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

Benoît Dessarthe, Aurélie Thedrez, Jean-Baptiste Latouche, Florian Cabillic, Aurélie Drouet, Pascale Daniel, Cécile Thomas de La Pintière, Véronique Catros and Olivier Toutirais

*J Immunol* 2013; 190:4868-4876; Prepublished online 25 March 2013;
doi: 10.4049/jimmunol.1202596
http://www.jimmunol.org/content/190/9/4868

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/03/25/jimmunol.1202596.DC1

References
This article cites 44 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/190/9/4868.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
CRTAM Receptor Engagement by Necl-2 on Tumor Cells Triggers Cell Death of Activated Vγ9Vδ2 T Cells

Benoît Dessarthe, Aurélie Thedrez, Jean-Baptiste Latouche, Florian Cabillie, Aurélie Drouet, Pascale Daniel, Cécile Thomas de La Pintière, Véronique Catros and Olivier Toutirais

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.

Received for publication September 14, 2012. Accepted for publication February 27, 2013.

This work was supported by grants from the Ligue Contre le Cancer, the Région Bretagne, and the Institut National du Cancer PL 2008-034. O.T. was supported by a grant from Innate Pharma.

Address correspondence and reprint requests to Dr. Véronique Catros or Dr. Olivier Toutirais, INSERM UMR991, Hôpital Pontchaillou, F-35033 Rennes, France (V.C.), or Laboratoire d’Immunologie, Centre Hospitalier Régional de Clemenceau, F-14033 Caen, France (O.T.). E-mail addresses: veronique.catros@univ- rennes1.fr (V.C.) or toutirais-o@chu-caen.fr (O.T.)

The online version of this article contains supplemental material.

Abbreviations used in this article: BrHPP, bromohydrin pyrophosphate; CRTAM, class I–restricted T cell–associated molecule; DNAM-1, DNAJ assembly molecule-1; HCC, hepatocellular carcinoma; IPP, isopentenyl pyrophosphate; Ncl, nectin-like; rhIFc, recombinant human IgG1 Fc.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00
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 cytotoxic 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 (Établissement Français du Sang, Rennes, France). The PBMCs were resuspended at 2 \times 10^7/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). The PBMCs were resuspended at 2 \times 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/functional assays. In some experiments, expanded Vγ9Vδ2 T cell lines 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 mastocyte), and RPMI 8226 (myeloma) 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-bisphosphate 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.

Vector construction

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-acetyltransferase 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.

Gene transfer procedure

H29/293 GPG packaging cells were transfected with the SFG vector encoding Necl-2 by the calcium chloride precipitation method. The Daudi,
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 Vγ9Vδ2 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 (Franklin Lakes, NJ); and CRTAM (210213) from R&D Systems (Lille, France). An unconjugated mAb against Necl-2 (3. E.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 Vγ9Vδ2 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 4% paraformaldehyde solution and permeabilized with PBS containing 0.1% saponin (Sigma-Aldrich) and 0.5% BSA (Sigma-Aldrich). Fixed cells were stained with the membrane dye PKH67 (Sigma-Aldrich) according to the manufacturer’s recommendations and cocultured with tumor cells in complete medium at an E:T ratio of 1:1 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.

**Cytotoxicity assay**

Expanded Vγ9Vδ2 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/10⁶ cells; Amersham) were cocultured with expanded Vγ9Vδ2 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 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. 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-
trations (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-CD3b mAb and analyzed by flow cytometry. The amount of lysosomes and autolysosomes in V9V82 T cells was evaluated by acridine orange (Sigma-Aldrich) staining (25). Briefly, cells were labeled with acridine orange (2 μg/ml) and conjugated anti-V62 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**

CRTAM expression was assessed by flow cytometry on resting or BrHPP-stimulated V9V82 T cells. In freshly isolated PBMCs, CRTAM was undetectable in Vδ2+ T cells (Fig. 1A). In contrast, stimulation with a single dose of BrHPP induced a strong up-regulation of CRTAM in the majority of V-9V82 T cells. Kinetic analysis showed that CRTAM expression was upregulated 6 h after BrHPP stimulation, with peak expression at 24 h (77% of CRTAM+ cells). Notably, CRTAM expression was transitional and decreased starting 48 h after stimulation. By comparison, the kinetic expression of the activation marker CD69 occurred earlier, with upregulation starting 2 h after the addition of phosphoantigen and sustained over time. Similar profiles of CRTAM expression were observed on the four distinct phenotypic subsets of V9V82 T cells: naive (CD45RA+ CD27+), central memory (CD45RA- CD27+), effector memory (CD45RA- CD27-), and terminally differentiated cells (CD45RA+ CD27-) (Fig. 1B). In addition, CRTAM expression was analyzed in polyclonal V9V82 T cell lines obtained by incubating PBMCs with BrHPP and IL-2 for 2 wk. No significant CRTAM expression was detected in expanded γδ T cells in a resting state (Fig. 2A). However, activation of expanded V9V82 T cells with PMA/ionomycin or immobilized anti-CD3 Abs strongly triggered CRTAM expression (Fig. 2B). Similarly, restimulation of V9V82 T cells with BrHPP resulted in CRTAM upregulation in a dose-dependent manner ≥300 nM BrHPP (Fig. 2C). Overnight coincubation of expanded V9V82 T cells with the immunogenic tumor cell lines 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 V9V82 T cells. Together, these data indicate that CRTAM expression is associated with TCR engagement.

**CRTAM expression in phosphoantigen-expanded Vγ9Vδ2 T cells is associated with effector functions**

We have investigated the functional properties of activated CRTAM+ V9V82 T cells. As previously observed, CRTAM expression is upregulated in V9V82 T cells stimulated with zoledronate-sensitized Raji or HT-29 cells (Fig. 3). Of interest, we observed that CRTAM expression in expanded V9V82 T cells was strongly associated with IFN-γ production and CD107α mobilization. As expected, V9V82 T cells coincubated with...
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) (17). 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 cells with a fluorescent dye. Following cell contact with CRTAM+ or CRTAM−preincubated with anti-CRTAM (10^4 MFI with rhIgFc-pretreated target cells, we measured membrane acquisition of CRTAM expression on the cell surface of Necl-2+ tumor cells was detected by immunofluorescence, suggesting that the presence of cell-surface CRTAM on tumor cells was not due to the binding of a soluble form of CRTAM (Fig. 4D).

**The CRTAM–Necl-2 interaction does not modify Vγ9Vδ2 T cell effector functions**

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).

**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 zolendronate-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. HuH7, HepG2, and RPMI 8226 tumor cell lines 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 Ctrl tumor cells − Annexin V MFI with Ctrl tumor cells]/Annexin V MFI with Ctrl tumor cells) 

\[\Delta \text{MFI} = \text{Annexin V MFI with Necl-2+ tumor cells} - \text{Annexin V MFI with Ctrl tumor cells}\]

\[\% \text{Annexin V staining inhibition in CRTAM+ γδ+ T cells} = \frac{\Delta \text{MFI}}{\text{Annexin V MFI with Ctrl tumor cells}} \times 100\]
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 zoledronate-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, NKT, 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 resting 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 Necl2+ 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 cells 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 the trogocytosis-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 recombinant CRTAM, revealing a specific CRTAM–Necl-2 interaction that 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.

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.

Acknowledgments
We thank C. Guillouzo (INSERM U991, Rennes, France) and E. Scotet (INSERM U892, Nantes, France) for providing the tumor cell lines; H. Sicard (Innate Pharma, Marseille, France) for providing the BrHPP; J.-J. Fournié and L. Baricault (UMR1037, Centre de Recherches en Cancérologie de Toulouse, Toulouse, France) for useful discussions; and the Centre de Ressources Biologiques of Centre Hospitalo-Universitaire Rennes for technical advice.

Disclosures
The authors have no financial conflicts of interest.

References


Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017
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.