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DNAX accessory molecule-1 (CD226) promotes human hepatocellular carcinoma cell lysis by Vγ9Vδ2 T cells

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Human Vγ9Vδ2 T lymphocytes can be activated by nonpeptidic antigens such as the mevalonate pathway-derived isopentenyl pyrophosphate or synthetic phosphoantigen such as bromohydrin pyrophosphate. They display a strong cytotoxic activity against several tumor types, including hepatocellular carcinoma (HCC). Little is known about the mechanisms underlying Vγ9Vδ2 T-cell recognition of tumor cells, but there is strong evidence that activating NK receptors play a role in γδ T-cell cytotoxicity. In this study, we showed that the two NK receptors DNAX accessory molecule-1 (DNAM-1) and CD96 were expressed by Vγ9Vδ2 T cells. The ligands Nectin-like-5 specific of both DNAM-1 and CD96, and also Nectin-2, an additional ligand of DNAM-1, were present on all HCC cell lines analyzed. Furthermore, we demonstrated by mAb-mediated masking experiments that cytotoxicity against HCC cells as well as IFN-γ production in γδ T cells were dependent on DNAM-1. Our experiments indicated that Nectin-like-5 but not Nectin-2 was involved in DNAM-1-dependent γδ T-cell functions. We did not reveal a role for CD96 in the killing of HCC cells. Finally, we showed by combined mAb-mediated blockade that DNAM-1 and NKG2D could cooperate in the cell lysis of HCC.

Key words: Antitumor immunotherapy · γδ T cells · Innate immunity · Nectins · NK receptors

Introduction

The prognosis of hepatocellular carcinoma (HCC) is poor with a 5-year survival rate of less than 5%. Surgical ablative therapy is the only curative treatment and may be suitable for a small group of HCC patients. Therefore, there is a pressing need for alternative antitumor therapies. A better understanding of tumor immunity has opened new perspectives to treat human malignant tumors [1]. Immunological approaches using innate antitumor effector cells are currently being evaluated in clinical trials [2]. Vγ9Vδ2 T cells represent the major population of human peripheral blood γδ T cells and display an *in vitro* non-MHC-restricted lytic activity against a broad panel of tumors including colon, kidney, liver, esophageal, small-lung cancers and myeloma [3–9]. The presence of tumor-infiltrating $V\delta2^+$ T cells in tumor-bearing livers of patients may support their potential role in tumor immunosurveillance of HCC [10]. Vγ9Vδ2 T cells recognize small nonpeptidic phosphorylated antigens such as isopentenyl

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pyrophosphate produced in mammalian cells through the mevalonate pathway [11]. Aminobisphosphonate such as zoledronate may activate Vγ9Vδ2 T cells indirectly by blocking the mevalonate pathway and consequently promoting intracellular accumulation of isopentenyl pyrophosphate [8]. Recent findings suggest that the mitochondrial F1-ATPase exposed at the surface of the stimulating cells could participate to phosphoantigen presentation and subsequent Vγ9Vδ2 T-cell activation [12, 13]. We and others have described that the synthetic phosphoantigen bromohydrin pyrophosphate (BrHPP, Innate Pharma, Marseilles, France) was able to vigorously expand Vγ9Vδ2 T cells owing a broad reactivity against tumor cell lines [4, 5]. However, until now, the mechanisms of γδ T-cell-mediated lysis of tumor cells are not completely understood. γδ T cells are able to recognize tumor cells in a TCR-dependent manner [14]. Moreover, NK cell receptors expressed by γδ T cells are known to play an important role in cytotoxicity of tumor cells. Among these, the activating NKG2D receptor has been widely investigated. This receptor interacts with MHC class I-related chain A and B (MICA/B) and UL16-binding protein (ULBP) and contributes to enhance killing of tumor cells by γδ T cells [4, 5, 15]. Recently, adhesion molecules that belong to the Nectin and Nectin-like (Necl) family have been described as ligands of receptors involved in cytotoxicity of NK cells [16]. The members of the Nectin/Necl family mediate epithelial cell junctions through both homophilic or heterophilic contacts [17]. It has been shown that Necl-5 (also called the poliovirus receptor or CD155) and Nectin-2 (also called the poliovirus-related receptor 2 or CD112) interact with the NK receptor DNAX accessory molecule-1 (DNAM-1 also called CD226) (Fig. 1) [18, 19]. The receptor CD96 (Tactile) was identified as an additional receptor for Necl-5 [20]. Since HCC is a potential candidate for γδ T-cell-based immunotherapy [5], there is interest to identify the NK receptors involved in the recognition of this tumor type. In the present paper, we have investigated the involvement of the two receptors DNAM-1 and CD96 in the lysis of HCC cells. The expression pattern of receptors and their ligands were analyzed on γδ T cells and HCC cells, respectively. For the first time, our data indicate that DNAM-1, but not CD96, plays a crucial role in the γδ T-cell-mediated cytolysis of HCC cells.

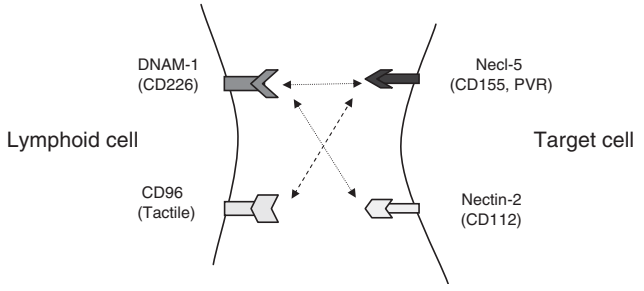

Figure 1. Interactions between NK receptors and Nectin/Necl molecules. The NK receptor DNAM-1 (CD226) expressed on lymphoid cells interacts with Necl-5 (CD155, poliovirus receptor) and Nectin-2 (CD112) on target cells. CD96 (Tactile) is an additional receptor for Necl-5.

Results

Expression of DNAM-1 and CD96 on γδ T cells

The expression of DNAM-1 and CD96 was assessed by flow cytometry on resting or BrHPP-stimulated γδ T cells. In freshly isolated peripheral blood leukocytes from donors, DNAM-1 and CD96 receptors are constitutively expressed on $CD3^+/\gamma\delta\ TCR^+$ cells (Fig. 2A). We also observed the expression of the two receptors on $CD3^+/\alpha\beta\ TCR^+$ cells (data not shown). After 15

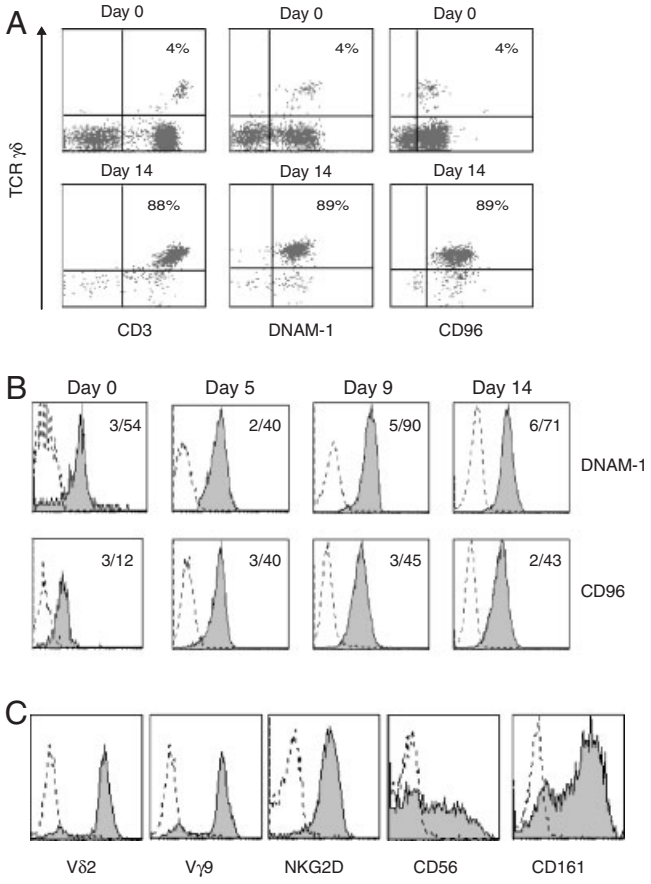

Figure 2. Expression of DNAM-1 and CD96 on γδ T cells. (A) Cell surface expression of DNAM-1 and CD96 was studied on γδ T cells freshly isolated from PBMC or on BrHPP-stimulated γδ T cells cultured for 2 wk. Double-staining was performed using mAb against CD3, DNAM-1, CD96 and pan-γδ TCR. Results are expressed in dot plot form. The proportions of cells expressing the cell surface marker in each dot plot are indicated. (B) DNAM-1 and CD96 expression on gated γδ T cells was analyzed by flow cytometry from day 0 to day 14 of culture of PBMC in the presence of BrHPP. MFI values of the background/marker staining are indicated on the histograms. (C) BrHPP-expanded γδ T cells were stained with anti-TCR Vγ9, TCR Vδ2, NKG2D, CD56 or CD161 mAb. The open histograms represent isotype controls and the filled ones are specific stainings. Data are representative of five independent experiments.

days with a single dose of BrHPP and in presence of IL-2, cultures were predominantly composed of γδ T cells that retain expression of DNAM-1 and CD96. Kinetic study showed a slight increase of DNAM-1 and CD96 expression over the culture (Fig. 2B). In addition, expanded Vγ9Vδ2 T cells also expressed the NK marker CD56 as well as the activating receptors NKG2D and CD161 (Fig. 2C).

Expression of Nectin-2 (CD112), Necl-5 (CD155) and NKG2D ligands on HCC cell lines

To evaluate the potential role of DNAM-1 and CD96 receptors in the recognition of tumor cells, we monitored the expression of their ligands on HCC cell lines. Phenotypic analysis indicated that all HCC cell lines express the ligands Nectin-2 and Necl-5 (Fig. 3). It was noted that the expression level of Necl-5 is higher as compared with Nectin-2. Interestingly, the Burkitt's lymphoma cell line Daudi does not express the ligands of DNAM-1 and CD96. We also investigated the expression of NKG2D ligands on HCC cell lines. MICA and MICB molecules are lacking or weakly expressed on HCC lines. Among

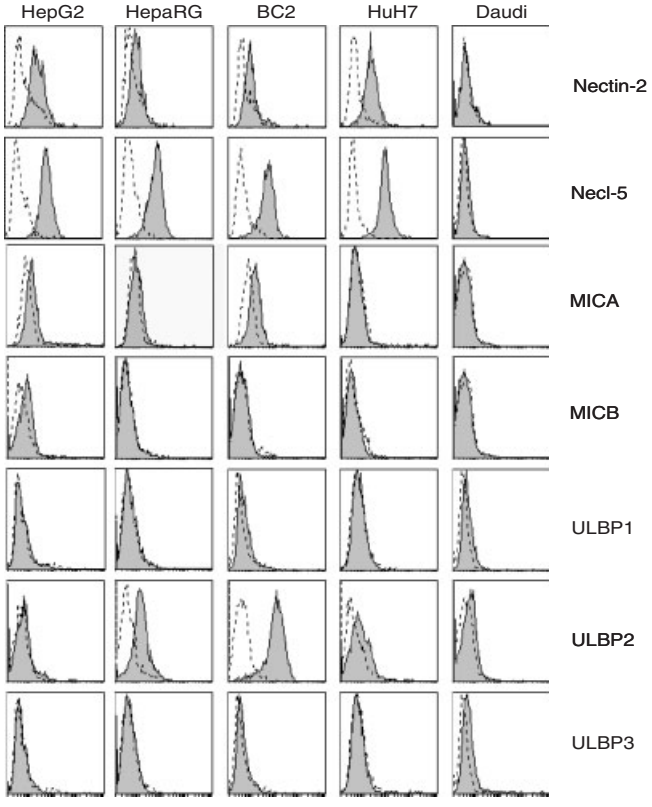

Figure 3. Expression of Nectin-2 (CD112), Necl-5 (CD155) and NKG2D ligands on tumor cell lines. HCC cell lines (HepG2, HepaRG, BC2 and HuH7) as well as the Daudi cell line were incubated with mAb directed against Nectin-2, Necl-5, MICA, MICB, ULBP1, 2, 3 (filled histograms) or control Ig (open histograms) and analyzed by flow cytometry. Data are representative of three independent assays.

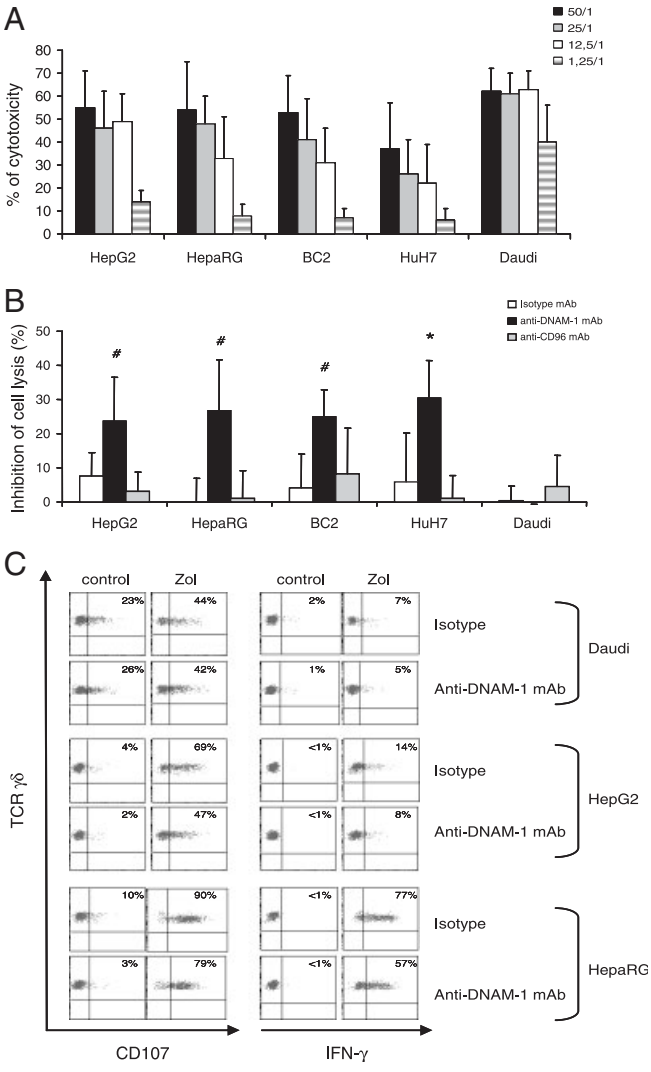

Figure 4. Inhibition of γδ T-cell-mediated cytotoxicity by anti-DNAM-1 antibody. PBMC from healthy donors were treated at day 0 with 3 μM BrHPP and cultured in the presence of 400 IU IL-2/mL for 2 wk. (A) Expanded γδ T cells were co-cultured for 4 h with 2×10^3 target cells previously labeled with ^{51}Cr in *E/T* of 50/1, 25/1, 12.5/1 and 1.25/1. Target cells were HCC cell lines (HepG2, HepaRG, BC2 and HuH7) and Daudi cell line. Data are from 11 independent experiments (mean ± SD). (B) Expanded γδ T cells were incubated with anti-DNAM-1 mAb, anti-CD96 mAb or isotype control for 45 min at room temperature and co-cultured for 4 h with 2×10^3 target cells previously labeled with ^{51}Cr in *E/T* of 50/1. Data are expressed in percentage inhibition of cell lysis and are from at least four independent experiments (mean ± SD). ♯ significantly different from isotype ($p<0.001$), ∗ significantly different from isotype ($p<0.05$). (C) Expanded γδ T cells were co-cultured with various target cells for 6 h at 37°C in a 1:1 ratio in the presence of anti-CD107-PE and 3 μM monensin. Target cells (Daudi, HepG2 and HepaRG cell lines) were pretreated or not with 5 μM zoledronate overnight (Zol). Viability of the cells was unchanged after this pretreatment. Following the co-culture, cells were stained with APC-labeled anti-γδ TCR and anti-PE IFN-γ mAb, and analyzed on a FACScalibur. The proportions of double-positive cells (γδ TCR^+/$CD107^+$ and γδ TCR^+/IFN-γ^+) are indicated in each dot plot. Data are from three experiments. Gamma-delta T cells cultured alone neither expressed CD107 nor IFN-γ while more than 65% of γδ T cells are positive for CD107 and IFN-γ staining following incubation of T cells with 300 nM BrHPP (data not shown).

ULBP molecules, only ULBP2 was clearly found on HepaRG and BC2 cell lines.

DNAM-1 is involved in effector functions of γδ T cells

We recently demonstrated that HCC tumors were sensitive to γδ T-cell-mediated cytotoxicity [5]. Here, we investigated the potential role of DNAM-1 and CD96 on HCC cell lysis. Cytotoxicity assays were performed with BrHPP-stimulated γδ T cells as effector cells in the presence of anti-DNAM-1 or CD96 mAb. As shown in Fig. 4A, BrHPP-expanded γδ T cells from donors display strong lytic activity against HCC cell lines. Lysis of HCC cell lines is significantly inhibited by anti-DNAM-1 mAb with a range of 23–31% of cell lysis inhibition (Fig. 4B). By contrast, blockade with anti-DNAM-1 mAb did not affect lysis of the Daudi cell line, a target cell line that lacks Nectin-2 and Necl-5 expression. In addition, incubation of effector cells with anti-CD96 mAb did not result in cell lysis inhibition. Then, to further define the contribution of DNAM-1 in the effector functions of γδ T cells, we evaluated the expression of CD107a, a marker associated with degranulation of cytotoxic cells, as well as the production of IFN-γ by intracytoplasmic cytokine staining. To improve the tumor cell recognition by γδ T cells, tumor cells were sensitized with the aminobisphosphate zoledronate. The incubation of γδ T cells with Daudi cells resulted in a significant up-regulation of CD107 expression while only a very low level of degranulation was obtained after co-incubation with HCC cell lines (Fig. 4C). CD107 expression on γδ T cells was dramatically increased when effector cells were cultured with pretreated zoledronate tumor cells. In these conditions, we also observed a significant increase of IFN-γ production by γδ T cells. Interestingly, when co-cultures between tumor cells and γδ T lymphocytes were performed in presence of anti-DNAM-1 mAb, we observed a significant decrease in both

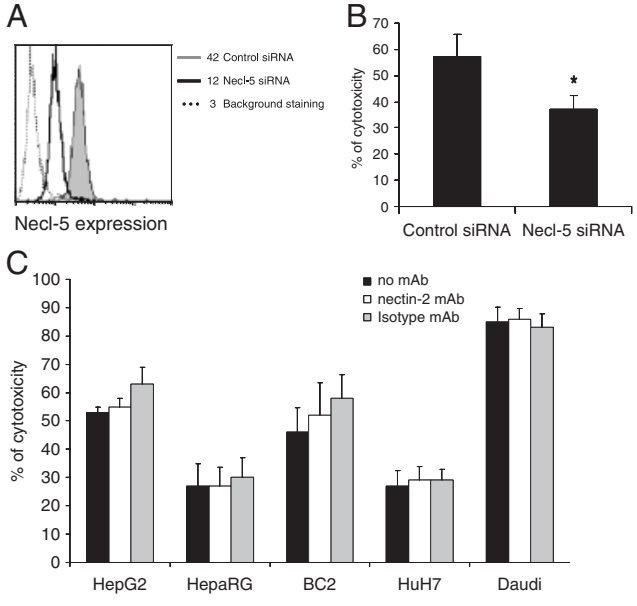

Figure 5. Necl-5 but not Nectin-2 triggers DNAM-1-dependent cytotoxicity. (A) HepaRG cells were electroporated with Necl-5-specific siRNA or control siRNA as described in the *Materials and methods*. Expression of Necl-5 was monitored by flow cytometry on day 3. MFI value of the background staining (dotted line) as well as sMFI values of the specific staining marker for transfected with control iRNA (filled gray), or Necl-5-specific siRNA (open black) are indicated on the histogram. (B) PBMC from healthy donors were treated at day 0 with 3 μM BrHPP and cultured in the presence of 400 IU IL-2/mL for 2 wk. Expanded γδ T cells were co-cultured for 4 h with CD155 siRNA or control siRNA-treated HepaRG cells previously labeled with ^{51}Cr in *E/T* of 50/1. Data are from three donors (mean ± SD). * significantly different from control siRNA treatment ($p<0.05$). (C) Expanded γδ T cells were co-cultured for 4 h with 2×10^3 target cells previously labeled with ^{51}Cr in *E/T* of 50/1. Target cells were HCC cell lines (HepG2, HepaRG, BC2 and HuH7) and Daudi cell line incubated or not with anti-Nectin-2 mAb or isotype control. Data are from three donors (mean ± SD).

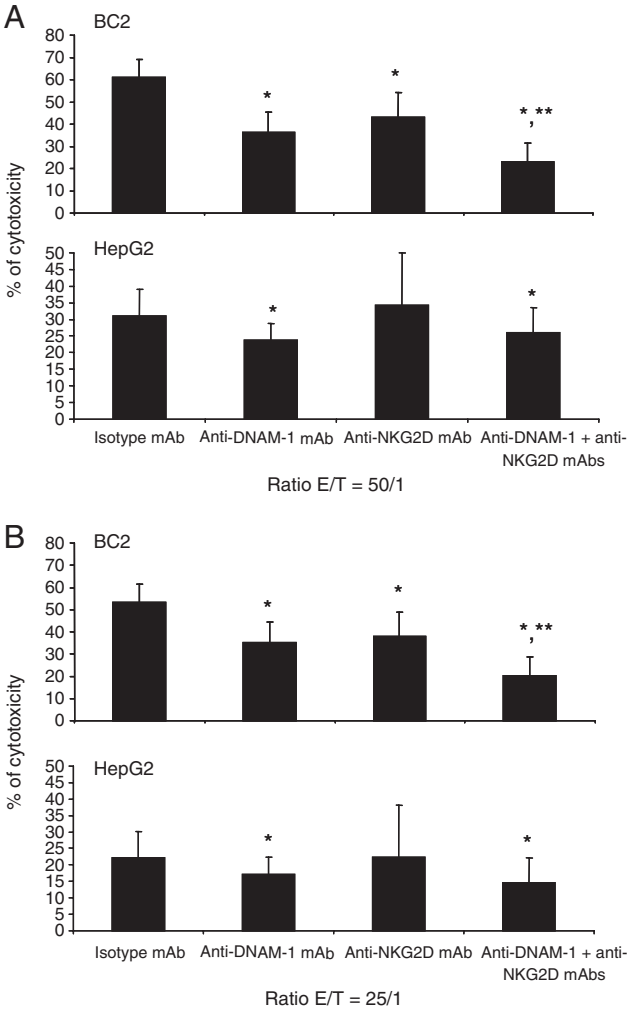

Figure 6. DNAM-1 and NKG2D cooperate in γδ T-cell-mediated lysis of HCC cell lines PBMC from healthy donors were treated at day 0 with 3 μM BrHPP and cultured in the presence of 400 IU IL-2/mL for 2 wk. Expanded γδ T cells were incubated with anti-DNAM-1 and/or anti-NKG2D mAb or isotype control for 45 min at room temperature and co-cultured for 4 h with 2×10^3 target cells previously labeled with ^{51}Cr in *E/T* of 50/1 (A) or 25/1 (B). Data are expressed in percentage of specific lysis and are at least from three independent experiments (mean ± SD), * significantly different from isotype mAb treatment ($p<0.05$), ** significantly different from anti-DNAM-1 mAb treatment ($p<0.05$).

CD107 expression and IFN-γ production for HepG2 and HepaRG but not for Daudi.

Necl-5 but not Nectin-2 is required for γδ T-cell-mediated killing of HCC cells

Next, we addressed the respective contribution of either Nectin-2 or Necl-5 in the DNAM-1-mediated cell lysis. We transiently transfected HepaRG cells with siRNA specific for Necl-5. Flow cytometry analysis of the specific siRNA-treated HepaRG cells demonstrated a marked decrease in Necl-5 expression 3 days after transfection (Fig. 5A). Interestingly, susceptibility to γδ T-cell-mediated lysis was significantly decreased for HepaRG cells transfected with Necl-5 siRNA compared with the cells transfected with nontargeting siRNA (Fig. 5B). We have also carried out blockade experiments with specific mAb in order to analyse the role of Nectin-2. As shown in Fig. 5C, anti-Nectin-2 mAb did not alter the cytotoxicity of γδ T cells against any HCC cell lines.

Relative contribution of DNAM-1 and NKG2D to γδ T-cell-mediated lysis of HCC cell lines

Since the NK receptor NKG2D was found to play a role in the killing of HCC tumor cells [5], we addressed the question whether DNAM-1 and NKG2D could have a synergic effect in the cytolytic activity of γδ T cells by incubating effector cells with anti-DNAM-1 and/or anti-NKG2D mAb. The lysis of BC2 cell line is reduced in the presence of anti-NKG2D mAb alone and simultaneous blockade of DNAM-1 and NKG2D resulted in a significant additional effect at both 50/1 and 25/1 effector to target ratios (*E/T*) (Fig. 6). The reduction was not shown for HepG2, a cell line that does not express NKG2D ligands.

Discussion

The capacity of γδ T cells to recognize human tumor cells in a nonrestricted MHC manner is well documented [3, 4, 7, 9]. However, the requirements for recognition of malignant cells are not completely understood. It has been described that γδ T cells may exert their cytotoxic activity *via* a TCR-dependent pathway and/or through activating NK receptors [21]. In a previous study, we had shown that γδ T cells were able to lyse HCC cells freshly isolated from tumors but not normal counterparts, emphasizing the potential usefulness of immunotherapy based on adoptive transfer of γδ T cells [5]. To gain insights into the mechanisms involved in tumor recognition, the role of two NK receptors DNAM-1 and CD96 in the lytic function of γδ T cells was investigated. DNAM-1 is a 65 kDa transmembrane glycoprotein physically and functionally associated with lymphocyte function-associated antigen-1 (LFA-1)[22]. DNAM-1 engagement by mAb-mediated cross-linking triggers tyrosine phosphorylation and its recruitment into the membrane rafts [23, 24]. Moreover, DNAM-1 functions such as adhesion are dependent on the phosphorylation of serine residue 329 in the cytoplasmic domain by the PKC [25].

Cytometric analysis reveals that DNAM-1 and CD96 receptors are constitutively expressed by γδ T cells from freshly isolated PBMC or after BrHPP-induced expansion. Expression of DNAM-1 and CD96 were also found on αβ T cells as previously described [23]. In parallel, we show that HCC cell lines expressed the ligands Nectin-2 and Necl-5 on their cell surface. The cellular distribution of two molecules was mostly overlapping although minor differences were observed for level expression. This result extends to HCC the data about Nectin-2 and Necl-5 expression in ovarian, colon and kidney tumors [18]. The role of DNAM-1 has been appreciated in mAb-dependent blocking experiments. Interestingly, masking the DNAM-1 receptor on γδ T cells inhibits the cytotoxicty against HCC cells as well as the production of cytokines by effector cells. As expected, effector functions of γδ T cells were not affected by anti-DNAM-1 antibody in the case of the Daudi cell line, which does not express the two DNAM-1 ligands. An involvement of DNAM-1 in the killing of tumor cells by NK cells has been previously demonstrated by antibody masking experiments with tumor cell lines from epithelial, neuronal or hematological origin but, until now, nothing has been reported for γδ T cells [26–28]. Conversely, using the anti-CD96 NK92.39 mAb, we have not been able to point a role of CD96 in the lysis of Necl-5-expressing HCC cells by γδ T effectors. Similarly, others groups did not succeed in demonstrating a contribution of CD96 to the NK-cell-mediated lysis of ovarian carcinoma or myeloma [27, 29]. Our experiments indicate that Necl-5 but not Nectin-2 is involved in the DNAM-1-dependent recognition of tumor cells by γδ T cells. Blockade ability of the anti-Nectin-2 mAb (R2.477) was not questioned since this mAb was able to block cytotoxic activity of NK cells against transfectant cells expressing Nectin-2 [19]. These results are consistent with data from Reymond *et al.* who have shown that DNAM-1 binds Necl-5 more efficiently than Nectin-2 and that Necl-5 but not Nectin-2 is the major ligand of DNAM-1 on endothelial cells [30]. Tahara-Hanaoka *et al.* have suggested that DNAM-1 interactions with Nectin-2 may be impaired by homophilic ligation of Nectin-2 [19]. We had already shown that the NK receptor NKG2D was involved in γδ T-cell-mediated lysis of HCC cells expressing the corresponding ligands [5]. In addition to these data, we report here an additive inhibitor effect of anti-DNAM-1 and anti-NKG2D mAb suggesting a cooperation of both receptors in triggering cytotoxic effector function of γδ T cells.

In conclusion, to our knowledge, it is the first report showing that DNAM-1 regulates cytotoxicity of γδ T cells *via* a specific interaction with Necl-5 expressed on HCC cells. Moreover, we also demonstrate that DNAM-1 engagement could induce IFN-γ production by γδ T cells. Regarding γδ T-cell-mediated cytotoxicity, it is likely that the overall lytic function of these cells reflects the net sum of multiple receptor–ligand interactions as illustrated here with DNAM-1 and NKG2D. In this regard, a recent paper by von Lilienfeld-Toal *et al.* shows that the natural cytotoxicity receptor NKp46 expressed by γδ T cells plays a role in their cytotoxic activity against myeloma [9]. Further studies are required to address the involvement of other NK receptor–ligand pairs in the recognition of HCC and to determine whether their expression is predictive of γδ T-cell therapy efficiency.

Materials and methods

γδ T-lymphocyte amplification

PBMC were isolated by density gradient separation (UniSep®, Novamed, Jerusalem, Israel) from blood samples of healthy donors ($n = 14$) (Etablissement Français du Sang, Rennes). PBMC were resuspended at 2.10^6/mL in RPMI 1640 (Eurobio, Les Ullis, France) supplemented with 10% FCS (Gibco Invitrogen Life Technologies, Cergy Pontoise, France), 1% L-glutamine, 50 μg/mL streptomycin and 50 IU/mL penicillin, referred to elsewhere as complete medium. Cells were treated once on day 0 with 3 μM BrHPP (IPH1101, Phosphostim™, a kind gift of Innate Pharma) and cultured in presence of 400 IU IL-2/mL (Proleukin® Novartis, Suresnes, France) for 2 wk. Every 3 days, fresh complete medium with additional 400 IU IL-2/mL was added.

Tumor cell lines and primary cultures

HCC tumor cell lines (HepG2, HuH7, BC2, HepaRG) were a gift from C. Guillouzo (INSERM U522, Rennes, France). Cells were cultured in complete medium with 0.5 μM hydrocortisone (Pharmacia, Guyancourt, France) and 0.5 μg/mL insulin (Sigma, Saint-Louis, USA).

Flow cytometry analysis

γδ T cells were stained by conjugated mAb against CD3 (UCHT1), IFN-γ (45.15), pan αβ (BMA031), pan-γδ (IMMU510), Vγ9 (IMMU360), Vδ2 (IMMU389), CD56 (NKH-1), CD161 (191B8) and NKG2D (ON72) purchased from Immunotech (Marseilles, France) and CD96 (NK92.39, HyCult). Mab against DNAM-1 (DX11), CD107 (H4A3) and pan-γδ (B1) were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Tumor cells were stained by mAb against MICA (159227), MICB (236511), ULBP1 (170818), ULBP2 (165903), ULBP3 (166510) purchased from R&D systems (Lille, France), Nectin-2 (R2.525, BD Biosciences) and Necl-5 (46.31, a kind gift from M. Colonna, Washington University, St Louis, MO, USA). Isotype-matched murine immunoglobulins from the corresponding manufacturer were used as negative controls. Data were analyzed on a FACSCalibur cytometer (BD Biosciences, Mountain View, USA).

CD107 mobilization assay and intracellular cytokine staining

Phosphoantigen-expanded γδ T cells were co-incubated with target cells at a ratio 1:1 in a final volume of 100 μL in round-bottomed 96-well plates in the presence of fluorochrome-conjugated anti-CD107 mAb or the corresponding IgG1 isotype. Positive control was obtained by stimulation of γδ T cells with 300 nM of BrHPP. Monensin was added 1 h into the incubation time. Following 6 h of co-culture, cells were fixed in a 4% paraformaldehyde solution and permeabilized with PBS containing 0.1% saponin and 0.5% bovine serum albumin. Finally, the cells were labeled with fluorochrome-conjugated anti-IFN-γ and anti-γδ TCR mAb, and analyzed by flow cytometry.

Cytotoxicity assays

Expanded Vγ9Vδ2 T cells were tested for cytotoxicity against allogeneic established HCC tumor cell lines and Daudi cell line in standard ^{51}Cr release assay. A total of 2×10^3 target cells labeled with ^{51}Cr sodium chromate (0.2 mCi/10^6 cells, Amersham) were co-cultured in complete medium in 96 U-bottomed well plates for 4 h with γδ T cells. The *E/T* ranged from 1.25/1 to 50/1. ^{51}Cr release was assessed in culture supernatants, using a Top-count gamma counter (Packard Instrument). Specific lysis (expressed as percentage) was calculated using the standard formula: ((mean experimental cpm-mean spontaneous cpm)/(mean maximum cpm-mean spontaneous cpm)) × 100. Results are the mean of assays performed in triplicate.

In blocking assays, the effector cells were previously treated with saturating concentrations of specific mAb against DNAM-1 (DX11), CD96 (NK92.39), NKG2D (149810, R&D systems) or isotype control mAb (MOPC-1) for 45 min. We also realized cytotoxic assays in the presence of anti-Nectin-2 mAb (R2.477, M. Lopez, INSERM UMR599, France). Inhibition of cell lysis was calculated according to the formula: percentage inhibition of cell lysis = (1- (% of specific lysis with mAb)/(% of specific lysis without mAb)) × 100.

siRNA transfection of tumor cells

To repress Necl-5 expression, a predesigned double-stranded siRNA (ON TARGETplus smart pool Human siRNA CD155; Dharmacon, Perbio-Science, Brebières, France) was used. A siCONTROL nontargeting siRNA pool (Dharmacon) was used as a negative control. SiRNA (60 nM) were transfected into HepaRG cells by electroporation using a pipette-type electroporator (MicroPorator MP-100, Labtech, Palaiseau, France) according to the manufacturer's instructions. Transfected cells were incubated into 12-well plates at 1×10^5 cells *per* well for 72 h. Expression of Necl-5 was monitored by flow cytometry.

Statistical analyses

Statistical analyses were carried out using the paired Student's *t*-test. A p-value < 0.05 was considered statistically significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **BrHPP:** bromohydrin pyrophosphate · **DNAM-1:** DNAX accessory molecule-1 · ***E/T*:** effector to target ratio · **HCC:** hepatocellular carcinoma · **MICA/B:** MHC class I-related chain A and B · **Necl:** Nectin-like · **PVR:** poliovirus receptor · **ULBP:** UL16-binding protein

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