International Immunology, Vol. 17, No. 9, pp. 1227–1237
doi:10.1093/intimm/dxh299

Heterotypic interaction of CRTAM with Necl2 induces cell adhesion on activated NK cells and CD8$^+$ T cells

Noriko Arase[1,2,3,4], Arata Takeuchi[1,2], Midori Unno[1], Satoshi Hirano[2], Tadashi Yokosuka[1,2], Hisashi Arase[2,3,4] and **Takashi Saito[1,2]**

[1]Laboratory for Cell Signaling, RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa 230-0045, Japan
[2]Department of Molecular Genetics, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan
[3]Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan
[4]PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

Keywords: cell adhesion, expression cloning, Ig superfamily, subtraction

Abstract

NK cells and CD8$^+$ T cells exhibit cytotoxicity and cytokine production upon recognizing target cells through cell–cell interaction. We screened the molecules involved in the recognition and regulation of these cells using cDNA subtraction between naive and activated NK cells. We identified class I-restricted T cell-associated molecule (CRTAM), a two Ig domain-bearing surface receptor, as a molecule rapidly and transiently expressed on NK cells and CD8$^+$ T cells upon activation. CRTAM is expressed as a dimer on the cell surface, and its expression is transcriptionally regulated. Using an expression-cloning system, we then further identified Nectin-like (Necl) molecule 2, a three Ig domain-containing receptor, as a ligand of CRTAM. While Necl2 mediates homotypic interaction, CRTAM interacts with Necl2 but not with CRTAM itself. The heterotypic CRTAM–Necl2 interaction has a higher affinity than the homotypic Necl2 interaction. Although there was no clear alteration in the cytotoxic function of the NK cells and CD8$^+$ T cells against the Necl2-expressing target cells, T cells expressing CRTAM tightly bound to Necl2-expressing cells. CRTAM$^+$ cells did not induce homotypic aggregation but they did exert strong heterotypic binding with Necl2$^+$ cells, which was inhibited by the addition of the CRTAM-Ig fusion protein. These results suggest that the heterotypic interaction between CRTAM and Necl2 plays an important role in the adhesion, interaction or migration of NK cells and CD8$^+$ T cells upon stimulation.

Introduction

NK cells contribute to immunity by mediating cytotoxicity and cytokine production upon recognizing tumor cells or virus-infected cells. Most inhibitory receptors recognize MHC class I molecules whereas activating receptors recognize various antigens including virus antigen (1–7). Many stimulatory receptors such as Ly49, KIR, NKp44, NKp46 and NKR-P1 associate with signaling adaptor molecules including DAP12, CD3ζ, or FcRγ, which contain immunoreceptor tyrosine-based activation motifs (ITAMs) (5). On the other hand, the inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within the cytoplasmic region and recruit SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP) or SH2-domain-containing protein tyrosine phosphatase (SHP) to these ITIMs for negative regulation (8). The balance of such stimulatory and inhibitory signaling determines whether NK cells are activated by target cells to mediate either cell lysis or cytokine production.

In addition to the NK activation receptors associated with ITAM-bearing adaptors, various other molecules are also involved in the regulation of NK cell activation. These surface molecules include 2B4 (CD244) and NTB-A that are associated with signalling lymphocyte activation molecule (SLAM)-associated protein (9), CD160-recognizing MHC class I, leukocyte function-associated antigen (LFA)-1 (10) and DNAM-1 (11, 12). Most of these surface receptors interact with the counter-receptor/ligand on the target cells. Stimulation through such adhesion molecules alone has been shown to activate NK cells. For example, an integrin LFA-1 mediates the adhesion with ICAM-1 and activates NK cells (13). Therefore, adhesion receptors also play a critical role in regulating the development and activation of NK cells. In addition, unlike T cell co-stimulation, there is no evidence for a dominant stimulatory receptor in NK cells, rather activation may instead be achieved by the summation or synergy of multiple different receptors.

Correspondence to: T. Saito; E-mail: saito@rcai.riken.jp

Received 21 May 2005, *accepted* 20 June 2005

Transmitting editor: M. Miyasaka

Advance Access publication 9 August 2005

In an attempt to identify new molecules regulating the activation of NK cells, we identified class I-restricted T cell-associated molecule (CRTAM) as one such molecule that is expressed on activated NK cells and $CD8^+$ T cells. Furthermore, we cloned Nectin-like (Necl) molecule 2 as a ligand for CRTAM. As a result, we found that the heterotypic interaction between CRTAM and Necl2 induces strong cell–cell adhesion, thus suggesting that CRTAM–Necl2 interaction may play a role in tissue localization and/or the recruitment of activated NK cells and CTLs.

Methods

Animals

C57BL/6 and Balb/c mice were purchased from Japan SLC (Shizuoka, Japan). RT-1 TCR-transgenic (Tg) mice were characterized elsewhere (14) and maintained in the animal facility of Chiba University. RT-1 is a CTL clone specific for the HIV gp160 V3 loop peptide P18IIIB, and restricted to H-$2D^d$ (15). Wistar rats were purchased from the Oriental Yeast Co. (Tokyo, Japan).

cDNA subtraction

Mouse NK cells were purified as described previously (16). In brief, sIg^-, $CD4^-$ and $CD8^-$ splenocytes from C57BL/6 mice were stained with PE-conjugated DX5 mAb (eBioscience, San Diego, CA, USA), and anti-$DX5^+$ cells were obtained using the MACS purification system (Miltenyi Biotech, Germany). $DX5^+$ NK cells were cultured in the presence of 2000 U ml^{-1} IL-2 (kindly provided by J. Hamuro, Ajinomoto Ltd, Japan) for 7 days. mRNA was extracted from purified NK cells using the mRNA Purification Kit (Amersham Bioscience, UK). cDNA was amplified with the SMART PCR cDNA Synthesis Kit (Clontech, Moutain View, CA, USA), and PCR-based cDNA subtraction was performed with the PCR-Select cDNA Subtraction Kit (Clontech).

Cell preparation

For PCR analysis freshly isolated NK cells were roughly purified as described above, stained with PE-conjugated–anti-NKR-P1C mAb (eBioscience) and then sorted by FACS Vantage (BD Bioscience, San Jose, CA, USA). NK cells were cultured as described above and used as LAK cells. sIg^- and $CD4^-$ splenocytes from C57BL/6 mice were stained with PE-conjugated–anti-CD8 (eBioscience) and sorted as $CD8^+$ T cells. For CTL assay splenic $CD8^+$ T cells were stained with MicroBeads-conjugated anti-CD8α mAb and then purified using a MACS system (Miltenyi Biotec).

Reverse transcription–PCR analysis

Total RNA was isolated from purified fleshly isolated NK cells, LAK cells and $CD8^+$ T cells, and semi-quantitative reverse transcription (RT)–PCR was performed using the Super Script One-Step RT–PCR Systems (Invitrogen, Carlsbad, CA, USA). The primers used for amplification were as follows: β-actin, sense primer (5′-TCTACAATGAGCTGCGTGTG-3′) and anti-sense primer (5′-GGTACGACCAGAGGCATACA-3′); CRTAM sense primer (5′-CGGGAGTTCTGTGAAGACGA-3′) and anti-sense primer (5′-GAGGGCTTGGGAGGAGAG-3′).

For real-time quantitative PCR, PCR was conducted using a SYBER green PCR kit (Qiagen) and analyzed by an iCycler (Bio-Rad Laboratories). The primers used for amplification were as follows: β-actin, sense primer (5′-TGGAATCCTGTGGCATCCATGAAAC-3′) and anti-sense primer (5′-TAAAACGCAGCTCAGTAACAGTCCG-3′); CRTAM, sense primer (5′-GTTTGCTGTTCTGGGTGCCGGTG-3′) and anti-sense primer (5′-TTTCTCCCGTGCAAGCCCTCGTG-3′). The transcription level of CRTAM was normalized to the amount of β-actin.

Establishment of anti-CRTAM mAb

Anti-mouse CRTAM mAb was established as follows (17). Ten-week-old Wistar rats were immunized with CRTAM-transfected NRK cells followed by immunization with CRTAM-Ig with Titer Max Gold (CytRx, Los Angeles, CA, USA). After the immunizations, lymph node cells were fused with the SP2/0 cell line, and clones (11–5 and 11E1) that stained CRTAM transfectants specifically were selected.

Retrovirus cDNA library

A retrovirus cDNA library was constructed as reported previously (16). In brief, cDNA was generated from mRNA purified from the B16 cell line using the Superscript plasmid system (Invitrogen) and it was cloned into *Sal*I and *Not*I sites of the pMxs retrovirus vector (18). Ligated cDNA was transformed into ElectroMAX competent cells (Invitrogen), and plasmids were purified using a MAXI prep plasmid purification kit (Qiagen).

Expression cloning of Necl2

A total of 2×10^7 2B4 cells were infected with a retrovirus cDNA library from B16 at an efficiency of 30%. Two days after infection, the cells were stained with CRTAM-Ig and PE-conjugated goat anti-human IgG, and the stained cells were purified using the FACS Vantage. After expansion of the purified cells, the cells were stained with CRTAM-Ig plus PE-conjugated anti-human IgG and FITC-conjugated anti-Fc receptor mAb (2.4G2; BD Pharmingen, San Diego, CA, USA), and cells stained with CRTAM-Ig, but not with 2.4G2 mAb, were obtained. Single-cell clones stained by CRTAM-Ig were isolated using a Clonocyte (BD Bioscience). Library-derived genes were amplified by PCR using sense primer (5′-GGTGGACCATCCTCTAGACT-3′) and anti-sense primer (5′-TTTATTTTATCGTCGATCGACC-3′). Amplified cDNA was cloned into the pMxs retrovirus vector, and the nucleic acid sequences were determined and thereafter the vector was used for generating transfectants.

Preparation of Ig fusion proteins and a FACS analysis

DNA fragments corresponding to the extracellular domains lacking the signal sequence of mouse CRTAM or Necl2 were inserted into either the *Xho*I site of a modified pME18S expression vector containing a mouse CD150 leader segment at the 5′ terminus, and the *Xho*I cloning site and the Fc segment of human IgG1 at the 3′ terminus. Various Ig fusion proteins including mutants were prepared using the following primers: CRTAM-Ig, sense primer (5′-AAT CTC GAG TTT CTG AAA ATG GAG ACC GTC ACG-3′) and anti-sense primer (5′-AAT CTC GAG ACC ACT CTT CCT CCG GGC-3′); CRTAM first Ig, sense primer (5′-AAT CTC GAG TTT CTG AAA ATG GAG ACC GTC ACG-3′) and anti-sense primer (5′-AAT CTC GAG TTT CAG CAC AAC CGA TTT CTC-3′); CRTAM second Ig,

sense primer (5′-AAT CTC GAG TTG CAC TAC GGG AGT TCT GTG-3′) and anti-sense primer (5′-AAT CTC GAG ACC ACT CTT CCT CCG GGC-3′); Necl2-Ig, sense primer (5′-TTT TTT TTT TGT CGA CCA GAA TCT GTT TAC TAA AGA CG-3′) and anti-sense primer (5′-TTT TTT TTT TGT CGA CGT GGT CCA CTG CCC C-3′); Necl2 first Ig, sense primer (5′-TTT TTT TTT TGT CGA CCA GAA TCT GTT TAC TAA AGA CG-3′) and anti-sense primer (5′-AAT CTC GAG GTT GAC TTC AAT CTC CTC CC-3′); Necl2 second + third Ig, sense primer (5′-TTT TTT TTT TGT CGA CCA GCT CTA CAC GGA CCC CC-3′) and anti-sense primer (5′-TTT TTT TTT TGT CGA CGT GGT CCA CTG CCC C-3′). Ig fusion constructs were transfected to Cos 7 cells and the fusion proteins in the culture supernatant were purified. The cells were incubated with saturating concentrations of various Ig fusion proteins or mAbs for 30 min on ice, followed by incubation with F(ab′)2 fragments of PE-conjugated goat anti-human IgG Fc or anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min. For staining with PE-labeled secondary antibody Ig fusion protein complex, 10 μg ml^{-1} Ig fusion proteins were mixed with 4 μg ml^{-1} PE-conjugated goat anti-human IgG Fc (Jackson ImmunoResearch) for 30 min on ice, followed by the addition of 30 μg ml^{-1} of normal human IgG (Cappel, Irvine, CA, USA) for 30 min. PE–anti-mCD25 mAb and PE–anti-mCD62L were from eBioscience. The stained cells were analyzed using a FACS Calibur™ (BD Bioscience).

Biochemical analysis of CRTAM

Transfectants and a cell line were surface biotinylated with EZ-link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Inc., Rockford, IL, USA) and lysed with 1% digitonin (Sigma–Aldrich, St Louis, MO, USA) lysis buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonylfluoride and 10 mM iodoacetamide (Sigma) at a concentration of 10^7 cells ml^{-1}. The lysate was precipitated with protein A-sepharose beads coated with Ig fusion protein. Next, the precipitated protein was loaded to 5–20% polyacrylamide gel (Atto Co., Japan) and subjected to SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA). The biotinylated proteins were detected using streptavidin-peroxidase (VECSTAIN Elite ABC kit; Vector Laboratories, Burlingame, CA, USA), Super Signal West Fetmo (Pierce) and LAS1000 (Fuji film, Japan).

Chemical cross-linking was performed as previously described (19, 20). Briefly, 2B4-CRTAM (1×10^6 cells ml^{-1}) was incubated in PBS with 1 mM Bis(sulfosuccinimidyl) suberate (BS^3) cross-linker (Pierce) at 14°C for 15 min. After the incubation, the reaction was stopped by the addition of 10 mM Tris–HCl (pH 7.5). Thereafter, the cells were washed, suspended in SDS-PAGE sample buffer and the lysates were subjected to SDS-PAGE.

Cytotoxic assay for NK cells

NK cells were purified and cultured as described above. The cytotoxicity of various number of NK cells against 1×10^3 NIH3T3 transfectants were analyzed by ^{51}Cr-release assays using standard techniques. A total of 1×10^5 NK cells were co-cultured with 1×10^4 YAC-1 transfectants in 96-well plates. After 24 or 48 h, culture supernatants were collected, and amounts of IFN-γ were measured by using mIFN-γ ELISA kit (BD Pharmingen).

Cytotoxic assay for CTLs

Purified $CD8^+$ T cells from RT-1 Tg mice were cultured with the peptide and irradiated whole spleen cells (35 Gy) from BALB/c mice for 2 or 3 days, and peptide-specific CTL was induced. The antigenic peptide P18IIIB (315–329: RIQRGPGRAFVTIGK) derived from HIV-IIIB was used at 1 μM (synthesized by T. Akizawa, Setsunan University, Osaka, Japan). For the CTL assay, various numbers of effector cells were cultured for 5 h in triplicate in 96-well round-bottom plates with 5×10^3 NIH3T3 target cells pre-incubated with or without P18IIIB peptides. Cytotoxicity was analyzed by standard ^{51}Cr-release assays and calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

Analysis of cell aggregation

A total of 1×10^5 2B4 transfectants labeled with 2 μg ml^{-1} CFSE dye (Molecular Probes, Eugene, OR, USA), according to manufacturer's instructions, were mixed and cultured with an equal number of 2B4 transfectants labeled with 0.5 μM CMTR dye (Molecular Probes) in 96-well plastic plates for 3 h. After incubation, fluorescence-labeled cells were visualized with an epi-fluorescence microscope, IX8 (Olympus, Japan). In the blocking experiment with CRTAM-Ig, 1×10^5 non-labeled 2B4 transfectants were cultured in a 96-well plastic plate for 3 h in the presence or absence of purified CRTAM-Ig or Necl2-Ig (1.25 μg ml^{-1}). The adhesion assays were performed as follows: 1.5×10^5 NIH3T3 transfectants were plated on 24-well plates the day before the assay. A total of 2×10^5 2B4 transfectants or $CD8^+$ T cells stimulated with immobilized anti-TCRβ mAb for 24 h were suspended and plated onto NIH3T3 transfectants. The plates were briefly centrifuged and cultured for 30 min at 37°C. Next, any floating cells were collected after gentle mixing, and counted.

Results

Cloning of CRTAM as a molecule expressed on activated NK cells

In order to find molecules that are involved in the recognition and effector function of NK cells, we tried to identify molecules that are up-regulated upon activation of NK cells by using PCR-based cDNA subtraction between naive and activated NK cells. Consequently, we identified several cDNAs that are up-regulated in activated mouse NK cells. CRTAM, which has been reported to be expressed on activated $CD8^+$ T cells and NKT cells (21), is one such gene. CRTAM belongs to the Ig superfamily and possesses two Ig-like domains. Indeed, the transcript of CRTAM was not observed in naive NK cells and $CD8^+$ T cells but it was strongly induced in both cells as well as LAK cells when NK cells and $CD8^+$ T cells were stimulated by cross-linking with anti-NKR-P1C and anti-TCR, respectively (Fig. 1). On the other hand, cytokine stimulation of these cells with IL-2 or IL-12 did not induce the transcript of CRTAM (data

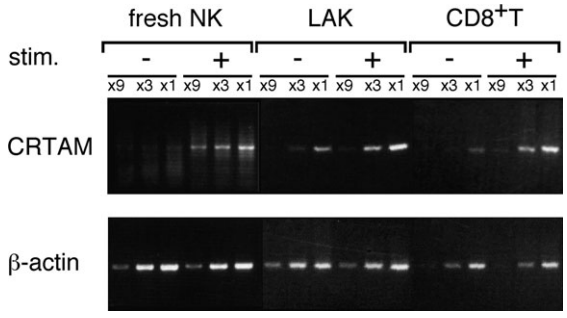

Fig. 1. Induction of the transcript of CRTAM in freshly isolated NK cells (fresh NK), IL-2-activated NK cells (LAK) and $CD8^+$ T cells. NK cells, IL-2-activated NK cells and $CD8^+$ T cells were stimulated with immobilized anti-NKR-P1 or anti-TCR mAb, for 8 h. The expression of CRTAM and β-actin was analyzed by semi-quantitative RT–PCR.

not shown). These results confirm that CRTAM is expressed in only a limited number of cells such as NK cells and $CD8^+$ T cells upon activation.

Characterization of CRTAM expression on NK cells and $CD8^+$ T cells

We analyzed the detailed expression kinetics of CRTAM expression. We thus established a monoclonal anti-CRTAM antibody and the expression of CRTAM on the cell surface was analyzed with mAb. The expression of CRTAM was induced upon stimulation and it reached a maximum between 6–12 h and 12–24 h after stimulation of NK cells (Fig. 2A) and $CD8^+$ T cells (Fig. 2B), respectively. The later kinetics on $CD8^+$ T cells are shown in Fig. 2(C) in comparison with other activation markers. CRTAM was rapidly induced and it reached a maximum level on day 1, and then almost disappeared on day 2. In contrast, early activation marker CD25 was induced

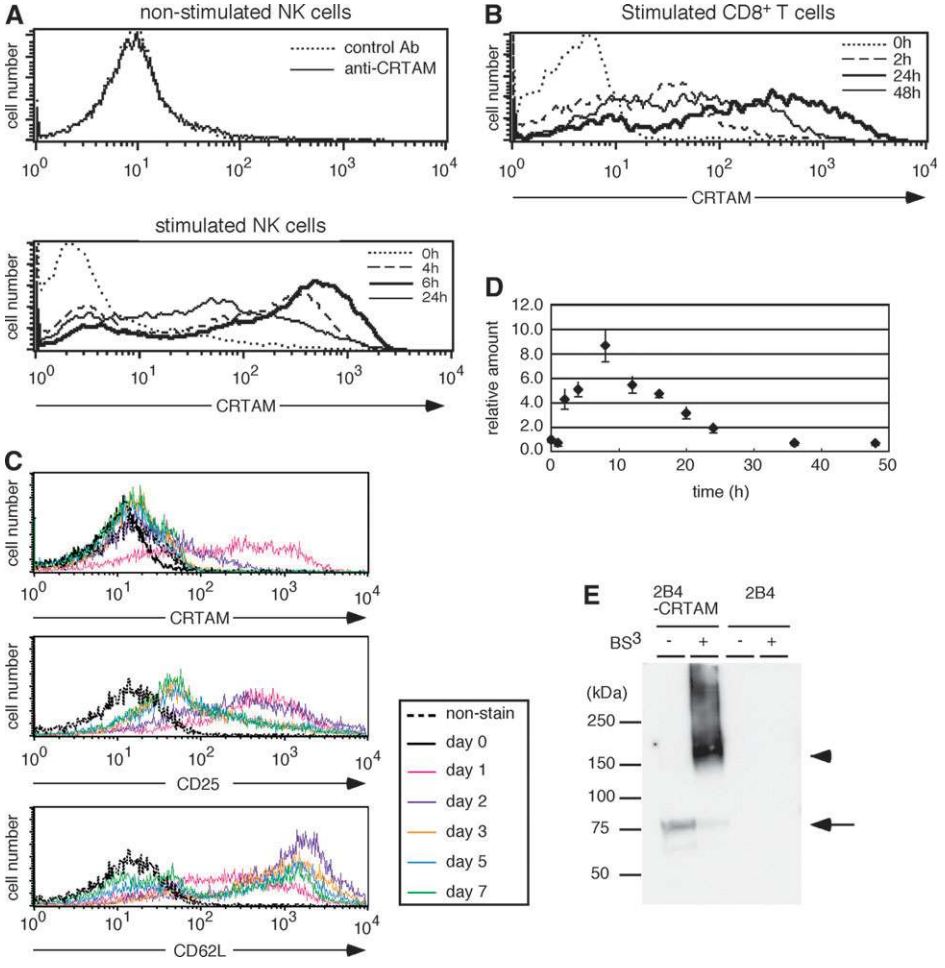

Fig. 2. Expression of CRTAM on activated NK cells and $CD8^+$ T cells upon stimulation. (A) Kinetics of the cell-surface expression of CRTAM on NK cells. Mouse NK cells were stimulated with immobilized anti-NKR-P1C mAb for indicated periods and were stained with anti-CRTAM mAb, 11-5. (B) Kinetics of the cell-surface CRTAM expression on $CD8^+$ T cells. Mouse $CD8^+$ T cells were stimulated with immobilized anti-TCRβ mAb, H57-597 for indicated periods and were stained with anti-CRTAM mAb. (C) Comparison of the expression kinetics between CRTAM, CD25 and CD62L on $CD8^+$ T cells. $CD8^+$ T cells were stimulated with immobilized anti-TCRβ mAb and the expression of each marker was analyzed by staining. (D) Kinetics of expression of CRTAM mRNA in $CD8^+$ T cells upon stimulation. $CD8^+$ T cells were stimulated similar to C and mRNA was analyzed by real-time RT–PCR for quantitation. (E) Dimer formation of CRTAM on the cell surface of T cells. CRTAM-transfected 2B4 cells (2B4-CRTAM) or parental 2B4 were treated (+) or untreated (−) with cross-linker BS^3, and blotted with anti-CRTAM antibody. Arrow and arrowhead at right margin indicate monomeric and dimeric form of CRTAM, respectively. Molecular weights are indicated at the left margin.

to a maximum level on days 1 and 2, and thereafter gradually decreased. CD62L was induced later, and its maximum expression was induced on days 2 and 3 (Fig. 2C). Therefore, CRTAM was found to be an early and transient activation molecule. This kinetics of the surface expression of CRTAM closely paralleled to the mRNA expression, thus demonstrating that CRTAM expression is predominantly regulated at the transcriptional level (Fig. 2D).

We next examined the biochemical nature of CRTAM on the cell surface. The simple cell lysate of CRTAM-transfected 2B4 T cell hybridoma (2B4-CRTAM) revealed only a monomeric form of CRTAM with ~80 kDa. However, when 2B4-CRTAM was treated by a cross-linker, BS^3, we observed a major band of ~160 kDa corresponding to a dimer of CRTAM, thus suggesting that CRTAM is expressed as a dimer with a weak interaction. No band was detected from parental 2B4 even with the cross-linker treatment (Fig. 2E). These results show that CRTAM is immediately and transiently expressed on both NK cells and $CD8^+$ T cells upon stimulation as a dimerized form on the cell surface.

Molecular cloning of Necl2 as a CRTAM ligand

We next intended to identify the ligand of CRTAM. For this purpose, CRTAM-Ig fusion protein (CRTAM-Ig) was generated to stain the CRTAM ligand on the cell surface. When various mouse tumor cell lines were stained with the CRTAM-Ig, B16 melanoma cells, both Hepa 1–6 hepatoma cells and the IC-21 macrophage cell line were positively stained with CRTAM-Ig (Fig. 3A). However, most of other tumor cell lines, including J774.1 or P388D1 macrophage cell lines, were not recognized by CRTAM-Ig (data not shown). These results indicate that some cell lines such as B16 melanoma express a putative CRTAM ligand.

We then utilized an expression-cloning approach to identify the CRTAM ligand. Because B16 melanoma was strongly stained for CRTAM-Ig, a retrovirus cDNA library was constructed from B16 cells, and used for expression cloning. A retrovirus cDNA library derived from B16 cells was transfected into 2B4 cells which is negative for CRTAM-Ig staining, and the cells positively stained with CRTAM-Ig were isolated by FACS. Thereafter, single-cell clones were obtained and the cDNA in

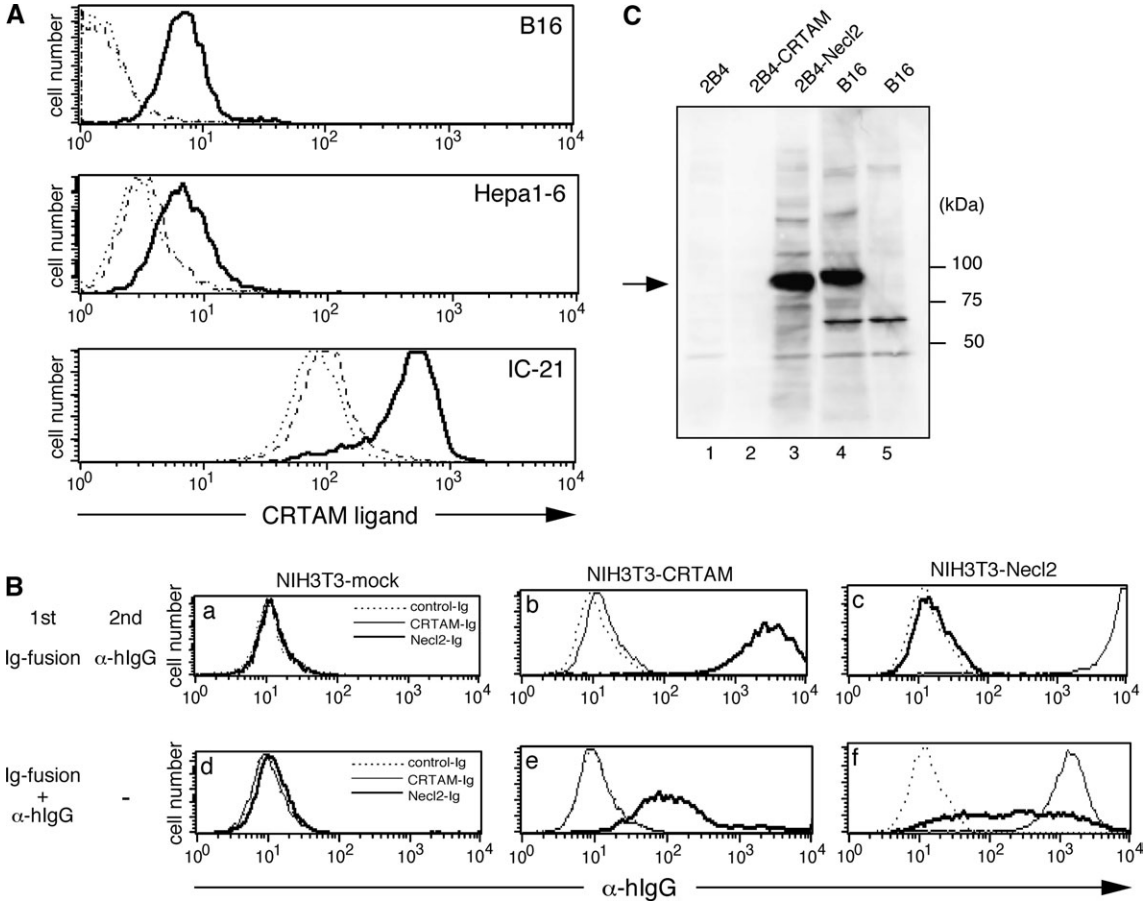

Fig. 3. Cloning and expression of Necl2 as a CRTAM ligand. (A) Staining of tumor cell lines with CRTAM-Ig. Several mouse tumor cell lines were stained with CRTAM-Ig (thick lines), control-Ig (dashed lines) or no Ig (dotted lines), followed by anti-human Ig antibody. (B) Expression of Necl2 on the cell surface and comparison between CRTAM–Necl2 versus Necl2–Necl2 interactions using CRTAM-Ig and Necl2-Ig. NIH3T3-mock (a and d), NIH3T3-CRTAM (b and e) and NIH3T3-Necl2 (c and f) were stained with CRTAM-Ig (thin lines), Necl2-Ig (thick lines) or control-Ig (dotted lines). Cells were stained with Ig fusion proteins followed by PE–anti-human-IgG Fc (a–c), or cells were stained with Ig fusion proteins pre-mixed with PE–anti-human IgG Fc (d–f). (C) Biochemical features of the cell-surface Necl2. 2B4 transfectants expressing CRTAM or Necl2 as well as B16 melanoma cells were surface biotinylated and the cell lysates were precipitated with CRTAM-Ig (lanes 1–4) or control-Ig fusion protein (lane 5) and analyzed on SDS-PAGE. Biotinylated proteins were visualized using chemiluminescence. Arrow indicates monomeric Necl2 (~90 kDa).

each clone was identified by PCR amplification using primers corresponding to the flanking retrovirus sequences. Subsequent sequencing clearly identified Necl2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM as a CRTAM ligand. Necl2 also belongs to the Ig superfamily and possesses three Ig-like domains in the extracellular region. Necl2 is broadly distributed in mouse tissues and is known to be a tumor suppressor (TSLC1) (22). Necl2 has been reported to mediate homotypic interaction and induce cell aggregation when expressed on fibroblasts (23).

Interaction between CRTAM and Necl2

When the cloned Necl2 was transfected into NIH3T3 cells, Necl2 cells, but not mock-transfected NIH3T3 cells, were clearly stained with CRTAM-Ig (Fig. 3B, a and c). To compare the binding affinity of the CRTAM–Necl2 heterotypic interaction with the Necl2 homotypic interaction, we also generated the Necl2-Ig fusion protein and stained CRTAM- and Necl2-transfected NIH3T3 cells. As predicted, CRTAM-transfectants were clearly stained with Necl2-Ig (Fig. 3B, b). In spite of the previous report that Necl2 mediates homotypic interaction (23), Necl2-transfectants were not stained with Necl2-Ig (Fig. 3B, c). However, when Necl2-Ig was pre-mixed with anti-human IgG Fc antibody to generate aggregated Ig fusion protein and then used for staining, Necl2-transfectants were significantly stained by the aggregated Necl2-Ig/anti-Ig (Fig. 3B, f). In contrast, CRTAM-transfectants were stained by neither CRTAM-Ig nor aggregated CRTAM-Ig/anti-Ig (Fig. 3B, b and e). Considering that the aggregated form of Ig fusion proteins may exhibit higher affinity to its ligand than simple dimeric Ig fusion proteins, these results suggest that the Necl2–CRTAM interaction has a higher affinity than the Necl2–Necl2 homotypic interaction, and that unlike Necl2, CRTAM may not exert a homotypic interaction.

We analyzed Necl2 on the cell surface of the transfectants by surface biotinylation, and pull-downed with CRTAM-Ig. Necl2-transfected 2B4 cells (2B4-Necl2) and B16 melanoma cells were lysed with 1% digitonin and pull-down assay was performed using CRTAM-Ig. As shown in Fig. 3(C), Necl2 was precipitated as a 90-kDa protein from the lysates of both 2B4-Necl2 and B16 cells but not parental 2B4.

Analysis of the domains responsible for the CRTAM–Necl2 interaction

Necl2 has three Ig-like domains in the extracellular region whereas CRTAM has two as illustrated in Fig. 4(A). To determine the domains of CRTAM and Necl2 responsible for mutual binding, we constructed various Ig fusion proteins of CRTAM and Necl2 based on the structure of Ig domains—Necl2-Ig with first Ig-loop and second + third Ig-loop as well as CRTAM-Ig with a first Ig-loop and second Ig-loop. The CRTAM-transfectants were stained with Necl2-Ig with an N-terminal first Ig-loop while those with Necl2-Ig with a second + third Ig-loop of Necl2 were not stained (Fig. 4B), thus demonstrating that the first Ig domain is mainly responsible for binding. On the other hand, Necl2 transfectants were stained

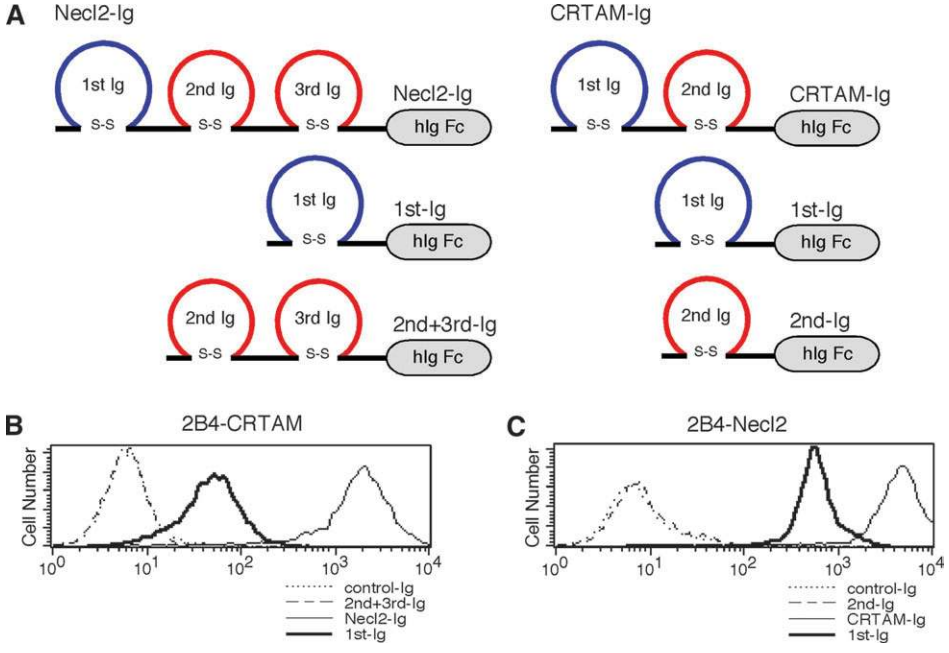

Fig. 4. Domain structure responsible for CRTAM–Necl2 interaction. (A) Structure of CRTAM-Ig, Necl2-Ig and their deletion mutants. Illustrated CRTAM- and Necl2-Ig mutants containing one or two Ig-like domains were constructed. Blue and red domains indicate IgV- and IgC-like domains. (B) N-terminus Ig domain of Necl2 is responsible for CRTAM binding. 2B4-CRTAM was stained with various Necl2-Ig fusion proteins and analyzed on FACS. Thin line: native extracellular region (Necl2-Ig); thick line: N-terminal Ig domain (1st-Ig); dashed line: two C-terminal Ig domains (2nd + 3rd-Ig); dotted line: control-Ig. (C) N-terminus Ig domain of CRTAM is responsible for Necl2 binding. 2B4-Necl2 was stained with CRTAM-Ig fusion proteins and analyzed on FACS. Thin line: native extracellular region (CRTAM-Ig); thick line: N-terminal Ig domain (1st-Ig); dashed line: second Ig domain (2nd-Ig); dotted line: control-Ig.

with CRTAM-Ig bearing first Ig-loop but not second Ig-loop (Fig. 4C). These data indicate that the N-terminal Ig domain (V-like) of both CRTAM and Necl2 are responsible for the heterotypic interaction.

CRTAM–Necl2 interaction is dispensable in the NK and CTL function in vitro

We originally cloned CRTAM as a molecule specifically expressed in activated NK cells. We therefore analyzed the function of CRTAM expressed on NK cells. NK cells expressed CRTAM on the cell surface upon co-culture with target cells YAC-1 (Fig. 5A) or NIH3T3 (data not shown). We then analyzed the cytotoxicity of NK cells from C57BL/6 mice against a Necl2-expressing target cell. As shown in Fig. 5(B), NK cells exerted a similar cytotoxicity against Necl2- and mock-transfected targets. Similarly, the cytotoxicity of NK cells against B16 melanoma cells did not change in the presence or absence of CRTAM-Ig (data not shown). Furthermore, there was no significant difference in IFN-γ production by NK cells upon stimulation with YAC-1–Necl2 or parental YAC-1 cells (Fig. 5C), in spite of the fact that the expression of Necl2 on these target cells was very high (Fig. 5D).

We next analyzed the effect of the CRTAM–Necl2 interaction on the function of effector $CD8^+$ CTLs. $CD8^+$ T cells from HIV gp160-specific RT-1 TCR Tg mice were used as antigen-specific CTLs as previously described (14). CTLs were induced by the stimulation of RT-1 TCR Tg $CD8^+$ T cells with specific peptide plus irradiated splenocytes for 2 or 3 days. Similar to anti-TCR stimulation (Fig. 2C), CRTAM expression on RT-1 $CD8^+$ T cells reached a maximum on day 2 after stimulation, and then decreased (Fig. 6A). Cytotoxic function of these CTLs upon days 2 or 3 stimulation against Necl2-transfected gp160-expressing NIH3T3 cells was analyzed. In spite of the high expression of CRTAM on CTL especially on day 2, there was no significant difference in the cytotoxicity against the control or Necl2-transfected target NIH3T3 cells by these CTLs either stimulated for 2 or 3 days (Fig. 6B). These results suggest that the CRTAM–Necl2 interaction does not clearly affect the effector function of NK cells and CTLs.

Induction of cell–cell adhesion through the CRTAM–Necl2 interaction

Since the cytotoxic function by NK cells or CTLs did not change in the presence of CRTAM–Necl2 interaction, we

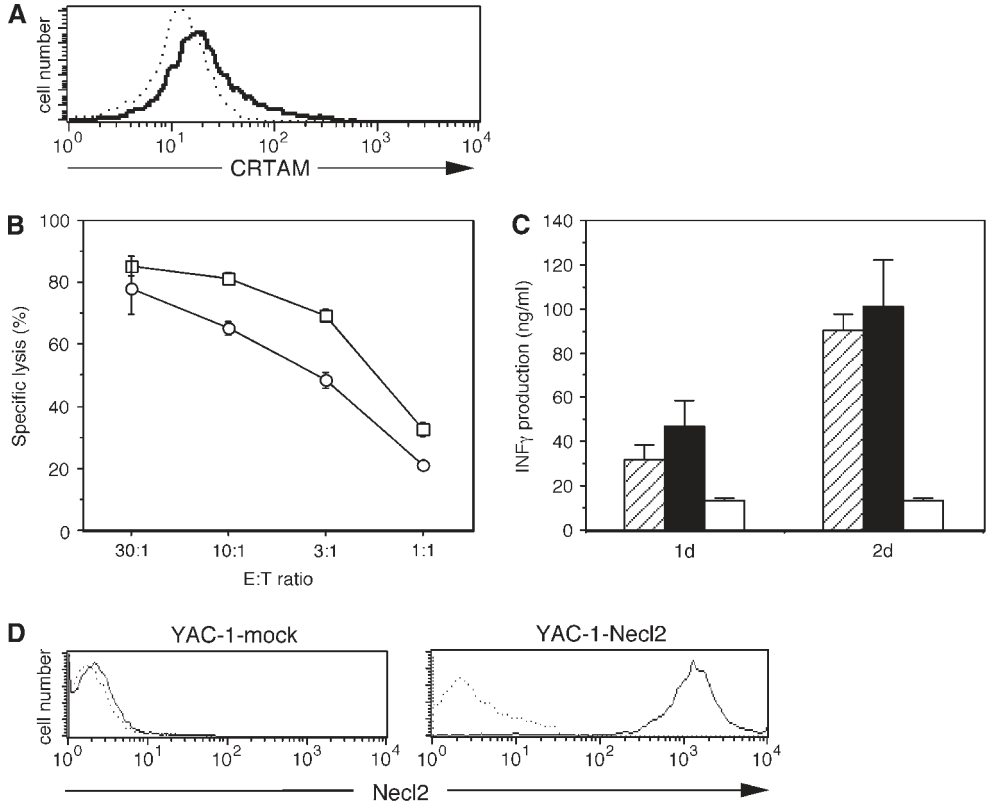

Fig. 5. Dispensable role of CRTAM–Necl2 interaction in effector function of NK cells. (A) CRTAM expression on tumor-activated NK cells. NK cells were co-cultured with YAC-1 or NIH3T3 (data not shown) for 12 h and were stained with anti-CRTAM mAb (thick line) or control mAb (dotted line). (B) Cytotoxicity of NK cells against NIH3T3-Necl2 (open circles) and NIH3T3-mock (open squares) were analyzed by ^{51}Cr-release assay. Specific lysis was calculated as described in the Methods. (C) IFN-γ production by NK cells upon stimulation with Necl2-expressing tumor cells. NK cells were co-cultured with YAC-1-Necl2 (filled bars), YAC-1-mock (hatched bars) or medium only (open bars) for 1 or 2 days. IFN-γ produced by NK cells was determined by ELISA. Data are presented as the mean ± SD. Similar results were obtained using 2B4-Necl2 cells as targets (data not shown). Representative data from three independent experiments are shown. (D) Expression of Necl2 on the cell surface of YAC-1 target cells. Mock- and Necl2-transfected YAC-1 cells were stained with CRTAM-Ig (thin lines) or control Ig (dotted lines).

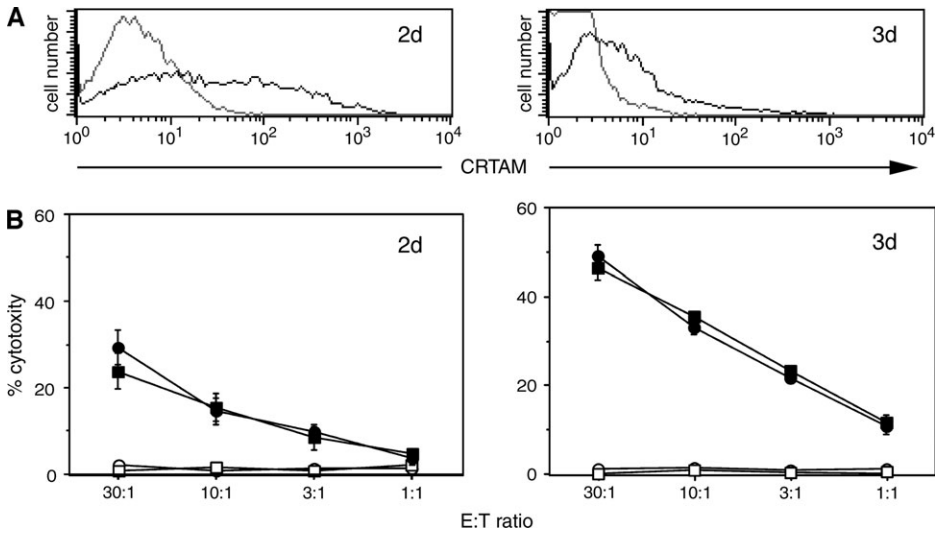

Fig. 6. Dispensable role of CRTAM–Necl2 interaction in the effector function of $CD8^+$ CTLs. (A) Expression of CRTAM on the cell surface of $CD8^+$ T cells upon stimulation with antigen peptide/APC. $CD8^+$ T cells from RT1 TCR-Tg mice were stimulated for 2 or 3 days and stained with Necl2-Ig (black lines) or control-Ig (gray lines). (B) Cytotoxicity of $CD8^+$ CTLs against antigen peptide-pulsed target cells. $CD8^+$ T cells from RT-1 TCR Tg mice were stimulated with irradiated BALB/c splenocytes and gp 160 peptide for 2 or 3 days, and the cytotoxicity against NIH3T3-Necl2 (circles) or NIH3T3-mock (squares) was analyzed. NIH3T3 transfectants were pulsed with peptide antigen (filled symbols) or not pulsed (open symbols) were used as targets. Data are presented as the mean ± SD. Representative data from three independent experiments are shown.

therefore analyzed whether this interaction induces cell–cell interaction between CRTAM- and Necl2-expressing cells. CRTAM-transfected 2B4 (2B4-CRTAM) and Necl2-transfected 2B4 (2B4-Necl2) were labeled with either CFSE (green) or CMTR (red), respectively, and these cells were mixed for 3 h. Whereas CMTR-labeled 2B4-Necl2 aggregated by themselves in all combinations (Fig. 7A, b, c and d), 2B4-CRTAM did not aggregate by themselves (Fig. 7A, a), but instead intensively co-aggregated with CMTR-labeled 2B4-Necl2 (Fig. 7A, c). CFSE-labeled 2B4-Necl2 but not CFSE-labeled 2B4 aggregated with CMTR-labeled 2B4-Necl2 (Fig. 7A, b and d). 2B4-CRTAM did not aggregate with 2B4 or with 2B4-CRTAM (data not shown). These data clearly suggest that CRTAM-expressing T cells specifically bind to Necl2-expressing cells.

To further analyze the differential interaction between CRTAM–Necl2 and Necl2–Necl2, we analyzed the inhibitory effects of CRTAM-Ig and Necl2-Ig on the Necl2-mediated homotypic cell aggregation. 2B4-Necl2 formed aggregation through the homotypic interaction within 3 h in the culture (Fig. 7B). The addition of CRTAM-Ig dramatically blocked the aggregation of Necl2-transfectants. In contrast, the addition of an equal amount of Necl2-Ig failed to perturb the aggregation. These results support the idea that the CRTAM–Necl2 interaction has a higher affinity than the homotypic interaction of Necl2.

The heterotypic interaction between CRTAM and Necl2 may reflect the heterotypic interaction between $CRTAM^+$T cells and Necl2 expressing other type of cells such as fibroblasts. Accordingly, we tested the binding of CRTAM-expressing T cells to Necl2-expressing NIH3T3 cells. First, NIH3T3 cells adhered to the plate and 2B4 T cells were added to NIH3T3 cells and the non-adherent fraction was counted (Fig. 7C). Indeed, 2B4-CRTAM specifically bound to NIH3T3-Necl2, but not to parental NIH3T3 cells. The addition of CRTAM-Ig significantly inhibited the binding of 2B4-CRTAM to 3T3-Necl2 (Fig. 7C). 2B4-Necl2 also bound to NIH3T3-Necl2, though less efficiently, in comparison to the binding of 2B4-CRTAM to NIH3T3-Necl2 (data not shown). Furthermore, we analyzed similar heterotypic cell binding using activated normal $CD8^+$ splenic T cells. Indeed, similar to CRTAM-transfected 2B4, activated $CD8^+$ T cells specifically bound to NIH3T3-Necl2 and the binding was significantly blocked by the addition of CRTAM-Ig (Fig. 7D), thus demonstrating that activated normal $CD8^+$ T cells expressing endogenous CRTAM bind to Necl2-expressing cells.

Collectively, these results indicate that the heterotypic CRTAM–Necl2 interaction induces cell–cell adhesion/aggregation, particularly between $CRTAM^+$T cells and $Necl2^+$ epithelial/fibroblast cells.

Discussion

In the present study, we described the identification of a pair of mutually interacting adhesion molecules, CRTAM and Necl2. We identified CRTAM as specifically induced activation molecule on NK cells and $CD8^+$ T cells using the cDNA subtraction method, and further Necl2 as a CRTAM ligand using an expression-cloning method.

As we intended to isolate the molecules specifically expressed in activated NK cells, CRTAM is one of such molecules that expressed highly restricted types of immune cells—activated NK cells, activated $CD8^+$ T cells and activated mast cells (our unpublished results). CRTAM is expressed very early after stimulation and transiently redundant on NK and CTLs. The transcript starts as early as 2 h, reaches the maximum at 6–12 and 12–24 h for NK cells and CTLs, respectively, thereafter decreases and disappears

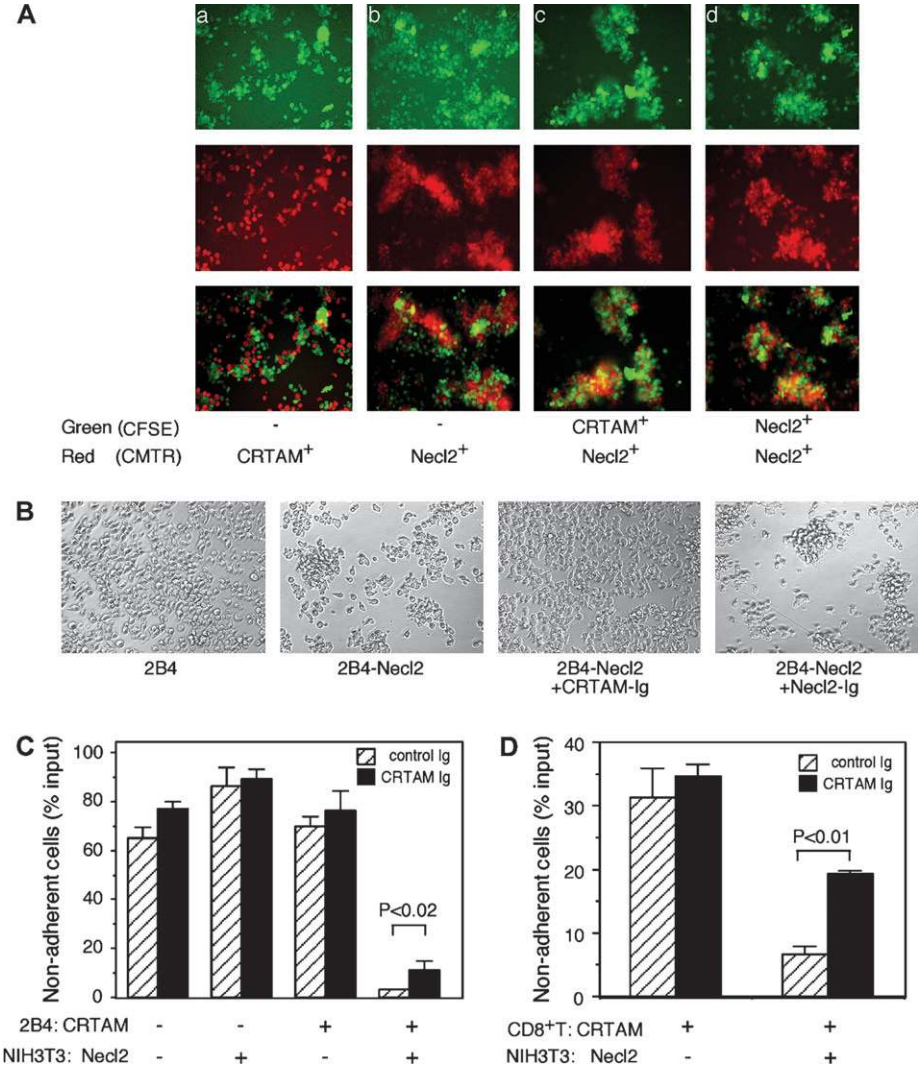

Fig. 7. Cell–cell adhesion induced by the CATAM–Necl2 interaction. (A) Heterotypic and homotypic interaction by CRTAM- and Necl2-expressing 2B4 cells. Single-cell suspension of CMTR-labeled 2B4-Necl2 (red) or CMTR-labeled 2B4-CRTAM was cultured with CFSE-labeled 2B4, 2B4-CRTAM, or 2B4-Necl2 (green) for 3 h. Cell aggregation of these cells was visualized by fluorescence microscopy. (B) Blocking of homotypic Necl2-aggregation by CRTAM-Ig but not Necl2-Ig. Single-cell suspensions of 2B4 or 2B4-Necl2 were incubated for 3 h in the presence or absence of either CRTAM-Ig or Necl2-Ig (1.25 μg ml^{-1}). (C) Cell–cell adhesion between 2B4-CRTAM and NIH3T3-Necl2 transfectants. NIH3T3 or NIH3T3-Necl2 were adhered on the plate and 2B4 or 2B4-CRTAM were added in the culture and incubated for 30 min in the presence of 3 μg ml^{-1} of CRTAM-Ig (closed bars) or control-Ig (hatched bars). Thereafter, non-adherent cells were counted and the proportion of cells released from adherent cells was calculated. Representative data from four independent experiments are shown. *P*-values are shown. (D) Cell–cell adhesion between activated normal $CD8^+$ T cells and Necl2-expressing fibroblast cells. Purified $CD8^+$ T cells were stimulated with anti-TCR mAb and cultured for 30 min with NIH3T3 or NIH3T3-Necl2 adhered on the plate in the presence of CRTAM-Ig (closed bars) or control-Ig (hatched bars). Similar to (C), the proportion of cells released was calculated as described above. Representative data from four independent experiments were shown. *P*-values are shown.

at 48 h. We found that the kinetics of protein and transcript of CRTAM was parallel, thus indicating that the expression is strictly regulated on the transcriptional level in these cells. The kinetics of CRTAM expression are therefore quite different from other activation markers such as CD25, CD69 and CD62L. This unique feature of such an early and transient expression of CRTAM on NK cells and $CD8^+$ T cells may suggest that such expression plays an important role in the early stage of cell activation. The reason why CRTAM expression is quickly down-regulated could be to prove specific cell–cell adhesion through the CRTAM–Necl2 interaction and to avoid any unnecessary non-specific adhesion, or alternatively, related to transient cell movement/recruitment. Although CRTAM mRNA was detected in mast cells, we failed to detect CRTAM protein in these cells, thus raising a possibility that there might be additional cell type-specific regulation of CRTAM expression.

As a CRTAM ligand, we cloned Necl molecule 2 (Necl2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1) by expression cloning using a retroviral cDNA library. Necl2 has a cell-surface molecule with three Ig domains and it belongs to the family of Nectin and Necl molecules that contribute to various types of

cell–cell adhesion through Ca^{2+}-independent interaction. In contrast to the restricted tissue expression of CRTAM, Necl2 is widely expressed in mouse tissues including the brain, testis, gallbladder, liver and pancreas but not in fibroblasts or endothelial cells, [(23), and N. Arase, unpublished results]. Necl2 is involved in the homotypic and heterotypic interaction with Nectin and Necl family molecules thus inducing cell–cell adhesion and the expression of Necl2 alone induces spontaneous aggregation (23–25). We found that the counter-receptor, CRTAM, also functions as an adhesion molecule. Our critical findings on CRTAM–Necl2 binding is 2-fold. One is that CRTAM mediates only the heterotypic interaction with Necl2 and not homotypically by itself. The other is that the heterotypic CRTAM–Necl2 interaction has a higher affinity than the homotypic Necl2–Necl2 interaction, thus suggesting that Necl2 preferentially binds to CRTAM and CRTAM can therefore interfere with homotypic Necl2 binding. Nectin–Necl family molecules bearing three Ig-like domains mediate homotypic adhesion as well as heterotypic interaction such as Necl2–Nectin-3 (23). In addition, CD226/DNAM-1 and CD96 have been recently shown to bind to Necl5 and Nectin-2 (12, 26), heterotypic CRTAM-Necl2 adhesion represents heterophillic cell–cell interaction between different cell types, e.g. lymphocytes and epithelial cells. Since some macrophage cell lines as well as thioglycolate-induced peritoneal macrophages (our unpublished results) express Necl2, such CRTAM–Necl2 interaction might therefore be involved in T cell–antigen-presenting cell (APC) interaction.

These characteristics and the expression patterns of CRTAM and Necl2 suggest that CRTAM–Necl2 interaction seems to play an important role in the regulation of CTL and NK cell function. However, cytotoxicity and IFN-γ production by NK cells were not affected by the expression of Necl2 on target cells. Although it has been shown that engagement of an adhesion molecule such as LFA-1 is sufficient to trigger NK cell activation (10), CRTAM–Necl2 interaction is not sufficient for both immediate cytokine secretion and cytotoxicity in our system. Similarly, no difference was seen in the cytotoxicity of antigen-specific CTLs against Necl2-transfected and untransfected targets. Furthermore, we found that the development of CTLs by stimulating naive $CD8^+$ T cells with $Necl2^+$APC was similarly induced to that with $Necl2^-$ APC (our unpublished results). Using transfected NK cell line, the transfection of mouse CRTAM into NKL cells also failed to affect the cytotoxicity against Necl2-expressing targets, although $CRTAM^+$NKL showed intensive aggregation with Necl2 transfectants (our unpublished results). We also analyzed cytotoxicity by transiently activated NK cells on which the maximum CRTAM expression was induced upon stimulation with immobilized NKR-P1C mAb against Necl2-transfected 3T3 cell line. However, the activated $CRTAM^+$NK cells showed no significant difference in cytotoxicity against mock- and Necl2-transfectants (our unpublished results). Collectively, CRTAM expressed on activated NK cells and CTLs do not appear to be directly involved in the cell activation and effector function *in vitro*. However, the condition of the *in vitro* culture may not reflect the *in vivo* situation. Cell–cell contact is extensive in an *in vitro* culture without these adhesion molecules, which might overcome the requirement of specific cell–cell binding. The requirement of CRTAM–Necl2 interaction regarding the *in vivo* function remains to be determined using either Tg or gene-knockout mice.

Although we did not observe a functional contribution of the CRTAM–Necl2 interaction on *in vitro* cytotoxicity by NK cells and $CD8^+$ T cells, it might have such a function *in vivo*. Since Necl2 is involved in cell–cell interaction and is also expressed ubiquitously (23, 27, 28), while it is also localized in basolateral plasma membrane in the case of epithelial cells, NK cells or $CD8^+$ T cells hardly interact with such Necl2-expressing epithelial cells, even after expressing CRTAM on the cell surface upon activation. However, under invasive situations such as tumorgenesis or severe infection, where the tissue structure is disrupted and normal cell–cell junction with adherent and tight junctions cannot be orderly maintained, Necl2-expressing cells, such as epithelial cells, may emerge at such sites where lymphocytes could have contact. NK cells or $CD8^+$ T cells may migrate into such tumor or inflammatory sites and thus be activated to express CRTAM. As a result, CRTAM-expressing NK or CTLs might stay transiently within the tumor/inflammatory site and exhibit an efficient cytotoxicity against tumor/infected target cells through specific cell–cell adhesion.

Necl2 is also known to be a tumor suppressor gene (TSLC1), which inactivates tumors in nude mice (22) and is frequently inactivated in many non-small cell lung cancers (22, 29). Although the mechanism of anti-tumor activity is still largely unknown, a lack of Necl2 may induce a disruption of cell polarity and adhesion, thus resulting in neoplastic growth. The cytoplasmic domain of Necl2 contains two important motifs, a protein 4.1-binding motif near the transmembrane region and a PDZ-binding domain at the C-terminus, and recruits intracellular adaptors (28, 30, 31). One of the protein 4.1 family molecule DAL-1 acts to anchor Necl2 to the actin cytoskeleton, and plays a crucial role in the tumor suppression mediated by Necl2 (32). The PDZ-domain of Necl2 binds to MPP3 and Pals2; the former functions to organize cell junction and mediates tumor suppression (33) while the latter is involved in the localization of other transmembrane protein(s) (23).

On the other hand, CRTAM has a candidate sequence of a PDZ-binding site at the C-terminus (30), and a possible serin phosphorylation site at the same position as that of DNAM-1 that is essential for LFA-1-mediated co-stimulatory signals for T cells and NK cells (34). This serine residue has also been reported to play a role in the recruitment of DNAM-1 into lipid raft in T cells. Accordingly, CRTAM might be involved in the regulation of NK cell and CTL activation.

In addition, the CRTAM–Necl2 interaction could be in part related to immune surveillance. An abnormal appearance of Necl2 in disrupted tissues and its recognition by CRTAM-expressing NK cells or CTLs in tumor sites may suggest that Necl2 is a possible target of immune surveillance which thus acts as a type of 'danger signal'. In addition to such local emergencies, considering the distribution of Necl2, especially on epithelial cells but not on endothelial cells, the CRTAM–Necl2 interaction might therefore be involved in the recruitment of activated NK and $CD8^+$ T cells to peripheral tissues under physiological conditions, which is now under investigation using CRTAM-deficient mice.

Acknowledgements

We thank S. Taki and S. Yamasaki, for discussion, C. Ishihara, M. Sakuma and S. Tanaka for technical help and H. Yamaguchi for secretarial assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Abbreviations

APC	antigen-presenting cell
BS^3	Bis(sulfosuccinimidyl) suberate
CRTAM	class I-restricted T cell-associated molecule
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
SHIP	SH2-domein-containing inositol polyphosphate 5-phosphatase
SHP	SH2-domein-containing protein tyrosine phosphatase
LFA	leukocyte function-associated antigen
Necl	nectin-like
RT	reverse transcription
Tg	transgenic

References

1 Cerwenka, A. and Lanier, L. L. 2001. Natural killer cells, viruses and cancer. *Nat. Rev. Immunol.* 1:41.
2 Diefenbach, A. and Raulet, D. H. 2002. The innate immune response to tumors and its role in the induction of T-cell immunity. *Immunol. Rev.* 188:9.
3 Moretta, A., Bottino, C., Vitale, M. *et al.* 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* 19:197.
4 Colucci, F., Di Santo, J. P. and Leibson, P. J. 2002. Natural killer cell activation in mice and men: different triggers for similar weapons? *Nat. Immunol.* 3:807.
5 Lanier, L. L. 2003. Natural killer cell receptor signaling. *Curr. Opin. Immunol.* 15:308.
6 Yokoyama, W. M., Kim, S. and French, A. R. 2004. The dynamic life of natural killer cells. *Annu. Rev. Immunol.* 22:405.
7 Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B. and Lanier, L. L. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296:1323.
8 Veillette, A., Latour, S. and Davidson, D. 2002. Negative regulation of immunoreceptor signaling. *Annu. Rev. Immunol.* 20:669.
9 Tangye, S. G., Lazetic, S., Woollatt, E., Sutherland, G. R., Lanier, L. L. and Phillips, J. H. 1999. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J. Immunol.* 162:6981.
10 Barber, D. F. and Long, E. O. 2003. Coexpression of CD58 or CD48 with intercellular adhesion molecule 1 on target cells enhances adhesion of resting NK cells. *J. Immunol.* 170:294.
11 Shibuya, A., Campbell, D., Hannum, C. *et al.* 1996. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* 4:573.
12 Bottino, C., Castriconi, R., Pende, D. *et al.* 2003. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J. Exp. Med.* 198:557.
13 Barber, D. F., Faure, M. and Long, E. O. 2004. LFA-1 contributes an early signal for NK cell cytotoxicity. *J. Immunol.* 173:3653.
14 Yokosuka, T., Takase, K., Suzuki, M. *et al.* 2002. Predominant role of T cell receptor (TCR)-alpha chain in forming preimmune TCR repertoire revealed by clonal TCR reconstitution system. *J. Exp. Med.* 195:991.
15 Takahashi, H., Houghten, R., Putney, S. D. *et al.* 1989. Structural requirements for class I MHC molecule-mediated antigen presentation and cytotoxic T cell recognition of an immunodominant determinant of the human immunodeficiency virus envelope protein. *J. Exp. Med.* 170:2023.
16 Shiratori, I., Ogasawara, K., Saito, T., Lanier, L. L. and Arase, H. 2004. Activation of natural killer cells and dendritic cells upon recognition of a novel CD99-like ligand by paired immunoglobulin-like type 2 receptor. *J. Exp. Med.* 199:525.
17 Akashi, S., Saitoh, S., Wakabayashi, Y. *et al.* 2003. Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J. Exp. Med.* 198:1035.
18 Arase, H., Saito, T., Phillips, J. H. and Lanier, L. L. 2001. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2). *J. Immunol.* 167:1141.
19 Lopez, M., Aoubala, M., Jordier, F., Isnardon, D., Gomez, S. and Dubreuil, P. 1998. The human poliovirus receptor related 2 protein is a new hematopoietic/endothelial homophilic adhesion molecule. *Blood* 92:4602.
20 Miyahara, M., Nakanishi, H., Takahashi, K., Satoh-Horikawa, K., Tachibana, K. and Takai, Y. 2000. Interaction of nectin with afadin is necessary for its clustering at cell-cell contact sites but not for its cis dimerization or trans interaction. *J. Biol. Chem.* 275:613.
21 Kennedy, J., Vicari, A. P., Saylor, V. *et al.* 2000. A molecular analysis of NKT cells: identification of a class-I restricted T cell-associated molecule (CRTAM). *J. Leukoc. Biol.* 67:725.
22 Kuramochi, M., Fukuhara, H., Nobukuni, T. *et al.* 2001. TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. *Nat. Genet.* 27:427.
23 Shingai, T., Ikeda, W., Kakunaga, S. *et al.* 2003. Implications of nectin-like molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in cell-cell adhesion and transmembrane protein localization in epithelial cells. *J. Biol. Chem.* 278:35421.
24 Biederer, T., Sara, Y., Mozhayeva, M. *et al.* SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297:1525.
25 Masuda, M., Yageta, M., Fukuhara, H. *et al.* 2002. The tumor suppressor protein TSLC1 is involved in cell-cell adhesion. *J. Biol. Chem.* 277:31014.
26 Tahara-Hanaoka, S., Shibuya, K., Onoda, Y. *et al.* 2004. Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112). *Int. Immunol.* 16:533.
27 Wakayama, T., Ohashi, K., Mizuno, K. and Iseki, S. 2001. Cloning and characterization of a novel mouse immunoglobulin superfamily gene expressed in early spermatogenic cells. *Mol. Reprod. Dev.* 60:158.
28 Fukami, T., Satoh, H., Fujita, E. *et al.* 2002. Identification of the Tslc1 gene, a mouse orthologue of the human tumor suppressor TSLC1 gene. *Gene* 295:7.
29 Fukuhara, H., Kuramochi, M., Fukami, T. *et al.* 2002. Promoter methylation of TSLC1 and tumor suppression by its gene product in human prostate cancer. *Jpn. J. Cancer Res.* 93:605.
30 Songyang, Z., Fanning, A. S., Fu, C. *et al.* 1997. Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* 275:73.
31 Fukuhara, H., Kuramochi, M., Nobukuni, T. *et al.* 2001. Isolation of the TSLL1 and TSLL2 genes, members of the tumor suppressor TSLC1 gene family encoding transmembrane proteins. *Oncogene* 20:5401.
32 Yageta, M., Kuramochi, M., Masuda, M. *et al.* 2002. Direct association of TSLC1 and DAL-1, two distinct tumor suppressor proteins in lung cancer. *Cancer Res.* 62:5129.
33 Fukuhara, H., Masuda, M., Yageta, M. *et al.* 2003. Association of a lung tumor suppressor TSLC1 with MPP3, a human homologue of Drosophila tumor suppressor Dlg. *Oncogene* 22:6160.
34 Shirakawa, J., Shibuya, K. and Shibuya, A. 2005. Requirement of the serine at residue 329 for lipid raft recruitment of DNAM-1 (CD226). *Int. Immunol.* 17:217.