RESEARCH PAPER

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Targeting EpCAM (CD326) for immunotherapy in hepatoblastoma

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Keywords: EpCAM (CD326), γδ T cells, hepatoblastoma, immunotherapy, PBMC, therapeutic antibodies

Abbreviations: ADCC, antibody dependent cell cytotoxicity; CDDP, cisplatin; EpCAM, epithelial cell adhesion molecule; E:T, effector-to-target; HB, hepatoblastoma; IL-2, interleukin 2; PE, phycoerythrin; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte

Hepatoblastoma (HB) is the most common liver cancer in children. Recurrence of HB after chemotherapy and surgery is frequent among high-risk patients and is associated with chemoresistance. Immunotherapy may improve poor treatment outcomes in HB patients. Cytotoxic leukocytes of the innate and adaptive immune system including different populations of cytotoxic T cells play a major role in fighting developing tumors. In this setting, monoclonal antibodies may be employed to specifically direct immune responses toward tumor cells. We addressed this issue by using humanized antibodies that recognize the cell surface molecule EpCAM (CD326, overexpressed in hepatic tumor cells) to enhance immune responses against HB. EpCAM was constantly expressed on HB cells and its expression was independent of previous therapy based on the DNA-damaging agent cisplatin. Co-culture assays performed with two well-described HB cell lines and tumor tissue cultures demonstrated that tumor cell lysis by γδ T cells can be dramatically augmented by applying EpCAM-specific monoclonal antibodies. These data emphasize the value of antitumor immune responses and encourage adapting immunotherapeutic regimens to improve the outcome of high risk HB.

Introduction

Hepatoblastoma (HB) is the most common solid liver tumors in children and account for approximately 1.5% of all pediatric malignancies.[1–3] A complete resection of the tumor represents the only curative chance for young patients. However, 60% of the HB are not resectable due to their advanced stage at diagnosis, and chemotherapy based on the DNA-damaging agent cisplatin (CDDP) is needed.[4] Despite aggressive chemotherapeutic treatment post-surgery, recurrence is frequent among high-risk HB patients.[5] Experimental approaches such as targeted therapy based on the multikinase inhibitor sorafenib are being tested to ameliorate disease outcome.[6] Furthermore, combined transplantation of liver and peripheral blood stem cells may be a valid therapeutic option for recurrent HB.[7]

Pathological HB appears as an embryonal malignancy manifesting epithelial differentiation with mesenchymal components. HB cells secrete the α-fetoprotein (AFP) and are present on their surface a high density of cell adhesion molecules like glypican 3 and CD326 (best known as epithelial cell adhesion molecule, EpCAM). Using immunohistochemistry and immunoelectron microscopy, the expression of EpCAM was observed in 70–80% of HB cases, in up to 100% of cells in epithelial tumor areas.[8,9] EpCAM is expressed on a variety of human tissues and is overexpressed by some neoplasms including hepatic tumor, breast cancer, ovarian cancer and gallbladder carcinoma.[10–13] EpCAM is involved in cell adhesion, intracellular signaling, migration, proliferation and differentiation.[14] The overexpression of CD326 in hepatic malignancies is associated with a poor survival rate[13] as CD326 promotes tumor progression by activating the expression of proto-oncogene *c-MYC*.[15] EpCAM is a well-known target for therapeutic antibodies against epithelial tumors.[16] Antibodies achieve their therapeutic effect by blocking EpCAM-mediated signal transduction and or by promoting antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[17] The immune cells underlying these effects include natural killer (NK) cells as well as subpopulations of T cells.

γδ T cells are part of the innate immune system and represent a minor subset (1–10%) of peripheral T cells.[18] Up to 90% γδ T cells of the peripheral blood bear a T cell receptor (TCR) that is composed of Vγ9 and Vδ2 chains.[19] These unconventional T cells lack classical restriction on major histocompatibility complex (MHC)[20] and are activated via their TCR or NKG2D

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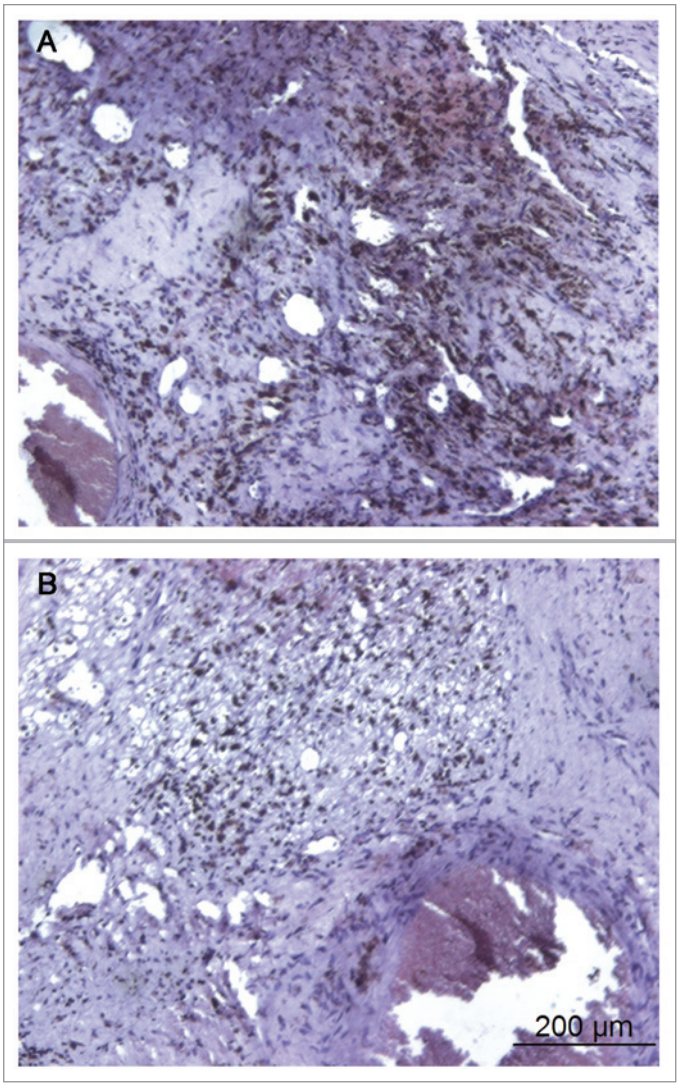

Figure 1. Staining for tumor-infiltrating leukocytes. Slices of a hepatoblastoma tissue sample revealed infiltrating CD45+ cells by DAB-based immunohistochemistry. Shown are two regions of the same tissue.

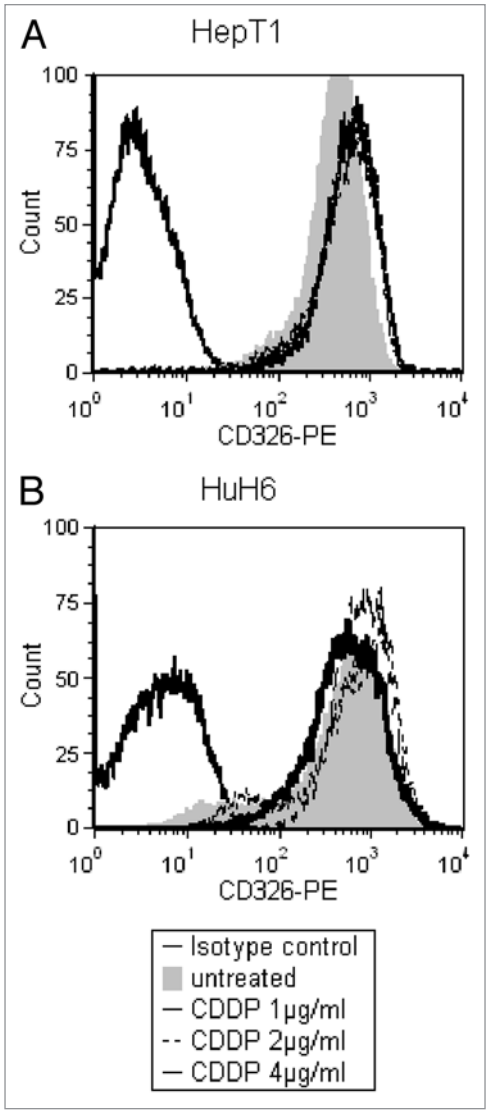

Figure 2. Expression of EpCAM on hepatoblastoma cells treated with cisplatin. (**A and B**) HepT1 (**A**) and HUH6 (**B**) hepatoblastoma (HB) cells were incubated with the indicated concentrations of cisplatin (CDDP) for 48 h. Flow cytometry with CD326-specific antibodies revealed constant expression of EpCAM on live HB cells regardless on previous treatment.

molecules.[21,22] γδ T cells are known for their capacity of lysing various tumor cells in vitro.[23,24]

The present study aimed at analyzing the value of EpCAM as a target for the immunotherapy of HB. An immunotherapeutic approach involving antibodies and γδ T cells was investigated in HB cells previously exposed to CDDP.

Results

EpCAM expression by HB cells in vitro and in vivo. To assess whether immunotherapy could be a promising tool for treating HB we screened for tumor infiltrating lymphocytes (TILs) in vivo. CD45 is a tyrosine-protein phosphatase expressed on leukocytes and is overexpressed in up to 50% of HB cases, as revealed by a gene expression analysis (http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-1851, data not shown). Slices from tumors explanted from a patient with epithelial HB were stained for the detection of CD45, revealing immune cells infiltrating the tumor parenchyma (**Fig. 1**). These leukocytes may play a prominent role during immunotherapy.

Since CD326 is highly expressed by HB, it represents a possible target for immunotherapeutic applications.[8,25] As demonstrated by flow cytometry, HUH6 and HepT1 HB cells express EpCAM on the cell surface (**Fig. 2**). There was no difference in the expression of this tumor marker regardless of the previous treatment with concentrations of CDDP ranging from 1 µg/mL to 4 µg/mL (for 48 h). As these results were obtained from monolayer cultures, we intended to mimic the in vivo setting more realistically by generating tumor spheroids. For this purpose, HB cell lines were cultured on ultra-low attachment surface plates at a starting cell density of 100,000 cells per well over a period of

7 d. Whereas CD326 is homogenously expressed on cells grown in monolayers, spheroids seemed to contain a subpopulation of cells that express EpCAM at comparatively low levels (**Fig. 3**). Similar observations were done analyzing tissue samples derived from xenografted HB cells. Immunofluorescence microscopy revealed large areas of the tumor expressing EpCAM and few tumor regions that were not recognized by the anti-EpCAM antibody, suggesting the presence of two HB cell differentiation states (**Fig. 4**).

EpCAM as a target for anti-HB immunotherapy. Since EpCAM expression is higher on HB than on normal liver cells, we sought out whether it could serve as a target for immunotherapy. For this purpose, peripheral blood mononuclear cells (PBMCs) and γδ T cells were incubated with HB cells, respectively, in the presence of different concentrations of EpCAM-specific antibodies (**Fig. 5**). PBMCs at a E:T ratio of 2:1 did not effectively kill HUH6 or HepT1 cells. The addition of the mono-specific antibody MT201 resulted in a slight increase in tumor cell lysis (reduction of tumor cell viability of 25%). Lysis was significantly higher in cultures treated with the bi-specific antibody MT110, which—at odds with MT201—concurrently binds EpCAM and CD3. At the highest concentration tested (50 μg/mL) only 25% of tumor cells remained alive. γδ T cells are more efficient at lysing HB cells than PBMCs as at an equal cellular ratio a 25% lower tumor cell viability was measured. With increasing concentration of MT110, a prominent killing of HB cells was achieved. We next analyzed the efficiency of γδ T cells at lysing tumor cells within xenograft-derived tissue slices, both in the presence and in the absence of MT110 (**Fig. 6**). These tumors were derived from HUH6 cells that had previously been transfected with Gaussia luciferase (GLuc). Hence, viable cells in tissue slices secreted GLuc into the culture media. Tumor slices accommodated for 8 h in culture released within the first hour of co-culture with γδ T cells an enzymative activity ranging between 9,000 to 15,000 RLU/sec, depending on the weight of the slice. At the end of co-incubation period, the enzyme activity released in the medium of antibody-free co-cultured increased up to 2-fold. In MT110 containing co-cultures, 50% of the initial GLuc activity was observed. These two different types of co-culture, i.e., cells in suspension and tissue slides, emphasize the positive effect of the bi-specific antibody MT110 on tumor recognition and lysis by γδ T cells.

A possible option for HB-directed immunotherapy would be constitute by the adoptive transfer of activated and expanded autologous γδ T cells, either as freshly isolated populations or upon cryopreservation. γδ T cells expanded in culture in the presence of 400 nM zoledronic acid and 200 IU/mL interleukin-2 (IL-2) were compared with cryopreserved cells from the same isolation in a killing assay with HB cells (**Fig. 7**). Expanded γδ T cells lysed up to 25% HB cells at a E:T ratio of 4:1. The addition of 5μg/mL MT110 significantly enhanced tumor cell lysis, an effect that was similar in freshly expanded and cryopreserved γδ T cells. Cryopreservation did not have any negative impact on the capacity of γδ T cells to lyse target cells, irrespective of the presence of MT110.

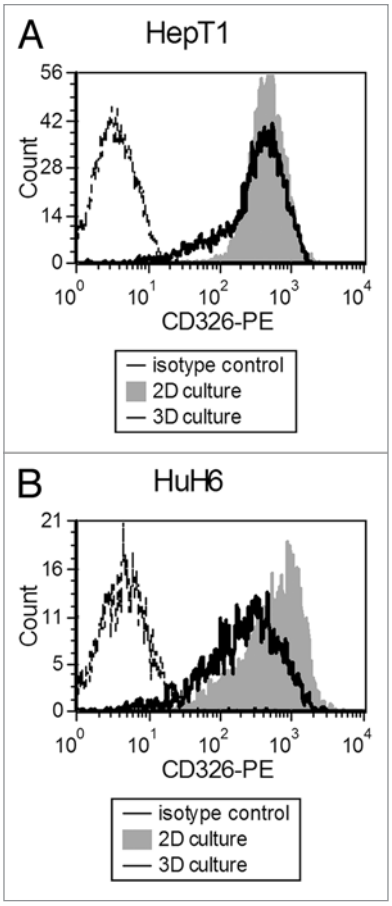

Figure 3. Expression of EpCAM on hepatoblastoma cells cultured spheroids. HepT1 and HUH6 hepatoblastoma (HB) cells were cultured on culture plates (2D) or as spheroids (3D). Flow cytometry revealed a lower expression of EpCAM on a subpopulation of hepatoblastoma (HB) cells cultured as spheroids as compared with a homogeneous staining observed for cells cultured on plates.

Discussion

New successful regimens to treat HB have been developed over the past 40 y. Despite all such efforts, survival rates of patients suffering from high-risk HB remain poor (3-y survival = 69%).[26] New strategies against advanced HB are needed, since the low overall response to chemotherapy and the high incidence of metastasis impair disease outcome.[26] We chose EpCAM as a target for immunotherapy because it is a well-known tumor antigen.[8] Moreover, a reduction of EpCAM expression due to chemotherapy is not expected. We indeed demonstrated that EpCAM is constantly expressed on cultured HB cells irrespective of chemotherapeutic treatment. Gene array analysis performed with hepatic tumor tissue from HB patients after several rounds of cytostatic drug application confirm our results in vivo (http://www.ebi.ac.uk/arrayexpress/experiments/E-EXP-1851). Histologically, EpCAM expression appeared to be distributed heterogeneously within the tumor.[8,9] Our experiments also showed a differential expression of EpCAM on malignant cells forming 3D

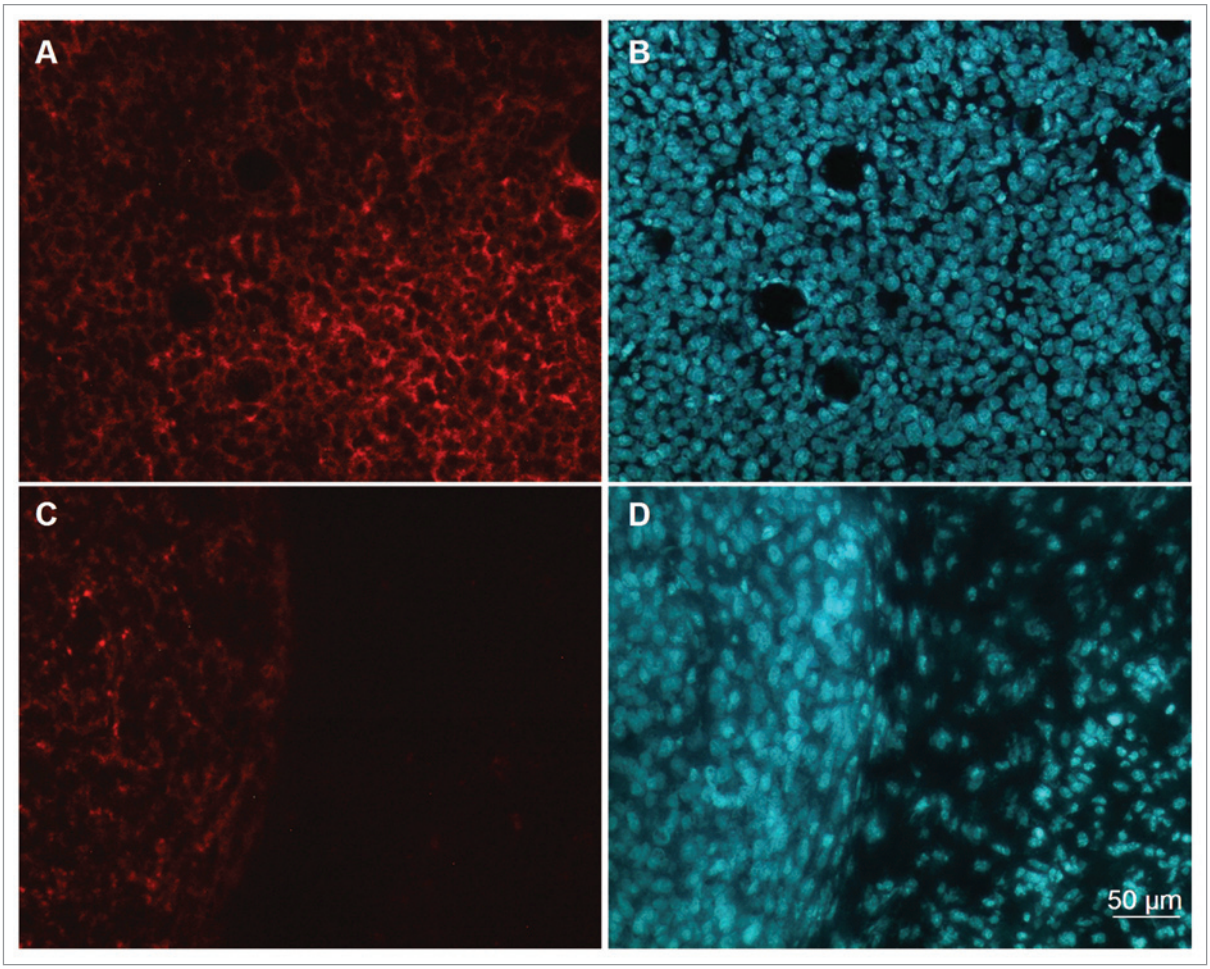

Figure 4. Expression of EpCAM in xenograft-derived hepatoblastoma tissue. Intensive cell membrane staining for EpCAM (red) was observed with the CD326-specific antibody AEC-125-PE in immunofluorescence on hepatoblastoma (HB) tissue slice. Nuclear staining with Hoechst 33347 revealed tumor cells not expressing EpCAM.

spheroids in cultured or generating tumors in vivo. We assume that the expression of EpCAM in HB cells is under epigenetic control or driven by WNT signaling, as observed in other cells.[27,28]

In spite of a heterogeneous expression pattern, EpCAM represents a convenient target for therapeutic antibodies. Only a slight enhancement of cell lysis was monitored when MT201/adecatumumab was administered to co-cultures of HB and PBMCs. Although an ADCC was expected,[29] at the low E:T ratio that we employed, the number of $CD16^+$ cells (which are responsible for the ADCC) may have been insufficient. The other antibody that we tested, MT110, is generated by the bBi-specific T-cell engager (BiTE) technology and binds both EpCAM and CD3 molecules on T cells. This dual specificity ensures not only the specific recruitment of $CD3^+$ T cells like cytotoxic αβ T cells and γδ T cells to $EpCAM^+$ tumor cells but also their activation, through the cross-linking of co-stimulatory complexes.[29] MT110-dependent activation induces T-cell proliferation and the release of cytotoxic granule proteins like perforin and granzymes.[30,31] Indeed, tumor cell lysis was more efficient when PBMs or γδ T cells were cultured with HB cells in the presence of MT110, as compared with co-cultures with MT201 or no antibody. The enormous capacity of this antibody to direct the activity of cytotoxic T cells to different tumor cells in vitro and in vivo has been shown elsewhere.[29,30,32,33] Therefore, BiTE MT110 stands out as a promising tool for treating $EpCAM^+$ tumors like HB. One of the advantages of administering antibodies is the represented by the direct activation of effector cells that infiltrate and surround the tumor. This may allow for the circumvention of inhibitory signals coming from stromal cells.[34] As in this study we analyzed the impact of MT110 in vitro, its efficiency at redirecting cytotoxic effector cells toward $EpCAM^+$ hepatic tumor cells in vivo remains to be determined. It is yet unclear tumor infiltration by antibodies and lymphocytes may be as strong as in vitro, because it depends not only on interstitial pressure but also on liver perfusion.

Titration experiments revealed that 5 µg/mL of the antibody were sufficient to initiate efficient cell killing in vitro by γδ T cells, as cell lysis could not be significantly enhanced by higher antibody concentrations. Mouse experiments demonstrated that increasing concentrations of MT110 can either efficiently induce tumor elimination or even prevent tumor formation.[33] Combining the adoptive transfer of γδ T cells and the administration og MT101 antibody in orthotopic HB xenografts in mice

may provide proof-of-principle for this therapeutic concept in vivo.

The impressive antitumor activity of activated γδ T cells, especially in combination with the bispecific antibody MT110, led to a nearly complete elimination of tumor cells in vitro. Since γδ T cells can be amplified and retain their cytotoxic activity also after prolonged cryopreservation periods, this unconventional, minor T-cell subset could be especially suitable for adoptive T-cell transfer against HB. The use of the T-cell engaging antibody MT110 after liver transplantation or autologous adoptive T-cell transfer, for instance against metastases or to prevent recurrence, also appears as a therapeutic option. So far the clinical testing of EpCAM-antibodies like catumaxomab has not been extended to situations of pathological liver. However, side effects can be expected, as transient higher expression of EpCAM was observed during cirrhosis or in liver regenerating post-hepatectomy. Mild toxicity was observed despite EpCAM expression in normal liver cells including biliary duct and hepatic stem cells (http://clinicaltrials.gov/). We will therefore consider an antibody therapy targeting EpCAM for subjects at a very high risk of relapse upon hematopoetic stem cell transplantation following liver transplantation. This concept has already been proposed in the treatment of particular cases of HB.[35]

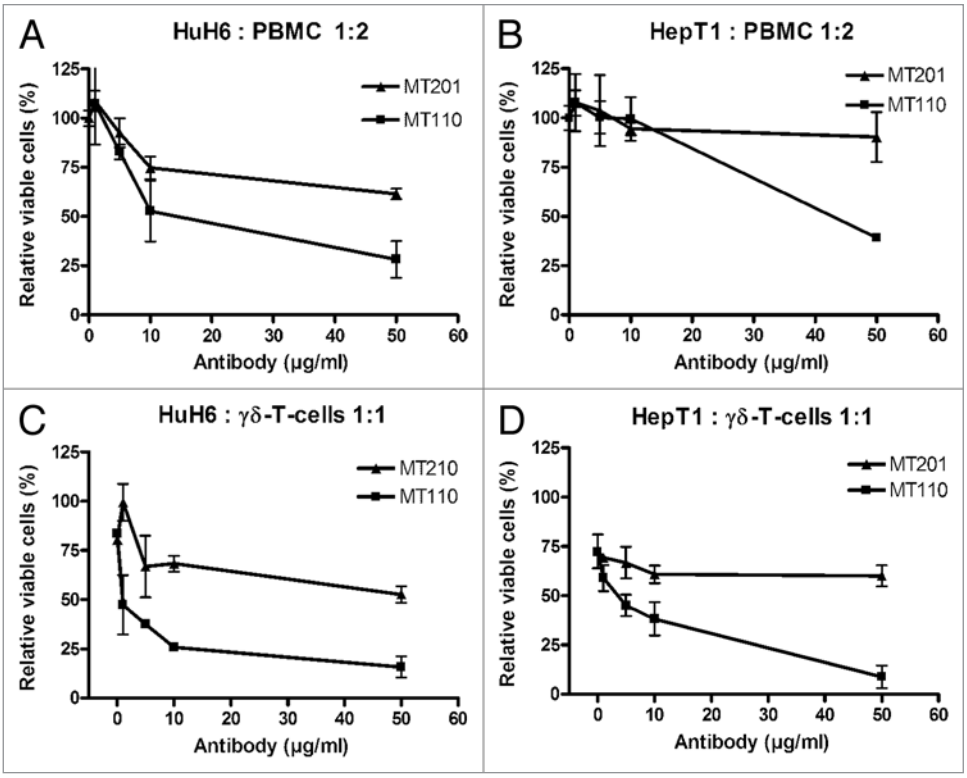

Figure 5. Lysis of hepatoblastoma cells with lymphocytes in the presence of EpCAM antibodies. (**A–D**) HUH6 (**A and C**) and HepT1 (**B and D**) hepatoblastoma (HB) cells were cultured with peripheral blood mononuclear cells (PBMCs) (**A and B**) or γδ T cells (**C and D**) at the indicated E:T ratio. Antibodies targeting EpCAM (MT201 and MT110) were added in antibody-dependent cell cytotoxicity (ADCC) assays at concentrations ranging from 5 μg/mL to 50 μg/mL. Shown is the mean cell viability ± SD of triplicates related to control cultures without antibodies and lymphocytes. Tumor cell lysis increased with antibody concentration and was significantly higher for MT110 than for MT201.

Materials and Methods

Cell cultures. HUH6 and HepT1 HB cells were derived from a mixed HB and from a multifocal embryonal HB, respectively.[36,37] Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) L-glutamine and 1% penicillin/streptomycin (all from Biochrom AG) on plastic culture dishes (Greiner-Bio One GmbH). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and were proved to be Mycoplasma negative. Spheroid cultures were grown in 200 μL culture medium on ultra-low attachment surface plates (Corning Inc.–Life Sciences) over a period of 7 d.[38] Some cultures were incubated with increasing concentrations of cisplatin (CDDP, Neocorp AG)—ranging from 1 μg/mL to 4 μg/mL—for 48 h.

Isolation of PBMCs and γδ T cells. Blood samples were obtained as buffy coats from the Centre for Transfusion

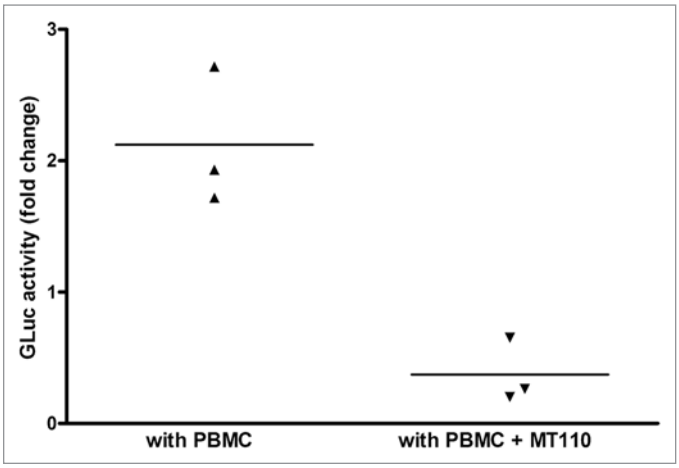

Figure 6. Lysis of hepatoblastoma cells with PBMCs within tissue slices. Tissue slices from HUH6-derived tumors were incubated with γδ T cells and 5 μg/mL MT110 antibody for 20 h. Changes in the number of viable tumor cells in the tissue slice were estimated by the relative activity of constitutive expressed GLuc. Each dot represents one cultured tissue slice.

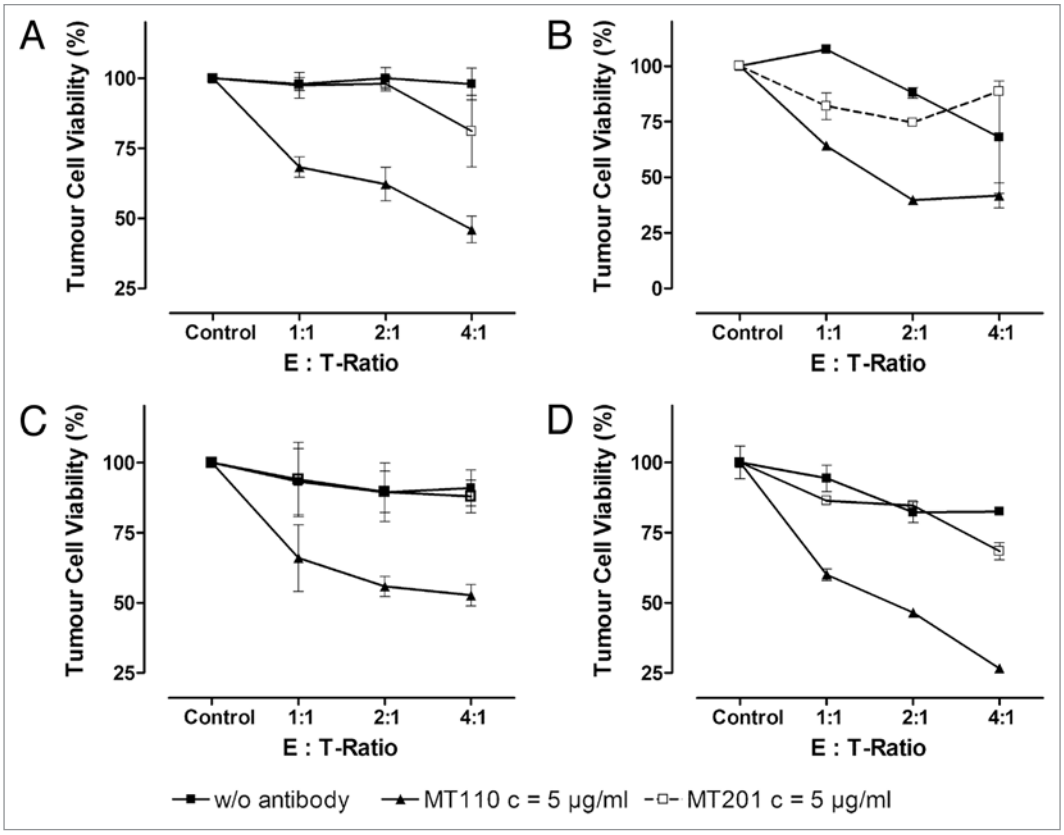

Figure 7. Lysis capacity of freshly isolated γδ T cells vs. cryopreserved γδ T cells. (**A-D**) HepT1 (**A and B**) and HUH6 (**C and D**) hepatoblastoma (HB) cells were incubated with increasing numbers of freshly prepared (**A and C**) or cryopreserved (**B and D**) γδ T cells in the presence or absence of 5 µg/mL MT110 and MT201 antibodies. Cell viability was measured by MTT assay after 24 h. All data points represent mean values of triplicates.

Medicine Tubingen. Donors were healthy volunteers of less than 30 y and were serum-negative for CMV, HIV, HCV, HBV and syphilis. PBMCs were isolated by density gradient centrifugation using Biocoll polymer solution (Biocoll Separating Solution, Biochrom AG). γδ T cells were subsequently isolated by negative MACS isolation according to the manufacturer's instructions (TCRγδ$^+$ isolation kit human, Miltenyi Biotec GmbH, 130–092–892). The purity of γδ T cells after negative MACS isolation, as estimated by flow cytometry using an anti-TCRγδ-PE antibody (Miltenyi Biotech), was > 99%. To expand γδ T cells, PBMCs were plated on plastic culture dishes in RPMI-1640 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin, as previously described.[39] After MACS isolation, γδ T cells were either cultured for another 4 d or immediately frozen and cryopreserved in liquid nitrogen for 4 mo. TCRγδ$^+$ cells were then thawed and cultured in fresh medium supplemented with IL-2 and zoledronic acid for 3 d. Cytotoxicity assays were performed 24 h after PBMC isolation.

Cytotoxicity assays. Tumor cells were cultured in 96-well plates (Becton Dickinson GmbH) under the conditions described above at a cell density of 10,000 cells per well in 50 µL culture medium. A suspension of PBMCs or γδ T cells was added in an equal medium volume containing increasing numbers of effector cells (E:T ratio ranging from 1:1 to 4:1). Humanized antibodies (BiTE antibody MT110 and MT201/adecatumumab, from Amgen administered up to a final concentration of 50 µg/mL. Cell viability was assessed by 24 h lasting 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity assays (Sigma). Twenty-five µL MTT solution (5 mg/mL dissolved in PBS) were added to each well 6 h prior to the end of the assay. To stop the reaction, 100 µL/well lysis solution (10% SDS in 0.1 N HCl) were added and plates were further incubated in the dark at room temperature overnight. Cell viability was assessed by measuring the absorption at 570 nm using Milenia Kinetic Analyzer (DPC Bierman). All assays were performed in triplicates. Relative cell viability was calculated upon normalization based on tumor cell cultured in the absence of effector cells and background of cultures containing no cells (only culture medium) or effector cells only, as controls.

Flow cytometry. Flow cytometry analyses were performed according to established protocols, as described elsewhere.[40] Briefly, 1 million cells were stained with anti-CD326-PE (Miltenyi Biotech). 20,000 events were collected on BD LSR II (Becton Dickinson GmbH) and analyzed using BD FACSDivaTM software and FCS Express 3 Flow Cytometry (De Novo Software).

Tumor tissue and tissue slice cultures. Xenografts derived from HUH6 cells with stable expression of Gaussia luciferase (GLuc) (pCMV-GLuc, NEB) were developed, as described

previously.[41] Spare tumor tissue was sliced in culture media with two scalpels as longitudinal sections of 2–3 mm thickness. Tissue slices were adapted to culture for 8 h. The sections were then placed in fresh culture media and incubated for 1 h until GLuc activity was assessed.[42] One million γδ T cells cells were added to the slice cultures and incubated for 20 h. Where indicated, 5 μg/mL MT110 were added. Gluc activity secreted in 1 h was measured after media was changed. The GLuc activity was expressed as relative light units per second (RLU/s) and was related to the initial activity for each cultured tumor slice. This paired analysis overcomes the bias due to different cell numbers in different tissue slices.

Immunofluorescence and histochemistry. Xenografts were derived from HUH6 and HepT1 cells, as described elsewhere.[41] Tumor specimen from a patient suffering of HB and receiving CDDP-based chemotherapy was obtained after informal consent. Sections of 4 μm from formalin fixed and paraffin embedded tissue were stained using the ABC method (Vector Stain). Human leukocytes were detected with the anti-CD45 antibody (BioGenex). EpCAM was detected with the antibody clone HEA-125 (Miltenyi). Nuclei were counterstained with Hoechst 33347 (Sigma). Microscopy was performed on a Zeiss Axio Scope epifluorescence microscope with an MRC5 camera (Carl Zeiss). Images were processed using AxioVision 4.8.1 software.

Statistics. Cell viability was analyzed by two-way ANOVA and Bonferroni post-hoc test (GraphPad Prism 4.00; GraphPad Softwares Inc.). Differences in relative gene expression were analyzed by Student's t-test. All numeric data are expressed as mean ± SD p values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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