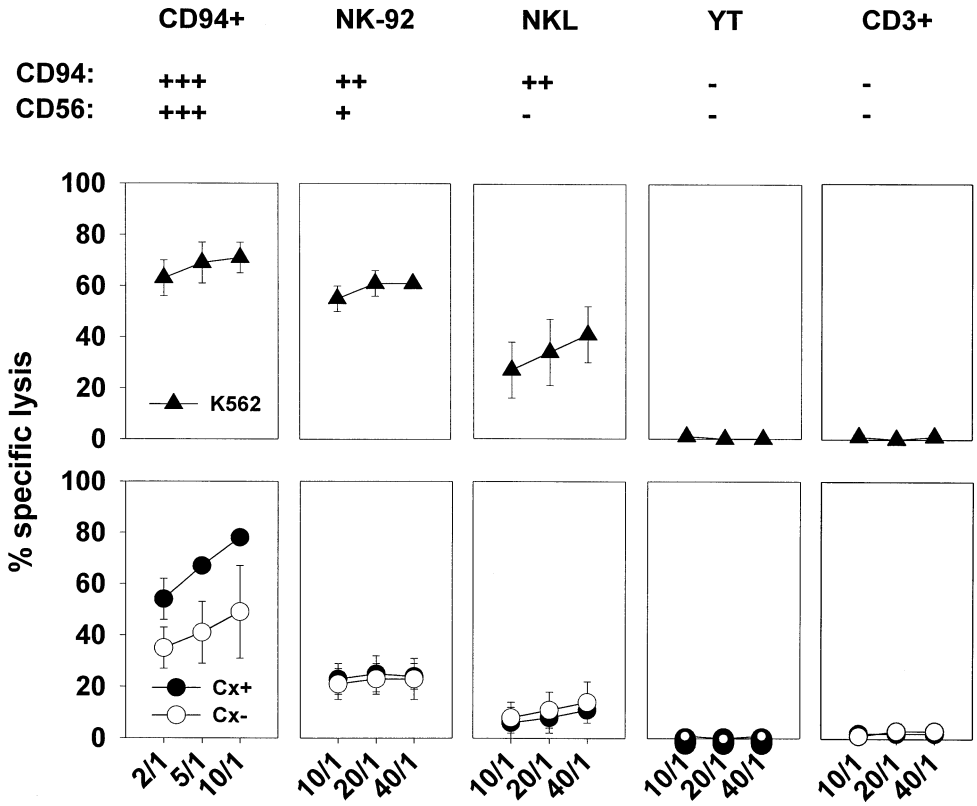


Fig 3. Comparative cytolytic activity of CD94-positive, primary natural killer (NK) cells, NK-92, NKL, YT cell lines and CD3-positive, primary T cells. K562, Hsp70 membrane–positive Cx+ and negative Cx− cells were used as target cells in a 4-hours ^{51}Cr cytotoxicity assay. The spontaneous release for each target cell was less than 20%. It is important to note that effector to target cell ratios ranged from 10:1 to 2:1 in case of CD94-positive, primary NK cells and from 40:1 to 10:1 in case of NK cell lines. Mean values of 3 independent experiments are shown.

Table 3 Phenotypic characterization of K562, Hsp70-positive Cx+ and Hsp70-negative Cx− target cells. Cells were stained with fluorescein isothiocyanate or phycoerythrin-conjugated antibodies

Antibody clone	Specificity	Positively stained cells (%)		
		K562	Cx+	Cx−
W6/32	MHC I	0 ± 0	99 ± 1	99 ± 1
L243 B8.12.2	HLA DR	0 ± 0	8 ± 3	6 ± 2
cmHsp70.1	Hsp70	42 ± 8	93 ± 3	18 ± 6
ICAM	CD54 (ICAM)	99 ± 0	69 ± 6	54 ± 5
VCAM	CD106 (VCAM)	not tested	9 ± 3	10 ± 3
NC1	CD57 (HNK1)	0.2 ± 0.1	12 ± 4	14 ± 3
NCAM 16.2	CD56 (NCAM)	0 ± 0	19 ± 7	17 ± 4
UIC2	MDR-1 p-gp	1 ± 0	68 ± 8	64 ± 7
AMO1	MICA	89 ± 4.5	3.5 ± 0.5	5 ± 0
87G	HLA-E	0 ± 0	6 ± 2	5 ± 2
TP25.99	HLA G	0 ± 0	2 ± 3	3 ± 2

Mean values of at least 3 different experiments are shown.

and HLA-G, as nonclassical MHC molecules mediating interaction with C-type lectin receptors (Borrego et al 1998), were not found on any of the tested tumor cell lines. A comparison of K562 and Cx+ and Cx− cell lines revealed differences in the MHC class-I, MDR-1, and MICA expression. In contrast, Cx+ and Cx− tumor sublines exhibited identity in all tested markers with 1 exception, the Hsp70 membrane expression.

The cytolytic response, mediated by different NK cell types was tested against all 3 target cell lines K562, Cx+ and Cx− cells. It is important to note that the effector to target cell ratios (E:T) ranged from 10:1 to 2:1 in the case of CD94-positive, primary NK cells and was 4-times higher (40:1 to 10:1) in all other effector cell lines. K562 cells were lysed by CD94-positive NK cells, NK-92, and NKL cells. At a defined E:T ratio of 10:1, CD94-positive cells showed the strongest cytolytic activity against K562 cells

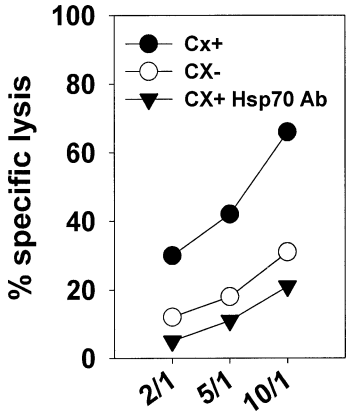


Fig 4. Hsp70-specific antibody inhibits the increased lysis of Cx+ tumor cells. After labeling, Cx+ and Cx− tumor target cells were incubated for 15 minutes with Hsp70-specific monoclonal antibody (cmHsp70.1, 5 μg/mL) and used as targets for CD94-sorted natural killer cells. Lysis of Cx− cells remained unaffected after antibody treatment (data not shown). The spontaneous release for each target cell was less than 20%. Effector to target cell ratios were 10:1, 5:1, and 2:1.

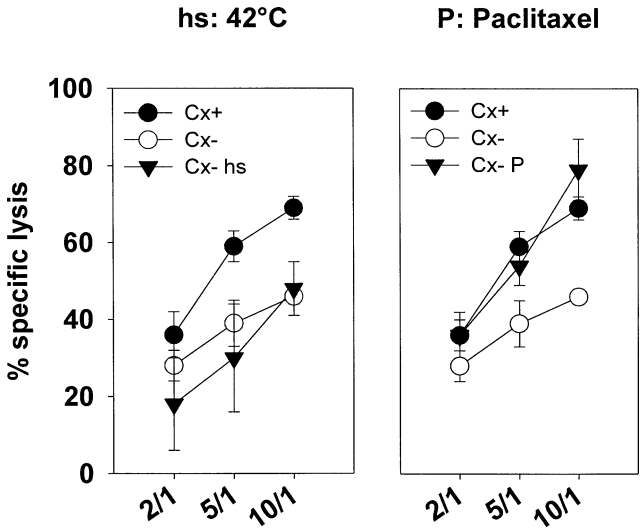


Fig 5. Increased Hsp70 membrane expression after treatment with paclitaxel (P) increases lysis of Cx− tumor target cells. Cx+ and Cx− cells were either heat shocked at the nonlethal temperature of 42°C for 1 hour (hs) or incubated with a nonlethal dose of paclitaxel (1 μM) for 1 hour. The major histocompatibility complex class I, class II, and Hsp70 phenotype of the tumor target cells before and after treatment are summarized in Table 4. Cx− tumor cells exhibited an upregulated Hsp70 expression after P treatment that correlates with an increased sensitivity to lysis. Nonlethal heat shock neither increases Hsp70 membrane expression nor lysis of Cx− cells. The spontaneous release for each target cell was less than 20%. Effector to target cell ratios were 10:1, 5:1, and 2:1.

with 71%. In contrast only half of the K562 cells were killed by NK-92 cells at this E:T ratio. K562 cells were also killed by NKL cells but to a much lesser extent as compared with primary NK cells. YT cells and primary T cells did not lyse K562 cells at all. The MHC I-positive and Hsp70-positive Cx+ and Cx− tumor cell lines were only lysed by CD94-positive, primary NK cells; however, with respect to the differential Hsp70 membrane expression, lysis of Cx− cells was significantly weaker compared with that of Cx+ cells. Apart from primary NK cells, the NK cell lines were unable to kill Cx+ and Cx− target cells. This might be because of the inhibitory signal mediated by the MHC class-I expression. Only CIK cells exhibited a weak but indistinguishable lysis of Cx+ and Cx− cells, at an E:T ratio 4-times higher than that of primary NK cells (data not shown).

Because Cx+ and Cx− tumor cells differ only with respect to the Hsp70 membrane expression, we speculated that differences in lysis mediated by TKD-stimulated CD94-positive primary NK cells is due to Hsp70 that appears to function as an activating signal. CD94 has been found to be involved in the interaction of NK cells with Hsp70 protein (Gross et al 2003). Although all NK cell lines were positive for CD94, they showed different cytotoxicity against Hsp70-positive tumor target cells. Therefore, we suggest that the differences in cell kill might be associated with the cell surface density of CD94.

Table 4 Phenotypic characterization of viable Hsp70-positive Cx+ and Hsp70-negative Cx− target cells either untreated, after nonlethal heat shock (hs, 42°C for 1 h, 37°C for 12 h), or treatment with the nonlethal concentration of paclitaxel (P, 1 μM for 2 h, 37°C for 12 h; Gehrmann et al 2002)

		Positively stained cells (%)					
Antibody	**Specificity**	**Cx+**	**Cx−**	**Cx+hs**	**Cx−hs**	**Cx+P**	**Cx−P**
W6/32	MHC I	98 ± 1	97 ± 1	98 ± 2	99 ± 1	98 ± 2	99 ± 1
L243	HLA DR	7 ± 3	5 ± 2	4 ± 3	5 ± 2	3 ± 3	5 ± 4
cmHsp70.1	Hsp70	89 ± 6	21 ± 5	85 ± 3	19 ± 4	87 ± 8	79 ± 4

Cells were stained with fluorescein isothiocyanate or phycoerythrin-conjugated antibodies. Mean values of at least 3 different experiments are shown. Untreated and treated cells were used as target cells for CD94-sorted natural killer cells in cytotoxicity assays.

Cells with high (+++, mfi > 300) expression levels of this receptor showed an increased kill of Hsp70-positive tumor cells. NK cells with moderate (++, mfi 150–300) or low (+, mfi < 10) expression levels in this marker did not show any Hsp70-specific kill, indicating an essential role of the protein density of CD94 for Hsp70 reactivity. Study of Michaelsson (2002) revealed that HLA-E, presenting leader peptides from Hsp60, results in increased sensitivity to lysis mediated by NK cells due to a loss of recognition by CD94/NKG2A inhibitory receptors. Because Cx+ and Cx− tumor cells both exhibited a comparable but very low HLA-E surface expression pattern and K562 cells completely lack expression of HLA-E, an involvement of HLA-E in the recognition by CD94-positive, primary NK cells was excluded. Similar to CD94, CD56 was upregulated in CD94-sorted, primary NK cells after stimulation with TKD. Because CD56 mediates homophilic cell adhesion, we speculated about a role of CD56 in the cytolytic response against Hsp70-positive tumor target cells. However, Cx+ and Cx− cells revealed identical CD56 expression levels (Multhoff et al 1997), whereas K562 cells lack CD56 expression (Table 3). In contrast to this finding, Cx+ and K562 cells, but not Cx− cells, were lysed by primary TKD-stimulated NK cells. Therefore, the involvement of CD56 in the cytolytic mechanism of Hsp70-positive tumor target cells remained to be elucidated. Concomitant with the finding that stimulation of NK cell lines NK-92, NKL, and YT with TKD did not increase the cell surface expression of CD94 and CD56, the cytolytic activity against Hsp70 membrane–positive tumor cells was also not enhanced.

To prove that indeed Hsp70 provides the target structure for CD94-sorted, TKD-stimulated NK cells, antibody blocking studies were performed. As illustrated in Figure 4, the increased lysis of Cx+ tumor cells could be reduced to the degree of Cx− cell lysis by Hsp70-specific antibody. In contrast, lysis of Cx− cells remained unaffected after antibody incubation (data not shown). These findings were further supported by the fact that a paclitaxel-induced increase in membrane-bound Hsp70, especially on Cx− cells, correlated with an increased sensitivity toward lysis mediated by NK cells (Fig. 5). The Hsp70 phenotype together with the MHC class-I and II expression in Cx+ and Cx− tumor cells before and after

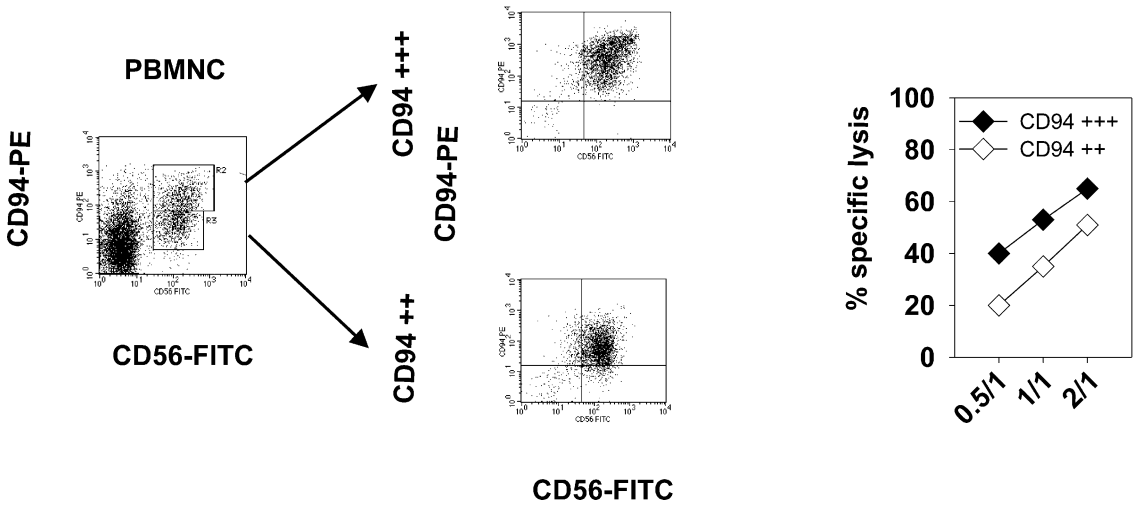


Fig 6. Comparative cytolytic activity of CD94 high (+++) and CD94 low (++) expressing natural killer (NK) cells. Peripheral blood mononuclear cells were separated in CD94 high (+++) and CD94 low (++) expressing NK cells by flow cytometry cell sorting. Both subpopulations were stimulated with low dose interleukin-2 (100 IU/mL) and TKD (2 μg/mL) for 4 days and used as effector cells in a 4-hours ^{51}Cr cytotoxicity assay. Major histocompatibility complex class I- and Hsp70-positive tumor cells were used as target cells. The spontaneous release for each target cell was less than 20%. Effector to target cell ratios were only 2:1, 1:1, and 0.5:1.

nonlethal heat shock (42°C) or treatment with a nonlethal concentration of paclitaxel (1 μM) are summarized in Table 4. Heat was unable to induce an increased membrane expression of Hsp70 and thus lysis of Cx− cells remained unaltered (Fig. 5, left graph). However, after paclitaxel treatment (Fig. 5, right graph), Hsp70 expression was significantly upregulated in Cx− tumor cells. Concomitantly the percentage-specific lysis drastically increased. Because upregulation of membrane-bound Hsp70 in Cx+ cells was only marginal, no upregulated cytotoxicity of Cx+ cells was observed (data not shown). In summary, these findings clearly indicate that Hsp70 provides the target structure for TKD-stimulated, CD94-positive primary NK cells.

In an effort to further elucidate the role of CD94 in the cytolytic activity, more detailed additional cell-sorting experiments were performed. As demonstrated in Figure 6, primary NK cells consist of 2 subgroups that differ with respect to their CD94 mfi cell surface expression pattern. By cell sorting through CD94, 2 subpopulations with CD94 high (+++, mfi >300), and CD94 intermediate (++, mfi 150–300) mfi were generated. Both subpopulations were incubated separately with low-dose IL-2 and TKD for 4 days. A representative flow cytometric profile of both populations is illustrated in the middle panel of Figure 6. The CD94 high population was also strongly positive for CD56, whereas the CD94 intermediate population was weakly positive for CD56. Both subpopulations were tested as effector cells in a 4-hours cytotoxicity assay using MHC class-I and Hsp70-positive tumor cells as targets. As illustrated in Figure 6, the CD94 high population showed an increased killing activity of Hsp70-positive tumor cells compared with the CD94 intermediate population.

Finally, the role of the 14 amino acid peptide TKD, as a naturally occurring sequence of the Hsp70-protein, in the stimulation of the NK cell activity and CD94 expression was analyzed in comparison with a mutated 14-mer peptide that exhibits 1 amino acid sequence exchange at position 1 (T-L). Both peptides were tested under identical conditions (2 μg/mL for 4 days) with respect to their immunostimulatory efficacy on CD94-sorted, primary NK cells. Interestingly, only TKD but not *L*KD in combination with low-dose IL-2 (100 IU/mL) was able to induce Hsp70 reactivity toward Cx+ tumor cells. Furthermore, an increase in CD94 mfi was also only observed with TKD-stimulated NK cells (Fig. 7). Taken together, these findings support our hypothesis that indeed the mfi of the CD94 expression is a relevant marker for the Hsp70 reactivity.

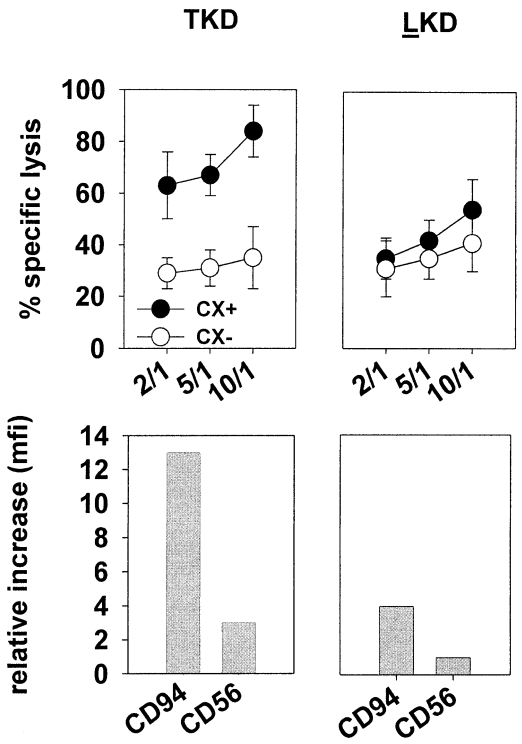


Fig 7. The 14-mer Hsp70-peptide TKD, but not the 14-mer *L*KD, exhibiting 1 amino acid exchange at position 1 (T to L), stimulates Hsp70 reactivity concomitant with an increase in the mean fluorescence intensity of CD94 on natural killer cells. Lysis of Cx+ tumor cells was significantly enhanced as compared with that of Cx− cells. The spontaneous release for each target cell was less than 20%. Effector to target cell ratios were 10:1, 5:1, and 2:1.

ACKNOWLEDGMENTS

The authors thank Dr M.J. Robertson for providing NKL cell line, Dr T. Whiteside for providing YT cell line, and Lydia Rossbacher and Gerald Thonigs for excellent technical assistance. The authors thank Dr M. Klouche (Department of Clinical Chemistry) for providing leukapheresis products. This study was supported by BMBF-grant (BioChance 0312338), by EU-grant TRANSEUROPE (QLRT 2001 01936), by multimmune GmbH, and Schering AG.

REFERENCES

Bauer S, Groh V, Wu J, Steinle A, Philips JH, Lanier LL, Spies T. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285: 727–729.

Biassoni R, Cantoni C, Falco M, et al. 1996. The human leukocyte antigen (HLA)-C-specific ''activatory'' or ''inhibitory'' natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med* 183: 645–650.

Borrego F, Kabat J, Kim DK, Lieto L, Maasho K, Penta J, Solana R, Coligan JE. 2002. Structure and function of major histocompatibility complex (MHC) class I specific receptors expressed on human natural killer (NK) cells. *Mol Immunol* 38: 637–60.

Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG confers protection from natural killer cell-mediated lysis. *J Exp Med* 187: 813–818.

Braud VM, Allan DS, O'Callaghan CA, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391: 795–799.

Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE. 1997. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J Exp Med* 185: 795–800.

Cantoni C, Bottino C, Vitale M, et al. 1999. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J Exp Med* 189(5): 787–796.

Colonna M, Navarro F, Bellon T, et al. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med* 186: 1809–1818.

Colonna M, Samaridis J. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 268: 405–408.

Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, Carson WE, Caligiuri MA. 2001. Human natural killer cells: a unique innate immunoregulatory role for the $CD56^{bright}$ subset. *Blood* 97: 3146–3151.

Cosman D, Fanger N, Borges L, Kubin M, Chin W, Peterson L, Hsu ML. 1997. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity* 7: 273–282.

Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14: 123–133.

Gehrmann M, Pfister K, Hutzler P, Gastpar R, Margulis B, Multhoff G. 2002. Effects of antineoplastic agents on cytoplasmic and membrane-bound heat shock protein 70 (Hsp70) levels. *Biol Chem* 383: 1715–1725.

Gong JH, Maki G, Klingemann HG. 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia* 8: 652–658.

Gross C, Hansch D, Gastpar R, Multhoff G. 2003. Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. *Biol Chem* 384: 267–279.

Gunturi A, Berg RE, Forman J. 2003. Preferential survival of CD8 T and NK cells expressing high levels of CD94. *J Immunol* 170: 1737–1745.

Jacobs R, Hintzen G, Kemper A, Beul K, Kempf S, Behrens G, Sykora KW, Schmidt RE. 2001. $CD56^{bright}$ cells differ in their KIR repertoire and cytotoxic features from $CD56^{dim}$ NK cells. *Eur J Immunol* 31: 3121–3126.

Lanier LL. 1998a. NK cell receptors. *Annu Rev Immunol* 16: 359–393.

Lanier LL. 1998b. Activating and inhibitory NK cell receptors. *Adv Exp Med Biol* 452: 13–18.

Lanier LL, Chang C, Philips JH. 1994. Human NKR-P1a. A disulphide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *J Immunol* 153: 2317–2323.

Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol* 136: 4480–4486.

Lanier LL, Ruitenberg JJ, Philips JH. 1988. Functional and biochemical analysis of CD16 antigen on NK cells and granulocytes. *J Immunol* 141: 3478–3485.

Lanier LL, Testi R, Bindl J, Phillips JH. 1989. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med* 169: 2233–2238.

Lazetic S, Chang C, Houchins JP, Lanier LL, Philips JH. 1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J Immunol* 157: 4741–4745.

Lee N, Llano M, Carretero A, Ishitani A, Navarro F, Lopez-Botet M, Geraghty DE. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci U S A* 95: 5199–5204.

Lian RH, Maeda M, Lohwasser S, Delcommenne M, Nakano T, Vance RE, Raulet DH, Takei F. 2002. Orderly and nonstochastic acquisition of CD94/NKG2 receptors by developing NK cells derived from embryonic stem cells in vitro. *J Immunol* 168: 4980–4987.

Ljunggren HG, Kärre K. 1990. In search of the missing self: MHC molecules and NK recognition. *Immunol Today* 11: 237–244.

MacDonald HR, Engers HD, Cerottini JC, Brunner KT. 1974. Generation of cytotoxic T lymphocytes in vitro. *J Exp Med* 140: 718–730.

Michaelsson J, de Matos CT, Achour A, Lanier L, Kärre K, Söderström K. 2002. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. *J Exp Med* 1403–1414.

Moretta A, Vitale M, Bottino C, et al. 1993. P58 molecules as putative receptors for major histocompatibilty complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med* 178: 597–604.

Moser C, Schmidbauer C, Gürtler U, Gross C, Gehrmann M, Thonigs G, Pfister K, Multhoff G. 2002. Inhibition of tumor growth in mice with severe combined immunodeficiency is mediated by heat shock protein 70 (Hsp70)-peptide-activated, CD94 positive natural killer cells. *Cell Stress Chaperones* 7(4): 365–373.

Multhoff G, Botzler C, Jennen L, Schmidt J, Ellwart J, Issels R. 1997. Heat shock protein 72 on tumor cells. A recognition structure for natural killer cells. *J Immunol* 158: 4341–4350.

Multhoff G, Mizzen L, Winchester CC, Milner CM, Wenk S, Kampinga HH, Laumbacher B, Johnson J. 1999. Heat shock protein 70 (Hsp70) stimulates proliferation and cytolytic activity of NK cells. *Exp Hematol* 27: 1627–1636.

Multhoff G, Pfister K, Gehrmann M, Hantschel M, Gross C, Hafner M, Hiddemann W. 2001. A 14-mer Hsp70 peptide stimulates natural killer (NK) cell activity. *Cell Stress Chaperones* 6(4): 337–344.

Navarro F, Liano M, Bellon T, Colonna M, Geraghty DE, Lopez-Botet M. 1999. The ILT2 (LIR2) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cell. *Eur J Immunol* 29: 277–283.

Pende D, Biassoni R, Cantoni C, et al. 1996. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140–kDa disulphide-linked dimer. *J Exp Med* 184: 505–518.

Pende D, Parolini S, Pessino A, et al. 1999. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J Exp Med* 190(10): 1505–1516.

Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, Biassoni R, Moretta A. 1998. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med* 188(5): 953–960.

Robertson MJ, Cochran KJ, Cameron C, Le JM, Tantravahi R, Ritz J. 1996. Characterization of a cell line, NKL, derived from an aggressive human natural killer leukemia. *Exp Hematol* 24: 410–415.

Schmidt-Wolf IGH, Negrin RS, Kiem HP, Blume KG, Weissman IL.

1991. Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. *J Exp Med* 174: 139–149.

Schmidt-Wolf GD, Negrin RS, Schmidt-Wolf IGH. 1997. Activated T cells and cytokine-induced CD3+ CD56+ killer cells. *Ann Hematol* 74: 51–56.

Trinchieri G. 1989. Biology of natural killer cells. *Adv Immunol* 47: 176–187.

Vitale M, Bottino C, Sivori S, et al. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med* 187(12): 2065–2072.

Voss SD, Daley J, Ritz J, Robertson MJ. 1998. Participation of the CD94 receptor complex in costimulation of human natural killer cells. *J Immunol* 160: 1618–1626.

Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. 1995. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and functional transfer. *Immunity* 3: 801–809.

Wu J, Song Y, Bakker AB, Bauer S, Spies T., Lanier LL, Philips JH. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 285: 730–732.

Yodoi J, Teshigawara K, Nikaido T, et al. 1985. TGGF (IL-2)-receptor inducing factor(s). I. Regulation of IL-2 receptor on natural killer-like cell line (YT cells). *J Immunol* 143: 1623–1630.

The Cell Surface-Localized Heat Shock Protein 70 Epitope TKD Induces Migration and Cytolytic Activity Selectively in Human NK Cells[1]

Robert Gastpar,[2]* Catharina Gross,[2]* Lydia Rossbacher,* Joachim Ellwart,† Julia Riegger,* and Gabriele Multhoff[3]*

Profiling of surface-bound proteins uncovers a tumor-selective heat shock protein 70 (Hsp70) membrane expression that provides a target structure for human NK cells. Hsp70 peptide TKD (TKDNNLLGRFELSG; aa 450–463) was found to enhance the cytolytic activity of NK cells. In this study, we demonstrate that TKD-activated $CD3^-CD56^+CD94^+$ NK cells are selectively attracted by Hsp70 membrane-positive tumor cells, and supernatants derived thereof. Hsp70 membrane-negative tumors failed to attract these NK cells. The capacity to migrate was associated with a substantial lytic activity against Hsp70-positive tumor cells. Because NK cell migration was independent of cell-to-cell contact, the involvement of a soluble factor was assumed. Interestingly, synthetic Hsp70 protein and Hsp70 peptide TKD, mimicking surface-bound Hsp70, initiates migration of NK cells in a concentration-dependent (1–5 μg/ml), highly selective, and chemokine-independent manner. In summary, our results indicate that Hsp70 peptide TKD not only stimulates cytolysis but also chemotaxis in $CD3^-CD56^+CD94^+$ NK cells. *The Journal of Immunology,* **2004, 172: 972–980.**

Unlike T and B cells, NK cells express neither clonally restricted, genetically diverse TCRs nor Abs. The interaction with target cells depends on an interplay between germline-encoded inhibitory receptors, with specificity for certain MHC class I alleles, and activating receptors whose ligands are less well characterized (1–3). Classical and nonclassical MHC class I molecules were characterized as interacting partners for killer cell Ig-like receptors, Ig-like transcripts, and C-type lectin receptors. According to the missing-self theory, target cells lacking MHC class I expression are susceptible to NK cell-mediated cytotoxicity, because they fail to provide the ligand for inhibitory receptors (1, 2, 4). We identified a heat shock protein 70 (Hsp70)[4] epitope (TKDNNLLGRFELSG; aa 450–463) on tumor cells as a recognition site for NK cells. To examine the role of membrane-bound Hsp70 in more detail, we investigated the ability of tumor cells with differential Hsp70 membrane expression pattern to attract effector cells. NK cells circulate in the peripheral blood or reside in the spleen and liver but are also present in thymus, bone marrow, and lymph nodes. Following viral and bacterial infections, NK cells accumulate in extrahematic organs (5–7). In addition, NK cells efficiently infiltrate murine tumors, especially those lacking host MHC class I molecules due to defects in transporter-associated Ag processing or β_2-microglobulin genes (8, 9). In vitro, NK cell migration is inducible by several chemokines and cytokines including macrophage-inflammatory protein-1α, IFN-γ-inducible protein 10, TNF-α, and IL-2 (10–17). Chemokine knockout mice showed severe defects in the migration of NK cells into solid tumors (11). Apart from chemokines, less is known about additional stimuli, for the recruitment of NK cells. Therefore, in the present study we intended to identify tumor cell-derived factors involved in NK cell migration.

Several groups reported on a plasma membrane localization of molecular chaperones on tumor cells (18–20). We detected Hsp70, the major stress-inducible member of the HSP70 group on a number of different tumor cells, but not on normal tissues (21). By cell sorting via Hsp70-specific Ab, we generated colon carcinoma sublines with a stable high and low Hsp70 cell surface expression. Apart from Hsp70, the sublines exhibited an identical expression pattern of other cell surface markers, including MHC and adhesion molecules (22). The Hsp70 epitope exposed to the extracellular milieu could be identified as the amino acid sequence TKDNNLLGRFELSG (TKD; aa 450–463), which is part of the C-terminal domain of Hsp70 (23, 24). We identified NK cells as being relevant effector cells for the recognition of membrane-bound Hsp70 on tumor cells (21, 22, 25–27). Incubation of NK cells with Hsp70 peptide TKD results in an up-regulated CD94 expression that is associated with an enhanced lytic activity against Hsp70 membrane-positive tumor target cells (24). Binding studies revealed a firm interaction of Hsp70 and Hsp70 peptide TKD to CD94-positive NK cells (28). In this study, we studied the migratory capacity of Hsp70-activated NK cells toward Hsp70 membrane-positive and -negative tumor cells and supernatants derived thereof. In an effort to identify a tumor-derived soluble factor, different Hsp70-related peptides, including TKD, were tested as potential attractants for Hsp70-reactive effector cells.

Materials and Methods

Peptides and proteins

The following 14-mer peptides TKDNNLLGRFELSG (TKD; aa 450–463), TKDNNLLGKFELTG (Hsc70 14-mer; aa 450–463), and

*Department of Hematology and Oncology, University Hospital Regensburg, Regensburg, Germany; and †Institute of Molecular Immunology, GSF National Research Center for Environment and Health, Munich, Germany

Received for publication July 31, 2003. Accepted for publication November 5, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the European Union project TRANSEUROPE, by Bundesministerium für Bildung und Forschung (BioChance; 0312338), and by multimmune GmbH.

[2] R.G. and C.G. contributed equally to this work.

[3] Address correspondence and reprint requests to Prof. Dr. Gabriele Multhoff, Department of Hematology and Oncology, University Hospital Regensburg, Franz-Josef Strauss Allee 11, D-93053 Regensburg, Germany. E-mail address: gabriele.multhoff@klinik.uni-regensburg.de

[4] Abbreviation used in this paper: Hsp70, heat shock protein 70.

0022-1767/04/$02.00

AADNKSLGQFNLDG (DnaK 14-mer; aa 447–460) were produced by F-moc synthesis (fluorenylmethoxycarbonyl/*t*-butyl-based solid-phase peptide chemistry method; Bachem, Bubendorf, Switzerland), as previously described (24). The purity of each peptide was >96%.

Human rHsp70 protein was purchased from Stressgen (SPP-75; Victoria, British Columbia, Canada).

Cell culture

The adherent growing human pancreatic carcinoma cell line Colo357 was separated into two sublines, $Colo^+$ and $Colo^-$, by FACS according to their Hsp70 plasma membrane expression using an $Hsp70^{FITC}$ Ab specific for the epitope presented to the surface (clone cmHsp70.1; multimmune, Regensburg, Germany). The resulting sublines exhibit either high ($Colo^+$) or low ($Colo^-$) Hsp70 plasma membrane expression. Similarly, the human colon carcinoma cell line CX2 was separated into sublines with stable high (CX^+) and stable low (CX^-) Hsp70 plasma membrane expression, as previously described (22). The cell lines were maintained mycoplasma free in RPMI 1640 medium containing 10% heat-inactivated FCS (Life Technologies, Eggenstein, Germany) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate, and were harvested by treatment with trypsin/EDTA (all cell culture reagents were purchased from Life Technologies).

Supernatants were collected from tumor cells cultured for 48 h in a humidified atmosphere, at 37°C and 5% CO_2. Then, supernatants were centrifuged at 300 × *g* to remove cell debris, and filtered through a 0.2-μm filter (Costar, Corning, NY).

Selection of CD94-positive and CD94-negative cells

CD94-positive and -negative cells were selected from PBMC derived from healthy human donors using CD94-biotin Ab (HP3-D9; Ancell Immunology Research Products, Bayport, MN) and anti-biotin magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 1×10^8 cells/ml were incubated with 5 μg of biotin-conjugated CD94 Ab for 30 min at 4°C. After extensive washing with MACS buffer (PBS, 0.5% BSA, and 2 mM EDTA), cells were incubated with anti-biotin magnetic microbeads for 15 min at 4°C. Afterward, cells were washed and separated using a LS/VS column (Miltenyi Biotec).

PBL, CD94-positive, and CD94-negative cells were incubated for 4 days in RPMI 1640 medium (Life Technologies) supplemented with heat-inactivated FCS (10%) plus 100 IU/ml IL-2 and 2 μg/ml Hsp70 peptide TKD at 37°C, in a humidified atmosphere containing 5% CO_2.

Migration assay

Migration assays were performed in a Transwell cell culture system (Costar; Corning) in triplicate. The upper and lower compartments were separated by a tissue culture polycarbonate membrane (polyvinyl-pyrrolidone free; Nucleopore, Pleasanton, CA), 6.5 mm in diameter, with a pore size of 5 μm. Tumor target cells (CX^+, CX^-, $Colo^+$, $Colo^-$), cell culture supernatants, and peptides were placed in a total volume of 600 μl of RPMI 1640 medium containing 10% FCS in the lower compartment. Then, 2×10^6 PBL or CD94-positively sorted cells stimulated with TKD were labeled with sodium [^{51}Cr]chromate (100 μCi; NEN-DuPont, Boston, MA) for 2 h. After extensive washing, labeled cells were counted, and 0.2×10^6 cells were resuspended in 100 μl of RPMI 1640 medium supplemented with 10% FCS and carefully added to the upper compartment. rIL-15 (10 ng/ml; Immunex, Seattle, WA) served as a positive control for chemotaxis (29). After a 4-h incubation period in a humidified incubator at 37°C and 5% CO_2, the cell suspension in the lower compartment was harvested, and radioactivity was measured on a gamma counter (Packard Instruments, Meriden, CT). The number of migrated cells was determined according to the following equation: % specific migration = (experimental value − spontaneous value)/(maximum value − spontaneous value) × 100.

Flow cytometry

Directly conjugated Abs ($CD3^{FITC}$, $CD16/56^{PE}$, $CD56^{FITC}$ (BD Biosciences, Heidelberg, Germany); $NKG2A^{PE}$ (Immunotech Coulter, Marseille, France); $CD94^{PE}$ (Ancell); $CD94^{FITC}$ (BD PharMingen, San Diego, CA)) were added to cell suspensions (1×10^5 cells), incubated for 20 min on ice, washed, and analyzed on a FACSCalibur instrument (BD Biosciences). The percentage of single- or double-positively stained cells was defined as the number of specifically stained cells minus the number of cells stained with an isotype-matched control Ab.

Cytotolysis assay

CX^+, CX^- and $Colo^+$, $Colo^-$ tumor target cells (3×10^3) were incubated for 1 h with sodium [^{51}Cr]chromate (100 μCi; NEN-DuPont), extensively washed, and plated with effector cells at indicated E:T ratio. After a 4-h incubation period at 37°C and 5% CO_2, supernatants were harvested, and radioactivity was determined by gamma counting. The percentage of specific lysis was calculated according to the following equation: % specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. The spontaneous release was <15% for each target cell.

Results

TKD-stimulated PBL selectively infiltrate Hsp70-positive tumor cell clusters

Previously, we reported that incubation with Hsp70 peptide TKD and low-dose IL-2 increases the cytolytic activity of NK cells against tumor target cells expressing Hsp70 on their cell surface (24). The role of membrane-bound Hsp70 as a target structure for NK cells has been demonstrated using HLA-identical colon carcinoma sublines CX^+ and CX^- that differ profoundly with respect to their capacity to express Hsp70 on the plasma membrane (22). To obtain further insight into the interaction of TKD-stimulated effector cells and Hsp70-positive tumor target cells, a long-term coincubation experiment was set up. TKD-stimulated PBL were added to exponentially growing tumor cell subpopulations. After a 24-h incubation period, the morphology of cocultures and controls, omitting PBL, was analyzed by light microscopy (Fig. 1). The inserts represent a 2.5-fold higher magnification of a selected cell cluster. When cultivated in the absence of TKD-stimulated PBL, both Hsp70-positive CX^+ and Hsp70-negative CX^- cells showed a similar morphology, forming adherent spheroidal cell clusters (control). In contrast, the morphology of CX^+ cells cocultured with TKD-stimulated PBL (+PBL) differed significantly from that of CX^- cells, indicating that activated PBL were attracted by Hsp70-positive CX^+ tumor cells. Almost all CX^+ cell clusters were infiltrated by effector cells, whereas Hsp70-negative CX^- cell clusters remained unaffected. In addition, a significantly reduced viability was observed in CX^+ cells compared with CX^- cells after coincubation with TKD-activated PBL (data not shown). These findings are in line with results from cytotoxicity assays showing that Hsp70-positive target cells are more susceptible to lysis mediated by TKD-stimulated effector cells, as compared with Hsp70-negative target cells (22, 25).

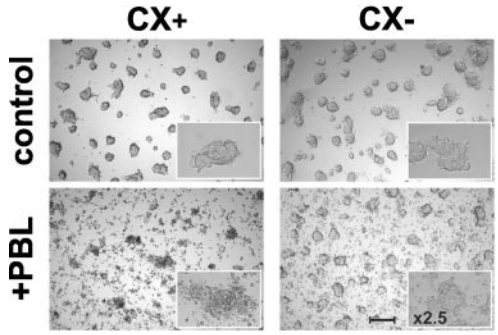


FIGURE 1. Infiltration of Hsp70-positive tumor cell clusters by TKD-activated PBL. Hsp70 membrane-positive CX^+ and -negative CX^- cells (0.1×10^6 cells/well) were seeded in 96-well flat-bottom plates and grown for 48 h. Tumor cells were coincubated with either medium or TKD-stimulated PBL for another 24 h. Light-microscopic analysis is shown of tumor cells only (control; *upper row*) or tumor cells coincubated with TKD-activated PBL (+PBL; *lower row*) at a magnification of ×5 on a Zeiss (Oberkochen, Germany) Axiovert 25 microscope; small *insets* show a 2.5-fold magnification of one representative cell cluster. The scale bar in the *lower right panel* indicates 100 μm.

TKD-stimulated PBL migrate to Hsp70 membrane-positive tumor cells and supernatants derived thereof

The increased sensitivity of Hsp70-positive tumor target cells mediated by TKD-stimulated NK cells has been demonstrated by using HLA-identical colon carcinoma sublines CX^+ and CX^- with differential Hsp70 membrane expression. In this study, an additional tumor model system was established using the human pancreatic carcinoma cell line Colo357. Initially, Colo357 cells express Hsp70 on ~50% of the cells. Identical with the CX^+CX^- cell separation, Hsp70 high ($Colo^+$)- and low ($Colo^-$)-expressing sublines were generated by cell sorting via an Hsp70 mAb (22). Fig. 2*A* illustrates the percentages of Hsp70 positively stained cells in CX^+ (90%) and CX^- (20%), and in $Colo^+$ (73%) and $Colo^-$ (34%) sublines after cell sorting. The migratory capacity of TKD-activated PBL was tested toward these different tumor sublines and against supernatants derived thereof. The cell density of the effector and tumor cells in the lower compartment, the pore size and pore distribution, and the coincubation time were optimized in kinetic studies (data not shown). In a first set of experiments, CX^+, CX^- and $Colo^+$, $Colo^-$ cells, with differential Hsp70 membrane expression pattern, were cultured in the lower compartment for 48 h. Then, TKD-activated PBL were added carefully to the upper compartment and after a 4-h coincubation period, specific migration was measured. As depicted in Fig. 2*B*, migration of TKD-activated PBL, derived from five independent healthy human volunteers, toward Hsp70 plasma membrane-positive CX^+ (11%) and $Colo^+$ (10%) was higher as compared with target cells with low Hsp70 membrane expression (CX^-, 5%; $Colo^-$, 6%). Differences in migration toward CX^+, CX^- cells was more pronounced as compared with $Colo^+$, $Colo^-$ tumor cells. This finding is due to the fact that differences in the Hsp70 cell membrane expression in the positive and negative sublines was also weaker in the Colo cell system. Migration to 10 ng/ml of the cytokine IL-15, serving as a positive control, was 15% (data not shown). This is in line with previously published data (29).

It has been shown that coincubation of IL-2-activated NK cells and K562 target cells results in effector-target cell conjugates that trigger chemokine secretion and chemoattraction of additional NK cells (30). To rule out chemoattraction induced by direct cell-to-cell contact, cell-free supernatants derived from 2-day tumor cell cultures, were used. Interestingly, the migration assay toward supernatants of Hsp70 membrane-positive CX^+ and $Colo^+$ cells resulted in significant migration (12%), whereas that toward CX^- and $Colo^-$ cells did not induce migration (3 and 6%, respectively) (Fig. 2*C*). This suggests that migration of TKD-activated PBL might be induced by a soluble factor that is secreted equally well by Hsp70 membrane-positive CX^+ and $Colo^+$ tumor cells. Recent data provide evidence for a tumor-specific release of members of the HSP70 family (31), indicating that the soluble factor released by CX^+ and $Colo^+$ tumor cells might be Hsp70 or an HSP-related protein.

It is also important to note that incubation of PBL with low dose IL-2 plus Hsp70 peptide TKD is a prerequisite for the induction of the migratory capacity, because unstimulated PBL or PBL cultured with IL-2 alone did not migrate toward Hsp70 membrane-positive and -negative tumor target cells, or supernatants derived thereof (data not shown).

$CD3^-CD56^+CD94^+$ NK cells exhibit migratory and cytolytic activity against Hsp70 membrane-positive tumor cells

According to the preceding findings, we were interested in the phenotype and functional properties of both the migrated and nonmigrated cell populations. Therefore, after a 4-h migration assay

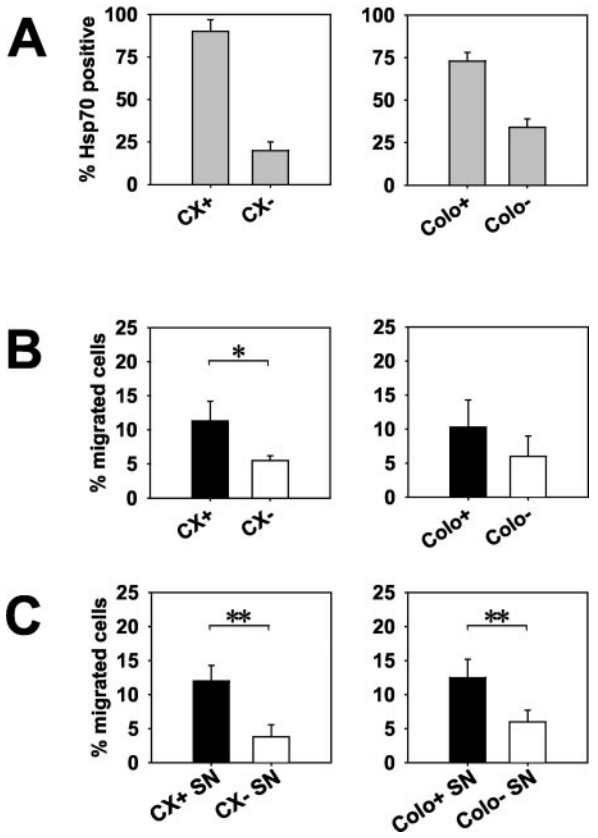


FIGURE 2. TKD-activated PBL migrate toward Hsp70-positive target cells and supernatants (SN) derived thereof. *A*, Phenotypic characterization of the Hsp70 membrane expression on human colon carcinoma cell sublines CX^+ and CX^- and human pancreatic carcinoma cell sublines $Colo^+$ and $Colo^-$. The sublines were generated by FACS using $Hsp70^{FITC}$ Ab (clone cmHsp70.1), which recognizes the Hsp70 surface epitope. The data represent the mean percentage (±SEM) of Hsp70-positively stained cells of at least eight independent experiments. *B*, Migration of TKD-activated PBL to CX^+, CX^- and $Colo^+$, $Colo^-$ tumor cells. Migration was tested in a Transwell system with two compartments separated by a polycarbonate membrane with a pore size of 5 μm. In the lower compartment, CX^+, CX^- or $Colo^+$, $Colo^-$ cells (0.2×10^6 cells per well) were grown for 48 h in 600 μl of RPMI 1640 medium containing 10% FCS. PBL, stimulated with TKD (2 μg/ml) plus low-dose IL-2 (100 IU/ml) for 4 days, were harvested, labeled with sodium [^{51}Cr]chromate (100 μCi), and transferred into the upper chamber of the Transwell system. Following a 4-h coincubation period, radioactivity was determined in PBL that migrated to the lower chamber. Specific migration was calculated as described in *Materials and Methods*. Results are shown as the mean ± SEM of at least five independent experiments. Statistically significant (Student's *t* test), *, $p < 0.05$. *C*, In the next set of experiments, 600 μl of cell-free culture supernatant (SN) of CX^+CX^- and $Colo^+Colo^-$ tumor cells cultured for 48 h were administered in the lower compartment. Following a 4-h coincubation period with PBL, radioactivity was determined in the migrated cells of the lower chamber. Specific migration was calculated as described in *Materials and Methods*. Results are shown as the mean ± SEM of at least five independent experiments. Statistically significant (Student's *t* test), *, $p < 0.05$; **, $p < 0.005$.

toward supernatants of Hsp70 membrane-positive tumor cells, nonmigrated cells from the upper compartment and migrated cells from the lower compartment were collected and immunophenotyped. The initial population (PBL) consisted of 34% $CD3^+$ T cells, 11% $CD3^+CD16/56^+$ NKT cells, and 29% $CD3^-CD16/56^+$ NK cells (Fig. 3*A*). The nonmigrated cells showed no significant differences in the percentage of $CD3^+$ T cells (36%), and $CD3^+CD16/56^+$ NKT cells (7%). However, the $CD3^-CD16/56^+$

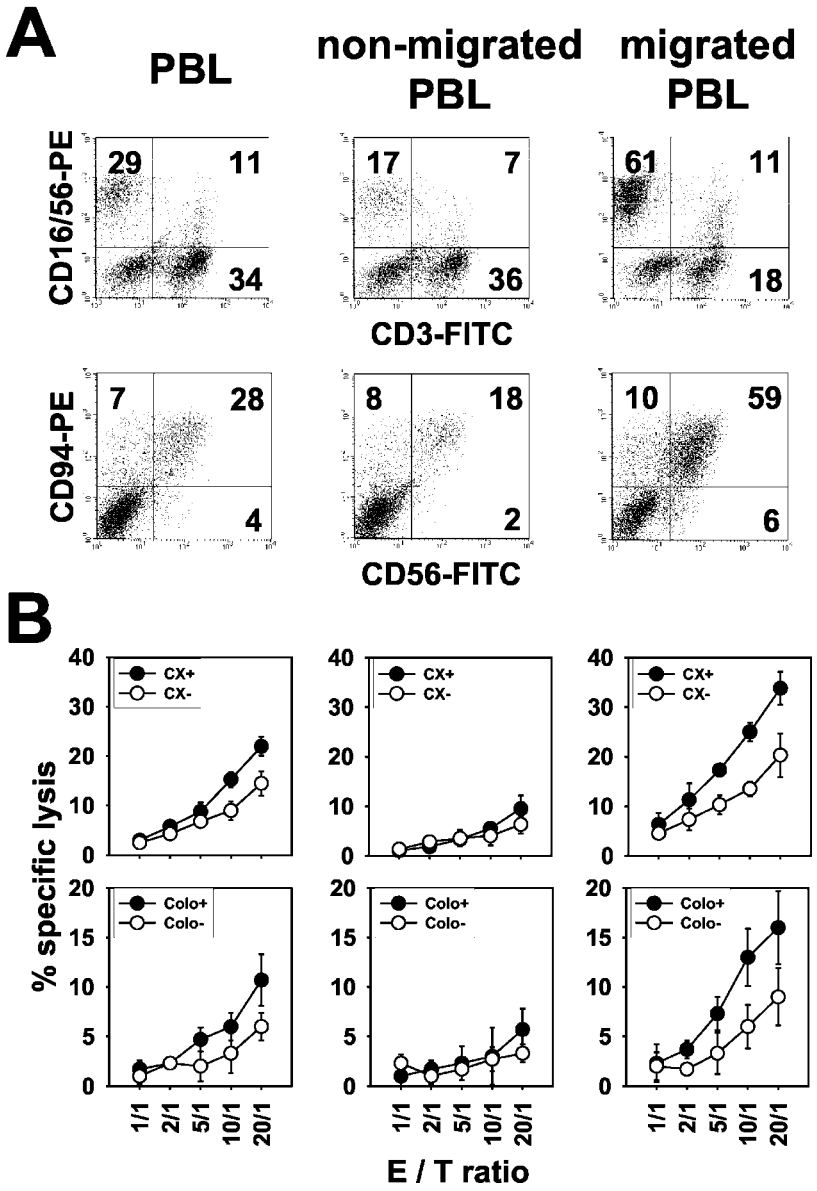


FIGURE 3. NK cells migrate and exhibit lytic activity against Hsp70-positive tumor target cells. A 4-h migration assay was performed using TKD-stimulated PBL against supernatants of Hsp70-positive CX^+ tumor cells to determine the phenotype and the lytic activity of nonmigrated and migrated cells in comparison with the initial PBL population. *A*, Nonmigrated PBL (*middle panel*), and migrated cells (*right panel*) were harvested and analyzed by multiparameter flow cytometry using $CD3^{FITC}$, $CD16/56^{PE}$, $CD56^{FITC}$, and $CD94^{PE}$ Abs, and a double staining was performed with $CD94^{FITC}$ and $NKG2A^{PE}$ Abs. The phenotype of each cell population was compared with the initial PBL population (*left panel*) that had been stimulated with TKD for 4 days. Numbers in the dot plot diagrams indicate percentage of positively stained cells for each quadrant. Data of one representative result of five independent experiments is shown. Increase in $CD56^+CD94^+$ cells was always doubled in the migrated cell population if compared with the initial PBL population. *B*, Concomitantly, the cytolytic activity of the three effector cell populations (initial PBL, nonmigrated PBL, and migrated PBL) was tested in a standard 4-h chromium release assay. Specific lysis of the effector cells was assessed against CX^+ and $Colo^+$ tumor target cells with high Hsp70 surface expression (●) and CX^- and $Colo^-$ tumor target cells with low Hsp70 surface expression (○). E:T ratios ranged from 1:1 to 20:1. Data represent the mean values of four independent experiments.

NK cell subpopulation shrank from 29 to 17%. In contrast, a significant increase in the percentage of $CD3^-CD16/56^+$ NK cells was observed (from 29 to 61%) within the migrated cell population, whereas the amount of $CD3^+$ T cells was found to be reduced by half (from 34 to 18%). These results suggest that predominantly $CD3^-CD16/56^+$ NK cells were attracted by supernatants derived from Hsp70 membrane-positive tumor cells.

Recently, we demonstrated an increase in cell surface density of the C-type lectin receptor CD94 after TKD stimulation, selectively on NK cells (28, 32). Furthermore, the enhanced lysis of tumor target cells with Hsp70 plasma membrane expression by TKD-stimulated NK cells was blockable in the presence of CD94 Ab (32). This suggests an involvement of CD94 in the interaction of NK cells with Hsp70-expressing tumor cells. Therefore, the role of CD94 was studied in more detail with respect to the migratory capacity. Compared with the original population, the percentage of $CD56^+CD94^+$ cells was markedly reduced in the nonmigrated cell population (from 28 to 18%). Concomitantly, a drastic increase of $CD56^+CD94^+$ cells was found in the migrated cell population (from 28 to 59%; Fig. 3*A*). The results of five independent migration assays confirmed this finding; the amount of $CD56^+CD94^+$ cells was always twice as high in the migrated cell population if

compared with the initial PBL and three to four times higher if compared with the nonmigrated cell population. No significant changes were observed in the $CD56^-CD94^+$ (7 vs 8 vs 10%) and in the $CD56^+CD94^-$ cell population (4 vs 2 vs 6%), indicating that predominantly $CD56^+CD94^+$ NK cells were attracted by cell culture supernatants of CX^+ cells. Multiparameter staining using $CD94^{FITC}$- and $NKG2A^{PE}$-specific Abs revealed that the migrated cells were double positive for CD94 and NKG2A.

To correlate phenotype and function, cytolysis assays were performed using nonmigrated and migrated cells as effectors. The initial cell population (PBL) exhibited only a moderately increased lytic activity against Hsp70-positive CX^+ and $Colo^+$ tumor cells at an E:T ratio of 20:1 (Fig. 3*B*, *left graph*). Nonmigrated cells consisting predominantly of T cells showed a very weak lytic activity against all four tumor sublines (Fig. 3*B*, *middle graph*). Interestingly, the migrated cell population showed a very strong lytic activity against Hsp70-positive tumor target cells (CX^+ and $Colo^+$) and a weaker activity against Hsp70-negative tumor target cells (CX^- and $Colo^-$) (Fig. 3*B*, *right graph*). Because this effect is associated with an increase in the percentage of $CD3^-CD56^+CD94^+$ NK cells, we speculate that these effector cells might be responsible for migration and lytic activity.

To further confirm this hypothesis, functional tests were performed using MACS-sorted effector cell populations. Fig. 4 illustrates the phenotypic characterization of CD3-sorted PBL that had been stimulated with TKD for 4 days, directly after cell separation. The cytolytic activity of both cell populations was tested against Hsp70-positive tumor target cells (CX^+, $Colo^+$, and K562). K562 cells are susceptible to NK cell-mediated lysis due to a missing MHC class I expression that might trigger inhibitory NK cell receptors, and due to the Hsp70 membrane expression as a trigger for CD94 (our unpublished observation). The $CD3^+$ T cell population ($93 \pm 10\%$ $CD3^+CD16/56^-$) showed no lytic activity (Fig. 4*A*). In contrast, the $CD3^-$ cell population consisting of $44 \pm 22\%$ $CD3^-CD16/56^+$ NK cells lysed Hsp70-positive tumor target cells to a significant extent (Fig. 4*A*). Regarding these findings, $CD3^+$ T cells were excluded as mediators of the cytolytic response against Hsp70-positive tumor target cells.

Preceding migration assays indicated that the phenotype of the migrated cells might be $CD3^-CD56^+CD94^+$. To analyze the role of CD94 in more detail, TKD-stimulated PBL derived from three independent donors were separated by MACS using a CD94 Ab. The CD94-depleted population ($4 \pm 2\%$ $CD56^+CD94^+$) showed a negligible lysis of Hsp70-positive tumor target cells (Fig. 4*B*), whereas the CD94-enriched population ($58 \pm 9\%$ $CD56^+CD94^+$) showed a strong lytic activity against CX^+, $Colo^+$, and K562 cells. Compared with the CD3 separation, following CD94 enrichment, the cytolytic activity was slightly increased. The CD94-enriched cell population consists of $CD3^-CD16/56^+$ NK cells, $CD3^+CD16/56^+$ NKT cells, and $CD3^+CD16/56^-$ T cells. To define which subpopulation is responsible for the Hsp70-mediated cytotoxicity, $CD94^+$ cells were further separated into a $CD94^+CD3^-$ ($79 \pm 19\%$ $CD56^+CD94^+$) and a $CD94^+CD3^+$ cell subpopulation ($70 \pm 7\%$ $CD56^+CD94^+$). A strong lytic activity against Hsp70-positive CX^+ cells was mediated by the $CD94^+CD3^-$ population (Fig. 4*C*). Due to the fact that the $CD3^+$ T cell population still contains $28 \pm 8\%$ $CD56^+CD94^+$ NK cells, a weak cytolytic activity was also seen in this population. In contrast to Hsp70-positive tumors, lysis of Hsp70-negative counterparts (CX^-, $Colo^-$) was $<10\%$.

Taken together, these results support our migration data showing that $CD3^-CD56^+CD94^+$ NK cells are responsible for the migratory and lytic activity against Hsp70 membrane-positive tumor cells.

Hsp70 protein and Hsp70 peptide TKD stimulate migration in $CD3^-CD56^+CD94^+$ NK cells

Earlier, we have shown that not only Hsp70-positive tumor cells but also supernatants derived thereof were able to induce NK cell migration. Because supernatants of Hsp70 membrane-negative tumor cells were unable to induce migration, attraction appeared to be associated with Hsp70 membrane expression. Therefore, we speculate about a soluble factor that might be released by Hsp70 membrane-positive tumor cells.

The Hsp70 epitope recognized by NK cells is identical with the 14-aa peptide TKD, residing in the C-terminal domain (24). Therefore, in addition to full-length Hsp70 protein, the cell surface-localized peptide TKD, mimicking the NK cell recognition site in a soluble form, was tested in migration assays. As shown in Fig. 5*A*, maximal migratory capacity of NK cells was detected if 10 μg/ml Hsp70 protein diluted in fresh medium was subjected to the lower compartment. A comparable migration of NK cells was induced by 1 μg/ml TKD peptide. In summary, these data indicate that Hsp70 protein and Hsp70 peptide TKD both exhibited similar chemotactic activity, which is comparable with that of supernatants derived from Hsp70 membrane-positive tumor cells.

In our previous experiments, the chemotactic factor was placed in the lower compartment, and the effector cells were placed in the upper compartment. To rule out chemokinesis or random cell mobility, the following experiments were performed. One criteria for directed chemotaxis is migration along a positive gradient. If the chemoattractant is equally distributed (no gradient), or in case of a negative gradient, active migration should therefore be inhibited. Fig. 5*B* demonstrates that migration of CD94-positive cells in response to 1 μg/ml TKD was observed only in a positive TKD-gradient (16%). In the presence of equal TKD concentrations in the upper and lower compartments, migration was inhibited (5%; kinesis I). Similar results were seen in a negative gradient (3%; kinesis II). These results demonstrate that migration in response to TKD was rather chemotaxis than chemokinesis or random cell mobility.

To determine whether TKD-related peptides also stimulate CD94-positive NK cells to migrate, two peptides derived from members of the HSP70 group ($Hsc70_{450-463}$ and $DnaK_{447-460}$) were used at concentrations ranging from 0.1 to 10 μg/ml (Fig. 5*C*). Interestingly, TKD-activated CD94-positive NK cells were selectively attracted by TKD, whereas no migration was induced by HSP70 peptides derived from Hsc70 and DnaK. Because $Hsc70_{450-463}$ (TKDNNLLG**K**F EL**T**G) and $DnaK_{447-460}$ (**AA**DN**KS**LG**Q**F**N**L**D**G) differ from TKD (TKDNNLLGRFELSG) by 2- and 7-aa exchanges (indicated by bold type and underlining), respectively, the induction of migration mediated by TKD has to be considered to be specific. Furthermore, the migratory response to TKD was concentration dependent, showing an optimal concentration range between 1 and 5 μg/ml. Concentrations >5 μg/ml resulted in a reduced migratory activity of NK cells. In contrast to CD94-positive NK cells, CD94-negative T cells showed no migration toward the HSP70 peptides at any concentration. Taken together, these findings demonstrate a concentration-dependent, specific attraction of CD94-positive NK cells by Hsp70 peptide TKD.

Discussion

NK cells circulate in the blood vessel system but also reside in solid tissues. Mobilization, recruitment, and migration of NK cells and cytotoxicity are induced in response to a variety of inflammatory stimuli (7, 12, 14–16). Several mouse tumors, including B16

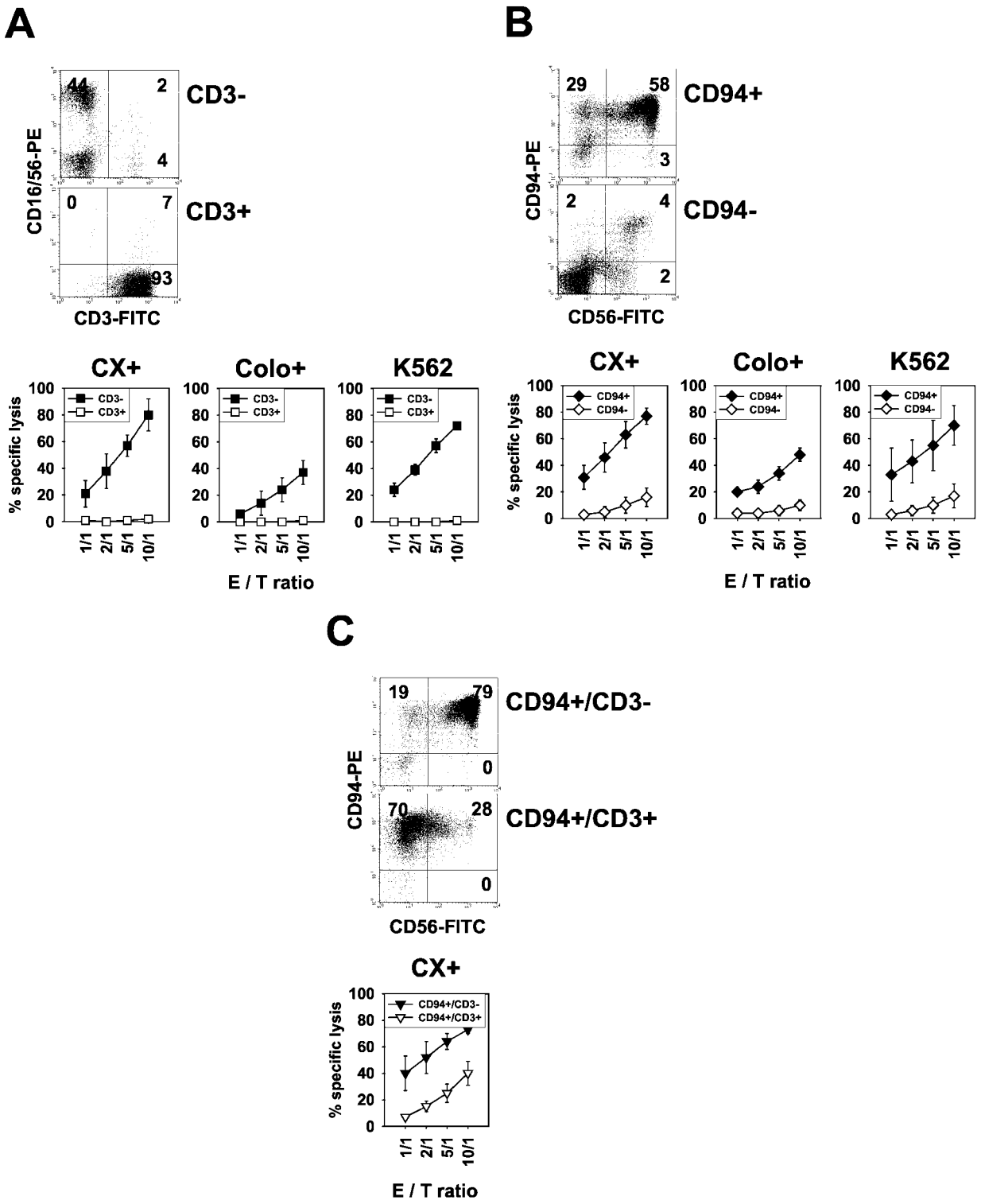


FIGURE 4. Hsp70-positive tumor target cells are lysed selectively by $CD3^-CD56^+CD94^+$ NK Cells, but not by $CD3^+$ T cells. *A*, Separation of PBL of three independent donors in a CD3-negative ($CD3^-$; *upper dot plot*) and CD3-positive ($CD3^+$; *lower dot plot*) subpopulation by MACS using CD3 Ab. Separated cells were stimulated for 4 days with TKD (2 μg/ml) plus IL-2 (100 IU/ml) and phenotyped using $CD3^{FITC}$ and $CD16/56^{PE}$ Abs. The dot plot diagrams of one representative separation is illustrated. Numbers in the dot plot diagram represent the mean percentage values of positively stained cells of three experiments. Concomitantly, the cytolytic activity of both effector cell populations ($CD3^-$, ■; $CD3^+$, □) was tested in a standard 4-h chromium release assay. Specific lysis of effectors was assessed against Hsp70 membrane-positive CX^+, $Colo^+$, and K562 tumor target cells. E:T ratios ranged from 1:1 to 10:1. The mean ± SEM from three independent separations is shown. *B*, Separation of PBL of three independent donors in CD94-positive ($CD94^+$; *upper dot plot*) and CD94-negative ($CD94^-$; *lower dot plot*) subpopulation by MACS using CD94 Ab. TKD-stimulated subpopulations were phenotyped using $CD56^{FITC}$ and $CD94^{PE}$ Abs. Concomitantly, the cytolytic activity of both effector cell populations ($CD94^+$, ◆; $CD94^-$, ◇) was tested against Hsp70 membrane-positive CX^+, $Colo^+$, and K562 tumor target cells. *C*, The CD94-positive subpopulations were further separated in a CD3-negative ($CD94^+CD3^-$; *upper dot plot*) and CD3-positive ($CD94^+CD3^+$; *lower dot plot*) cell population by MACS using CD94 and CD3 Ab. TKD-stimulated effector cells were phenotyped separately using $CD56^{FITC}$ and $CD94^{PE}$ Abs. Concomitantly, the cytolytic activity of both effector cell populations ($CD94^+CD3^-$, ▲; $CD94^+CD3^+$, △) was tested against Hsp70 membrane-positive CX^+ tumor target cells. Due to low cell numbers, cytolytic activity against K562 cells could not be tested.

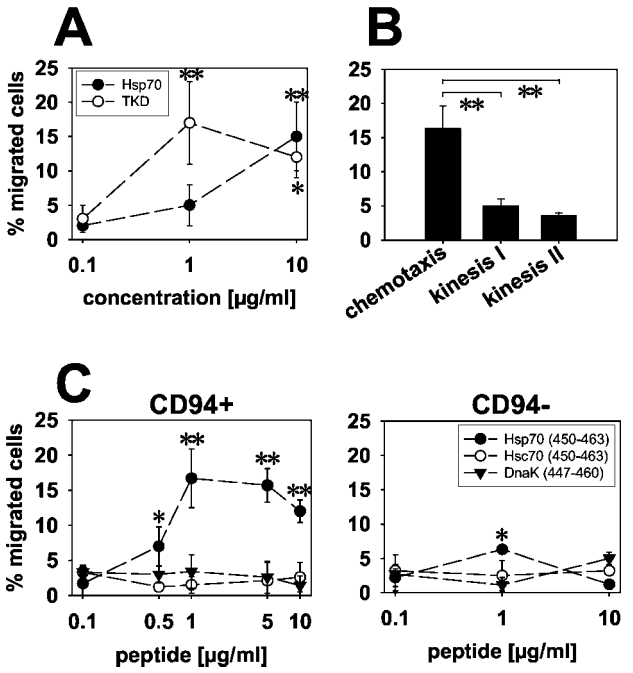


FIGURE 5. TKD induces migration selectively in CD94-positive NK cells. *A*, Migration of CD94-positive NK cells is inducible by human recombinant Hsp70 and TKD. CD94-positive NK cells were derived from MACS. Following stimulation with TKD (2 μg/ml) plus IL-2 (100 IU/ml) for 4 days, migration toward medium supplemented with Hsp70 (●) or TKD (○) was tested at concentrations ranging from 0.1 to 10 μg/ml. Results are shown as the mean ± SEM of three independent experiments. Statistically significant differences (Student's *t* test), *, $p < 0.05$; **, $p < 0.005$. *B*, TKD induces chemotaxis but not chemokinesis. Migration of $CD94^+$ NK cells, stimulated with TKD (2 μg/ml) plus IL-2 (100 IU/ml) for 4 days, was tested in a positive gradient (migration; 1 μg/ml TKD in the lower compartment), equal gradient (kinesis I; equal concentrations of 1 μg/ml TKD in the upper and lower compartments), or a negative gradient (kinesis II; 1 μg/ml TKD only in the upper compartment). Results are shown as the mean ± SEM of three independent experiments. Statistically significant differences between chemotaxis and chemokinesis (Student's *t* test), **, $p < 0.005$. *C*, Migration of CD94-positive NK cells to TKD is concentration dependent and highly specific. TKD-activated CD94-positive NK cells were tested for their capacity to migrate toward TKD at concentrations ranging from 0.1 to 10 μg/ml TKD (*left panel*; ●). Two closely related peptides derived from Hsc70 (aa 450–463; ○) and DnaK (aa 447–460; ▲) were included as controls. All peptides were diluted in RPMI 1640 medium containing 10% FCS. Identical experiments were performed with the CD94-depleted T cell subpopulation ($CD94^-$; *right panel*). Results are shown as the mean ± SEM of five independent experiments. Statistically significant differences compared with initial values (Student's *t* test), *, $p < 0.05$; **, $p < 0.005$.

melanoma, MCA 102 sarcoma, lung carcinoma, MADB106 mammary carcinoma, and RMA-S lymphomas have been found to be infiltrated with NK cells (11, 33–36). In these models, attraction could be associated with chemokines, a large family of small proteins involved in lymphocyte trafficking, and cytokines (37, 38).

In the present study, we record on a cytokine- and chemokine-independent stimulus inducing selective migration of NK cells. Incubation of PBL with Hsp70 peptide TKD enhances the cytolytic activity against Hsp70-positive tumor target cells. Coincubation and migration assays revealed infiltration of TKD-stimulated PBL selectively into Hsp70 membrane-positive, but not into Hsp70 membrane-negative tumor cell clusters. Therefore, we hypothesized that predominantly Hsp70-positive tumor cells possess chemotactic properties. To answer the question whether cell-to-cell contact or a soluble factor is responsible for migration, in additions to cells, supernatants of Hsp70 membrane-positive and -negative tumor cells were tested with respect to their capacity to induce migration. Our results indicate that a soluble factor is secreted by Hsp70 membrane-positive tumor cells. Recent data demonstrate Hsp70 can be released by tumor cells expressing Hsp70 on their cell surface (31, 39). Although the molecular nature of the chemotactic factor secreted by Hsp70 membrane-positive tumor cells remained elusive, we could demonstrate that NK cells were attracted by Hsp70 protein and TKD peptide in a tumor cell-free setting. Due to the fact that migration was found only toward a positive gradient, chemokinesis or random cell mobility appears to be unlikely.

A comparative phenotypic characterization of the migrated and nonmigrated cell population revealed that predominantly $CD3^-CD56^+CD94^+$ NK cells but not CD3-positive T cells migrated toward supernatants of Hsp70 membrane-positive tumor cells. The C-type lectin receptor CD94 is covalently linked to members of the NKG2 family (40). CD94/NKG2A, an inhibitory, and CD94/NKG2C, an activating receptor, are recognizing HLA-E. It appeared that cell surface homeostasis of the inhibitory receptor is independent of functional signaling (41). CD94 Ab blocking (32), and binding studies (28) indicate that, apart from HLA-E, presenting leader peptides of HLA-A, -B, and -C molecules, Hsp70 and Hsp70 peptide TKD provide an additional positive ligand for an activating CD94 receptor. This finding is further supported by the fact that, although CX^+ and CX^- tumor sublines are lacking HLA-E cell surface expression (42), Hsp70 membrane-positive tumor cells are lysed significantly better by $CD94^+$, migrated NK cells as compared with their Hsp70 membrane-negative counterparts. Cell sorting via CD3 and CD94 further confirmed these findings. Although, in addition to CD94, the expression of NKG2A was comparably up-regulated in the migrating cell population, these NK cells showed a strong cytolytic activity against Hsp70 membrane-positive target cells. Because, irrespective of the NK cell activity (inhibitory/activatory), NKG2A was always coexpressed with CD94 (data not shown), the specificity of this Ab for inhibitory NK cells is doubtful. Additional experiments will elucidate the molecular nature of the coreceptor for NK cells with Hsp70 reactivity.

Interestingly, migration of CD94-positive NK cells toward Hsp70 peptide TKD appears to be highly selective, because closely related HSP70 peptides with 2- or 7-aa exchanges were unable to stimulate migration. This observation is in line with our previous findings, that the stimulation of the cytolytic activity of NK cells is also restricted to TKD and Hsp70 protein, the major stress-inducible member of the HSP70 family. Other members of this group, including the constitutively expressed Hsc70 (84% homology to Hsp70) or the *E. coli*-derived Hsp70 homolog DnaK (50% homology to Hsp70), failed to stimulate cytotoxicity (32). Furthermore, protein database BLAST search revealed that the 14-mer sequence TKD is a highly variable region within the Hsp70 sequence and thus is not found in any other protein sequence registered so far (43). In this study, the uniqueness of this sequence has been demonstrated with respect to its migratory function. However, TKD stimulation was found to be a prerequisite to generate sensitivity for the chemotactic signal. No migration was observed if the effector cells were not prestimulated with TKD plus low-dose IL-2.

The role of chemokines in the migration of TKD-activated $CD3^-CD56^+CD94^+$ NK cells could be ruled out for several reasons. No differences in the expression pattern of chemokine receptors was found following stimulation of NK cells with TKD

(data not shown). Differences in the chemokine secretion pattern correlating with Hsp70 membrane expression was also unlikely, because NK cell attraction was also possible with TKD peptide in a tumor cell-free setting. Because Hsp70 membrane-positive and -negative tumor sublines were generated by cell sorting of one parental tumor cell line, both sublines exhibited an identical expression of MHC and adhesion molecules (22).

Adoptive transfer of TKD-stimulated NK cells in tumor-bearing mice revealed that predominantly Hsp70-positive tumors were eliminated (44–46). In this report, we show directed migration of NK cells to Hsp70-positive tumor cells, and supernatants derived thereof, and to TKD peptide. Therefore, it was speculated that killing of Hsp70-positive tumors in vivo might be related to an enhanced migratory capacity of CD94-positive NK cells.

It is known that IL-2-activated NK cells can induce regression of established lung and liver tumors (47–50). However, the antitumor effect is often limited, because primary tumors and metastases are not efficiently infiltrated by NK cells. The prestimulation of CD94-positive NK cells with TKD in combination with low-dose IL-2 results in cytolytic active effector cells with the ability to migrate toward Hsp70-positive tumors that have to be defeated. Therefore, our findings might have further clinical implications with respect to the development of an NK cell-based cellular immunotherapy.

Acknowledgments

We thank Gerald Thonigs for excellent technical assistance. Special thanks to Dr. Markus Becker (Institute of Pharmacy, Regensburg, Germany) for critical discussions and to Institute of Clinical Chemistry (Prof. G. Schmitz and Prof. Dr. M. Klouche (University Hospital Regensburg)) for providing us with leukapheresis products.

References

1. Lanier, L. L. 1992. NK cell receptors. *Annu. Rev. Immunol. 16:359.*
2. Moretta, L., E. Ciccone, M. C. Mingari, R. Biassoni, and A. Moretta. 1994. Human natural killer cells: origin, clonality, specificity, and receptors. *Adv. Immunol. 55:341.*
3. Fan, Q. R., L. Mosyak, C. C. Winter, N. Wagtmann, E. O. Long, and D. C. Wiley. 1997. Structure of the inhibitory receptors for human natural killer cells resemble haematopoietic receptors. *Nature 389:96.*
4. Ljunggren, H.-G., and K. Kärre. 1990. In search of the missing self: MHC molecules and NK recognition. *Immunol. Today 11:237.*
5. Whiteside, T. L., and R. B. Herberman. 1994. Role of human natural killer cells in health and disease. *Clin. Diagn. Lab. Immunol. 1:125.*
6. Holmberg, L. A., K. A. Springer, and K. A. Ault. 1981. Natural killer activity in the peritoneal exudate of mice infected with *Listeria monocytogenes*: characterization of the natural killer cells by using a monoclonal rat anti-murine macrophage antibody (M1/70). *J. Immunol. 127:1792.*
7. McIntyre, K. W., and R. W. Welsh. 1986. Accumulation of natural killer and cytotoxic T large granular lymphocytes in the liver during virus infection. *J. Exp. Med. 164:1667.*
8. Glas, R., K. Sturmhöfel, G. J. Hämmerling, K. Kärre, and H.-G. Ljunggren. 1992. Restoration of a tumorigenic phenotype by β_2-microglobulin transfection to EL-4 mutant cells. *J. Exp. Med. 175:843.*
9. Franksson, L., E. George, S. Powis, G. Butcher, J. Howard, and K. Kärre. 1993. Tumorigenicity conferred to lymphoma mutant by major histocompatibility complex-encoded transporter gene. *J. Exp. Med. 177:201.*
10. Lövik, G., J. T. Vaage, C. Naper, H. B. Benestad, and B. Rolstad. 1995. Recruitment of alloreactive natural killer cells to the rat peritoneum by a transfected cell line secreting rat recombinant interleukin-2. *J. Immunol. Methods 179:59.*
11. Smyth, M. J., J. M. Kelly, A. G. Baxter, H. Korner, and J. D. Sedgewick. 1998. An essential role for tumor necrosis factor in natural killer cell-mediated tumor rejection in the peritoneum. *J. Exp. Med. 188:1611.*
12. Pilaro, A. M., D. D. Taub, K. L. McCormick, H. M. Williams, T. J. Sayers, W. E. Fogler, and R. H. Wiltrout. 1994. TNF-α is a principal cytokine involved in the recruitment of NK cells to liver parenchyma. *J. Immunol. 153:333.*
13. Maghazachi, A. A. 1991. Tumor necrosis factor-α is chemokinetic for lymphokine-activated killer cells: regulation by cyclic adenosine monophosphate. *J. Leukocyte Biol. 49:302.*
14. Salazar-Mather, T. P., J. S. Orange, and C. A. Biron. 1998. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein-1α (MIP-1α)-dependent pathways. *J. Exp. Med. 187:1.*
15. Allavena, P., G. Bianchi, C. Paganin, G. Giardina, and A. Mantovani. 1996. Regulation of adhesion and transendothelial migration of natural killer cells. *Nat. Immunol. 15:107.*
16. Taub, D. D., T. J. Sayers, C. R. Carter, and J. R. Ortaldo. 1995. α and β chemokines induce NK cell migration and enhance NK cell mediated cytolysis. *J. Immunol. 155:3877.*
17. Nemlander, A., E. Saksela, and P. Hayry. 1983. Are "natural killer" cells involved in allograft rejection? *Eur. J. Immunol. 13:348.*
18. Ferrarini, M., S. Heltai, M. R. Zocchi, and C. Rugarli. 1992. Unusual expression and localization of heat-shock proteins in human tumor cells. *Int. J. Cancer 51:613.*
19. Tamura, Y., N. Tsuboi, N. Sato, and K. Kikuchi. 1993. 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. *J. Immunol. 151:5516.*
20. Shin, B. K., H. Wang, A. M. Yim, F. Le Naour, F. Brichory, H. J. Jang, R. Zhao, E. Puravs, J. Tra, C. W. Michael, et al. 2003. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. *J. Biol. Chem. 278:7607.*
21. Multhoff, G., C. Botzler, M. Wiesnet, E. Müller, T. Meier, W. Wilmanns, and R. D. Issels. 1995. A stress-inducible 72-kDa heat-shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. *Int. J. Cancer 61:272.*
22. Multhoff, G., C. Botzler, L. Jennen, J. Schmidt, J. Ellwart, and R. D. Issels. 1997. Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells. *J. Immunol. 158:4341.*
23. Botzler, C., G. Li, R. D. Issels, and G. Multhoff. 1998. Definition of extracellular localized epitopes of Hsp70 involved in an NK immune response. *Cell Stress Chaperones 3:6.*
24. Multhoff, G., K. Pfister, M. Gehrmann, M. Hantschel, C. Gross, M. Hafner, and W. Hiddemann. 2001. A 14-mer Hsp70 peptide stimulates natural killer (NK) cell activity. *Cell Stress Chaperones 6:337.*
25. Multhoff, G., C. Botzler, M. Wiesnet, G. Eissner, and R. D. Issels. 1995. $CD3^-$ large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72-kDa heat-shock protein on human sarcoma cells. *Blood 86:1374.*
26. Botzler, C., R. D. Issels, and G. Multhoff. 1996. Heat-shock protein 72 cell-surface expression on human lung carcinoma cells is associated with an increased sensitivity to lysis mediated by adherent natural killer cells. *Cancer Immunol. Immunother. 43:226.*
27. Botzler, C., H.-J. Kolb, R. D. Issels, and G. Multhoff. 1996. Noncytotoxic alkyllysophospholipid treatment increases sensitivity of leukemic K562 cells to lysis by natural killer cells (NK). *Int. J. Cancer 65:633.*
28. Gross, C., D. Hansch, R. Gastpar, and G. Multhoff. 2003. Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. *Biol. Chem. 384:267.*
29. Allavena, P., C. Giardina, G. Bianchi, and A. Mantovani. 1997. IL-15 is chemotactic for natural killer cells and stimulates their adhesion to vascular endothelium. *J. Leukocyte Biol. 61:729.*
30. Nieto, M., F. Navarro, J. J. Perez-Villar, M. A. del Pozzo, R. Gonzalez-Amaro, M. Mellado, J. M. R. Frade, C. Martinez, M. Lopez-Botet, and F. Sanchez-Madrid. 1998. Roles of chemokines and receptor polarization in NK-target cell interactions. *J. Immunol. 161:3330.*
31. Barreto, B., J. M. Gonzalez, E. Kabingu, A. Asea, and S. Fiorentino. 2003. Stress-induced release of HSC70 from human tumors. *Cell. Immunol. 222:97.*
32. Multhoff, G., L. Mizzen, C. C. Winchester, C. M. Milner, S. Wenk, H. H. Kampinga, B. Laumbacher, and J. Johnson. 1999. Heat shock protein 70 (Hsp70) stimulates proliferation and cytolytic activity of NK cells. *Exp. Hematol. 27:1627.*
33. Basse, P., R. B. Herberman, U. Nannmark, B. R. Johansson, M. Hokland, K. Wasserman, and R. H. Goldfarb. 1991. Accumulation of adoptively transferred adherent, lymphokine-activated killer cells in murine metastasis. *J. Exp. Med. 174:479.*
34. Van den Brink, M. R., M. L. Paloma, P. H. Basse, and J. C. Hiserodt. 1991. In situ localization of $3.2.3^+$ natural killer cells in tissues from normal and tumor-bearing rats. *Cancer Res. 51:4931.*
35. Fogler, W. E., K. Volker, K. L. McCormick, M. Watanabe, J. R. Ortaldo, and R. H. Wiltrout. 1996. NK cell infiltration into lung, liver, and subcutaneous B16 melanoma is mediated by VCAM/VLA-4 interaction. *J. Immunol. 156:4707.*
36. Kurosawa, S., G. Matsuzaki, M. Harada, T. Ando, and K. Nomoto. 1993. Early appearance and activation of natural killer cells in tumor-infiltrating lymphoid cells during tumor development. *Eur. J. Immunol. 23:1029.*
37. Baggiolini, M., and P. Loetscher. 2000. Chemokines in inflammation and immunity. *Immunol. Today 21:418.*
38. Zlotnik, A., and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity 12:121.*
39. Broquet, A. H., G. Thomas, J. Masliah, G. Trugnan, and M. Bachelet. 2003. Expression of the molecular chaperone Hsp70 in detergent resistant microdomains correlates with its membrane delivery and release. *J. Biol. Chem. 278:21601.*
40. Lazetic, S., C. Chang, J. P. Houchins, L. L. Lanier, and J. H. Phillips. 1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J. Immunol. 157:4741.*

41. Borrego, F., J. Kabat, T. B. Sanni, and J. E. Coligan. 2002. NK cell CD94/NKG2A inhibitory receptors are internalized and recycle independently of inhibitory signaling processes. *J. Immunol. 169:6102*.
42. Gross, C., I. K. Schmidt-Wolf, S. Nagaraj, J. Ellwart, L. Kunz-Schughart, and G. Multhoff. 2003. Hsp70-reactivity is associated with increased cell surface density of CD94 and CD56. *Cell Stress Chaperones. In press*.
43. Altschul, S., T. L. Madden, A. A. Schaeffer, J. Zhang, Z. Zhang, W. Miller, and H. Schild. 1999. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res. 25:3389*.
44. Botzler, C., J. Schmidt, A. Luz, L. Jennen, R. Issels, and G. Multhoff. 1998. Differential Hsp70 plasma-membrane expression on primary human tumors and metastases in mice with severe combined immunodeficiency. *Int. J. Cancer 77:942*.
45. Moser, C., C. Schmidbauer, U. Guertler, C. Gross, M. Gehrmann, G. Thonigs, K. Pfister, and G. Multhoff. 2002. Inhibition of tumor growth in mice with severe combined immunodeficiency is mediated by heat shock protein 70 (Hsp70)-peptide-activated, CD94-positive natural killer cells. *Cell Stress Chaperones 7:365*.
46. Multhoff, G., K. Pfister, C. Botzler, A. Jordan, R. Scholz, R. Burgstahler, and W. Hiddemann. 2000. Adoptive transfer of human NK cells in mice with severe combined immunodeficiency inhibits growth of Hsp70 expressing tumors. *Int. J. Cancer 88:791*.
47. Schwarz, R. E., N. L. Vujanovic, and J. C. Hiserodt. 1989. Enhanced antimetastatic activity of lymphokine-activated killer cells purified and expanded by their adherence to plastic. *Cancer Res. 49:1441*.
48. Vujanovic, N. L., S. Yasumura, H. Hirabayashi, W. C. Lin, S. Watkins, R. B. Herberman, and T. L. Whiteside. 1995. Antitumor activities of subsets of human IL-2 activated natural killer cells in solid tissues. *J. Immunol. 154:281*.
49. Whiteside, T. L., N. L. Vujanovic, and R. B. Herberman. 1989. Natural killer cells and tumor therapy. *Curr. Top. Microbiol. Immunol. 230:221*.
50. Yasumura, S., W. C. Lin, H. Hirabayashi, N. L. Vujanovic, R. B. Herberman, and T. L. Whiteside. 1994. Immunotherapy of liver metastases of human gastric carcinoma with IL-2 activated natural killer cells. *Cancer Res. 54:3808*.