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CRTAM Receptor Engagement by Necl-2 on Tumor Cells Triggers Cell Death of Activated Vγ9Vδ2 T Cells

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Human Vγ9Vδ2 T cells exert potent in vitro and in vivo antitumor activities, making them promising candidates for immunotherapy strategies. Recognition of tumor cells by Vγ9Vδ2 T cells requires engagement of the TCR and/or NK receptors. Recently, one of the novel NK receptors, the class I–restricted T cell–associated molecule (CRTAM), has been described to promote cytotoxic function of NK cells and to lead to IFN-γ secretion by CD8+ T cells through interaction with its ligand, Necl-2. A better understanding of the role of CRTAM in Vγ9Vδ2 T cell functions is highly relevant to optimize innate-like T cell–based cancer immunotherapy. In this article, we report that CRTAM is transiently expressed on activated Vγ9Vδ2 T lymphocytes following TCR engagement. However, CRTAM–Necl-2 interaction does not modify the cytotoxic function or IFN-γ secretion of Vγ9Vδ2 T cells. The expression of CRTAM in activated Vγ9Vδ2 T cells is quickly downregulated following interaction with Necl-2 on tumor cells. Of interest, CRTAM is concurrently acquired at the cell surface of Necl-2+ tumor cells through Vγ9Vδ2 T cell membrane capture. Finally, we highlight that coculture experiments with tumor cells expressing Necl-2 result in significant cell death of CRTAM+ Vγ9Vδ2 T cells. CRTAM-mediated cell death is dependent on an autophagic process, but not on apoptosis or necroptosis, as attested by the expression of characteristic markers and blocking experiments with specific inhibitors. On the basis of these findings, we propose that Necl-2 on tumor cells represents a new tumor counterattack mechanism and a potential target to improve efficiency of γδ T cell–based immunotherapy.
tumor suppressor gene tumor suppressor in lung cancer-1 (TSLC1) (19). The role of CRTAM has been described in the regulation of cell polarity and the retention of lymphocytes in lymph nodes (20, 21). Boles et al. (17) have shown that the engagement of CRTAM promotes the cytokine function of NK cells and leads to IFN-γ secretion by CD8+ T cells, suggesting that the CRTAM–Necl-2 interaction contributes to tumor recognition.

A better understanding of NK receptor–ligand interactions is highly relevant to improve innate-like T cell–based cancer immunotherapy. With this aim, we have characterized the role of CRTAM in Vγ9Vδ2 T cell functions. We show that CRTAM is transiently expressed in activated Vγ9Vδ2 T lymphocytes following TCR engagement. Unlike human NK and CD8+ T cells, the CRTAM–Necl-2 interaction is dispensable for both the cytotoxicity and the cytokine secretion of Vγ9Vδ2 T cells. Of interest, cell contact with Necl2-expressing tumor cells results in rapid downregulation of CRTAM expression and significant cell death of activated Vγ9Vδ2 T cells. Thus, our data reveal an unexpected role for Necl-2 as a promoting factor for tumor evasion of Vγ9Vδ2 T cell immunosurveillance.

Materials and Methods

γδ T cell culture

PBMCs were isolated by density gradient separation (UniSep; Novamed, Jerusalem, Israel) from blood samples of healthy donors (n = 15) recruited at the Blood Transfusion Center (Etablissement Français du Sang, Rennes, France). The PBMCs were resuspended at 2 × 10^6/ml in RPMI 1640 (Eurobio, Les Ulis, France) supplemented with 10% FCS (Life Technologies/Invitrogen Life Technologies, Cergy Pontoise, France), 1% L-glutamine, 50 μg/ml streptomycin, and 50 IU/ml penicillin, referred to elsewhere as complete medium. The cells were stimulated with 3 μM BrHPP (IPHI101; Phosphostim, a kind gift of Innate Pharma, Marseille, France) in the presence of 400 IU/ml IL-2 (Proleukin, Novartis, Suresnes, France) and maintained for 2 wk to generate polyclonal Vγ9Vδ2 T cell lines. Every 3 d, fresh complete medium supplemented with 400 IU/ml IL-2 was added. Resting Vγ9Vδ2 T cell lines were checked for purity by flow cytometry (>85% γδ+ CD3+ cells) and subsequently used in phenotype/function assays. In some experiments, expanded Vγ9Vδ2 T cells were stimulated with an immobilized anti-CD3 mAb (UCHT1, 1 μg/ml), with 20 ng/ml PMA and 0.5 μM ionomycin or with BrHPP (3 nM–3 μM).

Tumor cell lines

The K562 (myelogenous leukemia), Daudi and Raji (Burkitt’s lymphoma), and HT-29 (colon carcinoma) human cell lines were obtained from the American Type Cell Collection. The HCC cell lines (HepG2, HuH7, BC2, and HepaRG) were a gift from C. Guillouzo (INSERM U991, Rennes, France). The THP1 (acute monocytic leukemia), P815 (mouse mastocytoma), and RPMI 8226 (meloma) cell lines were a gift from E. Scotet (UMR S 892 Nantes-Angers, France). All tumor cell lines were maintained in complete medium except for the HCC cell lines, which were cultured in Williams’ Medium E with 10% FCS, 0.5 μM hydrocortisone (Pharmacia, Guyancourt, France), and 0.5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO). In some experiments, pretreatment of tumor cells with an amino-bisphosphonate or a statin was performed by incubating cells overnight with 50 nM zoledronate (Zometa, Novartis, France) or 10 μM mevastatin (Sigma-Aldrich), respectively. The cells were then extensively washed before use. In mAb cross-linking assays, the P815 cells were preincubated with mAbs at room temperature for 30 min before coculture with effector cells.

Gene transfer procedure

cDNAs encoding the full-length human Necl-2 protein were provided by V. Ossipow (Department of Biochemistry, University of Geneva, Switzerland) (22). Human cDNAs were cloned into a γ-retrovirus–derived SFG vector backbone. Dicistronic vectors containing the puromycin-N-acytetransferase open reading frame cloned downstream of an internal ribosome entry site (IRES-puro) were used to express the proteins. Vectors containing the IRES-puro sequence and multiple cloning sites were used as controls.

FIGURE 1. CRTAM is transiently expressed in Vγ9Vδ2 T cells following PBMC activation. (A) Kinetic study of CRTAM and CD69 expression in Vγ9Vδ2 T cells after PBMC stimulation with 3 μM BrHPP and culture with IL-2. Double staining was performed using mAbs against Vδ2, CRTAM (upper panel), or CD69 (lower panel). The proportion and mean fluorescence intensity (MFI) of CRTAM+ or CD69+ cells among Vδ2+ cells are indicated in the upper-right corner of each frame. The data are representative of three independent experiments. (B) Kinetic study of CRTAM expression in naïve (CD27+ CD45RA+), central memory (CD27+ CD45RA+), effector memory (CD27+ CD45RA−), and terminally differentiated effector memory (TEMRA; CD27− CD45RA−) Vγ9Vδ2 T cell subsets after PBMC stimulation, as in (A). Analyses were performed using mAbs against Vδ2, CRTAM, CD27, and CD45RA. The proportions of CRTAM+ cells in each subset of Vδ2+ cells are presented. The results from three independent experiments are shown (mean ± SEM).
Raji, and HT-29 cell lines were infected with cell-free retroviral supernatant in the presence of 8 µg/ml polybrene (Sigma-Aldrich) for 16 h, as previously described (23). Puromycin (Sigma-Aldrich) was added at 5–20 µg/ml to the medium for 1 wk to select cells expressing the vector-encoded puromycin-N-acetyltransferase.

**Flow cytometry analysis**

Fluorochrome-conjugated mAbs against the following Ags were used to stain the Vъγ9Vδ2 T cells: CD3 (UCHT1), pan-γδ TCR (IMMU510), Vδ2 (IMMU389), CD69 (TP1.55.3), CD27 (1A4CD27), CD45RA (ALBP11), and IFN-γ (45.15) from Immunotech (Marseille, France); CD107 (H4A3) from BD Biosciences (Lille, France). An unconjugated mAb against Necl-2 (3E.1) from MBL (Woburn, MA) and fluorochrome-conjugated secondary mAbs from Southern Biotech (Birmingham, AL) were used to stain tumor cells. Isotype-matched Igs from the corresponding manufacturer were used as negative controls. Flow cytometry analyses were performed using a FACSCalibur system (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

**CD107 mobilization assay and intracellular cytokine staining**

Expanded Vъγ9Vδ2 T cells in a resting state were cocultured at 37°C with tumor cells at an E:T ratio of 1:1 in a complete medium containing the fluorochrome-conjugated anti-CD107 mAb (or the corresponding isotype). Monensin (3 µM; Sigma-Aldrich) was added 1 h after beginning the assay. After incubation for 6 or 10 h, cells were labeled with fluorochrome-conjugated anti-γδ TCR and anti-CRTAM mAbs. Then, the cells were fixed in a 4% paraformaldehyde solution and permeabilized with PBS containing 0.5 mM EDTA before being analyzed by flow cytometry.

**Cytotoxicity assay**

Expanded Vъγ9Vδ2 T cells were tested for cytotoxicity against tumor cell lines, using a standard 51Cr release assay. Briefly, target cells labeled with 51Cr sodium chromate (0.2 mCi/10^6 cells; Amersham) were cocultured with expanded Vъγ9Vδ2 T cells in complete medium for 4 h at 37°C. The E:T ratios ranged from 1:1 to 20:1. 51Cr release was assessed in culture supernatants, using a TopCount gamma counter (Packard Instruments). The percentage of specific lysis was calculated using the following formula: [(mean experimental cpm − mean spontaneous cpm)/(mean maximum cpm − mean spontaneous cpm)] × 100. The results shown are the mean of assays performed in triplicates.

**Cell death measurement**

Expanded Vъγ9Vδ2 T cells were cocultured with target cells in complete medium at an E:T ratio of 1:1 for 4 h at 37°C. The cells were then labeled with fluorochrome-conjugated anti-pan-γδ TCR and anti-CRTAM mAbs. After two washes in ice-cold PBS, the cells were labeled with fluorochrome-conjugated Annexin V according to the manufacturer’s instructions and analyzed by flow cytometry. Annexin V staining on gated CRTAM+ or CRTAM−γδ T cells was analyzed. In the blocking assays, the target cells were previously treated with saturating concen-

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**FIGURE 2.** CRTAM is expressed on phosphoantigen-expanded Vъγ9Vδ2 T cells following exposure to various stimuli. Phosphoantigen-expanded Vъγ9Vδ2 T cells in a resting state were exposed to various stimuli. Surface expression of CRTAM was analyzed by flow cytometry using mAbs against pan-γδ TCR and CRTAM. The proportion and MFI of CRTAM+ cells in γδ+ cells are indicated in each quadrant. Expanded Vъγ9Vδ2 T cells were stimulated for 6 h with the following: (A) PMA/ionomycin, (B) immobilized anti-CD3 mAbs, or (C) increasing doses of BrHPP. (D) Expanded Vъγ9Vδ2 T cells were cocultured overnight with tumor cells untreated (Medium) or pretreated with zoledronate (Zol), mevastatin (Meva), or mevastatin plus zoledronate (Zol + Meva) (ratio 1:1). No stim, no stimulation. Kinetic study of CRTAM expression was performed in (B). The data are representative of three independent experiments.
tractions (20 μg/ml) of the recombinant human CRTAM/Fc chimera (R&D Systems) or with a control molecule [recombinant human IgG1 Fc (rhIgFc); R&D Systems] for 30 min at 4˚C. Some assays were performed with expanded Vγ9Vδ2 T cells pretreated for 30 min at 37˚C with the apoptosis inhibitor z-vad (100 μM), the necroptosis inhibitor necrostatin (100 μM), or the autophagic cell death inhibitors wortmannin (200 nM) or bafilomycin A1 (200 nM) before coculture with tumor cells. All inhibitors were purchased from Enzo Life Sciences (Farmingdale, NY).

Autophagic marker measurement

Expanded Vγ9Vδ2 T cells were cocultured with target cells in complete medium at an E:T ratio of 1:1 for 4 h at 37˚C. To detect the autophagosomes, the expression of the human microtubule-associated protein 1 L chain 3B (LC3B) was monitored using a specific fluorochrome-conjugated mAb (clone D11; Cell Signaling Technology, Danvers, MA) (24). The cells were stained with anti-Vδ2 TCR and anti-CRTAM mAbs, fixed with PBS containing 2% formol, and permeabilized in a 90% methanol solu-

FIGURE 3. CRTAM is associated with the effector functions of Vγ9Vδ2 T cells. Phosphoantigen-expanded Vγ9Vδ2 T cells in a resting state were cultured for 10 h with tumor cells (Raji or HT-29 cells) untreated or pretreated with zoledronate (Zol) (ratio 1:1). No stim, no stimulation. IFN-γ accumulation and CD107 mobilization assays were performed as described in Materials and Methods. The proportions of CRTAM+ IFN-γ+ or CRTAM+ CD107+ cells within the γδ+ cells are indicated in each dot plot. The data are representative of three independent experiments.

FIGURE 4. CRTAM is downregulated in Vγ9Vδ2 T cells and specifically acquired by Necl-2+ tumor cells. Phosphoantigen-expanded Vγ9Vδ2 T cells were harvested 24 h after CD3 stimulation and then cocultured with Raji or Daudi cells expressing Necl-2 or not (ratio 1:1). Ctrl, control. Kinetic expression of CRTAM (A) in Vγ9Vδ2 T cells and (B) in tumor cells was determined by flow cytometry, using mAbs against pan-γδ TCR and CRTAM. Before staining, the cells were washed once with ice-cold PBS containing 0.5 mM EDTA. The percentages of CRTAM+ cells (left panels) and the MFI of CRTAM (right panels) are shown. (C) Membrane cell transfer assay, as described in Materials and Methods. Vγ9Vδ2 T cells were labeled with the membrane dye PKH67 before being cocultured with Daudi Ctrl (upper panel) or Daudi Necl-2 (lower panel) for 1 h. The proportions of CRTAM+ PKH67+ cells among the γδ+ cells (left panel) and tumor cells (right panel) are presented in each dot plot. An overlay of PKH67 staining in CRTAM+ and CRTAM− Daudi Necl-2 cells is shown. (D) CD3-stimulated Vγ9Vδ2 T cells were incubated with Daudi Necl-2, Daudi Ctrl, or PMA/ionomycin (PMA/Iono). After 1 h, the cells and supernatants were collected. Cells were labeled for the expression of TCR-γδ and CRTAM (upper panels). Daudi Necl-2 cells were incubated with supernatants from different conditions for 1 h and labeled with mAbs against CRTAM (lower panels). For each condition, the percentages and MFI of CRTAM+ cells among the lymphocytes or tumor cells are indicated in the right corner of each dot plot.
tion. Finally, the cells were labeled with anti-IC3B mAb and analyzed by flow cytometry. The amount of lysosomes and autolysosomes in Vγ9Vδ2 T cells was evaluated by acridine orange (Sigma-Aldrich) staining (25). Briefly, cells were labeled with acridine orange (2 μg/ml) and conjugated anti-Vδ2 TCR mAb for 15 min before being analyzed by flow cytometry.

Statistical analyses
Statistical analyses were performed using GraphPad Prism 5.0 (La Jolla, CA). Comparison tests were carried out using unpaired and paired t tests. Statistically significant differences are indicated by *p < 0.05, **p < 0.001, and ***p < 0.0001.

Results
CRTAM is a phenotypic marker of Vγ9Vδ2 T cell activation
Cytokine production and CD107a mobilization. As expected, Vγ9Vδ2 T cells cocultured with Raji or Daudi or RPMI 8226 led to CRTAM expression, whereas coculture with poorly immunogenic tumor cell lines (Raji, K562, THP1, HT-29, HuH7, and HepG2) did not (Fig. 2D). Of note, sensitization of poorly immunogenic tumor cell lines with zoledronate reversed their inability to spontaneously induce CRTAM expression. As shown in Fig. 2E, treatment of sensitized HepG2 tumor cells with mevastatin, an inhibitor of the mevalonate pathway, decreased CRTAM expression in Vγ9Vδ2 T cells. Together, these data indicate that CRTAM expression is associated with TCR engagement.

Cytotoxicity assay performed with a standard 51Cr release assay. (A) Expanded Vγ9Vδ2 T cells were harvested 24 h after CD3 stimulation and then cocultured with target cells previously labeled with 51Cr at various E:T ratios for 4 h. The target cells were tumors cells expressing Necl-2 or not (Ctrl). (B) P815 cells were incubated with anti-CRTAM (10 μg/ml) and/or anti-pan-γδ TCR (1 μg/ml) or IgG1 isotype mAbs (1 μg/ml) for 30 min before labeling with 51Cr. P815 cells were then cocultured with expanded Vγ9Vδ2 T cells prestimulated with immobilized anti-CD3 mAbs, as described in (A). (C) Expanded Vγ9Vδ2 T cells in a resting state were cocultured for 4 h with target cells previously labeled with 51Cr (E:T ratio 1:1). Target cells were Raji or HT-29 cells expressing Necl-2 or not (Ctrl) that were untreated or pretreated with zoledronate (Zol). (D) Phosphoantigen-expanded Vγ9Vδ2 T cells in a resting state were cultured for 6 h with zoledronate-pretreated tumor cells (Raji or Daudi) expressing Necl-2 or not (Ctrl). The IFN-γ accumulation assay was performed as described in Materials and Methods. The percentages of CRTAM+ IFN-γ+ cells are indicated in each dot plot along with the MFI values. In (A)–(C), the results are from five, three, and two independent experiments (mean ± SEM), respectively. In (D), the data are representative of three independent experiments.
unsensitized tumor cell lines neither expressed CRTAM nor produced IFN-γ or CD107a.

**CRTAM is downregulated in Vγ9Vδ2 T cells and specifically acquired by Necl-2+ tumor cells**

The Daudi and Raji cell lines, which are target cells commonly used to study Vγ9Vδ2 T cell functions, do not express Necl-2 (Supplemental Fig. 1). To further evaluate the role of CRTAM, we genetically modified these two tumor cell lines to induce the constitutive expression of full-length Necl-2 (Supplemental Fig. 1). Coculture of expanded Vγ9Vδ2 T cells expressing CRTAM upon CD3 stimulation with Necl-2–expressing tumor cells specifically resulted in the rapid and dramatic downregulation of CRTAM expression in terms of both cellular frequency and level expression (Fig. 4A). In parallel, we observed the concomitant acquisition of CRTAM expression on the cell surface of Necl-2+ cells, but not control tumor cells (Fig. 4B). To investigate the mechanisms by which CRTAM is recovered by Necl-2+ tumor cells, we measured cell membrane transfer by staining Vγ9Vδ2 T cells with a fluorescent dye. Following cell contact with CRTAM+ or CRTAM− control tumor cells, we measured cell membrane transfer by staining Vγ9Vδ2 T cells with a fluorescent dye. Conditioning supernatants were then

![A](image)

**FIGURE 6.** The CRTAM–Necl-2 cell–cell interaction induces Vγ9Vδ2 T cell death. (A and B) Phosphoantigen-expanded Vγ9Vδ2 T cells in a resting state were cocultured for 4 h with zoledronate-pretreated tumor cells (Raji or Daudi) expressing Necl-2 or not (Ctrl) (ratio 1:1). The measurement of Vγ9Vδ2 T cell death was performed as described in Materials and Methods. Increases in Annexin V MFI in CRTAM+ or CRTAM−γδ+ T cells are shown (ΔMFI = Annexin V MFI with Necl-2+ tumor cells − Annexin V MFI with Ctrl tumor cells). The results are from at least three independent experiments (mean ± SEM). (C) Similar experiments were performed in the presence of blocking recombinant human CRTAM/Fc chimera (hCRTAM) or control recombinant human IgG1/Fc chimera (rhlgFc), as described in Materials and Methods. HuH7, HepG2, and RPMI 8226 tumor cells line naturally expressing Necl-2 were used. The percentage of Annexin V staining inhibition in CRTAM+ γδ+ T cells was calculated according to the formula: [(Annexin V MFI with hCRTAM+ γδ+ T cells) − (Annexin V MFI with Ctrl γδ+ T cells)]/2. In (C) and (D), the results are from three independent experiments (mean ± SEM). *p < 0.05, **p < 0.001, ***p < 0.0001.

Because CRTAM has been reported to be involved in the effector functions of activated NK and CTL in vitro, we analyzed the effects of the CRTAM–Necl-2 interaction on the functional properties of Vγ9Vδ2 T cells. Vγ9Vδ2 T cell lines that expressed high levels of CRTAM upon CD3 stimulation displayed similar cytotoxic activity against Necl-2−expressing target cells or control target cells (Fig. 5A). Next, we evaluated the response of the same effector cells to an anti-CRTAM mAb in a redirected killing assay against an FcγR+ P815 murine mastocytoma target. In the presence of anti-CRTAM mAb, neither the enhancement of CRTAM+ Vγ9Vδ2 T cell cytotoxicity nor a synergic effect with the anti-γδ TCR mAb was observed (Fig. 5B). Additional experiments were performed with resting Vγ9Vδ2 T cell lines not expressing CRTAM. We observed that Vγ9Vδ2 T cells cocultured with sensitized target cells that induced CRTAM during the cytotoxic assay elicited the same lytic response against Necl-2−expressing target cells or control target cells (Fig. 5C). Similarly, these effector cells produced equivalent levels of IFN-γ after incubation with Necl-2−expressing tumor cells or control tumor cells (Fig. 5D). Compa-
rable data were obtained in a CD107 mobilization assay (data not shown). Together, these results indicate that CRTAM is not an activating receptor for Vγ9Vδ2 T cells.

The CRTAM–Necl-2 interaction induces cell death of Vγ9Vδ2 T cells

The effects of the CRTAM–Necl-2 interaction on Vγ9Vδ2 T cell survival were examined using a flow cytometry–based Annexin V assay. Resting Vγ9Vδ2 T cell lines were incubated with zolendronate-sensitized Raji or Daudi cells for 4 h. The level of Annexin V staining increased in CRTAM+ Vγ9Vδ2 T cells following cell contact with Necl-2–expressing tumor cells compared with that following contact with control tumor cells (Fig. 6A, 6B). Of note, no staining in Vγ9Vδ2 T cells was observed in the same assays with the marker of cell permeabilization 7-aminoactinomycin D (data not shown). Higher Annexin V levels in CRTAM+ Vγ9Vδ2 T cells than in CRTAM− Vγ9Vδ2 T cells were also noted after coculture with tumor cell lines that naturally expressed Necl-2 (HuH7, HepG2, and RPMI 8226) (data not shown). Annexin V levels in CRTAM+ Vγ9Vδ2 T cells were significantly reduced by soluble recombinant human CRTAM (Fig. 6C). In addition, we showed that ligation of CRTAM with a specific Ab coated on P815 cells markedly increased cell death of activated CRTAM+ Vγ9Vδ2 T cells (Fig. 6D). This effect was enhanced after coengagement of CRTAM and the γδ TCRs.

Autophagy is involved in CRTAM-mediated cell death of Vγ9Vδ2 T cells

The mechanisms involved in CRTAM-mediated cell death were investigated using specific antagonists. Cell death of CRTAM+ Vγ9Vδ2 T cells following contact with Necl-2+ targets was significantly inhibited by wortmannin and bafilomycin A1, two inhibitors of autophagic cell death (Fig. 7A). In addition, expression of LC3B, a reliable marker of autophagolysomes, was examined. CRTAM+ Vγ9Vδ2 T cells displayed a higher expression of LC3B following cell contact with Daudi Necl-2 than with Daudi control, whereas such a difference was not observed for CRTAM− Vγ9Vδ2 T cells (Fig. 7B, 7C). Autophagy is a degradation process characterized by accumulation of lysosomal vacuoles. Thus, we used the vital dye acridine orange to quantify acidic vesicular organelles in the cytoplasm. In Fig. 7D, we showed an increased acridine orange staining in Vγ9Vδ2 T cells following cell contact with Daudi Necl-2, compared with Daudi control. Neither z-vad, an inhibitor of apoptosis, nor necrostatin, an inhibitor of necroptosis, had an impact on Vγ9Vδ2 T cell death.

Discussion

The expression of CRTAM has been predominantly described in activated NK, NK T, and CD8 αβ T cells (16). In this study, we document for the first time, to our knowledge, the presence of CRTAM in activated Vγ9Vδ2 T cells. Notably, we show that the expression of CRTAM on Vγ9Vδ2 T cells is induced by two specific TCR-dependent stimuli: synthetic phosphoantigens and direct contact with immunogenic tumor cells. The expression of CRTAM appears later than that of the activation marker CD69, with transient expression at similar levels in naive and memory Vγ9Vδ2 T cell subsets. In contrast, we recently reported the constitutive expression of two structurally related receptors, CD226/DNAM-1 and CD96, in Vγ9Vδ2 T cells (5). Because CRTAM expression was induced after activation, we expected to observe this receptor in cytotoxic and/or IFN-γ–producing Vγ9Vδ2 T cells. The particular pattern of CRTAM expression raised the question of its functional role in Vγ9Vδ2 T cells. In investigating the impact of the CRTAM–Necl-2 interaction on the effector properties of expanded Vγ9Vδ2 T cells, we did not notice any change in cytotoxic activity or IFN-γ production of CRTAM+ effector cells after challenge with Necl-2+ target cells, compared with control target cells. Of note, cytotoxic activity was evaluated with either Vγ9Vδ2 T cells expressing a high level of CRTAM upon anti-CD3 mAb stimulation or restimulating effector cells in which CRTAM expression was induced by contact with immunogenic tumor cells during the cytotoxic assay. In addition, cross-linking of CRTAM with a specific mAb did not affect the cytotoxic activity of effector cells. Our results lead us to conclude that the CRTAM in Vγ9Vδ2 T cells is not an activating receptor. Several authors have reported that CRTAM is not necessary for the cytotoxic function of activated CD8 αβ T cells in vitro mouse or human models (17, 18, 21, 26). Of note, Boles et al. (17) have observed that the CRTAM–Necl-2 interaction promotes IFN-γ production in human activated CD8 T lymphocytes, but not NK cells. Finally, CRTAM has been shown to contribute to the cytotoxicity of human, but not mouse, NK cells (17, 18). Collectively, these data indicate that the functional consequences of CRTAM engagement depend on the type of effector cells and/or the model species.
Of interest, we showed that Vγ9Vδ2 T cells quickly downregulate the expression of CRTAM after contact with Necl-2+, but not Necl-2−, tumor cells, suggesting that this process is mediated by ligand binding. CRTAM downregulation most likely does not require TCR engagement because similar levels of downregulation were obtained with Necl-2−-expressing Daudi and Raji cells that were recognized or not recognized by Vγ9Vδ2 T cells, respectively. In parallel with CRTAM downregulation, the concomitant acquisition of CRTAM by Necl-2+ tumor cells was observed, suggesting an intercellular transfer of cell-surface CRTAM from Vγ9Vδ2 T cells to tumor cells. The transfer of surface proteins from target to immune effector cell is a widespread physiological process (27). For example, Espinosa et al. (28) have reported that Vγ9Vδ2 T cells can acquire plasma membrane molecules from tumor cells. However, little is known regarding the intercellular transfer of surface molecules in the opposite way, from tumor cells to immune cells. Acquisition of NK cell inhibitory receptors or NKG2D by target cells has been documented (29, 30). Our data indicate that the process involved in intercellular CRTAM protein exchange displays three characteristics: 1) it is a rapid phenomenon, as CRTAM molecules were detected on tumor cells within 5 min after contact with Vγ9Vδ2 T cells; 2) it is not based on cleavage of CRTAM at the cell surface of Vγ9Vδ2 T cells or secretion of soluble CRTAM molecules because we do not observe the de novo presence of CRTAM in tumor cells after addition of supernatant from activated Vγ9Vδ2 T cells cocultured with or without tumor cells; and 3) it depends substantially on a tropocytosis-like mechanism, as we showed in cell membrane transfer experiments that Necl-2+ tumor cells specifically recovered components of CRTAM+ lymphocyte membranes. Other mechanisms could also be involved. A specific intercellular protein transfer through a membrane nanotube has been described (31). Alternatively, CRTAM molecules could be plucked from the Vγ9Vδ2 T cell surface and specifically acquired by tumor cells after binding with Necl-2 (27). Thus, further studies are required to determine the exact mechanism for CRTAM transfer to tumor cells.

Importantly, our work also reveals that CRTAM is involved in activated Vγ9Vδ2 T cell survival. Indeed, the interaction of CRTAM with tumor cells expressing Necl-2 either naturally or after transduction results in cell death of activated Vγ9Vδ2 T lymphocytes. This selective effect is inhibited by soluble human recombiant CRTAM, revealing a specific CRTAM–Necl-2 interaction. The vital dye 7-aminoactinomycin D was used to measure permeabilization of Vγ9Vδ2 T cell plasma membranes. Vγ9Vδ2 T cells were negative for 7AAD staining after coculture with Necl-2+ tumor cells, indicating that CRTAM–Necl-2 interaction does not induce necrosis of Vγ9Vδ2 T cells. To date, three main mechanisms of programmed cell death have been described: apoptosis, necroptosis (also called programmed necrosis), and cell death by autophagy (32). A role of apoptosis or necroptosis in CRTAM-mediated cell death was not observed in our study. Conversely, the contribution of an autophagic cell death mechanism was evidenced. Indeed, LC3B expression is increased in CRTAM+ Vγ9Vδ2 T cells following interaction with Necl-2−-expressing tumor cells. Autophagy is characterized by an active degradation of intracellular components through the lysosomal pathway (33). In agreement with these data, our results with acridine orange show a higher accumulation of acidic vesicular organelles in Vγ9Vδ2 T cells after coculture with Necl-2+ tumor cells (25). Autophagy is described as a cytoprotective process that plays essential roles in T cell development, activation, and proliferation (33). However, autophagy may also constitute a lethal mechanism that mediates autophagic cell death under certain conditions (32). Li et al. (34) have shown that growth factor withdrawal induces cell death through an autophagic pathway in CDD4+ T cells. Inhibitors of autophagy, such as Wortmannin or bafilomycin A1, allow discrimination between autophagic cell death and the cytoprotective autophagy (32, 35). Indeed, in the presence of autophagic cell death, a treatment by these inhibitors decreases cell death. In contrast, in the case of stress-induced cell death with cytoprotective autophagy, inhibitors of autophagy increase the cell death. In our experiments, Wortmannin and bafilomycin A1 reduce CRTAM-mediated cell death, demonstrating the triggering of autophagic cell death following CRTAM engagement. Additional studies are now needed to understand which signals contribute to autophagic cell death of activated Vγ9Vδ2 T lymphocytes. In this respect, the molecular events that control signaling through the CRTAM receptor are poorly understood.

The intracytoplasmic tail of CRTAM has three phosphorylation sites and a binding site that allow recruitment of PDZ-domain proteins, such as Scrib (36). Recent data from Yeh et al. (20) have shown that the regulation of cell polarity by CRTAM is controlled by Scrib. Because Necl-2 is widely distributed in various tissue types, the CRTAM–Necl-2 interaction could represent a critical pathway for Vγ9Vδ2 T cell homeostasis and could serve to limit or terminate an ongoing response by inducing cell death of activated lymphocytes (37). Inactivation of the TSLC1 gene, which leads to the suppression of Necl-2 expression, has been frequently described in various human cancers, including lung, cervical, and breast cancers (38). Conversely, Necl-2 was found to be expressed de novo in adult T leukemia or overexpressed in certain other tumors (39–43). Thus, similar to tumor-associated molecules, such as FasL or PD-L1, Necl-2 in tumor cells may represent a new tumor counterattack mechanism to evade immunosurveillance by Vγ9Vδ2 T cells (44).

In conclusion, our study reveals that CRTAM, which is transiently expressed in activated Vγ9Vδ2 T cells, appears dispensable for the effector functions of these cells. In addition, our work highlights that the CRTAM–Necl-2 interaction is critical for the control of Vγ9Vδ2 T cell survival. Ultimately, Necl-2 expression in tumor cells could be a factor that limits γδ T cell–mediated immunosurveillance against cancer. Therapeutic interventions aimed at reducing Necl-2–induced cell death may be of major interest in improving Vγ9Vδ2 T cell–based immunotherapy.

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Disclosures
The authors have no financial conflicts of interest.

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**Supplemental legend S1.** Flow cytometry analysis of Necl-2 expression on Raji and Daudi cells transduced with retroviral vectors encoding the full-length Necl-2 molecule or containing multiple cloning sites as control (Ctrl) (upper panels) and on RPMI 8826, HuH7 and HepG2 cell lines.
Dessarthe et al. Supplemental Figure S1