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Vγ9Vδ2 T Cell Response to Colon Carcinoma Cells[1]

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During analysis of CD8 T cells derived from ascites of a colon cancer patient, we isolated a Vγ9Vδ2 T cell clone showing strong reactivity against autologous tumor cell lines. This clone killed a large fraction of allogeneic colon carcinoma and melanoma cell lines, but did not affect a normal colon cell line, colon fibroblasts, or melanocytes. Tumor cell recognition was TCR and NKG2D dependent and induced TNF-α and IFN-γ secretion by the clone; accordingly, tumor targets expressed several NKG2D ligands, such as MHC class I chain-related gene A and UL16-binding protein molecules. Colon tumor recognition by Vγ9Vδ2 T cells was highly dependent on isopentenyl pyrophosphate production and ICAM-1 expression by target cells. Finally, similar reactivity patterns against colon carcinoma cell lines were observed using polyclonal Vγ9Vδ2 T cells of various origins, and Vγ9Vδ2 lymphocytes were present in the majority of colon tumor samples studied. Together, these results suggest that Vγ9Vδ2 T cells contribute to the natural immune surveillance against colon cancers. Therefore, this study provides a strong rationale for the use of Vγ9Vδ2 T cell agonists in immunotherapies targeting colon tumors. *The Journal of Immunology,* **2005, 175: 5481–5488.**

The role of tumor-infiltrating T lymphocytes (TIL)[3] in spontaneous tumor immunity and their potential use in immunotherapy have been subjects of intense investigation. Besides $\alpha\beta$ T lymphocytes, which represent the dominant TIL population in most tumors, the Vδ1 subpopulation of $\gamma\delta$ T cells frequently infiltrates some solid tumors, such as lung, kidney, breast, prostate, colon, ovarian carcinomas, and cutaneous malignancies (1–5). In contrast, Vγ9Vδ2 T cells, which predominate among $\gamma\delta$ PBL in healthy adults, have been rarely described in tumors (6).

Both Vδ1 and Vδ2 $\gamma\delta$ subsets have been implicated in tumor immunity based on their capacity to kill tumor cell lines in vitro in a TCR-dependent manner. Nonetheless, the tumor ligands recognized by these subsets are likely to be distinct. Indeed, although Vδ1 T cells derived from TIL kill a wide array of solid tumors (7–11), Vγ9Vδ2 T cells preferentially kill hemopoietic tumor cell lines, such as myeloma, lymphoma, and erythroleukemia (12–15). However, more recent studies reported recognition of some melanoma and renal carcinoma cell lines by Vγ9Vδ2 T cells and killing of autologous metastatic renal carcinoma cells (RCC) by autologous PBL-derived Vγ9Vδ2 T cell lines (16–18).

Tumor cell recognition by Vγ9Vδ2 T lymphocytes is mostly MHC unrestricted and is controlled by both TCR-dependent and TCR-independent signals. Several nonpeptidic phosphorylated compounds produced through the isoprenoid biosynthetic pathway and classically referred to as phosphoantigens can activate Vγ9Vδ2 T cells in a TCR-dependent fashion. Although natural and synthetic phosphoantigens (such as isopentenyl pyrophosphate (IPP), hydroxydimethylallyl pyrophosphate, and bromohydrin-pyrophosphate) readily stimulate Vγ9Vδ2 T cells in vitro, other compounds, such as aminobiphosphonates, sensitize target cells to Vγ9Vδ2 killing by promoting the accumulation of Vγ9Vδ2 agonists (e.g., IPP) in treated cells (19–27). The mode of recognition of phosphoantigen by Vγ9Vδ2 cells remains unclear, but their presentation by as yet undefined receptors is likely. Various receptors, such as the LFA-1 integrin, CD2 (19, 20), and NK receptors, both activatory, such as NKG2D, and inhibitory, were also reported to be important for tumor cell line recognition and lysis by Vγ9Vδ2 T lymphocytes (14, 18, 21–26).

The aim of the present study was to investigate the adaptive cellular immunity against colon carcinoma cells through analysis of colon tumor reactivity of $CD8^+$ T cells derived from tumor patients. Two colon carcinoma cell lines (CCCL) and $CD8^+$ T cells reactive to these tumor cells were derived from ascites of a metastatic colon carcinoma patient. Identification of Vγ9Vδ2 T cells within the tumor-reactive $CD8^+$ population prompted us to perform an in-depth analysis of Vγ9Vδ2 T cell reactivity against various CCCL and to study the modalities of CCCL recognition by Vγ9Vδ2 T cells and the frequency of these lymphocytes present within colon tumors. Our results suggest that Vγ9Vδ2 T cells infiltrate and recognize a significant fraction of human colon cancers and, therefore, may contribute to the immune surveillance of these tumors.

Materials and Methods

Cell lines

Tumor cell lines C4A (adherently growing) and C4S (growing in suspension) were established from an ascites of a stage IV colon cancer patient (C4). Other carcinoma cell lines used were SW620, SW1116, SW707, HT29, HTC116, LS180, SW480, CaCo2, Colo205, and SW1222 (gifts from J. Le Pendu, J. F. Chatal, C. Saï (Institut National de la Santé et de la Recherche Médicale, Unité 601, Nantes, France), and M. Denis (Unité Mixte de Recherche 539, Nantes, France)). The normal colon cell line CCL-241 was provided by American Type Culture Collection and was cultured in DMEM (Sigma-Aldrich) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), 2 mM L-glutamine

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[3] Abbreviations used in this paper: TIL, tumor-infiltrating lymphocyte; CCCL, colon carcinoma cell line; IPP, inositol pyrophosphate; RCC, renal carcinoma cell; RFI, ratio fluorescence intensity; MICA, MHC class I chain-related gene A; ULBP, UL16-binding protein.

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(Sigma-Aldrich), and 30 ng/ml epidermal growth factor. Mouse fibrosarcoma WEHI 164 clone 13, used for TNF production assays, was obtained from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). The B-EBV-transformed cell line LAZ 338 was a gift from T. Hercend (Vertex Pharmaceutical, Abingdon, U.K.). All tumor cell lines and LAZ 338 were cultured in RPMI 1640 (Sigma-Aldrich) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich).

Cell lines transfected by ICAM-1 cDNA

Tumor cell lines LS180 and Colo205 were transfected by electroporation using the AMAXA system according to the manufacturer's instructions with a full-length human ICAM-1 cDNA (gift from T. Yoshimasa, Kyoto University, Kyoto, Japan) inserted in pEF-BOS neoSE expression vector. The transfected cell lines were cultured in medium containing 0.4 mg/ml G418 (Sigma-Aldrich) for colo205 and 0.6 mg/ml G418 for LS180. These cell lines were later cloned by limited dilution or were sorted using an anti-ICAM-1 mAb coupled with anti-mouse mAb-labeled microbeads, according to the manufacturer's instructions (Miltenyi Biotec), to establish stable clones or cell lines highly expressing ICAM-1.

γδ T lymphocytes

Polyclonal TIL were obtained from a 12-day culture of tumor fragments in RPMI 1640 (Sigma-Aldrich) containing 8% AB human serum (local production), antibiotics, glutamine (as mentioned above), and 150 U/ml rIL-2 (Chiron). Polyclonal TIL were expanded in 96-well multiplates at 1000 TIL/well in the presence of irradiated feeder cells (1×10^4 LAZ cells and 10^5 PBL/well), 1 μg/ml PHA-L (Sigma-Aldrich), and rIL-2. TIL clones were derived by limiting dilution and then were expanded under the same culture conditions (27). The T cell clone C4-112 was derived from $CD8^+$ T cells present in the ascites of patient C4. The other Vδ2 populations were sorted from the TIL from colon and RCC cancer patients (C1 and R9, respectively) or from the PBMC of a healthy donor. $CD8^+$ T cells and Vδ2 T cells were purified using magnetic beads coated with CD8 mAb-conjugated microbeads or with an anti-Vδ2 mAb (IMMU389; Immunotech) and Ig anti-mouse-coated magnetic beads (Miltenyi Biotec).

Flow cytometry and blocking mAb

For T cell or tumor cell labeling, we used the following Abs: anti-CD94 (HP-3B1), anti-NKG2-A (Z199), anti-P70 or KIR3DL1/S1 (Z27), anti-KIR2DL2/S2 (Gl183), anti-KIR2DL1/S1 (EB6), and PE-conjugated or unconjugated anti-Vδ2 TCR (389) from Immunotech; PE-conjugated anti-NKRP1 (191B8) and FITC-conjugated anti-IFN-γ from BD Pharmingen; PE-conjugated anti-ILT2 (HP-F1) from Beckman Coulter; anti-CD16 (Leo Ac1), anti-PEN5 (5H10), anti-KIR3DL2 (DEC66), and anti-KIR2DS4 (FSTR) from E. Vivier (Centre d'Immunologie de Marseille-Luminy, Marseille, France); pan-γδ-TCR (IMMU510) ascites; an anti-NKG2D (149810) from R&D Systems; and anti-ICAM-1 (MCA 1615), ICAM-2 (MCA 1140), ICAM-3 (MCA 1485), and VCAM (MCA 907) from Serotec. Anti-MHC chain-related gene A (MICA) (M673), anti-MICB (M362), anti-UL16-binding protein 1 (ULBP1) (M295), anti-ULBP2 (M311), and anti-ULBP3 (M551) were gifts from D. Cosman (Amgen, Seattle, WA). The anti-mouse FITC- or PE-conjugated rabbit $F(ab')_2$ were obtained from Beckman Coulter, and the isotype controls (IgG or IgG2a) from Immunotech. Cell fluorescence was analyzed by flow cytometry using CellQuest software (BD Biosciences). The fluorescence intensity ratio (RFI) was calculated as follows: mean fluorescence intensity obtained with the test/mean fluorescence intensity obtained with the isotypic control. Intracellular cytokines were measured as previously described (28).

Analysis of T cell responses by measurement of TNF release

T lymphocytes were stimulated in duplicate or triplicate cultures by tumor cell lines at an E:T cell ratio of 3:1. Culture supernatants were tested 6 h later by a biological colorimetric assay using the TNF-sensitive mouse fibrosarcoma WEHI 164 clone 13, as previously described (29).

Analysis of T cell responses by cytotoxicity assay

Target cells were labeled with 100 μCi of $Na^{51}CrO_4$ (Oris Industries) for 1 h at 37°C. After washing, target cells were incubated with effector T cells for 4 h at 37°C. In blocking experiments, the effector or target cells were previously treated with various concentrations of specific or isotype control mAbs for 30 min at 4°C. The radioactivity released by target cells was measured, 4 h later, on a beta plate counter. The percentage of specific ^{51}Cr lysis was calculated using the following equation: percent specific lysis = $100 \times$ [(test release) − (spontaneous release)]/[(maximal release) − (spontaneous release)].

Pamidronate or mevastatin treatments

Colon tumor cell lines were cultured in 24-well, flat-bottom plates. Twenty-four hours later, the medium was changed to a medium containing pamidronate (Calbiochem) at various concentrations (50–150 μM) or containing 25 μM mevastatin (Sigma-Aldrich). After overnight incubation with pamidronate, cells were washed extensively and incubated with T lymphocytes. Mevastatin-treated tumor cells were mixed with T lymphocytes in the presence of 25 μM mevastatin, because the effect of this drug is rapidly reversible (12).

Results

The Vγ9Vδ2 TIL C4-112 clone recognizes and kills autologous and allogeneic tumor cell lines in a TCR-dependent manner

Two CCCL, C4A (adherent) and C4S (suspension), and $CD8^+$ T cells (C4 TIL) were derived from an ascites sample of a colon carcinoma patient. Because the $CD8^+$ TIL population produced TNF in response to autologous CCCL C4A and C4S (Fig. 1*A*), we cloned these TIL to isolate and characterize the tumor-reactive T cells. Several tumor-reactive clones were obtained, among which was the C4-112 $CD8^+$ clone. This clone expressed Vγ9Vδ2 TCR and CD8 (Fig. 1*B* and data not shown). As shown by TNF-α secretion and cytolysis assays, clone C4-112 recognized and killed the autologous and 7 of 11 allogeneic CCCL, but not the normal colon cell line tested (Fig. 1*C*). It also produced IFN-γ in response to the autologous tumor cell line (Fig. 1D). It also recognized four melanoma and three RCC lines: erythroleukemia (K562), ovary, and pancreatic tumor cell lines. By contrast, this clone did not recognize the breast, lung, or neuroblastoma tumor cell lines tested or various nontumor cell targets, such as a normal colon cell line, normal colon fibroblasts, and melanocytes (Fig. 1*C* and data not shown).

Lysis of CCCL by clone C4-112 was significantly inhibited by blocking anti-γδ TCR but not by an anti-HLA-I mAb (Fig. 1, *E* and *F*). Together, these results indicate the presence of Vγ9Vδ2 $CD8^+$ T cells in the ascites of colon carcinoma patient C4, which are able to recognize and kill a large fraction of CCCL in a TCR-dependent and HLA-I-independent fashion.

CCCL recognized by clone C4-112 are lysed by Vγ9Vδ2 T lymphocytes of various origins

To test whether CCCL recognition was a general property of Vγ9Vδ2 T cells, we generated several polyclonal Vγ9Vδ2 T cell lines after immunomagnetic sorting of Vδ2 cells from PBL of a healthy donor (81.65% $V\delta2^+$), RCC-infiltrating T cells (R9; 99.78% $V\delta2^+$), and another colon carcinoma patient (C1; 99.77% $V\delta2^+$). These polyclonal Vγ9Vδ2 T cell lines showed CCCL reactivity patterns very similar to that of clone C4-112, because they killed the same set of CCCL and to a similar extent as clone C4-112 (Fig. 2). Therefore, all Vγ9Vδ2 T cells, regardless of their origin and clonality, efficiently recognize a large fraction of CCCL.

Recognition of phosphoantigens by the C4-112 clone

Vγ9Vδ2 T cells can be activated by various low M_r nonpeptidic Ags (30–35). Among these, metabolites of the mevalonate pathway, such as IPP, have been shown to be tumor cell ligands of the Vγ9Vδ2 TCR (12, 36, 37). To determine whether CCCL recognition by the C4-112 Vγ9Vδ2 clone was due to IPP expression by these cell lines, we used mevastatin, an inhibitor of the mevalonate pathway, to block IPP synthesis by these cell lines. After mevastatin treatment, lysis of all CCCL was decreased by 50–90% (Fig. 3*A*). This indicates that production of mevalonate metabolites is required for CCCL recognition by Vγ9Vδ2 T cells. Because some CCCL were not recognized by the C4-112 clone, we asked whether this was due to a defect in IPP or other isoprenoid pathway metabolite synthesis. To address this, we studied Vγ9Vδ2-mediated killing of Vγ9Vδ2-susceptible and -resistant CCCL after

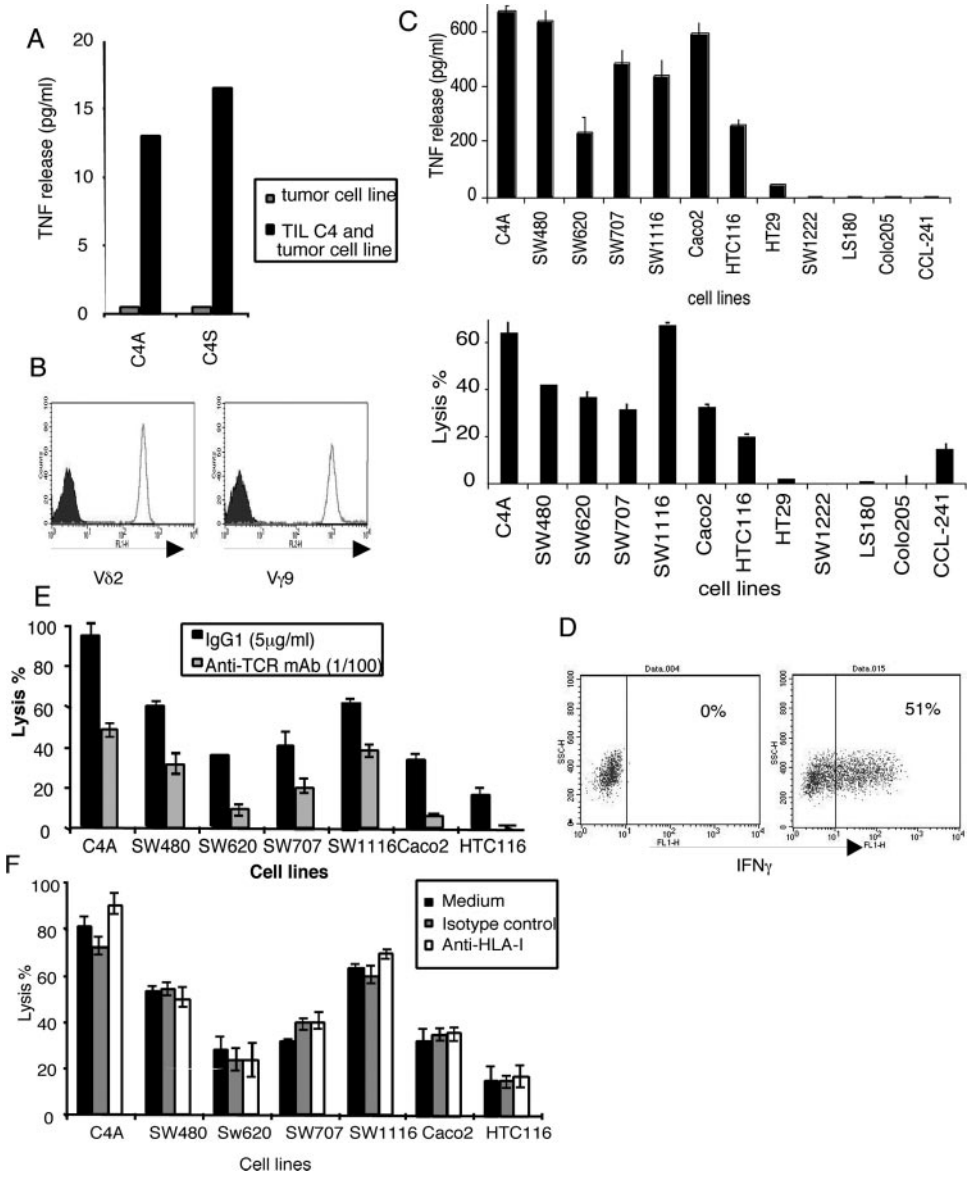

FIGURE 1. The C4-112 clone, derived from tumor cell-reactive TIL of a colon cancer patient ascites, expressed a Vγ9Vδ2 TCR and recognized a high fraction of CCCLs in a TCR-dependent and HLA-I-independent manner. *A*, The $CD8^+$ TIL from colon patient C4 produced TNF upon stimulation by the autologous tumor cell lines C4A and C4S. TIL stimulation was performed at an E:T cell ratio of 1:3. The TNF secreted was measured in the supernatant 6 h later by biological test. *B*, The C4-112 clone expressed a Vγ9Vδ2 TCR. Staining was performed using appropriate mAb, then was analyzed by flow cytometry. The filled histogram is the isotype control. The open histogram is the specific staining. *C*, The C4-112 clone recognized and killed 7 of 11 CCCLs. In the *upper figure*, the C4-112 clone was stimulated by various colorectal cell lines or by a normal colon cell line (CCL-241) at an E:T cell ratio of 1:3, and TNF was measured in the supernatant. The C4-112 clone and the tumor cells lines alone did not secrete TNF-α. In the *lower figure*, the C4-112 clone was stimulated by various[51] Cr-labeled colorectal tumor cell lines at an E:T cell ratio of 10:1.[51] Cr release was measured 4 h later in the supernatant. *D*, The C4-112 clone produced IFN-γ upon stimulation by the autologous tumor cell line C4A. C4-112 stimulation was performed at an E:T cell ratio of 1:3. The IFN-γ accumulated in C4-112 clone was measured 6 h later by intracellular staining. *E*, Colon carcinoma cell lysis is TCR dependent. The C4-112 clone was preincubated with an anti-TCR γδ mAb (1/100 ascites dilution) or IgG1 control (10 μg/ml) before performing the lysis assay on various tumor target cell lines as described in *C*. *F*, Colon carcinoma cell lysis is not HLA-I dependent. The C4-112 clone was preincubated with an anti-HLA-I mAb (1/100 ascites dilution), IgG1 control (10 μg/ml), or medium before performing the lysis assay on various tumor target cell lines as described in *C*.

treatment with pamidronate, an aminobiphosphonate known to precede IPP accumulation in treated cells. As shown in Fig. 3*B*, this treatment increased the lysis of cell lines C4A and SW1116 by 10 and 30%, respectively, and induced that of cell lines HT29 and SW1222 by clone C4-112, suggesting that recognition of most CCCLs correlates with the activity of the mevalonate pathway. The limited increase in lysis might be due to an already suboptimal IPP production in the absence of treatment or to another factor that limits the activation that results from T cell/CCCL interaction. Two cell lines, LS180 and Colo205, were not sensitized to C4-112 clone lysis upon pamidronate treatment, suggesting that either they were unresponsive to this drug or they lacked other components required for Vγ9Vδ2 T cell clone activation.

Lysis of CCCL by clone C4-112 depends, to a variable extent, on ICAM-1 expression

Accessory/adhesion receptors, such as CD2 and LFA-1, play an important role in Vγ9Vδ2 T lymphocyte activation (19, 20). We screened CCCL for LFA-3 (the CD2 counterreceptor); ICAM-1, ICAM-2, and ICAM-3 (LFA-1 counterreceptors); and VCAM

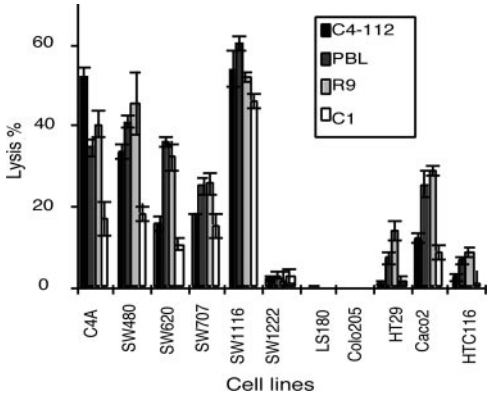

FIGURE 2. Vγ9Vδ2$^+$ T lymphocytes from various origins efficiently killed colon tumor cell lines C4A, SW1116, SW480, SW620, SW707, and Caco2, but not HT29, SW1222, LS180, Colo 205, and HTC116. Vγ9Vδ2 T cell populations were derived from a healthy donor blood (PBL) and from the TIL of colon and renal cell carcinomas (C1 and R9, respectively). These populations were sorted with anti-Vδ2 mAb (C1, PBL, and R9). Lysis by Vγ9Vδ2 T cell populations of the indicated tumor cell lines (colon cell lines C4A, SW480, SW620, SW707, SW1116, SW1222, LS180, Colo205, HT29, Caco2, and HTC116) was measured 4 h later as described in Fig. 1*C*.

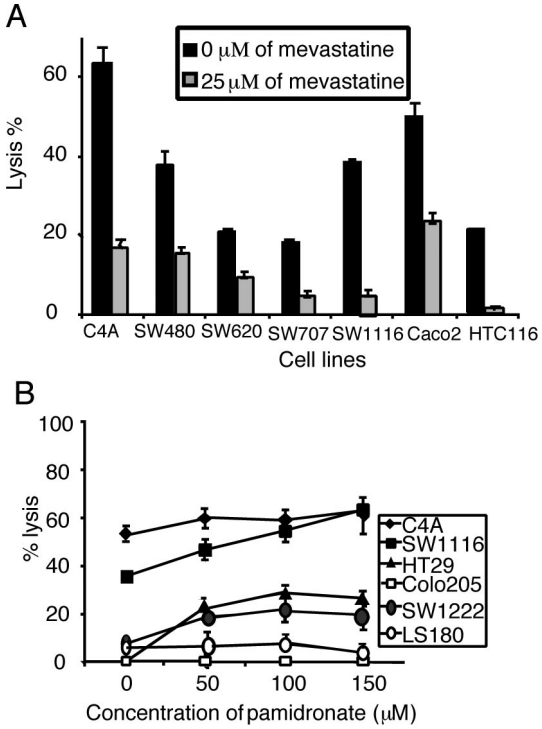

FIGURE 3. Mevastatin treatment of colon tumor cell lines decreased and pamidronate treatment increased their recognition by the C4-112 clone. Colon cell lines were not pretreated or were pretreated overnight with 25 μM mevastatin (*A*) or various concentrations of pamidronate (*B*), then mixed at an E:T cell ratio of 10:1 with the C4-112 clone for lysis measurement as described in Fig. 1.

(VLA-4 ligand) expression. None of these cell lines expressed ICAM-3 (data not shown). Most cell lines expressed LFA-3; although no clear correlation was found between LFA-3 expression levels and Vγ9Vδ2 lysis susceptibility, the most efficiently killed cell lines expressed LFA-3 at the highest levels. Most CCCL, including some of those killed by the C4-112 clone, expressed VCAM and ICAM-2 at low levels, and there was no clear correlation between the expression levels of these molecules and killing intensity. All CCCL recognized by the C4-112 clone, either spontaneously or after pamidronate treatment, expressed ICAM-1, and the correlation coefficient between this expression and Vγ9Vδ2 lysis was >0.75 (Fig. 4*A* and data not shown). The two cell lines that failed to be recognized even after pamidronate treatment (Colo205 and LS180) lacked ICAM-1 and VCAM (Fig. 4*A*). To address the contributions of ICAM-1 and VCAM to the recognition of colon tumor cell lines by the C4-112 clone, CCCLs were preincubated with blocking anti-ICAM-1, anti-VCAM mAb or an isotype control before the cytolysis assay. Anti-ICAM-1 mAb inhibited CCCL lysis to variable extents: >30% for four of seven colon tumor cell lines (C4A, SW1116, Caco2, and HTC116) and <10% for the other three cell lines (SW480, SW620, and SW707). The VCAM mAb, in contrast, had no or a very minor inhibitory effect on CCCL recognition (Fig. 4*B*). To determine whether the absence of lysis of LS180 and Colo205 CCCL by Vγ9Vδ2 T cells could be accounted for by lack of ICAM-1 expression, we transfected these cell lines with human ICAM-1 cDNA and established stable polyclonal and monoclonal transfectants expressing high levels of ICAM-1. ICAM-1 transfection induced both ICAM-1 and VCAM expression on Colo205 and LS180 (data not shown). Only ICAM-1 transfection in Colo205 dramatically increased its susceptibility to lysis by clone C4-112 after pamidronate treatment. In contrast, ICAM-1-transfected LS180 CCCL remained resistant to Vγ9Vδ2 T cell lysis even after pamidronate treatment (Fig. 5*A*). Lysis of pamidronate-treated ICAM-1-expressing colo205 cells was strongly inhibited by the anti-ICAM-1 mAb, but not by the anti-VCAM mAb (Fig. 5*B*). Together, these results demonstrate that ICAM-1 expression by CCCL contributes, although to a variable extent, to the recognition and lysis of these cells by Vγ9Vδ2 T cells.

The NKG2D coreceptor regulates CCCL recognition by the Vγ9Vδ2 C4-112 T cell clone

NKR are expressed by Vγ9Vδ2 T cells and can modulate the functions of these cells (14, 21, 22). We therefore studied the expression of common NKR using available mAbs NKG2D, NKRP1-A, CD94/NKG2A, ILT2, PEN5, KIR2DS4, KIR3DL1, and KIR2DL2/L3 by the C4-112 clone. The C4-112 clone expressed NKG2D, CD94/NKG2A, and KIR3DL1 (Fig. 6). The staining patterns obtained with ILT2- and KIR2DL2/S2-specific mAb were more heterogeneous, because only a small fraction of C4-112 cells were stained by these Ab (Fig. 6*A*). No labeling was observed with mAb directed against NKRP1-A, PEN-5, and KIR2DS4 NKR (data not shown). The NKG2D receptor is known to positively modulate the cytotoxicity of NK and T cell effectors (38). To determine whether this receptor contributed to the lysis of colon tumor cell lines by the C4-112 clone, we screened CCCL for the expression of several NKG2D ligands (MICA, MICB, and ULBP1, -2, and -3). All tumor cell lines expressed various levels of MICA, ULBP2, and 3, and most of them also expressed ULBP1 (Fig. 6*B*). None was MICB$^+$ (data not shown). The expression levels of NKG2D ligands correlated with Vγ9Vδ2 lysis susceptibility of CCCL. Furthermore, CCCL lysis by the C4-112 clone was significantly inhibited by blocking NKG2D-specific mAb (Fig. 6*C*). These data suggest that the TCR-dependent recognition of CCCL by Vγ9Vδ2 T cells is costimulated by NKG2D.

Colon tumors are frequently infiltrated by Vγ9Vδ2 T lymphocytes

Finally, because the C4-112 clone was isolated from ascites of a colon cancer patient, we asked whether solid colon carcinoma tumor samples also contained Vγ9Vδ2 T cells. TIL were isolated from fragments of colon tumor samples (C1–C38), cultured for a

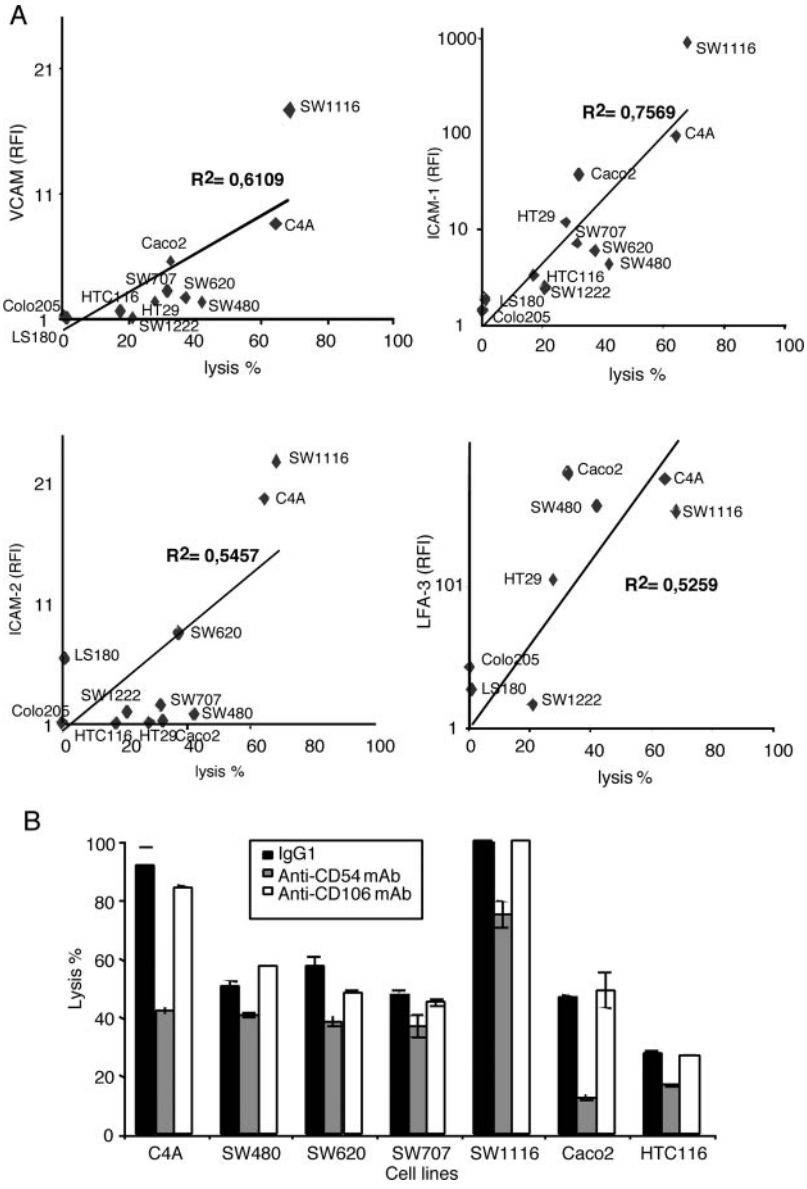

FIGURE 4. ICAM-1 expression seems to be important for colon tumor cell line recognition by the C4-112 clone. *A*, ICAM-1, ICAM-2, VCAM, and LFA-3 expression by colon tumor cell lines. Tumor cell lines were incubated with the relevant mAb and then with a PE-anti-mouse mAb and analyzed by flow cytometry. Simultaneously, these cell lines were labeled by ^{51}Cr and incubated with C4-112 clone, and lysis was measured as described in Fig. 1*C*. The results show simultaneously ICAM-1, ICAM-2, VCAM, and LFA-3 expression as the RFI and the percentage of lysis. *B*, The killing of colon tumor cell lines was inhibited by an anti-ICAM-1 mAb, but not by an anti-VCAM mAb. ^{51}Cr-labeled tumor cell lines were preincubated with a blocking anti-ICAM-1 or an anti-VCAM mAb (2 μg/ml) or with an isotypic control (2 μg/ml), and then cocultured with the C4-112 clone at an E:T cell ratio of 10:1. Lysis was measured as described in Fig. 1*C*.

few days in IL-2-containing medium, then expanded using polyclonal T cell stimulators and screened for the presence of Vδ2$^+$ T lymphocytes (Fig. 7). Vγ9Vδ2 lymphocytes were present in the majority (11 of 15) of colon TIL populations at frequencies ranging from 0.1–3%.

Discussion

In this work we describe a Vγ9Vδ2 TCR clone isolated from a colon cancer ascites, that efficiently kills, in a TCR-dependent and MHC-unrestricted fashion, autologous and allogeneic carcinoma cell lines, including a large fraction of CCCL. Similar tumor recognition patterns were observed with all Vγ9Vδ2 T cell lines tested regardless of their origins. Besides, CCCL killing efficiency by Vγ9Vδ2 T cells depended on both tumor expression levels of Vγ9Vδ2 TCR stimuli (such as mevalonate pathway metabolites), of tumor ligands engaging the NKG2D receptor, and of counter-receptors of adhesion molecules, such as ICAM-. We also showed that more than two-thirds of colon carcinoma tumors were infiltrated by Vγ9Vδ2 T cells. A previous study also reported the presence in these tumors of δ1$^+$ T cells recognizing autologous and allogeneic CCCL (7). This subpopulation was not investigated in our study.

To our knowledge, this is the first report describing the recognition and efficient killing of CCCL by Vγ9Vδ2 T cells. Among solid tumor cell lines, only some RCCs and melanomas had been shown to be targets for this T cell subset, either spontaneously or after pamidronate treatment (12, 17, 18). Because melanocytes were not killed by these cells, the lytic potential of Vγ9Vδ2 T cells appears selective for transformed epithelial cells, because it is for transformed hemopoietic cells, such as leukemia, myeloma, and lymphoma (12–15).

Human Vγ9Vδ2 T cells are activated in a TCR-dependent manner by several phosphorylated (34, 39) or aminated (40) alkyl molecules. Furthermore, the expression of IPP has been shown to correlate with the susceptibility of some tumor cell lines to Vγ9Vδ2 T cell lysis (12, 30–35). In this study data obtained through stimulation or inhibition of IPP biosynthesis by pamidronate and mevastatine, respectively, suggest that CCCL recognition might also correlate with IPP production. Consistent with the assumption that tumor cell recognition is due to increased expression and presentation of mevalonate metabolites is the finding that some tumor cell types show increased expression and function of the limiting enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (41, 42). Recently, new ligands of the Vγ9Vδ2 TCR were identified on

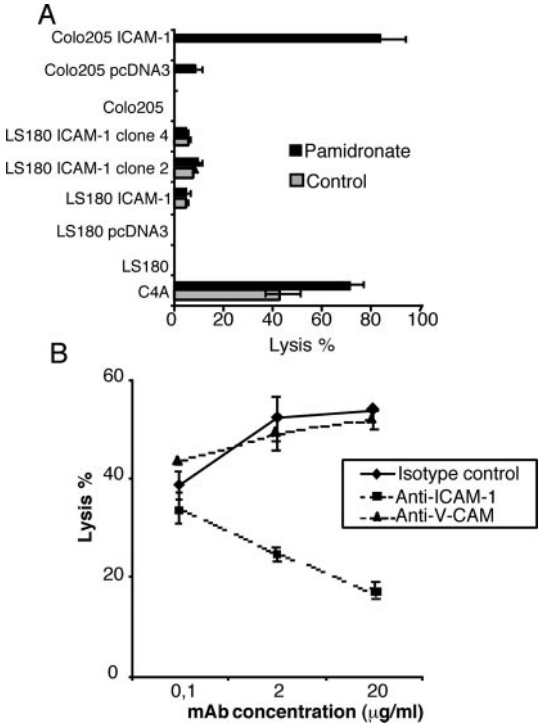

FIGURE 5. Induction of ICAM-1 by transfection and pamidronate treatment sensitized Colo205, but not LS180, colon tumor cell lines to lysis by the C4-112 clone. *A*, The percent lysis of Colo205 and LS180 cell lines or clones transfected with the ICAM-1 cDNA cloned in pcDNA3 or by pcDNA3 alone, after treatment, or not, with 100 μM pamidronate. Lysis of pamidronate-treated or untreated C4A is shown. *B*, Lysis of ICAM-1-transfected and pamidronate-treated Colo205 cell line is inhibited by an anti-ICAM-1 mAb, but not by an anti-VCAM mAb. Tumor cell lysis was measured as described above upon incubation with the mAb at the indicated concentration.

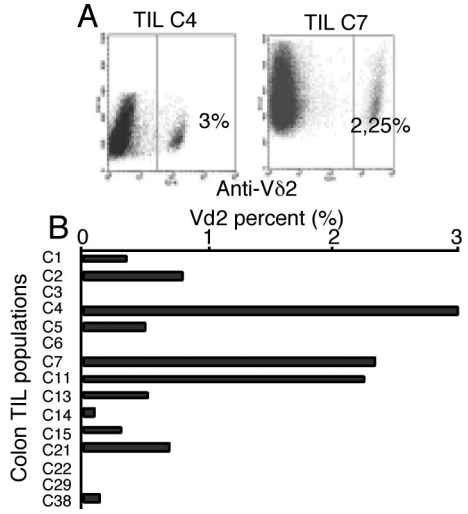

FIGURE 7. Fraction of Vδ2$^+$ lymphocytes among the TIL from colon carcinoma tumor samples (C1–C3 and C5–C38) or from ascites (C4). TIL populations were labeled with a PE-anti-Vδ2 mAb, and the fraction of δ2-positive cells was determined by flow cytometry. *A*, Example of dot-plot labeling of TIL populations C4 and C7. *B*, Percentage of Vδ2$^+$ T lymphocytes among the TIL from colon carcinoma tumor samples (C1–C3 and C5–C38) or from ascites (C4).

the surface of hemopoietic tumors and RCC lines, namely, an F_1-ATPase-related structure and one of its ligands, a delipidated form of apolipoprotein A1 (43). Although this work did not establish the relationships between recognition of these ligands and that of phosphoantigens on tumor cells by Vγ9Vδ2 T lymphocytes, it was hypothesized that a complex of these two molecules may bind phosphoantigens and present them to Vγ9Vδ2 TCR (43). It is possible that such a complex is exposed by colon carcinoma cells, making them susceptible to Vγ9Vδ2 TCR lysis.

It is known that Vγ9Vδ2 T cell activity is tightly regulated by NK-like receptors for MHC class Ia and Ib ligands. Accordingly, we showed that NKG2D contributed to CCCL lysis by Vγ9Vδ2 clones, and that, as previously described (18, 44, 45), CCCL expressed very high levels of several NKG2D ligands, such as MICA and ULBP1, -2, and -3. The Vγ9Vδ2 clone C4-112 also expressed several inhibitory receptors, such as KIR3DS1 and the C-type lectin receptor CD94/NKG2A. Because HLA-Bw4 and HLA-E, the

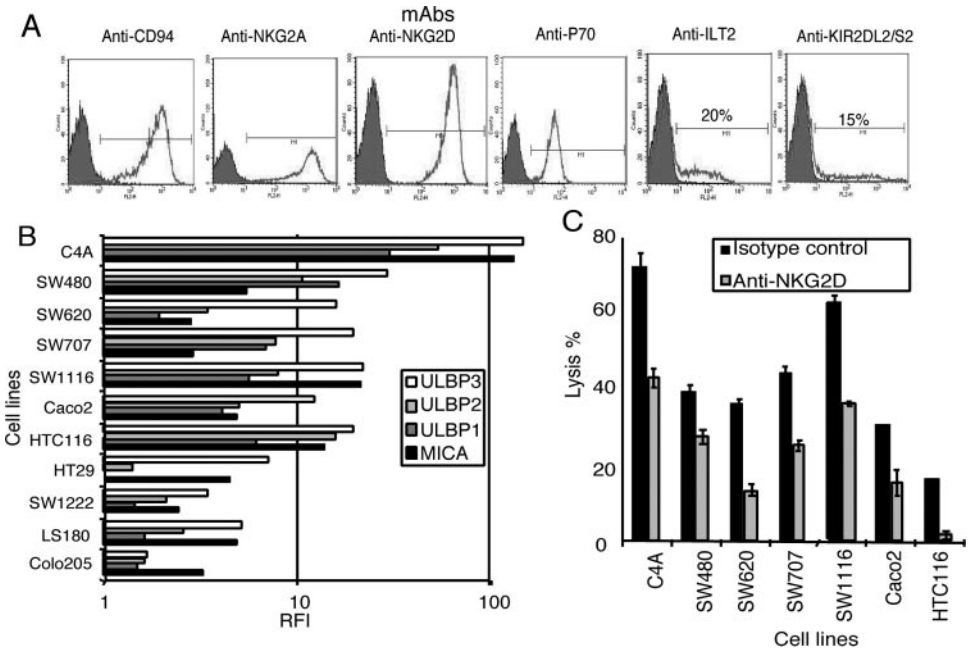

FIGURE 6. Potential role of NKR/NKR ligand interactions in colon carcinoma recognition by the C4-112 clone. *A*, Expression of NKRs by the C4-112 clone. Flow cytometric analysis of various NKR expression by the C4-112 clone. Negative results were obtained using the other NKR-specific mAbs: anti-CD16, anti-PEN5, anti-KIR2DS4, anti-KIR2DL1/S1, and NKRP1-A (data not shown). *B*, Expression of the NKR ligands ULBP1, -2, and -3 and MICA by CCCLs. The results are expressed as the RFI. No MICB expression by these cell lines was observed (data not shown). *C*, The killing of colon tumor cell lines was inhibited by an anti-NKG2D mAb. The C4-112 clone was preincubated with 4 μg/ml blocking anti-NKG2D mAb or with an isotypic control, and then lysis colon carcinoma was measured as described above.

respective ligands of these receptors, were expressed by the autologous tumor cell lines (data not shown), these two receptors might decrease the lysis efficiency of autologous CCCL. The fact that despite this, autologous CCCL were strongly lysed by clone C4-112 suggests that tumor cells expressed sufficiently high levels of activatory signals to overcome these inhibitory signals. It is still possible that variations in HLA-E and Bw4 expression by some other cell lines contribute to the varied susceptibility of CCCL to Vγ9Vδ2 T cell clone lysis. Nonetheless, we did not observe any correlation between the HLA-Bw4/Bw6 type of these cells and their lysis susceptibility.

A critical role of ICAM-1 molecule in the recognition of lung tumor and lymphoma cell lines by Vγ9Vδ2 T cells was previously suggested (19, 20). Our data show that ICAM-1 expression by colon carcinoma cells strongly contributes to the activation of Vγ9Vδ2 T cells, probably by strengthening effector/target cell interactions via LFA-1 engagement. We also addressed the roles of VCAM and ICAM-2, two other LFA-1 counterreceptors, and that of LFA-3, a ligand of the coactivatory TCR CD2, which was previously shown to be important in melanoma cell recognition by $\alpha\beta$ T cells (46). Our data do not suggest a key contribution of VCAM, ICAM-2, and LFA-3 molecules to CCCL recognition by Vγ9Vδ2 T cells, because several CCCL killed by the Vγ9Vδ2 T cells (spontaneously or after ICAM-1 expression) poorly expressed these molecules, and VCAM-specific mAb failed to inhibit CCCL killing by Vγ9Vδ2 T cells.

About one-third of CCCLs were not recognized by Vγ9Vδ2 T cells. For some of them, this could be explained by defective expression of the adherent molecule ICAM-1 (20) and/or by the inability to accumulate and/or present phosphoantigen ligands. However, one cell line (LS180) that spontaneously expressed NKG2D ligands remained resistant to lysis by the C4-112 clone even after ICAM-1, VCAM, and pamidronate treatment. This might be due to tumor cell unresponsiveness to pamidronate. Alternatively, it would suggest the existence of an additional requirement for CCCL recognition by Vγ9Vδ2 T cells, such as a minimal expression of LFA-3 or surface expression of ATP synthase or other Vγ9Vδ2 TCR ligands (43).

The presence of Vγ9Vδ2 T lymphocytes in the majority of colon cancer TIL populations and the response of this T cell subpopulation to many CCCL suggest that a natural immune response mediated by these lymphocytes contributes to the immunosurveillance of these tumors. Such observations may foster the development of novel alternative or adjuvant therapies targeting Vγ9Vδ2 T cells for the treatment of colon cancer patients. This might be achieved by stimulating Vγ9Vδ2 T cell activity in these patients through pamidronate and IL-2 injection, as recently performed in hematological malignancies (47). Alternatively, it could be achieved by passive transfer of ex vivo expanded autologous Vγ9Vδ2 T cells derived from cancer patients. These T cells could be obtained from patient blood, because PBL-derived Vγ9Vδ2 T cells exhibited a similar or even stronger reactivity than TIL to CCCL. Interestingly, because the mevalonate metabolites are required for cell survival and growth, their targeting on colon tumor cells will not have the risk of generating Ag loss tumor variants. In conclusion, our results show that colon carcinoma cancer patients are suitable candidates for active or passive immunotherapy targeting Vγ9Vδ2 T cells.

Disclosures

The authors have no financial conflict of interest.

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