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CD155 is involved in NK-cell mediated lysis of human hepatoblastoma *in vitro*

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1. ABSTRACT

NK cells are involved in the lysis of different solid tumors and leukemias. NK-activity is thereby regulated by activating and inhibitory receptors. Until now, nothing is known about the NK-activity against hepatoblastoma and the involved receptors. We tested NK cells for cytotoxicity against HB *in vitro*. Expression levels of activating NK ligands were analysed on 13 primary HB samples as well as on 3 HB cell lines. ALL HB cell lines showed low HLA-class-I-expression. CD155 expression was strong on primary HB samples and cell lines. NKG2D-ligands (MICA/B, ULBP1-3) were heterogeneous expressed in primary samples and cell cultures. There were no differences between the various histological subtypes. NK cells showed strong cytotoxicity *in vitro* which was significantly increased through interleukin-2 and –15 stimulation ($p<.0001$). Blockade of CD155 resulted in decreased lysis rates. Our findings show that NK cells exert high activity against hepatoblastoma *in vitro* and that CD155 is involved in the NK mediated killing of HB. The inclusion of a NK-based immunotherapy into novel treatment strategies might be a promising alternative especially for advanced tumors.

2. INTRODUCTION

Human Hepatoblastoma (HB) is the most common primary malignant liver tumor in children and infants. Impressive improvements of treatment results were achieved over the past decades. These mainly concern standard tumors. Standard risk HB-patients have a 3-year overall survival of 91% and within this group, disease free survival in stage I and II patients is above 95%. However, advanced HB, relapsed or metastasized tumors are still associated with a poor prognosis: Patients with a high risk HB show a 3-year overall survival of approximately 50%, and disease free survival in stage IV patients is only around 35% (1,2). In order to improve those data, additional approaches have become necessary especially since no essential progress could be achieved lately.

Natural killer (NK) cells as part of the innate immune system show the ability to respond to tumors and different pathogens (3,4). NK cell activity is thereby regulated by different activating and inhibitory receptors (reviewed in (5)). For some activating receptors no specific ligand on tumor cells has been characterised yet, e.g. for the natural cytotoxicity receptors NKp30, NKp44 und

Table 1. Clinical data of patients undergoing surgery for hepatoblastoma

Patient	Age [months]	Gender	Tumor extension (liver segments)	Subtype	Chemotherapy	Response	Surgical Procedure
1	9	f	IV, V, VI, VIII	embryonal/fetal	VP16/Carbo	PR	Right Trisegmentectomy
2	61.5	m	II, III, IV,bilateral lung mets	embryonal/fetal	HD VP16/Carbo+ St-cell-Tx	PR	Left Trisegmentectomy, Sternotomy
3	32.5	m	IV, V, VIII	mixed	PLADO	PR	Right Trisegmentectomy
4	18	f	IV-VIII	embryonal/fetal	HD VP 16/Carbo	PD	Right Trisegmentectomy
5	24	m	VII, VIII	fetal	IPA	PR	Right Hemihepatectomy
6	8.5	m	I, IV-VIII	fetal	PLADO	PR	Right Trisegmentectomy
7	33	m	I, V-VIII	embryonal/fetal	IPA	PR	Right Hemihepatectomy
8	5	f	IV-VIII	fetal	Ø	Ø	Right Trisegmentectomy
9	59	f	V-VIII,lung mets left, thrombus inf v.cava	embryonal	HD VP16/Carbo	PR	Right Hemihepatectomy, Sternotomy, Reconstruction v. cava
10	18	m	V-VIII	embryonal/fetal	IPA	PR	Right Hemihepatectomy
11	12	f	IV-VIII,bilateral lung mets	embryonal/fetal	HD VP16/Carbo+ St-cell-Tx	PD	Right Trisegmentectomy, Sternotomy
12	46	f	IV-VIII,bilat.lung mets, thrombus inf v.cava	embryonal/fetal	HD VP16/Carbo	PR	Right Trisegmentectomy, Sternotomy, Reconstruction v. cava
13	21	f	V-VIII	mixed	IPA	PR	Right Hemihepatectomy

Chemotherapy regimens: VP16, etoposid; Carbo, carboplatin; St-cell-Tx, stem cell transplantation; PLADO, combined cisplyatin/doxorubicin; IPA, combined Ifosfamid, cisplatin, doxorubicin; HD, high dose; Response: PR, partial response; PD, progressive disease.

NKp46. For other activating receptors specific ligands could be characterised on virus infected cells as well as tumor cells. The C-type lectin-like NKG2D receptor is expressed on NK cells as well as other cytotoxic lymphocytes (6). The human NKG2D ligands (NKG2DL) include the stress-inducible surface glycoproteins MICA and MICB, expressed on many epithelial tumors (7,8) as well as the UL 16 binding proteins (ULBP), a multigene family with several members (9,10,11). DNAM-1 (CD226) binds to CD112 and CD155 and can induce cytotoxicity or tumor rejection through interaction with its ligands (12,13). In other pediatric solid tumors, CD155 expression directly correlated with the susceptibility to NK cell-mediated lysis (14). Furthermore, assessment of the CD155 surface levels was considered as novel useful criterion to predict the susceptibility/resistance of tumor cells to NK-mediated killing in this study.

To date, there exists no knowledge on the expression of NK cell activating ligands as well as on the susceptibility to a NK-mediated lysis of hepatoblastoma. Therefore, we investigated the cytotoxic potential of NK cells from healthy donors against several hepatoblastoma cell lines with and without cytokine stimulation and analysed the expression profile of NK-ligands on cell lines as well as primary tumor samples.

3. MATERIALS AND METHODS

3.1. Patients

We analysed tissue samples from 13 patients undergoing liver resection for hepatoblastoma at the Department of Pediatric Surgery, University Children's Hospital Tuebingen, between April 2002 and August 2007. Mean age of the patients at surgery was 26.7 months (range 5-61.5, Table 1). Immediately after resection, the tumor samples were shock frozen and stored in liquid nitrogen until use. The study was done according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from the parents of the patients before operation.

3.2. Reagents and antibodies

The following anti-human mAbs were used: CD155 (unconjugated, Acris Antibodies GmbH, Hiddenhausen, Germany), BAMO1 (anti-MICA/B), AUMO1 (anti-ULBP1), BUMO2 (anti-ULBP2) and CUMO4 (anti-ULBP3; all unconjugated) as described previously (15). Unconjugated mAbs were detected using goat-anti-mouse-FITC (Dakocytomation, Hamburg, Germany) or goat-anti-mouse-PE (Jackson ImmunoResearch, West Grove, PA). For quantitative FACS-analysis we used a MEM-E/07 mAb (anti-HLA-E, Biozol, Eching, Germany) or a W6/32 mAb (anti-HLA-A/B/C), goat-anti-mouse mAb (FITC conjugated), and the Quifikit (all from Dakocytomation).

3.3. Tumor cells and culture conditions

For *in vitro* analyses we used the cell lines HUH6, HepT1, and HepT3. The cell line HUH6 originates from a mixed HB. The tumor presents chondroosteogenetic tissue and extra medullar haematopoiesis. Tumor cells express AFP and a caryotype with 48 chromosomes (16). The cell line HepT1 was derived from an embryonal HB. Tumor cells present an overexpression of cytokeratin 18 and 19, vimentine, α-fetoprotein (AFP), erythropoetin and the stem cell factor SCF. The caryotype is polyploid with 65-125 chromosomes per cell, deletions on 1p and 11q and a 6q15 translocation (17). The HepT3 cell line was derived from an epithelial tumor with embryonal predominance. This cell line has not been published so far.

All tumor cells were grown as monolayer in DULBECCO`s MEM medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, 1% glutamine and 2.5% HEPES buffer (Gibco, Eggenstein, Germany). The cells were grown at 37°C in a humidified atmosphere containing 5% carbon dioxide. All cells were mycoplasma negative.

3.4. RT-PCR analysis

Gene expression of NK cell interacting molecules on resected HB specimen was assessed using reverse transcriptase PCR. Tumor tissue (approximately 10mg) was homogenized. RNA-isolation was isolated using Silica gel membrane spin (Qiagen, Hilden/Germany). Synthesis of cDNA and PCR were realized in one step (Qiagen) using 1ug RNA at a final volume of 20ul. Forward and reverse primers of targeted genes (MWG, Ebersberg/Germany) were used at 10pmol/l, GAPDH (primers at 5 pmol/l, MWG) served as house keeping gene. Reverse transcription was carried out for 30 minutes at 50°C. One PCR cycle consisted of denaturation (1 min at 94°C), annealing (1.5 min at 72°C), and elongation (1 min). For GAPDH 30 cycles were used, for target genes 35. PCR products were analysed using electrophoresis (100V, 90 min) on a 1.5% agarose gel followed by visualisation with UV light after incubation with ethidium bromide. Quantitative analysis of gene expression levels was carried out for distinguished targets using the electropherogram method (Agilent 2100 Bioanalyzer) (18). The K562 cell line served as positive control for PCR analysis. Expression levels of target genes were standardized for each tumor to levels of the corresponding GAPDH expression.

3.5. Flow cytometry

Hepatoblastoma cells were detached from culture dishes using 0.25% Trypsin/EDTA and incubated with the primary unconjugated mAbs or the respective isotype control. The cells were then incubated with goat anti-mouse-PE or goat-anti-mouse-FITC conjugates and finally analyzed on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). For quantitative analysis of HLA class I expression, cells were incubated with a saturated concentration of unconjugated anti-HLA-ABC or anti-HLA-E and Isotypic Control for 30 min at 4°C. Cells were washed twice and then incubated with FITC-F(ab')2 conjugates (Dako) for 30 min at 4°C. Set-up- and Calibration-beads coated with known amounts of mouse antibodies (QIFIKIT, Dako) were stained with the same secondary antibody for the same time. Analysis was performed according to the manufacturer's instructions: set-up-beads were used to optimize the instrument settings, calibration beads were used for construction of the calibration curve (Mean Fluorescence Intensity (MFI) against Antibody-Binding Capacity (ABC)). ABC of HB cells was calculated by interpolation on the calibration curve. One antigen binding site was assumed per one surface HLA class I molecule.

3.6. Sequencing of HLA class I genes in HB cells

Identification of alleles of the human HLA class I genes was performed using the AlleleSEQR HLA Sequencing Kit (Abbott, Wiesbaden, Germany) as described previously [18]. Briefly, the genes were amplified in a PCR amplification mixture using a hot-start DNA polymerase, AmpliTaq Gold (Roche Molecular Systems, Pleasanton, USA). The resulting PCR amplicon was treated with Exo-SAP-IT (USB inc., Cleveland, USA), a combination of Exonuclease I and shrimp alkaline phosphatase, to remove unincorporated PCR primers and dNTPs. The treated amplicon served as DNA sequencing template in reactio using custom sequencing primers in a formulation with BigDye Terminators sequencing chemistry (Applera Corporation, Norwalk, USA). The DNA sequences were subsequently detected on an automated fluorescent DNA sequencer and data were processed with an allele typing software program (Assign SBT, Connexio Genomics, Applecross, Australia.).

3.7. Isolation of NK cells

Peripheral mononuclear cells (PMNC) from healthy donors were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were enriched for $CD56^+$ cells using CD56 beads (Miltenyi Biotec, Bergisch – Gladbach, Germany) according to manufacturer's instructions. $CD56^+$ cells were incubated overnight either without cytokines, with 1000 IE/ml IL-2, or with 10ng/ml IL-15.

3.8. Cytotoxicity assay

Cytolytic activity of NK cells was tested in a 2 hour BATDA [bis (acetoxymethyl) 2,2´:6´,2´´- terpyridine-6,6´´- dicarboxylate] europium release assay. Target cells were labelled with the fluorescence enhancing ligand BATDA (Wallac Oy, Turku, Finland) for 30 minutes at 37°C. After five washing steps, the target cell suspension was adjusted to 2 x 10^5 cells/ml and seeded into microplates (5000 target cells/well). Four different effector to target (E/T) cell ratios were used (20:1, 10:1, 5:1, 2.5:1) and tested in triplicates. Blocking of CD155 was performed by incubating the target cells with a CD155 antibody at a concentration of 1 ug/ml for 30 min before adding the effector cells. Specific lysis was calculated as follows: % specific lysis = (experimental release - spontaneous release) / (maximum release - spontaneous release) x 100.

3.9. Cytological analyses and immunofluorescence

Tumor cells were grown in chamber slides (Becton Dickinson, Hamburg, Germany) at $1x10^4$ cells per well. NK cells were added after 24 hours in ascending E/T ratios (2.5:1 to 20:1). Incubation was performed for 2 hours. After washing with PBS, the cells were fixated in formalin and incubated for 20 minutes with Phalloidin, 1:100, Alexis, ALX-350.268 (19,20). Counterstaining was performed with DAPI (Sigma-Aldrich, Seelze, Germany).

3.10. Statistical Analyses

Results from cytotoxicity assays (5 donors, all assays in triplicate) were statistically analysed using a student's t-test. Statistical significance was assumed for all $p<0.05$.

4. RESULTS

4.1. HB cells express NK cell-activating ligands.

Reverse Trancriptase PCR was used to assess expression of targeted genes. Furthermore, quantitative analysis was carried out using the electropherogram method. We observed strong expression levels of CD155 (Poliovirus receptor, PVR) in all analysed HB. MICA, MICB, and ULBP1 were diversely expressed in the specimen (Figure 1A), whereas ULBP2 expression was negative in all analysed HB. For quantification of the

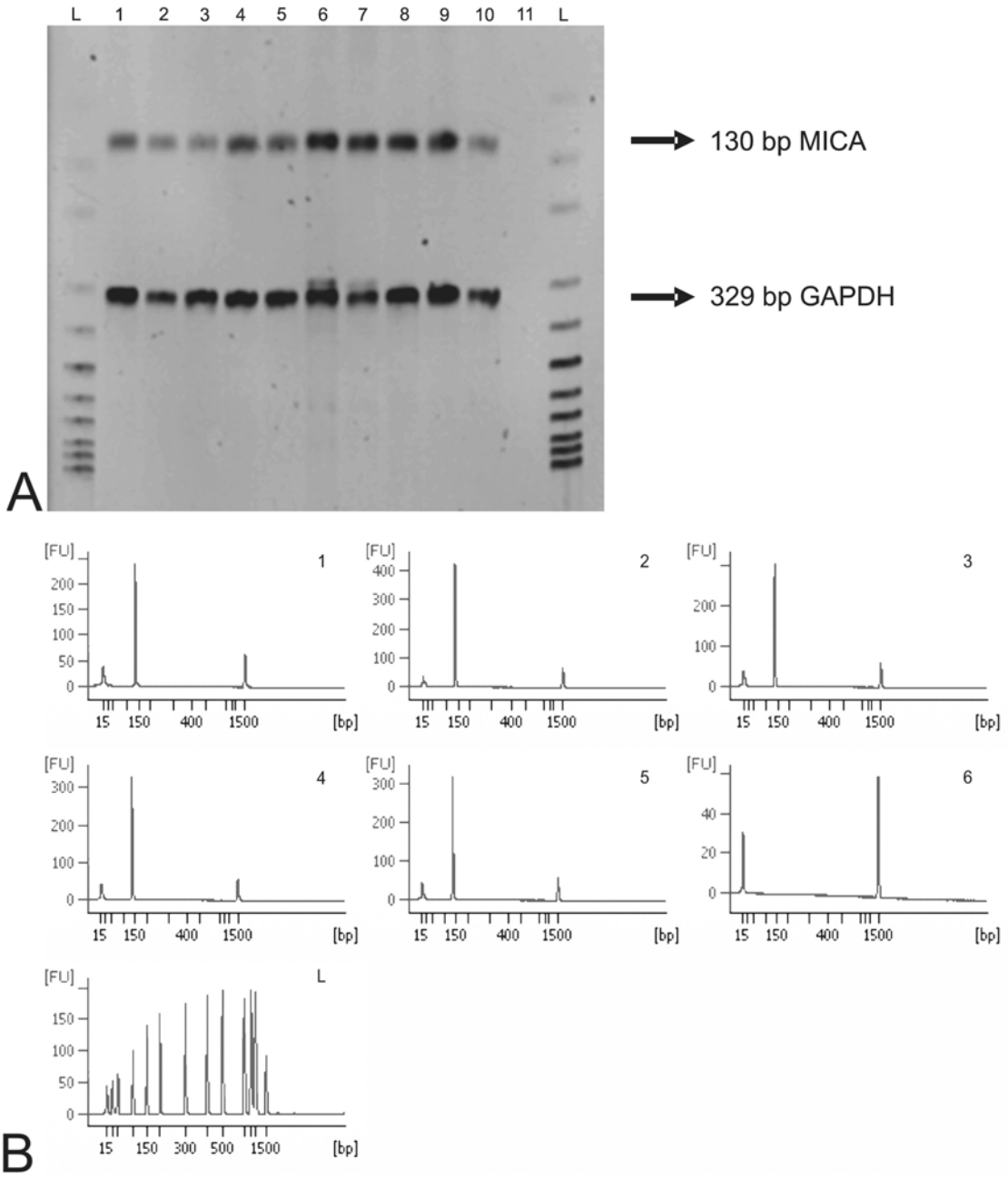

Figure 1. Examples for MICA gene expression on primary HB specimen. Primary resected HB were assessed using rT-PCR for expression of MICA. Gel electrophoresis was used for qualitative analysis. A) PCR products after gel electrophoresis revealing the bands for MICA (130 bp) and GAPDH (329 bp). The Different lanes are displaying various samples (L = DNA ladder, lane 1-9 = HB samples, lane 10 = positive control, lane 11 = negative control). B) Agilent electropherography was used for quantitative gene expression analysis. Results from electropherography displaying the MICA quantity in various samples (1-4 = HB samples, 5 = positive control, 6 = negative control, L = DNA ladder).

varying expression levels of NKG2D ligands MICA, MICB, and ULBP1 we used electropherography (Figure 1B). As a positive control we used K562 cells, which were highly positive for MICA, MICB, and ULBP1-3 in flow cytometry (data not shown). We found equally enhanced mRNA expression of MICA (mean 36.9%, range 19.6-109.7) compared to K562 cells (30.6%), MICB (mean 31.3%, range 11.4-58.9, K562: 27.3%), and ULBP1 (mean 15.2%, range 5.5-37.9, K562: 10.6%, Table 2). The cell lines showed lower mRNA-levels for these molecules in accordance to the lower expression in flow cytometry compared to K562.

Table 2.. Gene expression levels of NK cell associated molecules on resected HB as percentages of GAPDH-expression

	MICA	MICB	ULBP1
HB 1	34,28	20,79	6,9
HB 2	125,19	11,39	5,18
HB 3	19,63	16,98	4,36
HB 4	18,63	19,96	5,5
HB 5	41,73	26,29	8,7
HB 6	20,98	19,86	9,29
HB 7	28,46	28,7	6,29
HB 8	38,74	25,59	15,69
HB 9	46,45	52,4	23,03
HB 10	73,62	53,6	37,86
HB 11	30,49	24,95	36,27
HB 12	109,7	58,87	18,31
HB 13	16,76	48,06	19,66
HepT1	9,88	5,21	15,82
HUH6	7,03	6,34	16,55
HepT3	7,21	5,02	12,19
K562	30,64	27,25	10,56

Table 3. Surface expression of NK cell associated molecules on HB cell lines

	HepT1	HepT3	HUH6
MICA/B	11.2	5.0	2.3
ULPB1	1.0	1.0	1.0
ULBP2	1.2	1.0	0.9
ULBP3	4.0	1.0	0.9
CD155	15.9	5.0	4.8

Data are presented as the ratios of geometrical means of fluorescence intensities (stained/control). Positive expression was assumed for results > 1.5.

Table 4. HLA type of hepatoblastoma cell lines

HepT1		HepT3		HUH6	
A*0201	A*0201	A*0201	A*3002	A*0201	A*2402
B*3501	B*3501	B*1302	B*1801	B*3501	B*4001
Cw*0401	Cw*0401	Cw*0602	Cw*0501	Cw*0303	Cw*0304

4.2. HB cell lines show surface expression of CD155.

HB cell lines were next evaluated for surface expression of NK cell associated molecules using flow cytometry (Table 3). Ratios of geometrical means of fluorescence intensity and according controls were calculated. Quotients above 1.5 were considered as positive expression. Surface expression of MICA/B was positive in all cells whereas ULBP1-3 were negative in all cells except ULBP3 in HepT1 (Figure 2). We observed a strong CD155 expression in all three HB cell lines. Counting the binding loci per cell for CD155 mAb by quantitative flow cytometry we observed 12424 binding loci in HepT1, 4179 in HepT3 and 2940 in HUH6 cells.

4.3. Quantification of HLA expression levels on HB cell lines

Quantification of HLA surface expression on HB cells was performed by measuring the binding loci per cell for HLA-ABC and HLA-E by quantitative flow cytometry. For HLA-ABC we observed the strongest expression levels in HepT1 cells (4478 molecules/cell), lower levels in HepT3 cells (920 molecules/cell), and lowest levels in HUH6 cells (170 molecules/cell). All cell lines expressed less HLA class I than K562 cells (7736 molecules/cell) which are widely used as a standard NK target. For HLA-E we found comparable levels in HepT1 cells (278) and HUH6 cells (271). There was no detectable HLA-E expression on HepT3 cells. In order to detect KIR-mismatches between NK cell donors and cell lines HLA-typing was done of the cell lines and the NK cell donors (Table 4). All donors expressed Bw4-, Cw3- and Cw4-related alleles. Therefore, a KIR-mismatch and potential NK-alloreactivity was present in all NK-donor/target cell combinations.

4.4. NK cell mediated cytotoxicity against HB cells

Next, cytotoxic effects of NK cells against HB cells were investigated. Tumor cells were incubated with NK cells from 5 healthy donors with or without addition of IL-2 (1000 IU/ml) or IL-15 (10ng/ml). Cytotoxicity was increased in E/T ratios from 2.5:1 up to 20:1. In HepT1 cells mean cytotoxicity at E/T ratio of 20:1 was 54 % (+/- 5.4) without stimulation. IL-2 stimulation resulted in mean 99% (+/- 1.5, $p<0.0001$) toxicity, and IL-15 stimulation lead to a mean toxicity of 97% (+/- 0.7, $p<0.0001$). In HUH6 cells we observed 47% (+/- 8.0) toxicity of NK cells without stimulation, 86% (+/- 5.9, $p=0.0003$) after IL-2, and 89% (+/- 7.5, $p=0.0004$) after IL 15 stimulation. In HepT3 cells we observed 38% (+/- 5.9) toxicity of NK cells without stimulation (Figure 3). Differences between IL-2 and IL-15 stimulated NK cells were not significant in all cell lines (all $p>0.05$).

4.5. Anti CD155 monoclonal antibody (mAb) inhibits NK cell mediated cytotoxicity

Expression of the polio virus receptor (CD155) was observed on all resected HB specimen as well as on all HB cell lines. CD155 acts as a ligand for the DNAM-1 triggering receptor (CD226), whose interaction has activating effects on NK cells. Consecutively, we analysed the effects of anti CD155 monoclonal antibodies (mAb) on NK cell toxicity against HB. Anti CD155 mAb alone had

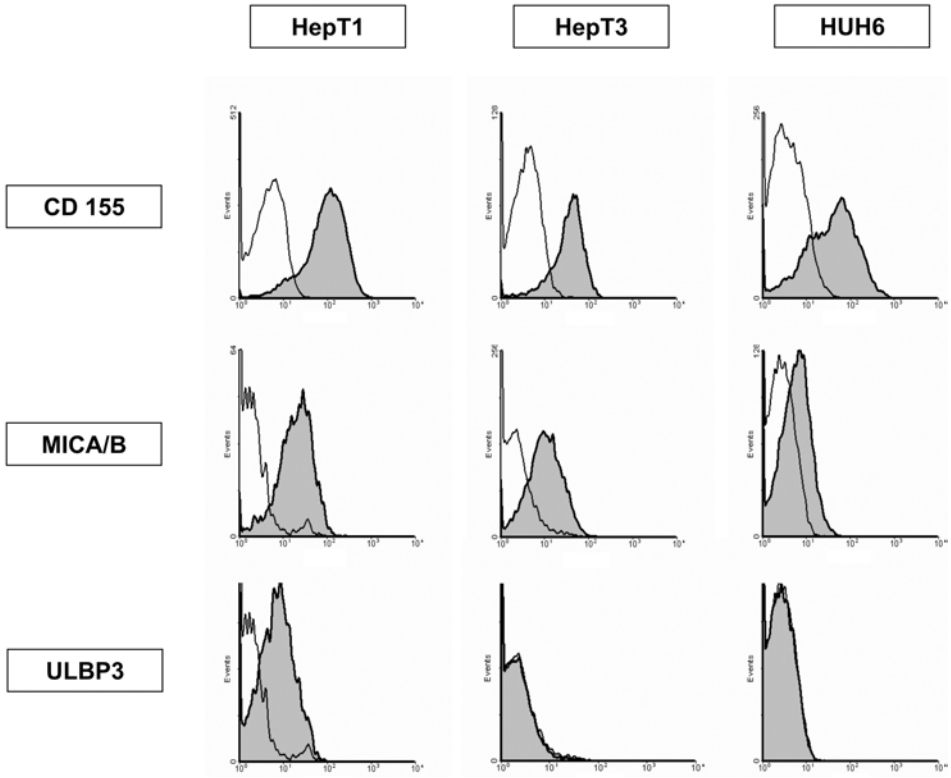

Figure 2. Surface expression of NK cell activating molecules on HB cell lines. Flow cytometry was used to determine surface expression of different activating NK ligands on HepT1, HUH6, and HepT3 cells. Open histograms, staining with control mAb, shaded histograms, staining with respective primary unlabeled mAbs; detection with goat-anti-mouse PE secondary antibody. Results displaying expression of CD 155 on all cell lines, MICA/B expression on all cell lines as well as ULBP3 expression on hepT1 cells.

no influence on the tumor cell growth. There were no cytotoxic or proliferative effects compared to control cells (all $p>0.05$). However, incubation with anti CD155 mAb reduced cytotoxicity of NK cells against all HB cell lines (Figure 4A). These effects were statistically significant in HepT1 and HUH6 cells, but not in HepT3 cells. At E/T ratio of 20:1, mean toxicity in HepT1 cells was reduced from 54% (+/- 5.4) to 35% (+/- 4.6, $p=0.0011$), in HUH6 cells from 47% (+/- 8.0) to 29% (+/- 5.7, $p=0.039$), and in HepT3 cells from 38% (+/-5.9) to 32% (+/- 4.4, p=0.212). Stimulation through IL-2 completely reversed the described anti CD155 mAb effects, indicating that CD155/DNAM-1 interactions play an important role in the lysis of hepatoblastoma by resting NK cells but not by highly stimulated NK cells (Figure 4B).

4.6. Cytological changes in HB cells incubated with NK cells

Fluorescence light microscopy, in analogy to the cytotoxicity assays, revealed a reduction in cell numbers in all cell lines after incubation with NK cells (Figure 5). This reduction was more marked after stimulation with interleukins. Thus, in some areas only NK cells and no tumor cells were seen. At higher magnification, tumor cells exhibited signs of apoptosis and cellular degradation after incubation with NK cells: cell volumes were decreased, the cell membrane and nuclear membrane were less clearly defined, and there was clumping and increased fluorescence of the heterochromatin and cytoplasm.

5. DISCUSSION

Hepatoblastoma (HB) is the most common primary malignant liver tumor in children. Despite impressive improvements of treatment results over the last years, advanced tumors are still associated with a poor outcome. This especially includes multifocal tumors, relapses and metastasized tumors (1,21). Several factors have been identified for this development (22,23). Recently it has been demonstrated that primary liver transplantation is associated with a much more favourable outcome in high risk HB compared to standard surgical resections (24).

Natural Killer (NK) cells as part of the innate immune system contain the ability to lyse neoplastic cells without the need for tumor-specific antigen recognition. The strategy of NK cell immunotherapy after haploidentical stem cell transplantation has been used in adults and children suffering from hematologic malignancies as well as solid tumors (25,26). The need for alternative treatment options for advanced hepatoblastoma (HB) has been formulated as consequence from the results of various international study trials (1,2,27). NK cells are increasingly analysed and used in preclinical and clinical

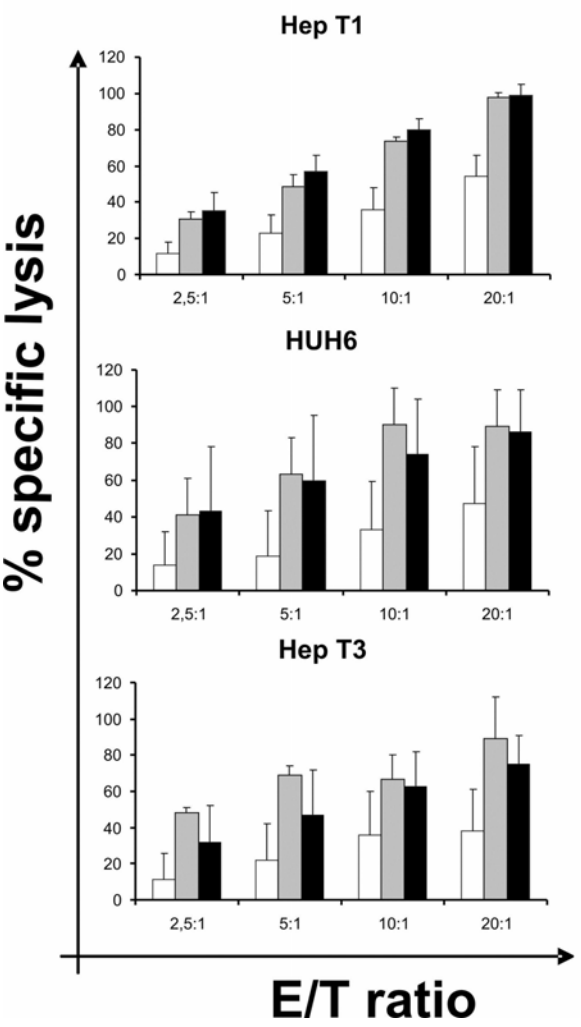

Figure 3. NK cell lead to lysis of HB cells in correspondence to increasing effector/target (E/T) ratios. HepT1, HepT3, and HUH6 cells were incubated for 2 hours with NK cells from healthy donors at increasing E/T ratios (2.5:1, 5:1, 10:1, 20:1). NK cells were used without stimulation as well as after stimulation with IL-2 or IL-15, respectively. Cellular cytotoxicity assays were performed in triplicate for each cell line as described in Materials and Methods. Columns, means of triplicates of all experiments performed (white columns, lysis using unstimulated NK cells; grey columns lysis of NK cells stimulated with IL-2; black columns, lysis of NK cells stimulated with IL-15); bars, SE. Differences between unstimulated cells and stimulated cells were significant for IL-2 and IL-15 in all cell lines. Results were not significantly different for cells stimulated with IL-2 or IL-15.

approaches against various malignancies. These studies include hematologic and solid tumors in adults as well as children. However, there are no data so far describing the susceptibility of HB towards NK cell-mediated immunotherapy.

We observed strong CD155 expression levels on all resected HB specimen as well as on all HB cell lines. These observations were seen in mRNA expression as well as surface expression analyses. Although there exist some reports on molecular expression levels in HB (28), the strong expression pattern of CD155 has never been described previously in this malignancy. CD155 mediates immunologically relevant processes such as NK cell driven killing of tumor cells (13,14,29). This mechanism is highly and constantly active in HB as we could demonstrate *in vitro*. We performed the blockade of CD155 in order to verify that the presence of CD155 is not only a morphological characteristic but also contains a functional relevance. This was confirmed through the observation of decreased NK cell toxicity using anti-CD155 monoclonal antibodies. As the lysis was not completely blocked it is clear that other mechanisms also contribute to NK cell mediated cytotoxicity in HB like interactions between NKG2D and MICA/B - which is expressed to a low amount on the tested cell lines - or activation by NCRs for which no specific ligands on tumor cells have been described so far. Beside this, there were no blocking effects of anti-CD155-antibodies after a high stimulation of the NK cells with IL-2. The fact that IL-2 stimulation significantly enhances NK cell activity is well known. In HCC, an increased TRAIL expression on liver NK cells after IL-2 stimulation has been identified as underlying mechanism (30). Taken together, CD155 seems to play a major role in NK cell mediated cytotoxicity in HB by resting NK cells. Apart from this immunologic effect, we thus identified CD155 as possible target for gene directed treatment approaches as it has been demonstrated lately for neuroblastoma *in vivo* (31).

We observed low levels of HLA class I genes in the qualitative and quantitative analysis of HB cell lines. Loss or downregulation of HLA class I expression is regularly observed in solid tumors and is regarded as major mechanism through which tumors escape control by HLA class I antigen-restricted, tumor antigen-specific cytotoxic T lymphocytes. On the other side, low levels of HLA class I seem to identify a high susceptibility of HB towards NK-cell driven immunologic approaches which may be further augmented by KIR mismatch constellations. There were killer immunoglobulin-like receptor (KIR) mismatches between NK cell donors and HB cell lines in all our experiments and this may have additionally enhanced the cytotoxic effects. A positive effect of KIR mismatch on NK cell mediated lysis of solid tumors has been previously described for melanoma and renal cell carcinoma cells (32). These facts have not yet been described in HB. Our observations seem to underline that HB might be susceptible to NK cell mediated immunotherapy irrespective of the advancement, histology, or risk stratification of the tumors. Whether or not the low HLA class I expression alone represents a generally favourable constellation still has to be analysed also in KIR-matched circumstances. Therefore, immunotherapeutic strategies with KIR-incompatible allogeneic NK cells might have a superior antineoplastic potential against solid tumors compared with approaches using autologous NK cells.

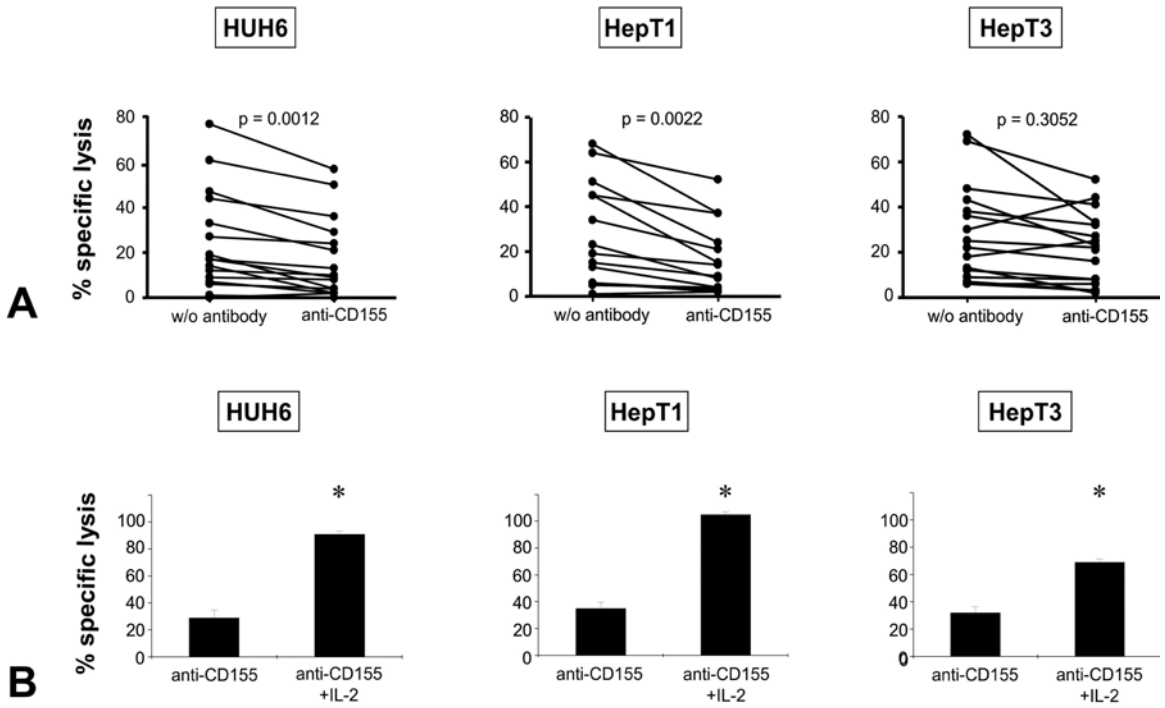

Figure 4. Incubation with anti-CD155 mAb reduces NK cell cytotoxicity against HB cell lines. HepT1, HepT3, and HUH6 cells were incubated with 1 µg/ml anti-CD 155 mAb for 30 min before adding NK cells. Incubation with NK cells was performed for 2 hours. A) Effects of unstimulated NK cells against HB cells without or with prior incubation of CD155 antibody. Summary of linked results for all E/T ratios in the different cell lines. Results were significant in HUH6 and HepT1 cells. B) Stimulation of NK cell with IL-2 significantly reverses the inhibitory effects of anti-CD155 mAb in all cell lines. Columns, means of triplicates of experiments performed with unstimulated or stimulated NK cells (E/T ratio 20:1); bars, SE. *, $p<0.05$, significant differences between unstimulated and stimulated cells for each cell line.

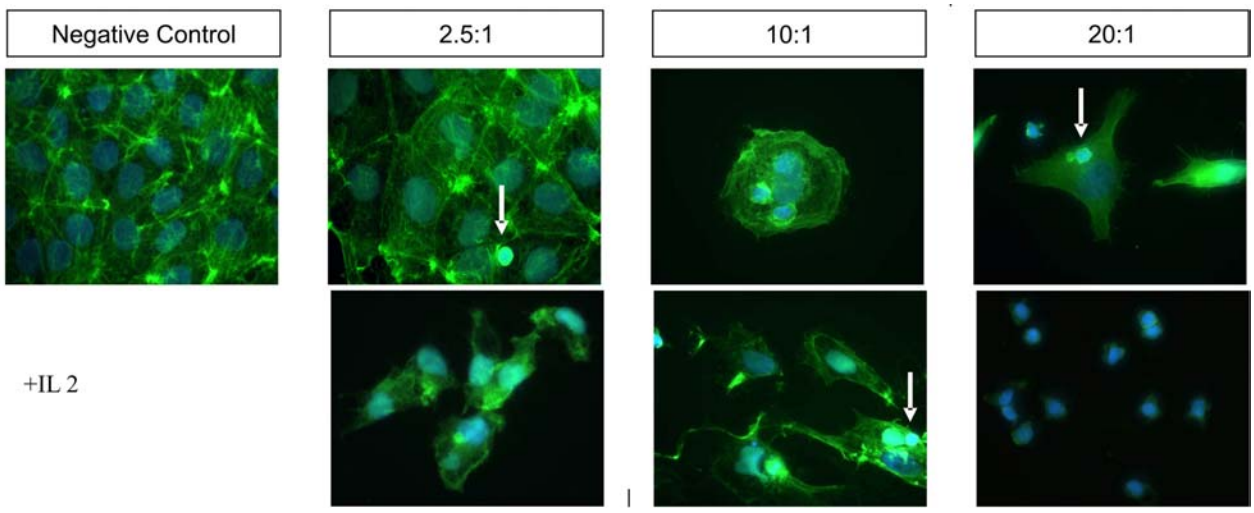

Figure 5. Immunofluorescence displaying cytological analysis of NK cell effects on HUH6 cells. Tumor cells were grown in chamber slides at 1x104 cells per well. NK cells were added after 24 hours in varying E/T ratios (2.5:1 to 20:1). Incubation was performed for 2 hours. After fixation, cells were incubated for 20 minutes with Phalloidin 1:100, counterstaining was performed with DAPI. Green fluorescence, tumor cell plasma; blue fluorescence, tumor cell nuclei; bright green fluorescence, NK cells (white arrows). Results are displaying NK cell effects without (upper lane) or with (lower lane) prior IL-2 stimulation at increasing E/T ratios. Observed effects include altered core/plasma relations, membranous disintegration and accentuated fluorescending signals of intracellular structures.

Inaba described the single case of a patient suffering from a recurrent metastatic HB in which a probable graft versus tumor effect was observed after a non-myeloablative hematopoietic stem cell transplantation from a HLA-matched unrelated donor (33). The course of this patient contains remarkable details since it was closely linked to full donor T-cell engraftment under which the advanced tumor regressed and serum alpha-fetoprotein levels decreased in concurrence with the onset of graft-versus-host disease (GVHD). The disease recurred when GVHD resolved under immunosuppressive treatment. Although the exact role of NK cells has not been investigated, this case contains strong evidence that there might be a susceptibility of HB towards allogeneic immune reactions.

HB cell lines showed only low mRNA expression levels of the human NKG2D ligands including the stress-inducible surface glycoproteins MICA, MICB, and UL16 binding Proteins (ULBP1 and ULBP2). Interestingly we observed partially discrepant results analysing cell lines and primary tumors, because we found significantly higher MICA and MICB mRNA expression levels in HB samples than in HB cell lines. There was no difference in ULBP expression between primary samples and cell lines. The MIC induction might result from the stress administered to the tumors via chemotherapy and subsequent apoptosis. Similar effects have been observed in tumor cells following heat shock and ionising radiation (34). Therefore, chemotherapy not only improves surgical resectability of HB but also might seem to increase the tumors' susceptibility to NK cell mediated immunotherapy. Armeanu described an increase of NKG2D ligand expression in hepatocellular carcinoma (HCC) cells through the histone deacetylase inhibitor (HDAC-I) sodium valproate (35). One of the cell lines used in this study was derived from a child with HCC. Based on our observations, HDAC-I thus might be a promising clinical application for the use of NK cells against HB, especially since some of the concerning substances are already being used for different purposes in children.

Another reason for the discrepant behaviour of solid tumors and cell cultures in the expression of NKG2DL might be the presence of stromal cells in the tumor samples, despite the fact that tumor stroma is generally referred to as being rather immunosuppressive. Future analyses should therefore also focus on clarifying this aspect.

NK cell mediated cytotoxicity was comparable in all HB cell lines irrespective of the histological subtype. Relevant cytotoxic effects were observed from effector to target ratios lying below those of other malignancies (34,36), which also contributes to the promising character of the presented results. In order to further evaluate the observations from this study, NK cell effects on HB should be evaluated *in vivo*. There are several animal models in which these future analyses seem realizable (37,38). In the clinical setting of high risk tumors, a combination of haploidentical stem cell transplantation and living related organ transplantation has to be considered as possible therapeutical goal; especially since the primary liver transplantation in high risk HB seems to produce superior results compared to high risk and extended surgical resections (24). This treatment strategy would contain essential aspects towards tumor control and immunosuppression.

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