The Exocytosis of Lytic Granules Is Impaired in Vti1b- or Vamp8-Deficient CTL Leading to a Reduced Cytotoxic Activity following Antigen-Specific Activation

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The Exocytosis of Lytic Granules Is Impaired in Vti1b- or Vamp8-Deficient CTL Leading to a Reduced Cytotoxic Activity following Antigen-Specific Activation

Ralf Dressel,* Leslie Elsner,* Peter Novota,*1 Namita Kanwar,†,2 and Gabriele Fischer von Mollard†

The exocytosis of cytotoxic proteins stored in lytic granules of activated CTL is a key event during killing of target cells. Membrane fusion events that are mediated by soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins are crucial, as demonstrated by patients with familial hemophagocytic lymphohistiocytosis type 4 who have mutations in the SNARE protein syntaxin-11 that result in an impaired degranulation of cytotoxic cells. We found an increased mRNA expression of the SNARE protein genes Vti1b and Vamp8 during Ag-specific activation of CTL from TCR-transgenic OT-I mice. Therefore, we investigated the cytolytic activity of CTL from TCR-transgenic OT-I mice. At 3 d as well as at 4 d of Ag-specific stimulation, the degranulation of CTL was significantly reduced in Vti1b and Vamp8 knockout mice, as determined by cell surface expression of the degranulation marker CD107a. After 3 d of Ag-specific stimulation, the cytolytic activity of Vti1b- and Vamp8-deficient CTL was reduced to ≈50% compared with heterozygous controls. However, 4 d after stimulation, the cytotoxic activity of Vti1b- as well as Vamp8-deficient CTL was not impaired anymore. The capacity of Vti1b- and Vamp8-deficient dendritic cells to process Ags and to stimulate the proliferation of CTL was not reduced, arguing against an indirect effect on the activation of CTL. These findings suggest a role of the SNARE proteins vti1b and vesicle-associated membrane protein 8 in the degranulation of CTL. However, a deficiency can apparently be compensated and affects only transiently the cytotoxic activity of CTL during their development to armed effector cells. The Journal of Immunology, 2010, 185: 1005–1014.

CTL are important effector cells in the immune system that kill virus-infected or malignant target cells after recognition of specific Ags displayed by MHC class I molecules. A key event during killing of target cells is the exocytosis of cytotoxic proteins, such as perforin and granzymes, stored in lytic granules of CTL and NK cells (1). Lytic granules are specialized secretory lysosomes in cytotoxic cells (2). The exocytosis of these intracellular vesicles is a complex and tightly regulated process (2–4). It requires the polarization of granules toward the immunological synapse. The lytic granules move along microtubules to the contact sites between CTL and target cells and dock at the plasma membrane before fusion. The final fusion step of vesicles in general is mediated by specific sets of soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins localized on both vesicular and target membranes (5). They form complexes of four α-helices, one from each SNARE subfamily called R-, Qa-, Qb-, and Qc-SNAREs (6).

A number of genetic diseases and animal models have been described that demonstrate a critical role of granule exocytosis for the function of cytotoxic cells. In cytotoxic cells of Hermansky-Pudlack syndrome type 2 patients, lytic granules are unable to move to the immunological synapse due to a lack of the adapter protein 3 complex that can be caused by a mutation in the gene AP3B1 (7). Exocytosis of lytic granules is defective in Griscelli syndrome type 2 patients (8) and ashen mice (9) due to mutations in the Rab27a or Rab27a gene, respectively. Myosin-IIA (10) and dynamin-2 (11) have been shown to be required for lytic granule exocytosis in NK cells and might have the same function in CTL. Lytic granule exocytosis is also impaired in familial hemophagocytic lymphohistiocytosis (FHL) type 3 patients who have mutations in the Munc13-4 protein encoded by the gene UNC13D (12). These defects impair the lytic granule maturation and exocytosis (13) before the final fusion of the vesicles with the plasma membrane. The importance of SNARE proteins for the fusion of lytic granules (14) has been demonstrated for the Qa-SNARE syntaxin-11. Mutations in the STX11 gene that are associated with a complete loss or truncate version of syntaxin-11 cause FHL type 4 (15). In NK cells derived from these patients, degranulation was impaired, even though the lytic granules did polarize toward the immunological synapse upon activation (15). This confirms that syntaxin-11 functions directly in the secretion of lytic granules. Interestingly, FHL type 5 that is caused by a defect in the STXBPA2 gene encoding Munc18-2, which binds to syntaxin-11 and has a role in regulating SNARE activity, and a deficiency leads to impaired lytic granule exocytosis (16,17).
Other SNAREs, which have been implicated in lysosomal trafficking and fusion of secretory lysosomes, are good candidates for analyzing of their role in lytic granule fusion. A SNARE complex consisting of vesicle-associated membrane protein (VAMP) 7, syntaxin-7, syntaxin-8, and vti1b functions in transport to the lysosome (18). VAMP8, syntaxin-7, syntaxin-8, and vti1b are involved in fusion with late endosomes (19). VAMP8 also has a role in exocytosis of organelles formed in the secretory pathway, such as exocrine granules and secretory lysosomes (20) [e.g., secretory granules from mast cells (21, 22)]. In plateau, VAMP8 is involved in exocytosis of dense granules, α-granules, and lysosomes (23). Recently, VAMP8 has also been suggested to be involved in granule exocytosis from CTL (24). In addition, VAMP7 has been reported to be essential for granule exocytosis by a NK cell line (25), but its role in primary killer cells has not been determined.

In this study, we show that the R-SNARE VAMP8 and the Qb-SNARE vti1b affect the function of peptide-specific CTL.

Materials and Methods

**Mice and genotyping**

**Vti1b** (26) and **Vamp8** (27) knockout mice as well as TCR-transgenic OT-1 mice (28) were maintained on a mixed genetic background of 129Svl and C57BL/6 strains. Heterozygous (Vti1b<sup>+/−</sup> and Vamp8<sup>+/−</sup>) TCR-transgenic animals were obtained by crossing Vti1b<sup>−/−</sup> and Vamp8<sup>−/−</sup> mice with OT-1 mice and used for further breeding. Only adult mice were used for experiments. All animal experiments had been approved by the local government and were in accordance with institutional guidelines for the welfare of animals. DNA was obtained for genotyping from tail biopsies. Primers specific for the **Vamp8** wild-type allele (5′-CCGAAACAGAC-AGAGGACTTG-3′) and 5′-CGCTGCCCAGACCCTTGCCC-3′ were used to amplify a 510-bp fragment. For the detection of the **Vti1b** mutant allele (5′-CGTTAGGAATGGAGCAGTTGAC-3′) and 5′-CGCTGCCCAGACCCTTGCCC-3′), the amplified PCR product for the wild-type allele (5′-ATACC-TTCTCTTGCACTATCCAG-3′ and 5′-TACAAATGTACAGTCTTCGGA-3′) were used to amplify a 510-bp fragment. For the detection of the **Vti1b** allele disrupted by a neomycin resistance cassette, a forward primer (5′-TACAAATGTATCGATTCTTCG-3′) that annealed to the **Vti1b** gene was used. The reverse primer was specific for the neo<sup>+</sup> cassette (5′-CCTCCGGTCGACATACCTTGG-3′). The amplified PCR product for the **Vti1b** mutant allele was 410 bp. The expression of the transgenic TCR was tested by flow cytometry on blood samples.

**Cell culture**

RMA cells (H2<sup>b</sup>) were used as target cells for CTL and maintained in NaHCO<sub>3</sub>-buffered DMEM supplemented with 10% FCS (Biochrom, Berlin, Germany), 2 mM l-glutamine, 1 mM sodium pyruvate, 50 μM l-α-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), and all cell culture material from Sarstedt (Nümbrecht, Germany), unless specified. To generate peptide-specific CTL, lymphocytes were obtained from spleen of TCR-transgenic mice (28) using a Tenbroeck homogenizer. Erythrocytes were removed from splenocytes by incubation for 5 min in lysis buffer (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA, pH 7.4–7.8). Then the cells were cultured in round-bottomed microtiter plates (Nunc, Wiesbaden, Germany) in fully supplemented DMEM in the presence of 20 ng/ml IL-2 (ImmunoTools, Friesoythe, Germany), 10−8 M 2-ME, and Ag. As Ag, either the OVA protein (Sigma-Aldrich) or the SINIFEKEL peptide (OVA, 257–264; Bachem Biochemica, Heidelberg, Germany) was used at the indicated concentrations. The cells were harvested and used as effector cells after 3–5 d or restimulated under the same conditions. For restimulation, irradiated splenocytes (25 Gv) of the haplotype H2<sup>b</sup> were added as feeder cells to the culture at a 1:1 ratio. In these experiments, the SINIFEKEL peptide was used as Ag.

**Separation, labeling, and coculture of CD8<sup>+</sup> T cells and dendritic cells**

To obtain CD11c<sup>+</sup> dendritic cells (DCs), spleens were injected with 500 μl collagenase (2 mg/ml), cut into pieces, and incubated in HEPS-buffered DMEM for 30 min at 37°C. Afterward, the cells were passed through a 100-μm cell strainer (BD Biosciences, Heidelberg, Germany) and washed with PBS. DCs were isolated using CD11c MicroBeads (130-052-001; Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The DCs were cultured in round-bottomed microtiter plates (2 × 10<sup>4</sup> CD11c-positive cells per well) in supplemented DMEM with 20 ng/ml IL-4, 20 ng/ml IFN-γ (both purchased from Immunotools), and 10−3 M 2-ME. On the next day, CD8<sup>+</sup> T cells (10<sup>5</sup> cells/well) were given to the DC cultures together with 20 ng/ml IL-2. The Ag (SIINFEKL or OVA) was added at the indicated concentrations. CD8<sup>+</sup> T cells were separated from splenocytes of TCR-transgenic mice using an indirect magnetic labeling system for the isolation of untouched CD8<sup>+</sup> CTL, according to the manufacturer’s instructions (130-090-859; Miltenyi Biotec). The CD8<sup>+</sup> T cells were additionally depleted from DCs using CD11c MicroBeads, to avoid trace contaminations with DCs. The purity of the isolated cell populations was always controlled by flow cytometry. In some experiments, splenocytes or isolated CD8<sup>+</sup> T cells were labeled by the dye CFSE (C-1157; Invitrogen, Karlsruhe, Germany) before culture. Between 5 and 10 × 10<sup>6</sup> cells/ml were incubated for 5 min with 5 μM CFSE in PBS/0.1% BSA at 37°C. After being washed three times with HEPS-buffered DMEM containing 10% FCS, the cells were cultured, as described above.

**51 Cr release assay**

Target cells were labeled by incubating 1 × 10<sup>6</sup> cells in 200 μl HEPS-buffered DMEM containing 100 μl FCS and 50 μCi Na<sup>141</sup>CrO<sub>4</sub> (Hartmann Analytic, Braunschweig, Germany) for 1 h at 37°C and washed three times with HEPS-buffered DMEM. Effector cells were added to 5 × 10<sup>5</sup> CFSE-labeled target cells in triplicate at various ratios in 200 μl HEPS-buffered DMEM with 10% FCS well of round-bottomed microtiter plates. Spontaneous release was determined by incubation of target cells in the absence of CTL. To allow for a peptide-specific killing, the respective wells were supplemented with 0.5 μg/ml SIINFEKL. To determine calcium dependency of killing, 2 mM EGTA and 4 mM MgCl<sub>2</sub> were added. The microtiter plates were centrifuged for 5 min at 37°C for 4 h, and then centrifuged again. Supernatant and sediment were separately taken to determine radioactivity in each well using a Wallac MicroBeta Trilux counter (PerkinElmer Life Sciences, Köln, Germany). Percentage of specific lysis was calculated by subtracting percentage of spontaneous 51 Cr release (29).

**Degranulation assay**

To determine degranulation of CTL (30), 5 × 10<sup>5</sup> RMA cells were incubated at 37°C in a 1:10 ratio with activated TCR-transgenic CTL in presence or absence of the SIINFEKL peptide (0.5 μg/ml). A FITC-labeled anti-CD107a mAb (clone 1D4B, rat IgG2a; BD Biosciences) or the respective isotype control was added to parallel vials. After 4 h, the cells were harvested, stained with an anti-CD8a mAb (clone CT-8a, rat IgG2a; Caltag Laboratories, Hamburg, Germany), and further analyzed by flow cytometry.

**Flow cytometry**

Flow cytometry was performed on FACScan or FACSCalibur flow cytometers with CellQuest or CellQuestPro software (BD Biosciences). Ten thousand to 50,000 cells per sample were counted. Lymphocytes were gated on the basis of forward scatter/size scatter characteristics. Blood samples were directly stained before lysis of erythrocytes with FACS lysing solution (BD Biosciences), according to the manufacturer’s instruction. Splenocytes, MACS-separated cell populations, or precultured CTL were resuspended in 100 μl PBS before being stained using 1 μg respective mAbs: anti-CD4 (clone CT-CD4, rat IgG2a), anti-CD8a (clone CT-CD8a, rat IgG2a), anti-CD11c (clone N418, hamster IgG), anti-CD69 (clone H1.2F3, hamster IgG), and anti–I–A<sup>β</sup> (clone 25-5-16S, mouse IgM). These mAbs as well as appropriate isotype controls were purchased from Caltag Laboratories. An anti-TCRβ<sub>5.1,5.2</sub> (clone MR9-4, mouse IgGl) was obtained from BD Biosciences. The cells were stained for 30 min at 4°C before being washed and resuspended in 200 μl PBS. The proliferation of CFSE-labeled cells was determined by the reduction of the fluorescence intensity of the dye in every cell division.

**Gene expression analysis**

For gene expression analyses, RNA was prepared from splenocytes or separated CD8<sup>+</sup> T cells before or after Ag-specific stimulation using TRI-ZOL reagent (Invitrogen), according to the manufacturer’s instructions. The extracted RNA was treated with RNase-free DNase I (RQ1; Promega, Mannheim, Germany) for 20 min at 37°C and purified by phenol-chloroform-isooamyl alcohol extraction. The reverse transcription of RNA was performed for 75 min at 37°C with Moloney murine leukemia virus reverse-transcriptase polymerase and random oligo primers (Promega) in a total volume of 25 μl. The expression levels of Vti1b and Vamp8 were measured.
transcripts were analyzed by real-time PCR assays using the following forward and reverse primers: Vti1b (5'-CATCTTGGTCGCTCTGTTGF-3' and 5'-AGGCCACAGCAGACAACAF-3') and Vamp8 (5'-CCCTCTCTCTTCCCTCCA-3' and 5'-TCTCTCAGGGATCAAG-3'). The mRNA expression of the housekeeping gene Hprt1 (5'-GTTGCTTG-TGCGATCTGCTCTTA-3' and 5'-GGGACGAGCACTGACATT-3') was always monitored as internal control. Amplification reactions were carried out in 96-well plates in 25 μl reaction volumes with the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The PCR reactions were preheated for 2 min at 95°C and for 10 min at 95°C, followed by 40 cycles of denaturation (15 s at 95°C) and amplification (1 min at 60°C). All reactions were performed in triplicates using an ABI 7500 Real Time PCR System. For the data analysis, the ABI 7500 SDS software (Applied Biosystems) was used. The variations in the cDNA concentration in different samples were normalized to the housekeeping gene Hprt1. The cycle threshold (ct) values obtained for the genes of interest were corrected by the ct value obtained for Hprt1 in the same sample. The relative level of transcripts was then expressed as Δct value (ct for Hprt1 minus ct for the gene of interest) and the fold-change of transcripts between naive and Ag-stimulated cells was calculated as 2^Δct at day 3 - 2^Δct at day 0.

Statistics

All data were analyzed using WinSTAT software (R. Fitch Software, Bad Krozingen, Germany). ANOVA was used to analyze designs involving two factors. The different factors were incorporated into two-way ANOVA involving interactions. Student t test was used for the analysis of two samples. A significance level of α = 0.05 was used. Adjustments for multiple comparisons were performed where appropriate due to subgroup testing.

Results

Vti1b and Vamp8 transcripts are up-regulated during the Ag-specific stimulation of CTL

TCR-transgenic mice are a convenient tool to investigate the function of CTL with defined Ag specificity. In OT-I mice, the vast majority of CD8+ T cells are specific for the peptide SIINFEKL derived from OVA and presented by H2Kb molecules (28). The cytotoxic activity of the CTL depends on the stimulation of naive CD8+ T cells with the Ag. Both the SIINFEKL peptide and the complete OVA can be added to cultures of splenocytes derived from OT-I mice to obtain activated CTL within 3–5 d (Fig. 1A). In the effector phase, the cytotoxic activity depends on the presence of the H2Kb-SIINFEKL complexes on target cells. Cells that express H2Kb, but not OVA, such as RMA cells, can be pulsed with the SIINFEKL peptide during a 4-h cytotoxicity test to allow for killing (Fig. 1A). The killing of targets is mediated by the calcium-dependent granule-exocytosis pathway because it is completely inhibited by EGTA, as shown in Fig. 1A. Therefore, this system allows for studying the biology of granule exocytosis in peptide-specific CTL.

To study the role of SNARE proteins in granule exocytosis, we compared the expression of Vti1b and Vamp8 transcripts in splenocytes and isolated CD8+ T cells derived from OT-I mice before and after stimulation with SIINFEKL for 3 d. The abundance of both transcripts was increased in splenocytes and in isolated CD8+ T cells after stimulation with the Ag (Fig. 1B).

The cytotoxic activity of Vti1b- and Vamp8-deficient CTL is impaired

We crossed OT-I mice with Vti1b and Vamp8 knockout mice to obtain CTL with defined Ag specificity. Splenocytes from these TCR-transgenic Vti1b+/+, Vti1b−/−, Vamp8+/+, Vamp8−/−, and wild-type OT-I mice were obtained and cultured in the presence of Ag (1 nM SIINFEKL) before being used as effector cells in 51Cr release assays against RMA target cells that were pulsed with the

FIGURE 1. Vti1b and Vamp8 mRNA expression increases during Ag-specific activation of CTL. A. A representative experiment is shown demonstrating the specific cytotoxic activity of Ag-stimulated CTL derived from TCR-transgenic OT-I mice. Splenocytes were stimulated for 4 d in the presence of IL-2 (20 ng/ml) either without Ag (w/o Ag), with 1 μM OVA protein, or with 1 nM SIINFEKL peptide. The cultured splenocytes were used as effector cells against RMA target cells (H2Kb). The means of specific lysis of triplicates plus SD at different E:T ratios (10:1 to 2.0:1) are shown as measured in a 51Cr release assay. During the 4-h cytotoxicity test, the SIINFEKL peptide (0.5 μg/ml) or no Ag (w/o Ag) was present. To confirm the granule exocytosis dependency of killing, EGTA was added to some samples. For the negative controls without Ag or plus EGTA in the test, only the results of the first three titer steps were determined. B. To determine the expression of the Vti1b and Vamp8 genes, mRNA was isolated from splenocytes of naive OT-I mice at day 0 and after culture for 3 d in presence of SIINFEKL, inducing maturation of armed effector CTL. To further restrict the expression analysis to CTL, CD8+ T cells also were purified by MACS separation at day 0 or 3. The expression levels of Vti1b and Vamp8 transcripts were determined in comparison with the housekeeping gene Hprt1 by real-time PCR assays. Means of Δct values (Hprt minus gene of interest) of triplicates and ΔΔct values (mean Δct at day 3 minus mean Δct at day 0) were calculated. The mean fold-change plus SD of transcripts from days 0 to 3 (2^ΔΔct) is shown as determined in three independent experiments.
FIGURE 2. The acquisition of full cytotoxic activity is decelerated in Vti1b- and Vamp8-deficient mice. A and C. Splenocytes of TCR-transgenic OT-I wild-type (wt), Vti1b−−, Vti1b+−, Vamp8+/−, and Vamp8−− mice were stimulated for 4 (A) or 3 d (C) with 1 nM SIINFEKL peptide. The cultured splenocytes were used as effector cells against RMA target cells in 51Cr release assays in presence of the SIINFEKL peptide (0.5 μg/ml). Each individual test was done in triplicates. The means of specific lysis plus SEM are shown to summarize these experiments. Controls that confirm the peptide and calcium dependency of killing were always performed, but are not shown in this summary. B and D. The proportion of specific CD8+ T cells expressing the transgenic TCR (belonging to the Vβ5.1,5.2 family) among the effector cells used in the cytotoxicity tests shown in A and C was determined by flow cytometry. The means plus SEM are shown for the various genotypes.

SIINFEKL peptide. Tests with CTL from wild-type OT-I indicated that a 4-d culture was optimal for the activation of the CTL under these conditions (data not shown). The cytotoxic activity of Vti1b- or Vamp8-deficient CTL was not impaired after 4 d of stimulation, as summarized in Fig. 2A. The killing of targets was peptide dependent and could be inhibited by EGTA (data not shown). The proportion of CD8+ T cells expressing the transgenic TCR (that can be stained with an anti-TCR Vβ 5.1,5.2 mAb) in the splenocyte cultures was similar for the various genotypes (Fig. 2B), excluding the possibility that an effect on cytotoxicity was masked by an increased proportion of Ag-specific CTL in the splenocyte cultures. We then tested the CTL activity after a 3-d culture. In these experiments, the cytotoxic activity of Vti1b- and Vamp8-deficient CTL was impaired, as summarized in Fig. 2C. The differences of heterozygous controls compared with Vti1b (p = 0.00017, ANOVA)- and Vamp8 (p = 0.01743, ANOVA)-deficient CTL were statistically significant. On average, the cytolytic activity of the CTL was reduced to ≈50–60% compared with heterozygous controls or wild-type CTL. This effect was not due to a reduced proportion of Ag-specific CTL in the splenocyte cultures derived from the knockout mice (Fig. 2D). Thus, the maturation of naive CTL to armed CTL appears to be decelerated in Vti1b- and Vamp8-deficient mice, but they are able to generate fully active CTL.

To confirm this finding, we restimulated CTL from TCR-transgenic Vti1b−− mice and Vti1b+/− controls after a first 4-d culture for additional 4 d and found now an almost identical cytotoxic activity (Fig. 3A) and proportion of specific CTL (Fig. 3B) with even less variation compared with the previous experiments (Fig. 2A). Two independent restimulation experiments comparing CTL from Vamp8−− and Vamp8+/− mice gave similar results (data not shown).

The Ag-specific degranulation of Vti1b- and Vamp8-deficient CTL is reduced

To determine an effect of SNARE protein deficiency on the degranulation of CTL, we performed Ag-specific degranulation assays. The CTL were exposed to RMA target cells in the presence or

FIGURE 3. The cytotoxic activity of Vti1b-deficient mature effector CTL is not impaired. A. Splenocytes of TCR-transgenic Vti1b−− (n = 9) and Vti1b+/− mice (n = 14) were stimulated for 4 d and restimulated for additional 4 d with 1 nM SIINFEKL peptide. The cultured splenocytes were used as effector cells against RMA target cells in 51Cr release assays in presence of the SIINFEKL peptide (0.5 μg/ml). Each individual test was done in triplicates. The means of specific lysis plus SEM at different E:T ratios (4:1 to 0.05:1) are shown to summarize these experiments. Controls that confirm the peptide and calcium dependency of killing were always performed, but are not shown in this summary. B. The proportions of specific CD8+ T cells expressing the transgenic TCR among the cells used as effectors in the tests shown in A were determined by flow cytometry. The means plus SEM are shown.
absence of the SIINFEKL peptide, and the expression of the degranulation marker CD107a (i.e., the lysosomal-associated membrane protein 1 [LAMP1]), that arrives at the cell surface with the membrane of degranulating cytotoxic vesicles was determined by flow cytometry on CD8+ T cells. Cell surface expression of CD107a has previously been used to compare the degranulation deficiency in various FHL subtypes (31). The percentage of cells with a higher CD107a expression in the presence of the SIINFEKL peptide was calculated as exemplified in Fig. 4A. In addition, we compared the mean fluorescence intensity (MFI) of CD107a in presence and absence of the SIINFEKL peptide. The degranulation (i.e., CD107a cell surface expression), of Vti1b- and Vamp8-deficient CTL was significantly impaired after 4 and 3 d of Ag-specific stimulation (Fig. 4B, 4C).

Thus, both SNARE proteins vti1b and VAMP8 appear to be involved in the degranulation of CTL, and a deficiency results transiently in a reduced cytotoxic activity during the maturation to armed effector CTL. A higher percentage of CD107a-positive cells and a higher MFI were observed after 4 d of Ag stimulation compared with 3 d for all genotypes. At later time points, the reduced degranulation appears not to be the limiting factor for the cytotoxic activity of the CTL.

The cell surface expression of CD107a/LAMP1 before degranulation was very low and similar in all genotypes (Table I). In addition, the overall expression level of CD107a/LAMP1 and the cytotoxic effector protease granzyme B was also unchanged in Vti1b- and Vamp8-deficient CTL compared with wild-type and heterozygous controls, as determined by immunoblotting (data not shown).

The processing of Ags and the stimulatory capacity of DCs are not affected by Vti1b or Vamp8 deficiency

One could assume that the stimulatory capacity of Vti1b- or Vamp8-deficient APCs might also be altered (32). It is conceivable that Ag processing is affected because it requires vesicular transport at several steps and VAMP8 has been described to regulate phagocytosis in macrophages and DCs (33). Thus, we added the OVA protein to the splenocyte cultures from the TCR-transgenic Vti1b- and Vamp8-deficient mice. The protein has to be taken up, processed, and cross-presented on H2Kb molecules of professional APCs to allow for stimulation of the SIINFEKL-specific CTL. For OT-I wild-type CTL, 1 μM OVA was required to obtain after 5-d CTL with a similar cytotoxic activity as after stimulation with 1 nM SIINFEKL peptide that was used in previous experiments. Therefore, the stimulatory effect of 1 or 0.2 μM OVA was tested in comparison with 1 nM SIINFEKL in Vti1b−/−, Vti1b+/−, Vamp8−/−, and Vamp8+/− mice. In these experiments, the CTL and APCs were derived from the same knockout or heterozygous animals.

When the cytotoxic activity against RMA cells pulsed with SIINFEKL was used as readout, no impairment of the Ag-processing capacity could be detected for Vti1b- and Vamp8-deficient CTL (Fig. 5). The lower Ag concentration (0.2 μM) resulted in a reduced cytotoxic activity of the CTL, but it was similar in knockout and

A

B

C

FIGURE 4. The Ag-specific degranulation of CTL is diminished in Vti1b- and Vamp8-deficient mice. A, Splenocytes derived from TCR-transgenic Vti1b−/− and Vti1b+/− mice were stimulated in vitro for 4 d with the SIINFEKL (1 nM) and then used as effector cells against RMA target cells in a 10:1 ratio in the presence or absence of the SIINFEKL peptide. An Ab against the degranulation marker CD107a or the respective isotype control was present during the assay. After 4 h, the cells were harvested, stained for CD8, and analyzed by flow cytometry. The histograms show the CD107a expression on CD8+ cells in the presence of peptide compared with absence of peptide (thin line) or presence (bold line) of SIINFEKL. Without peptide, the CD107a staining was similar to the isotype control (data not shown). The percentages of cells with an increased CD107a expression (i.e., degranulating cells) in the presence of peptide compared with absence of peptide were calculated and are indicated. B, A summary of experiments performed after 4 d of Ag-specific stimulation with CTL from TCR-transgenic mice Vti1b−/− (n = 12), Vti1b+/− (n = 15), Vamp8−/− (n = 10), Vamp8+/− (n = 7), and OT-I wild-type (wt, n = 7) is shown. The means of peptide-dependent CD107a-positive CTL plus SEM (left panel) and the ΔMFI plus SEM (right panel) of CD107a (MFI in presence of SIINFEKL minus MFI in absence of SIINFEKL) were calculated. Results of knockout mice and heterozygous controls were analyzed using unpaired Student t test, and significant differences are indicated. *p < 0.05; **p < 0.01. C, A summary of similar experiments performed after 3 d of Ag-specific stimulation with CTL from TCR-transgenic mice Vti1b−/− (n = 7), Vti1b+/− (n = 6), Vamp8−/− (n = 4), Vamp8+/− (n = 4), and OT-I wild-type (wt, n = 6) is shown.

Table I. The cell surface expression of CD107a/LAMP1 on CTL before degranulation is low and not affected by a Vti1b or Vamp8 deficiency

<table>
<thead>
<tr>
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<th>Day 3</th>
<th>Day 4</th>
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<tr>
<td>CD107a (MFI)</td>
<td>CD107a (%)</td>
<td>CD107a (MFI)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>3.3 ± 1.5</td>
<td>3.0 ± 1.1</td>
</tr>
<tr>
<td>Vti1b−/−</td>
<td>3.4 ± 2.1</td>
<td>3.9 ± 1.7</td>
</tr>
<tr>
<td>Vti1b+/−</td>
<td>3.1 ± 0.8</td>
<td>2.4 ± 1.8</td>
</tr>
<tr>
<td>Vamp8−/−</td>
<td>2.7 ± 1.2</td>
<td>4.1 ± 2.1</td>
</tr>
<tr>
<td>Vamp8+/−</td>
<td>3.6 ± 1.5</td>
<td>2.9 ± 0.9</td>
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Splenocytes of TCR-transgenic mice were stimulated in vitro for 3 or 4 d with the SIINFEKL peptide (1 nM). Afterward, the CD8+ CTL were analyzed by flow cytometry for cell surface expression of CD107a/LAMP1 in the absence of the SIINFEKL peptide. The percentage of positive cells and the specific MFI (anti-CD107a minus isotype control) were calculated. Means ± SEM of 4–12 independent experiments are given.
control mice. Importantly, also in this set up, the stimulation of the CTL was completely dependent on the Ag. In the effector phase, killing was always dependent on the presence of the SIINFEKL peptide on the RMA targets, and the cytotoxicity could be always completely inhibited by EGTA (data not shown).

The Ag-specific proliferation of CD8+ T cells was used as a second readout. The splenocytes were labeled with CFSE, and the dilution of the dye during cell division was determined by flow cytometry after gating on CD8+ T cells (Fig. 6A). We observed experimental variation between individual mice with respect to the number of cell divisions of knockout as well as heterozygous CTL. In the shown example, the Vti1b-deficient CTL proliferated more rapidly than the heterozygous control. However, in general, the proliferation of Ag-specific CTL was not altered by Vti1b or Vamp8 deficiency after 3 or 5 d of culture (Fig. 6B). The expression of the activation marker CD69 on CD8+ T cells was also not affected by the genotype (data not shown).

To exclude that contrary effects of the knockouts on CTL and APCs mask each other, we purified naive CD8+ T cells (>95% CD8 and TCR positive) from wild-type OT-I mice and cocultured them with DCs (>80% CD11c and I-Ab positive) separated from Vti1b--/ or Vamp8--/ mice. Due to a lack of APCs, the purified CD8+ T cells alone were not efficiently stimulated by SIINFEKL or OV A, as shown by a greatly reduced cytotoxic activity (Fig. 7A). However, Vti1b--/ and Vamp8--/ deficient DCs were able to stimulate the cytotoxic activity of purified Ag-specific CTL as well as heterozygous controls (Fig. 7A). Similarly, the genotype of DCs had no effect on the proliferation of purified Ag-specific wild-type CTL (Fig. 7B). When CD8+ T cells were separated from Vti1b--/ and Vamp8--/ deficient mice and stimulated for 5 d with SIINFEKL or OVA presented by wild-type DCs, no difference was found in their proliferation or cytotoxic activity compared with the respective heterozygous controls (data not shown). Thus, both vti1b and VAMP8 do not appear to affect the Ag processing or

FIGURE 5. The activation of CTL by an Ag that has to be processed is not impaired by Vti1b or Vamp8 deficiency. Splenocytes of TCR-transgenic Vti1b--/ and Vti1b+/+ (A) and Vamp8--/ and Vamp8+/+ mice (B) were cultured for 5 d in the presence of 1 nM SIINFEKL peptide, 1 or 0.2 μM OVA protein, or without Ag (w/o Ag). The cultured splenocytes were used as effector cells against RMA target cells in 51Cr release assays in presence of the SIINFEKL peptide (0.5 μg/ml). Each individual test was done in triplicates. The means of specific lysis plus SEM at different E:T ratios (10:1 to 0.2:1) are shown to summarize these experiments. The number of analyzed individual animals was four for Vti1b--/ and Vti1b+/+ and five for Vamp8--/ and Vamp8+/+ mice. Controls that confirm the peptide and calcium dependency of killing were always performed, but are not shown in this summary.
the stimulatory capacity of DCs. However, both SNARE proteins are involved in lytic granule exocytosis in CTL.

**Discussion**

Little is known about the specific SNARE proteins that are involved in the fusion of lytic granules with the plasma membrane of cytotoxic cells, allowing for the exocytosis of cytotoxic effector proteins, such as perforin and granzymes (14). A defective degranulation has been observed in NK cells of syntaxin-11–deficient FHL-4 patients (15). In line with this result, it was reported that a knockdown of syntaxin-11 in NK cells reduced their cytotoxic activity, whereas syntaxin-11 overexpression was able to augment cytotoxicity (34). Recently, a reduced exocytosis of lytic granules and an impaired cytotoxic activity of CTL from mice deficient for VAMP8 have been reported (24). We show in this study that Qb-SNARE vt1b is involved in the exocytosis of lytic granules from CTL and confirm this function for the R-SNARE VAMP8.

We investigated CTL from TCR-transgenic OT-I mice that allow for the functional analysis of peptide-specific CTL in defined stages of their activation. Despite these advantages, TCR-transgenic mice have previously not been used to study the role of SNARE proteins for cellular cytotoxicity. When comparing naive and Ag-specific activated CTL, we found an increased expression of vt1b and Vamp8 mRNAs, suggesting a functional role of the respective SNARE proteins in effector CTL. Therefore, we crossed OT-I mice with vt1b and Vamp8 knockout mice. The peptide-specific exocytosis of lytic granules from TCR-transgenic CTL of vt1b– and Vamp8-deficient mice was significantly impaired as measured by cell surface expression of the degranulation marker CD107a/LAMP1 at days 3 and 4 after activation of CTL. Notably, the degranulation of CTL from both knockout mice was compromised, but not completely blocked, suggesting a functional redundancy between vt1b or VAMP8, respectively, and other SNARE proteins. It is unlikely that the result of the degranulation assay was severely affected by a general misrouting of LAMP1 in vt1b– or VAMP8-deficient CTL. Misrouted LAMP1 usually appears at the plasma membrane (35), but cell surface expression of CD107a/LAMP1 before degranulation as well as the overall expression level was similar in vt1b– and VAMP8-deficient CTL compared with wild-type and heterozygous CTL. However, we cannot completely exclude that vt1b and VAMP8 might play a role in LAMP1
trafficking. Therefore, it is important that the cytolytic activity of Vti1b- and Vamp8-deficient CTL was reduced during Ag-specific activation. We found an impaired cytotoxic activity at day 3 after activation of CTL. The finding that the cytotoxic activity was normal at day 4 suggests that the granule fusion is not the limiting step for the cytolytic activity of fully stimulated CTL. Although the granzyme B expression levels were not reduced in Vti1b- or Vamp8-deficient CTL, we cannot exclude the possibility that other critical components of the lytic granules were reduced at day 3. Notably, in syntaxin-11–deficient NK cells from FHL-4 patients, IL-2 stimulation for 3 d was able to restore degranulation and cytotoxicity (15). Thus, compensatory mechanisms for single SNARE deficiencies might be stimulated by IL-2, which was also present in our experiments during the Ag-specific activation of CTL. Syntaxin-11–deficient FHL-4 patients have a less severe disease progression compared with perforin-deficient FHL-2 and Munc13-4–deficient FHL-3 patients (15). Thus, vti1b- or VAMP8-deficient patients, if existing, could have a rather mild immunological phenotype complicating their identification. However, the kinetics of CTL activation in vivo might nonetheless be critical to overcome some rapidly progressing infections. In future experimental studies, it will be interesting to investigate functional effects of parallel defects in more than one SNARE protein to determine which SNARE proteins can replace each other in lytic granule exocytosis. VAMP7 is a candidate for compensation of VAMP8 loss because both proteins are involved in degranulation of mast cells (23) and a NK cell line (25). The closest homolog of vti1b is vti1a, which has a role in early endosome fusion and retrograde transport from endosomes to the trans-Golgi network (19, 36).

Although the role of vti1b in lytic granule exocytosis has not been investigated before, data were recently reported for CTL from another Vamp8 knockout mouse line (24). These Vamp8-deficient CTL showed a normal immunological synapse and granule polarization upon target cell contact. Granzyme B and perforin expression levels were also normal in Vamp8-deficient CTL (24). Both

**FIGURE 7.** The capacity of DCs to activate naive CTL is not affected by Vti1b or Vamp8 deficiency. A, CD8+ T cells were purified from wild-type OT-I mice and cultured for 5 d without DCs (w/o DC) or with DCs from Vti1b−/−, Vti1b+/−, Vamp8−/−, and Vamp8+/− mice at a ratio of 5:1 in the presence of 1 nM SIINFEKL or 1 μM OVA. As in previous experiments, cultures without Ag did not give rise to activated CTL (data not shown). The cultured CD8+ T cells were used as effector cells against RMA target cells in 51Cr release assays in presence of the SIINFEKL peptide (0.5 μg/ml). Each individual test was done in triplicates. The means of specific lysis plus SEM at different E:T ratios (10:1 to 0.2:1) are shown to summarize these experiments. The number of analyzed individual animals was three for Vti1b−/− and Vti1b+/− and six for Vamp8−/− and Vamp8+/− mice. Controls that confirm the peptide and calcium dependency of killing were always performed, but are not shown in this summary. B, In parallel to the experiments shown in A, the naive CD8+ T cells were labeled at day 0 with CFSE before being cultured either with 1 nM SIINFEKL peptide, 1 μM OVA protein, or without Ag (w/o Ag). At day 5, the cells were harvested and stained with an anti-CD8 mAb. The percentage of proliferating CD8+ T cells was determined by flow cytometry, as shown in Fig. 6A. The means of the percentages of proliferating CD8+ T cells plus SEM are shown.
results suggest a normal biogenesis of lytic granules in these Vamp8-deficient mice. However, killing of P815 target cells was impaired in a redirected lysis assay with anti-CD3/CD28-activated CTL (24). In addition, degranulation, as measured by CD107a cell surface expression, and granzyme A and B secretion also were found to be reduced in Vamp8-deficient CTL (24). The results obtained in our Vamp8 knockout mouse line in general confirm these findings for a peptide-specific CTL activation. However, we show in this study, using a physiological trigger for the activation of CTL, that the killing defect in Vamp8-deficient CTL is transient and can be functionally compensated for during Ag-specific activation.

Because Ag-specific activation of CTL requires professional APCs, we investigated the stimulatory capacity of Vti1b- and Vamp8-deficient DCs. The stimulatory capacity of DCs from both knock-out strains was not impaired when proliferation and activation of cytotoxicity were measured with CTL from TCR-transgenic OT-I mice. This result was obtained with a peptide Ag (SIINFEKL) and with the OVA protein that has to be processed before presentation. Thus, the efficacy of OVA uptake and cross-presentation on MHC class I molecules was not affected by a deficiency of vti1b or VAMP8. VAMP8 has been reported to negatively regulate phagocytosis in macrophages and DCs (33). Similarly, syntaxin-11 has been reported to act as a negative regulator of phagocytosis in human monocytes and macrophages (37). In conclusion, we have no indication that results on the effector function of Vti1b- and Vamp8-deficient CTL were secondary due to a defect in stimulatory APCs.

Deficiencies in three different SNAREs, VAMP8, vti1b, and syntaxin-11, result in reduced lytic granule secretion and killing by CTL. These three SNAREs are capable of forming a complex because they belong to different SNARE subfamilies. VAMP8 is a R-SNARE; syntaxin-11, a Qa-SNARE; and vti1b, a Qb-SNARE. This means that only a Qc-SNARE is missing to form a SNARE complex of the conserved structure (6). A candidate would be the Qc-SNARE syntaxin-8, which is known to form a complex with VAMP8, vti1b, and syntaxin-7 (19). In exocytosis secretion, VAMP8 may function together with the Qa SNARE syntaxin-4 and syntaxosomat-associatated protein-23 (SNAP-23), which contributes both a Qb- and a Qc-SNARE helix (20). However, a replacement of syntaxin-4 with syntaxin-11 would not result in a functional SNARE complex. It is assumed that SNAREs with transmembrane domains are required on both membranes to exert mechanical force on the membrane, which may be required to overcome the energy barrier for fusion (6). However, neither SNAP-23 nor syntaxin-11 contains a transmembrane domain (38, 39). Therefore, syntaxin-11 cannot function together with SNAP-23, but requires separate Qb and Qc SNAREs, all of which contain transmembrane domains. The reduced exocytosis of lytic granules in Vti1b- and Vamp8-deficient CTL shown in this study indicates that VAMP8 and vti1b are partners for syntaxin-11 in the SNARE complex, which is involved in the fusion of lytic granules with the plasma membrane at the immunological synapse of cytotoxic cells. More studies are necessary to elucidate the Qc-SNARE of this complex.

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References


