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# CRTAM Confers Late-Stage Activation of CD8$^+$ T Cells to Regulate Retention within Lymph Node[1]

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**In vivo immune response is triggered in the lymph node, where lymphocytes for entry into, retention at, and migration to effector sites are dynamically regulated. The molecular mechanism underlying retention regulation is the key to elucidating in vivo regulation of immune response. In this study, we describe the function of the adhesion molecule class I-restricted T cell-associated molecule (CRTAM) in regulating CD8$^+$ T cell retention within the lymph node and eventually effector function. We previously identified CRTAM as a receptor predominantly expressed on activated CD8$^+$ T cells, and nectin-like molecule-2 (Necl2) as its ligand. In vivo function of CRTAM-Necl2 interaction was analyzed by generating CRTAM$^{-/-}$ mice. CRTAM$^{-/-}$ mice exhibited reduced protective immunity against viral infection and impaired autoimmune diabetes induction in vivo. Although Ag-specific CRTAM$^{-/-}$ CD8$^+$ T cells showed normal CTL functions in vitro, their number in the draining lymph node was reduced. Because CRTAM$^+$ T cells bound efficiently to Necl2-expressing CD8$^+$ dendritic cells (DCs) that reside in T cell area of lymph node, CRTAM may induce retention by binding to CD8$^+$ DCs at the late stage of activation before proliferation. The CRTAM-mediated late interaction with DCs induced retention of activated CD8$^+$ T cells in an Ag-independent fashion, and this possibly resulted in effective CTL development in the draining lymph node.** *The Journal of Immunology,* **2009, 183: 4220–4228.**

Naive lymphocytes circulate by continuously entering and egressing from lymphoid tissue, and those migratory events are important for immune surveillance. G protein-coupled receptor families, such as chemokine receptors or sphingosine-1-phosphatase (S1P)[4] receptor, regulate those migrations. Lymphocytes home into secondary lymphoid tissues via chemokines that are produced by lymphoid stromal cells as well as adhesion molecules such as integrins, and initiate Ag surveillance or adaptive immune responses. In contrast, lymphocyte egress is regulated mainly by S1P receptor (1). During lymphocyte activation, S1P receptor-1 ($S1P_1$) is down-regulated from the cell surface, and through this, the egress process is transiently shut down. As a result, activated lymphocytes are retained within the draining lymph node (LN) (2). In addition to entry and egress, whether there is a specific mechanism for lymphocyte retention within LN is largely unknown.

CTLs play critical roles in protection from viral infection and tumor growth. Activated Ag-specific CD8$^+$ T cells mature into CTLs by interacting with dendritic cells (DCs) within the draining LN, and the CTLs migrate to peripheral tissues, where protective CTL response is required (3, 4). CD8$^+$ T cells recognize and respond to Ag peptides displayed by MHC class I on APCs and target cells, and function to exert cytotoxicity or recruit and activate other immune cells. The activation of naive CD8$^+$ T cells is dependent on Ag-presenting DCs, and mature CD8$^+$ T cells proliferate substantially (~1000-fold) over a span of 5–8 days (5). Clonal expansion is tightly coupled to the differentiation of effector T cells that migrate to peripheral tissue, where they exhibit cytotoxicity and effector cytokine release (6). The majority (>90%) of Ag-specific effector cells are eliminated by apoptosis-induced contraction, and finally, Ag-experienced cells function as immunological memory (7, 8). The movement of primary T cells in LN was analyzed in detail by two-photon microscopy (9, 10). T cell priming by DCs occurs in three successive phases: T cells and DCs continuously encounter each other for a short time at the first activation phase and establish stable contact at the second phase. This interaction induces cytokine production and the transition to the third phase, where T cells are highly motile and proliferate rapidly. However, the mechanism underlying the retention of activated T cells within LN, which may induce maturation and migration to effector tissues, remains elusive.

We previously identified class I-restricted T cell-associated molecule (CRTAM) as an activation-induced surface receptor predominantly expressed on CD8$^+$ T cells, and nectin-like molecule-2

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[4] Abbreviations used in this paper: S1P, sphingosine-1-phosphatase; DC, dendritic cell; CRTAM, class I-restricted T cell-associated molecule; ES, embryonic stem; KO, knockout; LN, lymph node; mOVA, membrane-bound OVA; Necl2, nectin-like molecule-2; NP, nuclear protein; RIP, rat insulin promoter; $S1P_1$, S1P receptor-1; Tg, transgenic; WT, wild type.



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(Necl2)/TSLC1/CADM1 as its ligand (11–14). In this study, the physiological role of the CRTAM-Necl2 interaction was analyzed in CRTAM-deficient ($CRTAM^{-/-}$) mice. The mice exhibited impaired protective immunity against viral infection and diabetes. In the CTL-induced diabetes model, $CRTAM^{-/-}$ CTLs failed to induce diabetes. Whereas cytotoxicity and proliferation of $CRTAM^{-/-}$ CTLs were normal in vitro, their numbers in the draining LN were reduced, causing impaired immunity of effector tissues. Activated $CRTAM^{+}$ T cells were retained within LN during transient CRTAM expression by interacting with Necl2 on $CD8^{+}$ DCs and proliferated efficiently in vivo. These results suggest that CRTAM expression is critical for the accumulation of Ag-specific CTLs and their subsequent proliferation within the draining LN.

## Materials and Methods

### *Animals*

C57BL/6 mice were purchased from CLEA Japan. OT-I transgenic (Tg) mice (15) and rat insulin promoter (RIP)-membrane-bound OVA (mOVA) Tg mice, which express mOVA under the rat insulin promoter, were previously described (16). Necl2-deficient mice were generated by Y. Takai (Kobe University) and J. Miyoshi (Osaka Medical Center for Cancer and Cardiovascular Diseases) (manuscript in preparation). All animal experiments were performed in compliance with the institutional guidelines of the animal facility of RIKEN Yokohama Institute.

### *Generation of $CRTAM^{-/-}$ mice*

A genomic DNA clone containing CRTAM gene was isolated from a genomic library of R1 mouse embryonic stem (ES) cells (17). The targeting vector consists of a 2.5-kb genomic fragment, Neo gene flanked by two loxP sites (provided by H. Gu, Columbia University, New York, NY) (18) at 5′ and 3′ ends, a 1.2-kb genomic fragment, and HSV-tk gene in pBluescript II $SK^{+}$ (Stratagene) (Fig. 1*A*). ES cells were transfected with the linearized targeting vector by electroporation. Homologous recombination in ES clones was confirmed by Southern blot analysis with 5′- and 3′-specific DNA probes (Fig. 1*B*). The deletion of exon 1 was further confirmed by RT-PCR using primers crtam20U (5′-GTTTGCTGTTCTGGGT GCCGGTG-3′) and crtam592L (5′- TTTCTCCCGTGCAAGCCCTC GTG-3′). Three independent ES clones were subjected to aggregation with C57BL/6 blastocysts, followed by transfer into pseudopregnant Jcl:ICR mice. Chimera mice were crossed with C57BL/6 mice, and three mouse lines with germline transmission were established. $CRTAM^{-/-}$ mice were generated by intercrossing heterozygous mice. $CRTAM^{-/-}$ mice were backcrossed more than seven times into the C57BL/6 background. We also confirmed loss of CRTAM expression on the activated $CRTAM^{-/-}$ $CD8^{+}$ T cells by cell surface staining (Fig. 1*C*).

### *Cell preparation*

Splenic $CD8^{+}$ T cells were purified from splenocytes by staining with PE anti-B220, anti-CD4, anti-CD11b, anti-CD11c, anti-NK1.1, anti-Gr1, and anti-γδ TCR mAbs (eBiosocience), and negatively sorted by magnetic bead-conjugated anti-PE mAb and MACS system (Miltenyi Biotec).

### *In vitro cytotoxicity analysis*

To generate CTLs, purified $CD8^{+}$ T cells from OT-I Tg mice were stimulated with 3 μM OVA peptide (SIINFEKL) and 35.0 Gy irradiated spleen cells from C57BL/6 mice for 5 days. For CTL assay, various numbers of effector cells were cultured for 3 h in triplicate in 96-well round-bottom plates with $5 \times 10^3$ OVA-expressing EG7 target cells with or without Necl2 expression. Cytotoxicity was determined by standard $^{51}Cr$ release assay and calculated as follows: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

### *In vivo cell transfer experiments*

*Induction of diabetes.* Purified $5 \times 10^6$ OT-I $CD8^{+}$ T cells were adoptively transferred into RIP-mOVA Tg mice. Adoptively transferred mice were monitored for blood glucose levels every 5 days using Glutest Neo (Sanwa Kagaku).

*Competitive cell transfer system.* Equal numbers of $CRTAM^{+/-}$ ($Ly5.1^{+}$) or $CRTAM^{-/-}$ ($Ly5.2^{+}$) OT-I $CD8^{+}$ T cells were cotransferred into RIP-mOVA Tg mice that had been crossed with a Tg mice expressing GFP ubiquitously, and thus, all host origin cells are GFP positive and transferred cells were GFP negative. After days indicated, $CRTAM^{+/-}$ or $CRTAM^{-/-}$ T cells in various tissues were analyzed by staining for Ly5 markers, and we determined the ratio of $CRTAM^{+/-}$ vs $CRTAM^{-/-}$ OT-I $CD8^{+}$ T cells among GFP-negative cell population.

### *Histological analysis*

Pancreas and s.c. LN were frozen in liquid nitrogen within OCT (Tissue-Tek; Sakura Finetechnical). Sections (5 μm) were cut on a cryostat (Leica Microsystems), mounted on glass slides (Matsunami Glass), and fixed in paraformaldehyde for 30 min at room temperature. LN sections were incubated with anti-Necl2 (anti-mouse IGSF4A; R&D Systems) and anti-B220 (BD Biosciences), anti-mouse fibroblast (ER-TR7; AbD Serotec), or anti-CD11c Ab (BD Biosciences).

### *Blockade of cell migration into LN*

RIP-mOVA Tg mice were treated with purified anti-CD62L (L-selectin) Ab (clone Mel-14, 250 μg/mouse) i.p. 3 h after OT-I $CD8^{+}$ T cells were transferred.

### *FTY720- and BrdU-treated mice*

RIP-mOVA Tg mice were treated with FTY720 (1.0 mg/kg body weight; Novartis; licensed by Mitsubishi Pharma) i.p. 3 h after OT-I $CD8^{+}$ T cells were transferred, and were given drinking water containing FTY720 (2 μg/ml) for 5 days before tissue collection. BrdU (Sigma-Aldrich) was introduced through the drinking water (0.8 mg/ml) after transfer.

### *Analysis of cell adhesion*

Equal numbers of stimulated $CRTAM^{+/-}$ (PE-labeled) and $CRTAM^{-/-}$ (allophycocyanin-labeled) $CD8^{+}$ T cells ($1 \times 10^5$) were mixed and incubated with DCs for 3 h at 37°C in the presence or absence of anti-ICAM (Seikagaku) and anti-VCAM (Southern Biotechnology Associates) Abs. After incubation, DC-conjugated cells were analyzed by flow cytometry.

### *Influenza virus infection and titration*

Live influenza virus, A/Aichi/2/68 (H3N2) ($1 \times 10^4$ PFU), was intranasally inoculated into mice. Six days after infection, viral titers in lung were determined by performing plaque assay on Madin-Darby canine kidney cells (19). A confluent monolayer on six-well plates was infected with serial dilutions of lung homogenate for 1 h at room temperature, washed with PBS twice, and covered with MEM containing 1% agar and 5 μg/ml trypsin (Difco). After incubating at 35°C under 5% $CO_2$ atmosphere for 48 h, plaques were visualized with crystal violet and counted (20).

### *Analysis of virus-specific $CD8^{+}$ T cells*

Virus-specific $CD8^{+}$ T cells were analyzed by staining cells with FITC-CD8α (eBiosocience), PE-labeled pro5 MHC pentamer H-2Db/ASNEN MDAM as an influenza-specific peptide/MHC pentamer (Pro Immune), and ethidium monoazide (Molecular Probes). After irreversible photolytic coupling of ethidium monoazide to cellular DNA, cells were fixed with 2% paraformaldehyde and analyzed by flow cytometry.

### *In vivo CTL assay*

Whole splenocytes ($2 \times 10^7$ cells/ml) from C57BL/6 mice were incubated with 0.5 μM OVA peptide (SIINFEKL) or influenza virus nuclear protein (NP) 366–374 peptide (ASNENMDAM) for 2 h at 37°C. After washing, cells were labeled with graded concentrations of CFSE. Equal numbers of splenocytes carrying each peptide were mixed, and the mixture ($5 \times 10^6$ each) was injected i.v. into recipient mice. Fourteen hours after injection, spleen cells were collected and CFSE-positive cells were analyzed by flow cytometry. NP-specific killing was calculated as follows: percentage of killing = (1 − ((number of NP peptide-carrying cells ($CFSE^{high}$) in immunized mice)/(number of OVA peptide-carrying cells ($CFSE^{low}$) in immunized mice))/(number of $CFSE^{high}$ cells in normal mice)/(number of $CFSE^{low}$ cells in normal mice)) × 100 (21).

## Results

### *Defective accumulation of $CRTAM^{-/-}$ $CD8^{+}$ T cells in draining LN*

The physiological roles of CRTAM and its heterotypic interaction with the ligand Necl2 were investigated by generating $CRTAM^{-/-}$ mice (Fig. 1). $CRTAM^{-/-}$ mice were born without any

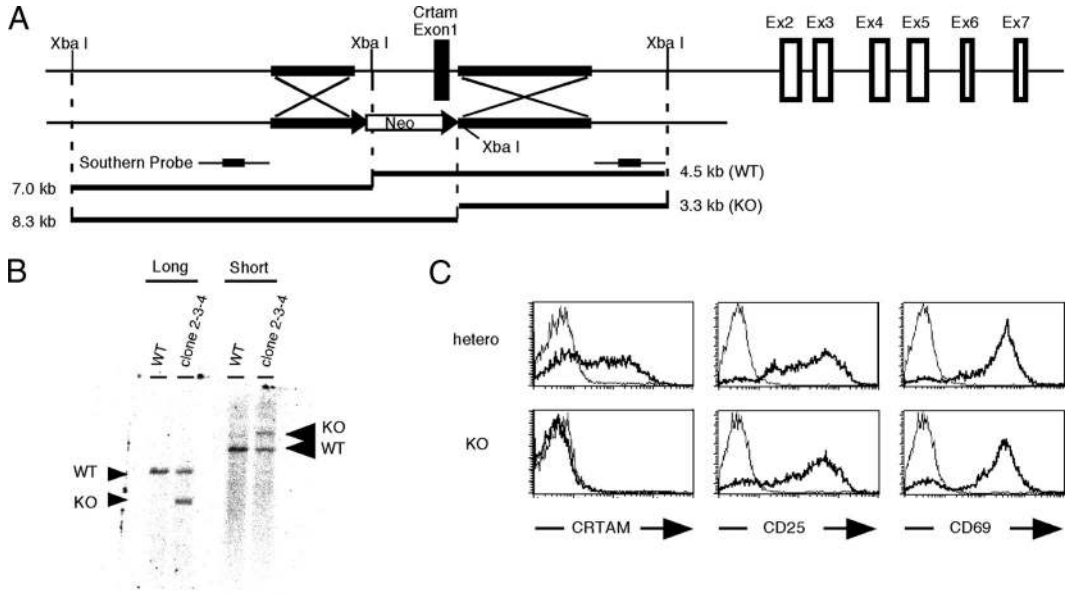


**FIGURE 1.** Generation of CRTAM$^{-/-}$ mice. *A*, The targeting construct designed to replace the first exon (containing the first ATG) of CRTAM gene with a Neo cassette. *B*, Southern blot analysis of recombination of CRTAM gene in an ES clone with homologous recombination. *C*, Expression of CRTAM and activation markers in CRTAM$^{-/-}$ T cells upon stimulation. CD8$^+$ splenic T cells from CRTAM$^{+/-}$ (hetero) and CRTAM$^{-/-}$ (knockout (KO)) mice were stimulated with anti-CD3 and anti-CD28 for 20 h; stained with Necl2-Ig, anti-CD25, and anti-CD69; and analyzed by flow cytometry.

macroscopic abnormalities and showed normal development of lymphocyte and myeloid cells (Fig. S1).[5]

We examined the in vivo impact of CRTAM expression in a diabetes model. We used Tg mice expressing mOVA under the insulin promoter (RIP-mOVA) as the system enables effective analysis of CD8$^+$ T cell function (16), because Ag-specific OT-I CD8$^+$ T cells have been shown to trigger autoimmune diabetes in RIP-mOVA mice (15, 22). Accordingly, OT-I CRTAM$^{+/-}$ or CRTAM$^{-/-}$ CD8$^+$ T cells were transferred into RIP-mOVA mice and diabetes incidence was compared. Whereas diabetes was induced in almost all recipients of OT-I CRTAM$^{+/-}$ CD8$^+$ T cells, OT-I CRTAM$^{-/-}$ CD8$^+$ T cells induced diabetes in only 27% of recipient mice (Fig. 2*A*).

The impairment may be attributed to either the reduced number of CD8$^+$ CTLs or impaired cytotoxic activity of CD8$^+$ T cells. Because CRTAM is predominantly expressed on activated CD8$^+$ T cells, we assumed the involvement of CRTAM in cytotoxic function. However, when we analyzed the effector cytotoxic function of OT-I CRTAM$^{+/-}$ and CRTAM$^{-/-}$ CTLs, in vitro cytotoxicity against OVA-expressing target cells (EG7) was similarly induced by both CTLs regardless of Necl2 expression on EG7 (Fig. S2*C*). Furthermore, when we examined the in vitro function of CD8$^+$ CTLs using CRTAM$^{-/-}$ OT-I mice, OT-I CRTAM$^{+/-}$ and CRTAM$^{-/-}$ CD8$^+$ T cells exhibited comparable responses in terms of proliferation, IFN-$\gamma$ (Fig. S2, *A* and *B*) and IL-2 production, cell survival, and cell death upon OVA-peptide/APC stimulation (data not shown). These results indicate that CRTAM$^{-/-}$ CD8$^+$ T cells exhibit comparable Ag-specific response and effector function in vitro to wild-type (WT) T cells. Thus, the CRTAM-Necl2 interaction may not be involved in the in vitro CTL function unexpectedly. We next analyzed lymphocyte infiltration into the pancreatic islet. Although histological examination revealed that lymphocyte infiltration was observed regardless of the transferred cell type (Fig. S3), the frequency of lymphocyte-infiltrated islets in the whole pancreas was much lower when CRTAM$^{-/-}$ CD8$^+$ T cells were transferred (Fig. 2*B*), suggesting that the impairment of diabetes induction by CRTAM$^{-/-}$ CD8$^+$ T cells is attributed to the reduction of islet-infiltrating lymphocytes.

To understand the basis of the difference in the number of islet-infiltrating T cells, we next analyzed the number of Ag-specific CD8$^+$ T cells in the draining LN and performed histological examination (Fig. 2*C*). Equal numbers of OT-I CD8$^+$ T cells from CRTAM$^{+/-}$ and CRTAM$^{-/-}$ mice were mixed and transferred into RIP-mOVA Tg mice. In the draining LN, the number of OT-I CD8$^+$ T cells gradually increased during the 3 days after transfer. Notably, we found that more CRTAM$^{+/-}$ OT-I CD8$^+$ T cells accumulated than CRTAM$^{-/-}$ cells (Fig. 2*C*, *upper left panel*). There was a clear decrease in the percentage of CRTAM$^{-/-}$ OT-I cells in the draining LN on days 2 and 3 after cell transfer (Fig. 2*C*, *upper right panel*), but no difference was observed in control LN during the whole period (Fig. 2*C*, *lower right panel*). These results suggest that the number of Ag-specific CD8$^+$ T cells decreased in the draining LN during the induction of diabetes when OT-I CRTAM$^{-/-}$ CD8$^+$ T cells were transferred, and indicate that CRTAM expression may be critical for the maintenance of Ag-specific CD8$^+$ T cells in the draining LN. However, when a large number of cells was transferred into RIP-mOVA Tg mice, percentages of OT-I CRTAM$^{-/-}$ cells became almost the same to the CRTAM$^{+/-}$ cells (Fig. 2*D*). This result indicates that CRTAM efficiently may function when small numbers of Ag-specific T cells were transferred, probably reflecting a physiological condition. Because we found that Necl2 is not expressed in the islets of pancreas (data not shown), CTL effector function may be exhibited independently of Necl2 expression in the tissue of effector sites.

### *CRTAM is not involved in entry into and egress from LN*

We then analyzed the molecular basis of the reduction in the number of Ag-specific CD8$^+$ T cells in the draining LN in the diabetes system. Because there are three stages of CD8$^+$ T cell movement, entry into, accumulation within, and egress from LN, we analyzed the possibility of CRTAM involvement at each stage.

[5] The online version of this article contains supplemental material.

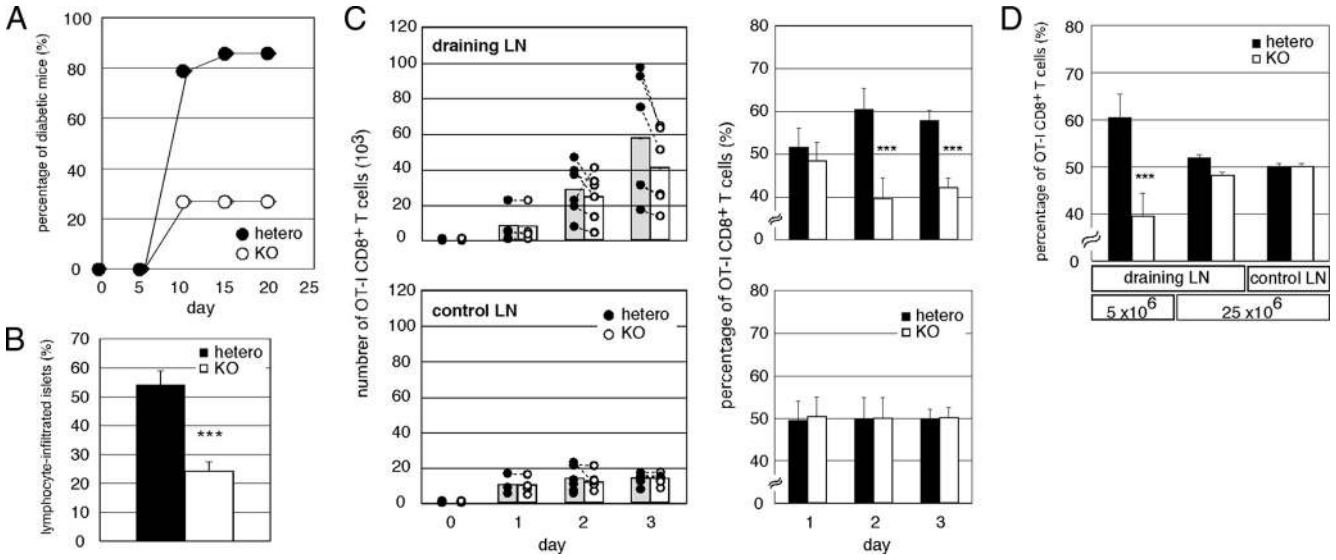


**FIGURE 2.** Impaired migration of $CD8^+$ T cells into inflammatory sites in $CRTAM^{-/-}$ mice. *A*, Impaired diabetes induction by $CRTAM^{-/-}$ T cells in RIP-mOVA Tg diabetes system. OT-I Tg $CRTAM^{+/-}$ (●) and $CRTAM^{-/-}$ (○) $CD8^+$ T cells were transferred into RIP-mOVA Tg mice, and diabetes induction was monitored for 4 wk by measuring blood glucose levels (hetero, $n = 18$; KO, $n = 19$). *B*, Frequency of lymphocyte-infiltrated islets in pancreas. In the system described in *A*, the mice were sacrificed 5 days after transfer, and pancreas was fixed and subjected to H&E staining. The frequencies of lymphocyte-infiltrated islets were quantified in whole pancreas. $CRTAM^{+/-}$ (■) and $CRTAM^{-/-}$ (□). *C*, Competitive migration analysis of $CRTAM^{+/-}$ and $CRTAM^{-/-}$ T cells in the diabetes model. Equal numbers of OT-I $CRTAM^{+/-}$ and $CRTAM^{-/-}$ $CD8^+$ T cells were transferred into RIP-mOVA Tg mice. *Left panels*, Show the cell number of individual animals (hetero, ●; KO, ○) in draining (*upper*) or control (*lower*) LN. *Right panels*, Indicate the percentage of either $CRTAM^{+/-}$ (■) or $CRTAM^{-/-}$ (□) cell types among total OT-I $CD8^+$ T cells within LNs at indicated day responses (***, $p < 0.001$; day 1, $n = 4$; day 2, $n = 6$; day 3, $n = 6$). Draining LN were renal LN in most cases, but pancreatic LN in some cases, and control LN are axillary LN. *D*, Transfer of higher dose of T cells overcomes the retention defect of the $CRTAM^{-/-}$ cells in the diabetes model. The mixture of the equal numbers (5 or $25 \times 10^6$) of OT-I $CRTAM^{+/-}$ (■) and $CRTAM^{-/-}$ (□) $CD8^+$ T cells was transferred into RIP-mOVA Tg mice. Two days later, the number of either type of OT-I $CD8^+$ T cells was quantified in the draining LN (renal) and control LN (axillary) ($n = 4$; ***, $p < 0.001$).

First, we checked cell migration under the nonstimulation condition. It has been shown that CCR7 and integrins, such as LFA-1 and $\alpha_4\beta_1$/VLA-4, are essential for lymphocyte entry (23, 24). However, the expression of these molecules on OT-I $CRTAM^{-/-}$ $CD8^+$ T cells was not altered (Fig. S4*A*). In addition, there was no difference in the number and proportion of $CD8^+$ T cells in various LNs in the steady state (Fig. S4*B*). Together with our observation that Necl2 is not expressed in high endothelial venule where lymphocytes enter LN (data not shown), these data suggest that CRTAM may not be involved in the entry and egress in LN under the steady state.

Next, to perform detailed analysis of the traffic of T cells in LN by using OT-I $CD8^+$ T cell transfer system, we first examined whether transferred OT-I $CRTAM^{-/-}$ $CD8^+$ T cells show normal distribution under the steady state (Fig. S5). We mixed equal numbers of OT-I $CD8^+$ T cells from $CRTAM^{+/-}$ and $CRTAM^{-/-}$ mice and transferred them into WT mice. Then, the number of OT-I $CD8^+$ T cells in LN was analyzed at various time courses. A sufficient number of OT-I T cells was detected at 3 h after transfer, and their number remained unchanged even at 24 h. The percentages of both cells were almost the same in the secondary lymphoid organs, lymph, and blood at 24 h after transfer. From these data, we confirmed that CRTAM is not involved in LN entry and egress under the steady state. Furthermore, these results indicate that the accumulation of OT-I $CRTAM^{+/-}$ $CD8^+$ T cells in the draining LN was induced only after the stimulation, consistent with the fact that CRTAM expression is induced upon T cell activation.

We next analyzed T cell migration under the stimulation condition in detail by blocking re-entry into or egress from LN. Regarding entry, we assumed that the reason that the number of OT-I $CRTAM^{-/-}$ $CD8^+$ T cells was not altered on day 1 when CRTAM expression reached a maximum (14) could be due to the presence of re-entered T cells. To this end, we blocked entry with anti-CD62L Ab (25) because the Ab is able to block the continuous supply of naive T cells and to synchronize Ag-specific T cell stimulation within the draining LN. Equal numbers of labeled OT-I $CD8^+$ T cells ($CRTAM^{+/-}$: CFSE and $CRTAM^{-/-}$: 5-(and 6-)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) were mixed and transferred into RIP-mOVA Tg mice. Three hours later, a sufficient number of Ag-specific T cells migrated to LN (Fig. S5), and anti-CD62L Ab was introduced into the peritoneal cavity. When the draining renal LN was analyzed 12 h later, both labeled OT-I T cells could be detected in the T cell area of LN, but the percentage of $CRTAM^{-/-}$ OT-I $CD8^+$ T cells was clearly lower than that of $CRTAM^{+/-}$ cells (Fig. 3, *A* and *B*). These results suggest that the defective accumulation of $CRTAM^{-/-}$ T cells in the draining LN may take place as soon as CRTAM expression is induced. Because activated OT-I T cells had not yet proliferated at this stage (data not shown), it was suggested that reduction of $CRTAM^{-/-}$ T cells in the draining LN was induced before cell proliferation.

We then analyzed egress of activated T cells from the draining LN. It has been shown that $S1P_1$ serves as an essential egress receptor and that its transient surface expression on activated T cells determines the egress from LN (1). It was also reported that CD69 negatively regulates $S1P_1$ expression (2). We confirmed normal $S1P_1$ and CD69 mRNA expression in $CRTAM^{-/-}$ $CD8^+$ T cells (Fig. S6) and normal CD69 expression on the surface of OT-I $CRTAM^{-/-}$ $CD8^+$ T cells in the draining LN when transferred into RIP-mOVA mice (Fig. S6). To directly examine the possibility of the involvement of CRTAM in the egress regulation, we blocked LN egress from LN by the treatment of mice with FTY720, a powerful S1P antagonist. If the reduction in the frequency of OT-I $CRTAM^{-/-}$ $CD8^+$ T cells within LN were due to

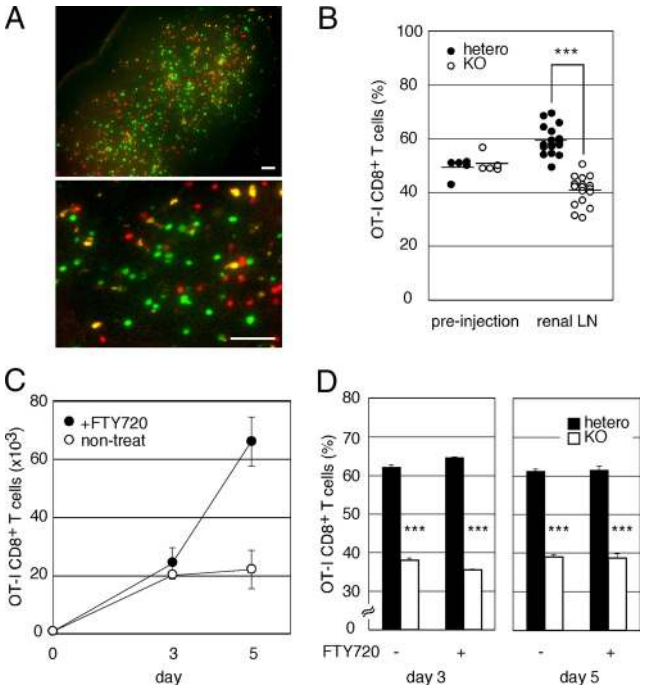


**FIGURE 3.** CRTAM functions for retention within, but not egress from the draining LN of activated T cells. *A*, Equal numbers of OT-I CRTAM$^{+/-}$ (CFSE-labeled cells; green) and CRTAM$^{-/-}$ CD8$^+$ T cells (CMTMR-labeled cells; red) were transferred into RIP-mOVA Tg mice. Three hours after the transfer, the recipient mice were treated with anti-CD62L Ab. Twelve hours later, draining renal LN was analyzed. Green dot indicates OT-I CRTAM$^{+/-}$ cells, and red dot indicates CRTAM$^{-/-}$ cells. *Upper panel*, low magnification; *lower one*, high magnification. Bars, 50 $\mu$m. *B*, CRTAM$^{-/-}$ OT-I CD8$^+$ T cells were less accumulated than CRTAM$^{+/-}$ cells in the draining LN. Labeled OT-I cells in the similar data of *A* were counted, and the percentage of heterozygous (●) and KO (○) cells was calculated. Seventeen fields were counted. *C* and *D*, CRTAM does not regulate cell egress from LN. OT-I cell transfer and analysis were performed in same way as Fig. 2*C*. Three hours after the transfer, recipient mice were treated with FTY720, and the treatment was continued until tissue preparation. *C*, Total number of OT-I cells in draining LN. The mixture of OT-I cells was transferred into RIP-mOVA Tg mice with (●) or without (○) FTY720 treatment. *D*, The percentage of each cell type in OT-I CD8$^+$ T cells on days 3 and 5 (hetero is represented by ■, and KO by □). ***, $p < 0.001$;

the enhanced egress, the number of OT-I CRTAM$^{-/-}$ cells would be the same as that of OT-I CRTAM$^{+/-}$ cells in recipient mice after the FTY720 treatment. The mixture of OT-I CRTAM$^{+/-}$ and CRTAM$^{-/-}$ CD8$^+$ T cells was transferred into RIP-mOVA Tg mice, and 3 h after the transfer, the recipient mice were treated with FTY720 to block lymphocyte egress. Total numbers of OT-I CD8$^+$ T cells were accumulated in the draining LN of FTY720-treated mice as compared with those of untreated mice (Fig. 3*C*). However, the percentage of Ag-specific CRTAM$^{-/-}$ T cells was similarly reduced regardless of FTY720 treatment as compared with CRTAM$^{+/-}$ T cells (Fig. 3*D*). These results suggest that OT-I CRTAM$^{-/-}$ CD8$^+$ T cells exhibit normal S1P$_1$-mediated egress from LN.

### *CRTAM regulates retention of CD8$^+$ T cells in LN*

Lastly, we investigated the involvement of CRTAM in CD8$^+$ T cell accumulation within the peripheral LN. Two closely related regulations, retention and proliferation, are involved in cell accumulation within the draining LN. If CRTAM contributes to one (or both) of these functions, the number of CRTAM$^{-/-}$ cells may decrease in the draining LN.

First, we investigated whether CRTAM is involved in cell retention within the draining LN. Because VCAM-1 expression in lymphatic sinusoids coincides with lymphocyte retention within inflamed LN (26), we addressed a similar possibility that the CRTAM-Necl2 interaction may function to retain T cells within the draining LN. To this end, we tried to identify Necl2-expressing cells that may interact with CRTAM$^+$ T cells and induce retention within LN. Although it has been previously reported that Necl2 is expressed on CD8$^+$ DCs (12) and naive T cells in spleen (27), it is still unclear which cells are the major source of the CRTAM ligand.

Immunohistological analysis revealed that Necl2 was widely expressed in the T cell area of LN, and some cells showed sporadically high expression (Fig. 4*A*). In LN of RAG-deficient mice, the basal level of Necl2 expression in the T cell area disappeared, but cells with high expression similarly remained (Fig. 4*B*, red cells), suggesting that Necl2 is expressed at very low levels in T cells and at high levels in some other cells. Because activated T cells showed immediate down-regulation of Necl2 expression (27), Necl2 on T cells may not function as a CRTAM ligand. The cells that strongly express Necl2 may be critical. Necl2 was not expressed on fibroblastic reticular cells as the T cell area meshwork (Fig. 4*B*, *upper panel*). Instead, most of the cells that strongly expressed Necl2 were CD11c$^+$ cells (Fig. 4*B*, *lower panel*). Although the majority of labeled T cells were localized without forming clusters, several OT-I T cells were found to be attached to morphologically DC-like cells (Fig. 4*C*). Because these cells express CD11c, these cells were confirmed to be DCs (Fig. S7). The frequency of the conjugate formation of CD8$^+$ T cells with DCs was higher in CRTAM$^{+/-}$ than in CRTAM$^{-/-}$ OT-I cells (Fig. 4*D*). We then examined identification of the DC subset expressing Necl2. Mouse DCs are basically defined by their expression of CD11c and the combination with other surface markers, such as CD4, CD8$\alpha$, and B220 (28), and Necl2 is expressed on CD8$^+$ DCs in both spleen and LN and on CD4$^-$CD8$^-$ DCs in LN (Fig. 4*E*). Thus, these DCs may interact with CD8$^+$ T cells and are involved in their function.

Therefore, we postulated that the interaction between CD8$^+$ T cells and CD8$^+$ DCs may induce the retention of CRTAM$^+$ T cells within the draining LN through the CRTAM-Necl2 interaction, and analyzed the interaction between these cells. Activated CRTAM$^{+/-}$ and CRTAM$^{-/-}$ CD8$^+$ T cells were labeled with different fluorescence markers, and equal numbers of these cells were mixed and cultured with CD8$^+$ or CD8$^-$ DCs. CD8$^+$ T cells that associated with DCs were analyzed in the presence or absence of blocking Abs to LFA-1 and VCAM-1, to clearly observe the contribution of CRTAM-Necl2 interaction. The association of CRTAM$^{-/-}$ CD8$^+$ T cells with CD8$^+$ DCs was severely impaired regardless of the presence of blocking Abs (Fig. 4*F*). In contrast, the association with CD8$^-$ DCs was not different between CRTAM$^{+/-}$ and CRTAM$^{-/-}$ T cells (Fig. S8). These results suggest that CRTAM$^+$ CD8$^+$ T cells bind to CD8$^+$ DCs for efficient retention. This also demonstrated that the CRTAM-Necl2 interaction appears to have unexpectedly high affinity, because the blocking of other integrin-mediated adhesions did not affect cell binding in these experiments. Furthermore, the results imply that once T cells are activated, neither DC activation nor specific Ag presentation is necessary for this interaction.

Next, we analyzed the effect of the CRTAM-Necl2 interaction on cell proliferation within LN as compared with its function in retention. Although CRTAM$^{-/-}$ CD8$^+$ T cells have normal proliferative ability in vitro (Fig. S2*A*), we addressed the possible

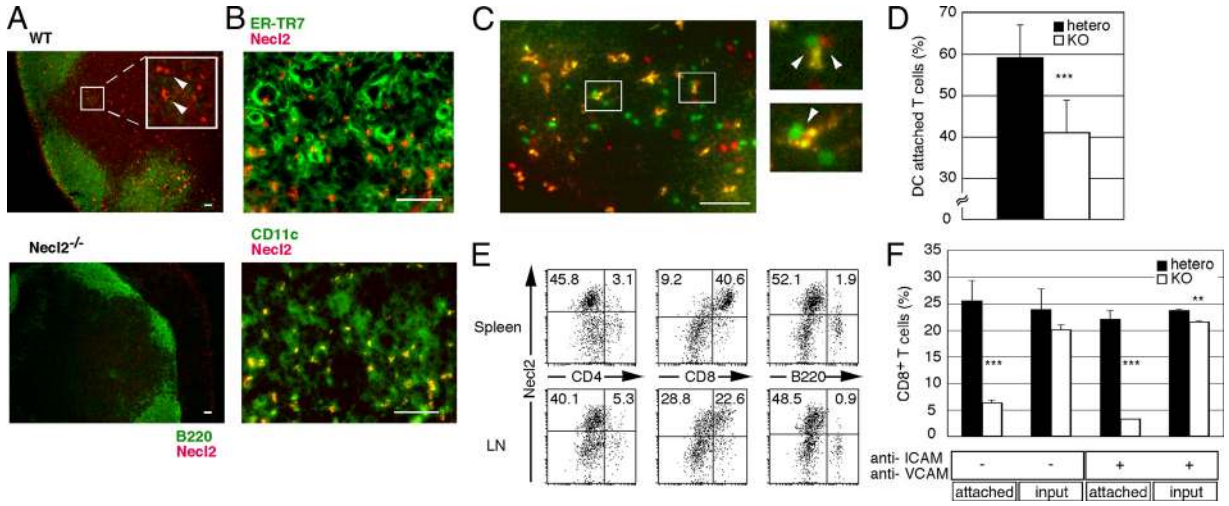


**FIGURE 4.** $CD8^+$ DCs strongly express Necl2 and efficiently bind to $CRTAM^+$ T cells. *A*, Immunohistology of LN. Frozen sections were stained with anti-Necl2 Ab (red) and anti-B220 Ab (green). *Inset*, Shows higher magnification of the indicated areas. Arrowheads represent cells strongly expressing Necl-2. Necl-$2^{-/-}$ LN was used as negative control staining (*lower panel*). Bars, 50 $\mu$m. *B*, RAG2-deficient LNs were stained with anti-Necl2 Ab (red) and anti-mouse fibroblast Ab (ER-TR7), which is a fibroblastic reticular cell marker (*upper panel*), or anti-CD11c Ab (*lower panel*). Bars, 50 $\mu$m. *C*, OT-I cell transfer and analysis were performed under the same protocol as Fig. 3*A*. DCs were detected by autofluorescence as well as CD11c staining (Fig. S7), and *right panels* show higher magnification of the indicated areas. Arrowheads indicate DCs attached to OT-I T cells. Bars, 50 $\mu$m. *D*, Ratio of T cells attached to DCs. The frequencies of each cell type were calculated for each field. We checked more than 15 fields and performed two independent experiments. *E*, CD11c-positive gated splenocytes (*upper panels*) and lymphocytes (*lower panels*) were developed Necl2-positive and $CD4^-$, $CD8^-$, or B220-positive cells, respectively. *F*, Specific binding of $CRTAM^+$ T cells to $CD8^+$ DCs. Equal numbers of stimulated $CRTAM^{+/-}$ (PE-labeled) or $CRTAM^{-/-}$ (allophycocyanin-labeled) $CD8^+$ T cells were incubated with $CD8^+$ DCs for 3 h in the presence or absence of other adhesion molecule-blocking Abs (anti-ICAM and anti-VCAM Abs). Frequency of activated T cells that associated with DCs was analyzed by flow cytometry. ***, $p < 0.001$.

function of CRTAM in T cell proliferation in vivo. To analyze T cell proliferation within the draining LN in the RIP-mOVA model, equal numbers of OT-I $CRTAM^{+/-}$ and $CRTAM^{-/-}$ $CD8^+$ T cells were mixed and transferred into RIP-mOVA Tg mice that had been treated with BrdU, and the draining LN was analyzed 3 days after transfer. The proportion of BrdU-positive proliferating $CRTAM^{-/-}$ cells was marginally reduced as compared with that of $CRTAM^{+/-}$ T cells under the competitive condition (Fig. 5*A*), suggesting that $CRTAM^{-/-}$ T cells may have a minimal defect in proliferation. This weak defect was also confirmed in proliferation experiments in which CFSE-labeled OT-I cells were transferred into RIP-Tg mice (Fig. 5*B*). $CRTAM^{-/-}$ $CD8^+$ T cells exhibited a slight delay in cell division particularly in the most rapidly dividing population when compared with $CRTAM^{+/-}$ cells in the draining LN. In contrast, no such difference was observed in the control LNs of unrelated sites. In addition, the number of dividing cells was also decreased in $CRTAM^{-/-}$ T cells in the draining LN (Fig. 5*B*). These results suggest that CRTAM contributes minimally to the efficient proliferation of $CD8^+$ T cells within the draining LN, but are not sufficient to explain the reduction in the number of $CRTAM^{-/-}$ T cells.

### *CRTAM mediates effective protective T cell responses in vivo*

Our data from the RIP-Tg diabetes model demonstrated that CRTAM is important for $CD8^+$ T cell accumulation within the draining LN through retention during the late phase of T cell activation. To confirm this result, we re-examined the critical role of CRTAM expression in the process of initial immune response, using a simpler and more general T cell transfer system.

A mixture of OT-I $CRTAM^{+/-}$ and $CRTAM^{-/-}$ T cells was transferred into normal recipient mice, and OVA peptide-pulsed splenic DCs were injected into footpad (Fig. 6). Three days after DC injection, both OT-I $CRTAM^{+/-}$ and $CRTAM^{-/-}$ $CD8^+$ T cells in the popliteal draining LN were analyzed. Similar to the results obtained in RIP-mOVA Tg system in Fig. 2*D*, when $5 \times 10^6$ OT-I cells were transferred, the percentages of OT-I $CRTAM^{+/-}$ and $CRTAM^{-/-}$ cells were not altered in the draining

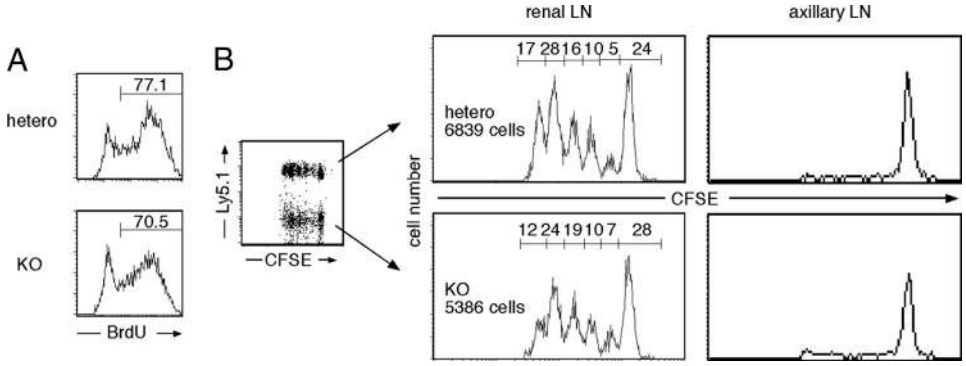


**FIGURE 5.** CRTAM may contribute minimally to proliferation within draining LN. OT-I cell transfer was performed in the same way as Fig. 2*C*. *A*, Recipient mice were given drinking water containing BrdU for 3 days. *B*, CFSE-labeled cells were transferred, and 2 days later, divided cells were analyzed.

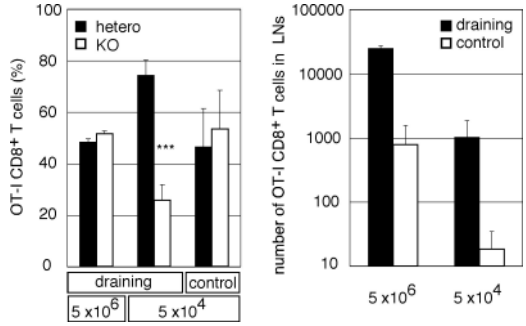


**FIGURE 6.** CRTAM functions effectively under physiological conditions. The effectiveness of CRTAM expression can be confirmed clearly when a small number of cells was activated in vivo. The cell mixture was prepared from OT-I Tg mice as in Fig. 2*C*, and $5 \times 10^6$ or $5 \times 10^4$ cells were transferred into recipient WT mice. Twenty-four hours later, OVA peptide-pulsed splenic DCs were injected into one side of footpad. Three days after DC injection, inguinal draining LN and control LN (opposite site of inguinal LN) were analyzed. *Left panel*, Shows percentage of each cell in OT-I $CD8^+$ T cells; *right panel*, shows total number of OT-I cells in LNs of both draining LN and control LN (***, $p < 0.001$; $5 \times 10^6$, $n = 4$; $5 \times 10^4$, $n = 6$).

LN. However, when $5 \times 10^4$ OT-I cells were transferred, the percentage of OT-I $CRTAM^{-/-}$ cells was clearly decreased in the draining LN (Fig. 6, *left panel*). In both cases, the number of OT-I T cells accumulated and increased within the draining LN when Ag-pulsed DCs were injected (Fig. 6, *right panel*). These results indicated that immune responses were induced in both situations with high and low number to T cells transferred, but CRTAM functions for retention within LN only when small number of T cells was transferred, which resembles physiological condition. This result confirmed the regulation in the diabetes model (Fig. 2*D*). These results suggest that the CRTAM-Necl2 interaction is important to establish the interaction between activated $CD8^+$ T cells and $CD8^+$ DCs before the cell proliferation phase, and imply that this interaction induces cell retention, particularly when a very small number of Ag-specific T cells relative to $CD8^+$ DCs is activated within the draining LN, which may represent the physiological condition.

Finally, we examined the function of CRTAM in protective immunity in vivo against viral infection in which CTLs play major roles. Because CTLs are important for the clearance of influenza virus (29, 30), we analyzed the protective immunity against influenza virus infection. $CRTAM^{+/-}$ and $CRTAM^{-/-}$ mice were intranasally infected with influenza virus, and virus clearance was analyzed in the lung. Viral titer at day 6 after infection was much higher in $CRTAM^{-/-}$ mice than in $CRTAM^{+/-}$ mice, indicating the impaired viral clearance in the lung (Fig. S9*A*). We also analyzed influenza-specific $CD8^+$ T cells in the lung and found that the number of influenza-specific $CD8^+$ T cells was decreased in $CRTAM^{-/-}$ mice, suggesting that sufficient number of $CRTAM^{-/-}$ T cells could not migrate to effector sites (Fig. S9*B*). In contrast, when the specific cytotoxic function was examined in spleen, $CRTAM^{-/-}$ mice had normal ability of virus-specific killing (Fig. S9*C*). These results are quite similar to those of the diabetes model, and suggest that CRTAM may mediate the same function in this virus infection model.

Together, these results demonstrate that CRTAM is critically involved in in vivo CTL mediated in protective immunity against viral infection through delivering sufficient CTLs.

## Discussion

We analyzed the in vivo function of the CRTAM-Necl2 interaction, which is a unique heterotypic binding specific to lymphocytes, by generating $CRTAM^{-/-}$ mice. Because CRTAM is expressed predominantly on activated $CD8^+$ T cells and we found that Necl2 is expressed strongly on $CD8^+$ DCs, analyses of $CRTAM^{-/-}$ T cells revealed the new functions of the CRTAM-Necl2 interaction between $CD8^+$ T cells and $CD8^+$ DCs for the retention of $CD8^+$ T cells in LN. Transient CRTAM expression upon Ag stimulation is critical for the accumulation of activated $CD8^+$ T cells within the draining LN, and consequently, for efficient development of effector cells. It is known that CD69 is similarly expressed at early stimulation period and down-regulates $S1P_1$ surface expression, resulting in the negative regulation of egress by retaining activated lymphocytes within the draining LN. Thus, both CRTAM and CD69 are immediate early markers of T cell activation, and both function in the retention of T cells within LN, albeit through a different mechanism, as follows: CRTAM functions in sustaining adhesion to DCs and CD69 functions in directly inhibiting egress.

The CRTAM ligand Necl2 is strongly expressed on $CD8^+$ DCs in spleen and LN (Fig. 4*E*). Because $CD8^+$ DCs reside in T cell area of LN and interact with $CD8^+$ T cells particularly for cross-presentation (31, 32), the expression of Necl2 on $CD8^+$ DCs may provide an effective system for interaction with $CD8^+$ T cells. However, the CRTAM-Necl2 interaction might not be important for cross-presentation directly, because CRTAM is transiently expressed in the late activation phase after stimulation. Therefore, it is reasonable that CRTAM may serve as a positive regulator of T cell retention within LN by interacting with Necl2 on $CD8^+$ DCs in the T cell area. It should be noted that DC activation and Ag presentation are not required for this interaction. Thus, it is postulated that $Necl2^+$ $CD8^+$ DCs provide a niche for Ag-specific $CD8^+$ T cells to maintain stable contact to induce signals for T cell proliferation and maturation during the CRTAM-expressing period after stimulation in the draining LN. Furthermore, the CRTAM-induced accumulation of $CD8^+$ T cells within the draining LN is mediated by retention rather than proliferation. The reduced cell number of $CRTAM^{-/-}$ $CD8^+$ T cells in the draining LN just 1 day after transfer was clearly observed. Because activated T cells had not proliferated at this stage, these results clearly indicate that part of activated $CRTAM^{-/-}$ $CD8^+$ T cells could not retain within the draining LN, and may spill out from LN before proliferation and maturation. Consistently, it is likely that the small reduction of BrdU uptake and the slight delay in cell division (Fig. 5) were caused by the failure of keeping activated T cells within the draining LN before proliferation (Fig. 7).

Our finding that the CRTAM-mediated retention in LN was optimized when a small number of Ag-specific $CD8^+$ T cells was present within the LNs is critical to elucidate the physiological function of CRTAM. We observed this in in vivo experiments (Fig. 6): $CRTAM^{+/-}$ $CD8^+$ T cells were retained effectively only when a small number of cells was transferred into recipient mice, and no difference was observed when large numbers of T cells were transferred. In the case of the RIP-mOVA Tg diabetes model, although no difference in the retention in LN was observed in $CRTAM^{-/-}$ T cells at an early time point (day 1) after cell transfer in vivo, the retention difference was readily observed when the continuous entry into LN was inhibited and T cell activation was synchronized by administering anti-CD62L Ab. A similar physiological situation may take place in the RIP-mOVA Tg system, where the number of Ag-specific T cells was very small in renal LN. Under normal conditions, immune responses start from a

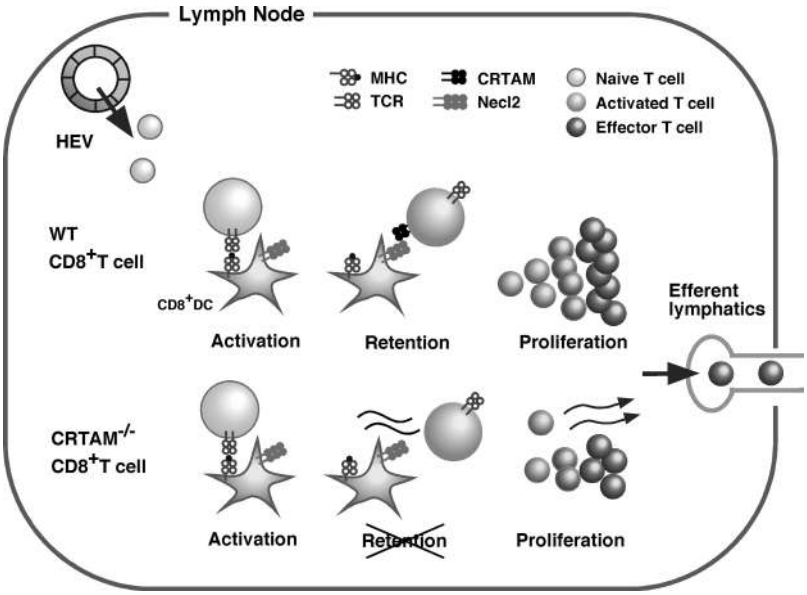


**FIGURE 7.** A model of the CRTAM-mediated retention mechanism of $CD8^+$ T cells in draining LN. Activated $CD8^+$ T cells transiently express CRTAM, and retain within a draining LN during this period by interacting with Necl2 expressed on $CD8^+$ DCs present in the T cell area of LN. Whereas activation is mediated by Ag/MHC-bearing $CD8^+$ DCs, the retention regulation is mediated by $Necl2^+$ $CD8^+$ DCs in an Ag/MHC-independent manner. This interaction induces retention and resulted proliferation and functional differentiation of T cells. In the case of CRTAM-deficient $CD8^+$ T cells, these T cells fail to retain within the draining LN due to the lack of the interaction with $Necl2^+$ DCs, and may spill out from LN to the periphery as immature stage of T cells before proliferation and differentiation.

small number of Ag-specific T cells in the draining LN, and the cells exhibit immediate and extensive clonal expansion until they reach a sufficient number to remove Ag/pathogen. Therefore, CRTAM may play an important role in T cell retention in the draining LN during physiological immune responses.

In contrast, CRTAM function may be attributed to the intracellular signaling capacity of CRTAM itself. CRTAM has a C-terminal PDZ binding site that may function in this signal transduction (14). Recently, it was reported that CRTAM is associated with Scrib, and influences the immune response through the maintenance of cellular polarity (27). This report strengthened the concept of signal transduction mediated by CRTAM. However, our results showed differences in terms of cytokine production and cell proliferation. We did not observe any differences in these functions in $CRTAM^{-/-}$ T cells (Fig. S2). We also examined IFN-$\gamma$ production carefully using $CD8^+$ DCs and $CD8^-$ DCs as APCs, because the heterogeneity of APCs could make a difference. However, both DCs induced equal levels of IFN-$\gamma$ production in both OT-I $CRTAM^{+/-}$ and $CRTAM^{-/-}$ $CD8^+$ T cells (our unpublished observation). Our results reveal that $CRTAM^{-/-}$ T cells can normally produce IL-2 and IFN-$\gamma$ in vitro. The basis of these differences is still unclear, but some differences, including genetic background of mice, might have an effect.

T cell priming by DCs in LN has been demonstrated to occur in three distinct phases (9). At the first phase, T cells form transient and multiple contacts with DCs. By phase II, T cells form long stable conjugates with DCs and begin to secrete IL-2 and IFN-$\gamma$. In phase III, T cells dissociate from DCs, migrate rapidly, and proliferate vigorously. CRTAM expression reaches a maximum at the late stage of phase II (16–24 h) after cytokine secretion begins. In this phase, termed "swarming" in another report, T cells move slowly in a looping pattern within a local area, and most cells swarm around DCs (10). It was suggested that the swarming action may augment previous Ag signals and may represent an additional checkpoint for further T cell maturation. However, the role of cell swarming in the late stage of phase II is still unclear. Our data strongly suggest that CRTAM plays an important role in the communication with $CD8^+$ DCs at this stage and in augmenting previously experienced Ag signals, as well as in enhancing efficient cell proliferation. CRTAM may not be directly involved in cytokine production because CRTAM expression is induced after signal induction of cytokine secretion.

In $CRTAM^{-/-}$ mice, part of the activated $CD8^+$ T cells may leave $CD8^+$ DCs before reaching sufficient activation and maturity due to the lack of the CRTAM-Necl2 interaction, and therefore, cannot proliferate to a sufficient cell number. This may result in impaired migration of CTLs to effector sites, thereby leading to impaired immunity against viral infection in lung and against diabetes in pancreatic islet. Because Necl2 expression was not detected in all these effector sites, CRTAM may regulate retention within the draining LN by interacting with Necl2-expressing $CD8^+$ DCs rather than migration to effector sites independently of Necl2 expression in the effector tissues.

Collectively, CRTAM-mediated regulation of retention within LN may provide a distinct, yet complementary mechanism to the $S1P_1$-mediated egress regulatory system for mature activated lymphocytes. Particularly, it is important that CRTAM functions at not the initial phase, but the late phase of T cell activation by modulating triggered responses. Thus, the modulation of CRTAM-mediated retention enhancement of CTLs may promote protective immunity against viral infection as well as prevent CTL-mediated autoimmunity.

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## Disclosures

The authors have no financial conflict of interest.

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