VAMP8-dependent fusion of recycling endosomes with the plasma membrane facilitates T lymphocyte cytotoxicity

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Cytotoxic T lymphocytes (CTLs) eliminate infected and neoplastic cells through directed release of cytotoxic granule contents. Although multiple SNARE proteins have been implicated in cytotoxic granule exocytosis, the role of vesicular SNARE proteins, i.e., vesicle-associated membrane proteins (VAMPs), remains enigmatic. VAMP8 was posited to represent the cytotoxic granule vesicular SNARE protein mediating exocytosis in mice. In primary human CTLs, however, VAMP8 colocalized with Rab11a-positive recycling endosomes. Upon stimulation, these endosomes rapidly trafficked to and fused with the plasma membrane, preceding fusion of cytotoxic granules. Knockdown of VAMP8 blocked both recycling endosome and cytotoxic granule fusion at immune synapses, without affecting activating signaling. Mechanistically, VAMP8-dependent recycling endosomes deposited syntaxin-11 at immune synapses, facilitating assembly of plasma membrane SNARE complexes for cytotoxic granule fusion. Hence, cytotoxic granule exocytosis is a sequential, multivesicle fusion process requiring VAMP8-mediated recycling endosome fusion before cytotoxic granule fusion. Our findings imply that secretory granule exocytosis pathways in other cell types may also be more complex than previously appreciated.

Introduction

Cytotoxic T lymphocytes (CTLs) together with natural killer cells can recognize and kill infected and neoplastic cells. Killing by these cell types is mediated by exocytosis of specialized secretory lysosomes, called cytotoxic granules, which contain the cytotoxic proteins perforin and granzymes (Trapani and Smyth, 2002; Stinchcombe and Griffiths, 2007; de Saint Basile et al., 2010). CTL activation is dictated by somatically recombined, clonally distributed T cell receptors (TCRs) that bind specific complexes of peptide major histocompatibility complex (pMHC) molecules on target cells. Upon engagement, CTLs can induce target cell death within minutes (Lopez et al., 2013). The immune synapse is a spatially organized site of contact between CTLs and target cells, supporting both directed exocytosis of cytotoxic granules and signaling required for cytokine production (Dustin and Long, 2010). For CTLs, induction of an immune synapse and full activation requires at least 10 pMHC complexes on the target cell (Purbhoo et al., 2004). However, three pMHCs are sufficient to facilitate transient interactions that support target cell killing without formation of a mature immune synapse (Sykulev et al., 1996; Faroudi et al., 2003; Purbhoo et al., 2004). Thus, CTL killing is rapid, extremely sensitive to antigen, and involves a complex intracellular signaling cascade that polarizes proteins required for cytotoxic granule exocytosis toward the target cell interface.

In eukaryotes, exocytosis as well as intracellular vesicle fusion processes is mediated by transmembrane proteins containing cytoplasmic SNARE domains (Jahn and Scheller, 2006; Südhof and Rothman, 2009). Typically, one R-SNARE protein residing on the vesicular membrane and three Q-SNAREs (Qa, Qb, and Qc) proteins residing on the target membrane can span the distance between two membranes, forming a parallel

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four-helical bundle that catalyzes membrane fusion (Sutton et al., 1998). When forming a SNARE complex, SNARE proteins evince remarkable specificity for their cognate SNARE partners, resulting in highly specific combinations of SNAREs at differing steps of vesicle fusion (Chen and Scheller, 2001; Un-germann and Langosch, 2005).

Whereas SNARE-dependent exocytosis has been extensively studied in the context of neurotransmitter release, current research shows that SNARE-dependent exocytosis is also critical for proper immunological function (Stow et al., 2006). Mutations in genes encoding the SNARE protein syntaxin-11 (Stx11) and SNARE complex regulators Munc13-4 and Munc18-2, as well as the gene encoding perforin, are all associated with development of an early onset, often fatal, hyperinflammatory syndrome termed hemophagocytic lymphohistiocytosis (HLH; Stepp et al., 1999; Feldmann et al., 2003; zur Stadt et al., 2005, 2009; Côté et al., 2009). Importantly, Stx11, Munc13-4, or Munc18-2 deficiency abrogates cytotoxic granule exocytosis and target cell killing by CTL (Bryceson et al., 2007; Côté et al., 2009; zur Stadt et al., 2009). In Stx11-deficient cytotoxic lymphocytes, cytotoxic granules polarize to the target cell interface but fail to fuse with the plasma membrane (Bryceson et al., 2007), suggesting that Stx11 mediates cytotoxic granule fusion for target cell killing. With the exception of Stx11, other members of the SNARE complex regulating cytotoxic granule fusion with the plasma membrane have not been clearly defined, although several SNARE-containing candidates have been proposed (Stow, 2013). Conjointly, Munc13-4 has also been shown to facilitate intermediate steps of cytotoxic granule maturation, promoting late endosome-to-lysosome fusion (Ménager et al., 2007), suggesting that multiple vesicle fusion steps regulate cytotoxic granule exocytosis and hence, several SNARE complexes might control cytotoxic granule exocytosis.

The vesicle-associated membrane protein (VAMP) family typically mediates fusion of vesicles with cognate, membrane-associated SNARE complexes, and several VAMPs have been implicated in lymphocyte cytotoxicity. Vamp2 (encoding VAMP2 or synaptobrevin-2) knockout mice display defective cytotoxic granule exocytosis (Matti et al., 2013). Moreover, VAMP2 colocalizes with cytotoxic granules, indicating that VAMP2 may serve as a vesicular R-SNARE for cytotoxic granule exocytosis. Concurrently, VAMP4, and VAMP7 also colocalize with cytotoxic granule proteins and siRNA-mediated knockdown in a human natural killer cell line exhibited deficits in lymphocyte cytotoxicity (Krzewski et al., 2011). Furthermore, in mice, VAMP8, an R-SNARE associated with exocytosis of secretory pathway organelles in multiple immune cell types, colocalized with the cytotoxic granule protein granzyme B. Vamp8 knockout mouse CTLs display reduced granule exocytosis and impaired target cell killing, also indicating that VAMP8 may be required for cytotoxic granule exocytosis (Loo et al., 2009; Dressel et al., 2010). Thus, how distinct VAMPs regulate cytotoxic granule trafficking and fusion for target cell killing is unclear.

Here, we studied the role of VAMP8 in cytotoxic granule fusion in primary human lymphocytes using structured illumination microscopy (SIM) and total internal reflection fluorescence (TIRF) microscopy. Examining endogenous as well as fluorescently tagged, ectopically expressed protein, we established that VAMP8 is associated with the recycling endosome compartment rather than with cytotoxic granules. Complimentary approaches revealed that recycling endosomes rapidly accumu-lated and fused at the immune synapse, preceding the arrival of cytotoxic granules. Strikingly, reduction of VAMP8 expression compromised both recycling endosome and cytotoxic granule fusion, delineating a VAMP8-dependent fusion step between recycling endosomes and the plasma membrane that brings Stx11 to the immune synapse for cytotoxic granule exocytosis. These insights provide a new mechanistic view of cytotoxic lymphocyte exocytosis and bear relevance to how VAMP8 may support regulated secretion in several other cell types.

## Results

### VAMP8 accumulates at the immune synapse of human CTL

We examined human CTL expression of VAMP8, which from studies of knockout mice has been suggested to represent the vesicular SNARE required for cytotoxic granule exocytosis. VAMP8 transcripts were expressed in human primary CD8+ T cells, and their levels increased upon anti-CD3 and anti-CD28 bead stimulation, as assessed by quantitative real-time PCR (qRT-PCR; Fig. 1 A). Upon anti-CD3 and anti-CD28 bead or superantigen staphylococcal enterotoxin A (SEA) stimulation, VAMP8 protein expression also increased (Fig. 1, B and C). Notably, in our model, increased expression of VAMP8 upon activation coincided with an increased capacity of the stimulated CTL to kill target cells (Pattu et al., 2012). As the proportion of unstimulated, bulk CD8+ T cells expressing perforin and other cytotoxic granule constituents varies from donor to donor depending on the distribution of different cellular subsets (Chiang et al., 2013), we used CD8+ T cells stimulated with beads or SEA that more homogeneously express cytotoxic granule constituents for all further experiments. Such cells are hereafter referred to as CTLs.

Studies in mice have indicated that VAMP8 is recruited to the immune synapse of CTLs (Loo et al., 2009). We found that numerous VAMP8-carrying vesicles also accumulated at the immune synapse of human CTL conjugated with SEA-pulsed target cells, as revealed by immunofluorescence microscopy and SIM (Fig. 1 D). SIM increases the spatial resolution of fluorescence microscopes to resolve colocalization within a resolution of 100 nm in x, y, and z direction (Gustafsson, 2005; Gustafsson et al., 2008). A similar accumulation of VAMP8 at immune synapses was observed upon expression of full-length VAMP8 fused to TFP (VAMP8-TFP) in target cell–conjugated CTL (Fig. 1 E). A twofold accumulation of endogenous and ectopically expressed TFP-tagged VAMP8, respectively (2.2 ± 0.2–fold and 2.0 ± 0.1–fold for endogenous VAMP8 vs. ectopically expressed VAMP8-TFP, n = 10 and 11 CTLs, respectively), toward target cells was observed. Notably, both VAMP8-TFP and VAMP8-mCherry constructs colocalized well with endogenous VAMP8, implying that these C-terminally tagged constructs properly localized to VAMP8-carrying vesicles (Fig. S1, A and B). These results suggest that VAMP8 may also play a role in cytotoxic granule exocytosis in human CTLs.

### VAMP8 colocalizes with recycling endosomes rather than perforin-containing cytotoxic granules

We set out to decipher how VAMP8 may facilitate cytotoxic granule exocytosis. In contrast to studies of mouse CTL (Loo et al., 2009), we found VAMP8 localization to be distinct from
that of granzyme B in unconjugated, fixed human primary CTLs using SIM (Fig. 2 A). Of note, VAMP8-carrying compartments were more numerous than those with granzyme B. Upon conjugation with SEA-pulsed target cells, VAMP8-carrying vesicles did not start to colocalize with those containing granzyme B (Fig. 2 B). Similarly, VAMP8-carrying vesicles did not colocalize with perforin-containing vesicles in unconjugated or conjugated CTLs (Fig. 2, C and D). Unexpectedly, we conclude that VAMP8 localized to a compartment distinct from cytotoxic granules in human CTLs.

Having established that VAMP8 did not colocalize with cytotoxic granules, we sought the identity of the endosomal compartment that did contain VAMP8. CTLs were transfected with VAMP8-TFP and various endosomal markers coupled to mCherry. CTLs were thereafter mixed with SEA-pulsed target cells, fixed, and imaged using SIM. Imaging revealed limited colocalization between VAMP8 and Rab5a, a marker of early endosomes (Rybin et al., 1996; Barysch et al., 2009), or Rab7a, a marker of late endosomes (Pearson’s coefficient r of 0.34/0.39 and 0.36/0.36, respectively; Figs. 3 and S2; Chavrier et al., 1990). However, a substantial degree of colocalization was observed between VAMP8 and Rab11a, a marker of recycling endosomes (Ullrich et al., 1996), as indicated by a Pearson’s coefficient of 0.68/0.72 in unconjugated and conjugated CTLs, respectively (Figs. 3 and S2). Overall, VAMP8 preferentially localized to recycling endosomes in CTLs, with its distribution not being drastically affected by stimulation. These findings raise questions of how VAMP8 may facilitate human lymphocyte cytotoxicity.

**VAMP8-carrying recycling endosomes traffic and fuse with the plasma membrane at immune synapses**

Although VAMP8 exhibited strong colocalization with recycling endosomes in our fixed cell experiments, it was unclear whether these VAMP8 vesicles were also trafficking through the recycling endosome compartment and fusing at the immune synapse. To examine the behavior of VAMP8-carrying vesicles...
icles, we used TIRF microscopy to visualize vesicle dynamics in the vicinity of the immune synapse. This technique allows real-time visualization of vesicles within 200 nm of the immune synapse (Campi et al., 2005; Douglass and Vale, 2005; Yokosuka et al., 2005). Transfection of CTLs with VAMP8-TFP showed rapid movement and subsequent accumulation of VAMP8-carrying vesicles in the TIRF plane (Video 1). To simultaneously monitor the dynamics of VAMP8 and recycling endosomes, CTLs were cotransfected with VAMP8-TFP and Rab11a-mCherry and allowed to form synapses with anti-CD3 and anti-CD28 antibody-coated coverslips. Shortly after sedimentation, the appearance and subsequent accumulation of both VAMP8- and Rab11a-containing vesicles could be observed at the immune synapse in the TIRF plane (Video 1 and Video 2). VAMP8-TFP and Rab11a-mCherry exhibited a striking colocalization in the TIRF plane (Fig. 4 A). In comparing the dwell times at the immune synapse, we found that there was no statistically significant difference between Rab11a-mCherry vesicles and VAMP8-TFP vesicles, with VAMP8 dwell time of 14.5 ± 0.7 s compared with 15.7 ± 0.9 s of Rab11a (Fig. 4 B). When we examined vesicle accumulation over time, we also found similar numbers of VAMP8 and Rab11a vesicles at immune synapses (Fig. 4 C).

Interestingly, several rapid fluorescence dispersion events, characteristic of vesicle fusion, were also observed in the TIRF plane (Video 2). Such events were defined as a sudden reduction in fluorescence within 400 ms (i.e., four images), which could be distinguished from movement of granules back into the cell (undocking) that typically lasted >1 s (see Materials and methods for analysis details). Cells exhibited a similar

Figure 2. VAMP8 does not colocalize with cytotoxic granule constituents in human CTLs. (A and B) SIM images depicting VAMP8 localization in unconjugated (A) or SEA-target cell-conjugated (B) human CTLs stained with anti-VAMP8 and anti-granzyme B (GzmB). (C and D) SIM images depicting VAMP8 localization in unconjugated (C) or SEA-target cell-conjugated (D) CTLs stained with anti-VAMP8 and anti-perforin. (C) SIM images of resting human CTL stained with anti-VAMP8 and anti-perforin. For SEA-target cell-conjugated CTLs, the Pearson’s coefficient for VAMP8 versus perforin was r = 0.28 (n = 10). Bars, 2.5 μm.
number of such fusion-like events for both VAMP8-TFP and Rab11a-mCherry (46.9 ± 6.4 vs. 54.0 ± 8.1; Fig. 4 D). Quantification of the cumulative data revealed that rapid fluorescence dispersion events occurred promptly after immune synapse formation (Fig. 4 E). Together, the concerted appearance and fusion-like events, characterized by the quickly forming lateral diffusion clouds, of VAMP8-TFP and Rab11a-mCherry seen in our live imaging (Video 2), clearly indicated that VAMP8 and Rab11a traffic in the same vesicle that may fuse with the plasma membrane subsequently.

To formally demonstrate fusion of VAMP8-carrying recycling endosomes with the plasma membrane, we transfected CTLs with a construct encoding VAMP8 tagged with C-terminal, luminal 3×FLAG and TFP. Upon fusion of VAMP8-carrying vesicles, the FLAG-tag will be exposed to the extracellular milieu. Addition of the anti-FLAG antibody to the extracellular
Figure 4. VAMP8-carrying recycling endosomes accumulate and fuse at immune synapses. (A–E) Bead-stimulated human CD8+ T cells were transfected with VAMP8-TFP and mCherry-Rab11a encoding constructs and imaged 24 h after transfection. (A) Selected live-cell TIRF microscopy images of VAMP8-TFP and mCherry-Rab11a in a transfected CTL in contact with an anti-CD3- and anti-CD28-coated coverslip. Bar, 2.5 µm. (B) Mean dwell time of VAMP8-TFP and mCherry-Rab11a vesicles in the TIRF plane per cell (n = 15, paired t test, P > 0.05). (C) Mean VAMP8-TFP and mCherry-Rab11a vesicle accumulation over time in the TIRF plane per cell (n = 15). (D) Mean fluorescence dispersion events for VAMP8-TFP and mCherry-Rab11a vesicles in the TIRF plane per...
milleu followed by flow cytometry and gating on TFP-expressing cells revealed some constitutive expression of VAMP8 on the plasma membrane (Fig. 4, F and G). Upon stimulation with anti-CD3 antibodies, VAMP8 expression on the plasma membrane rapidly increased threefold. Furthermore, to study the characteristics of individual recycling endosome fusion events, CTLs were transfected with a construct encoding VAMP8 tagged with C-terminal, luminal pHluorin and mCherry (Fig. 4 H). pHluorin is a pH-sensitive variant of GFP that has been used to study vesicle fusion of VAMP2-carrying vesicles in neuronal and endocrine cells (Miesenböck et al., 1998). Transfected cells were imaged by TIRF microscopy. Some vesicles first docked at the immune synapse before undergoing rapid fusion and dispersion of VAMP8 and pHluorin signal (Fig. 4 H, left; and Video 3). In contrast, many vesicles fused immediately after reaching the plasma membrane, sometimes with pHluorin signals remaining clustered before dispersion (Fig. 4 H, right; Fig. S3; and Video 3). Altogether, these results demonstrate that numerous VAMP8-carrying recycling endosomes are rapidly recruited to and fused with the plasma membrane at CTL immune synapses.

**VAMP8-containing recycling endosome fusion precedes perforin-containing cytotoxic granule fusion**

To gain further insights to the relationship between VAMP8-carrying recycling endosome activity and cytotoxic granule exocytosis at human immune synapses, CTLs were cotransfected with VAMP8-TPF and perforin-mCherry and allowed to form a synapse with anti-CD3 and anti-CD28 antibody-coated coverslips. CTLs were imaged in real-time by TIRF microscopy. Interestingly, in live-cell imaging, multiple VAMP8-carrying recycling endosomes rapidly appeared in the TIRF plane followed by fewer perforin-containing vesicles (Fig. 5 A and Video 4). Comparing the dwell times of both VAMP8 and perforin vesicles in the TIRF plane, the former resided significantly shorter than the latter (14.5 ± 0.8 vs. 117.4 ± 18.9 s for perforin-containing vesicles, respectively; Fig. 5 B). The accumulation of VAMP8-carrying recycling endosomes outnumbered that of perforin-containing cytotoxic granules at the immune synapse (Fig. 5 C). At the start of the 6-min recording, VAMP8-carrying vesicles showed rapid accumulation that subsided with time. By comparison, perforin-containing vesicles appeared later in the TIRF plane. Comparing fusion-like events revealed by imaging of VAMP8-TPF and perforin-mCherry, CTLs exhibited many more VAMP8 versus perforin fusion-like events per cell (41.6 ± 7.6 vs. 2.4 ± 0.1, respectively; Fig. 5 D). As previously noted, the VAMP8-carrying vesicle fusion-like events occurred promptly after immune synapse formation and then gradually subsided with time (Fig. 5 E). Similar results were also found upon cotransfection of CTL with VAMP8-TPF and granzyme B–mCherry (Fig. S4 and Video 5). In summary, VAMP8-carrying recycling endosome fusion preceded cytotoxic granule exocytosis, implying that recycling endosome fusion with the plasma membrane might be VAMP8 dependent and represent a prerequisite for cytotoxic granule exocytosis.

**VAMP8 knockdown ablates recycling endosome and subsequent cytotoxic granule fusion**

To determine whether VAMP8 regulates human CTL vesicle exocytosis, we attempted to knockdown the expression of VAMP8 by transfection of CTL with four VAMP8-specific siRNAs. Western blot and densitometric analyses showed that VAMP8 expression was reduced by all siRNAs (Fig. 6, A and B). siVAMP8#2 consistently provided the strongest knockdown of VAMP8 expression (with a knockdown efficiency of 70.8 ± 12.6). Staining of transfected cells with a VAMP8-specific antibody confirmed that the expression of VAMP8 was uniformly reduced in CTLs (Fig. S5, A and B). siVAMP8#2 was therefore used for all the following experiments.

To test the involvement of VAMP8 in recycling endosome fusion, VAMP8 knockdown was performed in the TIRF microscopy of CTLs cotransfected with Rab11a-mCherry and control siRNA or VAMP8 siRNA. Knockdown of VAMP8 did not significantly change the mean dwell time of Rab11a-carrying vesicles (17.8 ± 0.5 vs. 21.1 ± 2.4; Fig. 6 C). However, in contrast to cells transfected with control siRNA, VAMP8 siRNA-transfected cells showed a very strong reduction of Rab11a fusion-like events (42.4 ± 5.3 vs. 8.3 ± 1.9; Fig. 6 D and Video 6). Consequently, our results indicate that VAMP8 is a vesicular SNARE required for fusion of recycling endosomes with the plasma membrane.

Studies of knockout mice have implicated VAMP8 in many forms of secretory granule secretion (Paumet et al., 2000; Wang et al., 2004; Ren et al., 2007; Loo et al., 2009; Dressel et al., 2010). Despite not being localized to cytotoxic granules in human CTL, we examined whether siRNA-mediated VAMP8 knockdown would affect cytotoxic granule exocytosis. First, we performed TIRF microscopy on CTL transfected with VAMP8 siRNA and the cytotoxic granule marker granzyme B–mCherry to visualize cytotoxic granule trafficking and fusion. After sedentation of CTLs, cytotoxic granules could be observed fusing at the immune synapse in the TIRF plane (Fig. 6 E and Video 7). Knockdown of VAMP8 did not significantly change the mean dwell time of granzyme B–containing vesicles (Fig. 6 F). In contrast to cells transfected with control siRNA, VAMP8 siRNA–transfected cells showed a strong reduction (>87%) in granzyme B–mCherry fusion-like events (Fig. 6 G and Video 7). Moreover, VAMP8 knockdown also reduced degranulation as measured by induced surface expression of CD107a (also known as lysosome-associated membrane protein 1 [LAMP-1]; Fig. 6, H and I). These experiments revealed that VAMP8...
is required for both recycling endosome and cytotoxic granule fusion with the plasma membrane. Although our results suggested that VAMP8 is required for an early fusion event obligatory to cytotoxic granule exocytosis, it was not clear whether VAMP8-mediated fusion of recycling endosomes with the plasma membrane is required for TCR signaling and immune synapse formation or, further downstream and more specifically, the assembly of the plasma membrane SNARE complex for cytotoxic granule fusion.

**VAMP8 regulates Stx11 deposition at immune synapses but not proximal TCR signaling**

To further dissect the mechanism whereby VAMP8 facilitates lymphocyte cytotoxicity, we first studied the impact of VAMP8 knockdown on TCR signaling in human CTLs. CTLs were transfected with control siRNA or VAMP8 siRNA, labeled with Fluo-4 and Fura red Ca²⁺-sensitive dyes, and assessed by ratiometric flow cytometry. Knockdown of VAMP8 did not significantly alter intracellular Ca²⁺ mobilization after TCR stimulation (Figs. 7 A and S5 D), which is crucial for lymphocyte cytotoxicity (Maul-Pavicic et al., 2011). Similarly, knockdown of VAMP8 did not reduce phosphorylation of the MAPK extracellular signal–regulated kinase (ERK) after TCR stimulation (Fig. 7, B and C; and Fig. S5 E). ERK phosphorylation has been implicated in cytotoxic lymphocyte granule polarization (Jenkins et al., 2009). Thus, VAMP8 was not required for proximal TCR signals leading to intracellular Ca²⁺ mobilization and phosphorylation of ERK. To study actin dynamics, CTLs were cotransfected with recombinant, fluorescently tagged actin (LifeAct-GFP) and control siRNA or VAMP8 siRNA and subsequently imaged by TIRF microscopy (Fig. 7, D and E; and Fig. S5 F). In line with the experiments negating a role for VAMP8 in proximal TCR-induced Ca²⁺ mobilization and phosphorylation of ERK, knockdown of VAMP8 did not affect established F-actin architectural phases during immune synapse formation (Rak et al., 2011). An even distribution of F-actin was initially observed upon contact with the coverslip. After cell spreading and within ~40 s, distribution of F-actin around the cell periphery was observed. This annular distribution was accompanied by the appearance of granule-sized clearance in the actin meshwork and normal cell spreading (Fig. 7, E and F). In conclusion, our results indicated that VAMP8-mediated fusion with the plasma membrane is not required for proximal TCR-induced signaling and actin reorganization.

We have recently shown that Stx11 localizes to recycling endosomes in human CTLs (Halimani et al., 2014). We therefore postulated that knockdown of VAMP8 might change Stx11...
dynamics and interfere with downstream cytotoxic granule exocytosis. To test this hypothesis, CTLs were cotransfected with Stx11-mCherry and control siRNA or V AMP8 siRNA. CTLs were subsequently imaged using TIRF microscopy (Figs. 7 G and S5 G and Video 8). Knockdown of V AMP8 led to an increase in dwell time of Stx11-carrying vesicles (Fig. 7 H). Paralleling this, knockdown of V AMP8 led to a cumulative increase in the number of Stx11-carrying vesicles as well as the overall Stx11 fluorescence intensity in the TIRF plane (Fig. 7, I and J). Remarkably, Stx11 vesicle fluorescence dispersion events were greatly reduced in V AMP8 knockdown compared with control CTLs (10 ± 3.9 vs. 1.3 ± 1.26, respectively; Fig. 7 K). V AMP8 and, even more so, Stx11 fluorescence dispersion events were predominately focused toward the center of the immune synapse (Fig. 7, L–N) suggesting a degree of recycling endosome exocytosis polarization. With respect to the radial distribution of Stx11, V AMP8 knockdown resulted in an increased central clustering of Stx11-carrying vesicles at the immune synapse (Fig. 7 O), possibly reflecting a microtubule-organizing complex–coordinated polarization of recycling endosomes toward
Figure 7. VAMP8 knockdown does not affect proximal T cell signaling, but regulates Stx11 vesicle trafficking and fusion at immune synapses. (A–K) Bead-stimulated human CD8+ T cells were transfected with siRNA, as indicated, as well as LifeAct-GFP (D–F) or Stx11-mCherry (G–K). (A) CTLs were loaded with Ca2+-sensitive Fluo-4 and Fura red dyes and assessed by flow cytometry and stimulated as indicated. iono., ionomycin. (B and C) Western blot of CTL lysates after transfection and stimulation as indicated. Blots for phospho-ERK (pT202/pY204), VAMP8, and loading control GAPDH are shown for one representative donor. (C) Densitometry analysis of phospho-ERK signaling in five individual donors. Mean values are indicated, with bars representing standard error. (D–K) Time-lapse images of LifeAct-GFP (D–F) or Stx11-mCherry (G–K) expression in siCTRL or siVAMP8#2 transfected cells. (G) Representative images from each donor. Scale bars = 10 μm. (H) Stx11 vesicle dwell time was determined by measuring the time between two consecutive vesicle fusions. **p < 0.01. (I) Syntactic Stx11 accumulation per cell was calculated as the number of vesicles times the dwell time. (J) Syntactic Stx11 accumulation per cell was calculated as the number of vesicles times the dwell time. (K) Stx11 fluorescence dispersion was calculated as the number of vesicles times the dwell time. (L–O) VAMP8 distribution was measured by quantifying the number of vesicles within the center and periphery of the cell. (L) Representative image of VAMP8-TFP expression in a transfected cell. Scale bars = 10 μm. (M) VAMP8 dispersion event distribution was determined for siCTRL and siVAMP8#2 transfected cells. **p < 0.01. (N) VAMP8 dispersion event distribution was determined for siCTRL and siVAMP8#2 transfected cells. (O) Syntactic VAMP8 dispersion was calculated as the number of vesicles times the dwell time.
the immune synapse. With VAMP8 knockdown, Stx11 deposition and diffusion in the membrane would be reduced, resulting in an increased central clustering of Stx11. Hence, our results suggest that Stx11 deposition into the plasma membrane is specifically mediated by VAMP8 recycling endosome fusion.

**Discussion**

The mechanisms of lymphocyte cytotoxicity have not been fully elucidated (de Saint Basile et al., 2010). In studying the R-SNARE candidate VAMP8, we made several unexpected discoveries. Super high resolution imaging of both endogenous and ectopically expressed VAMP8 in primary human CTLs established that VAMP8 localizes to the Rab11a-positive recycling endosome compartment rather than to cytotoxic granules. Complimentary approaches demonstrated that VAMP8-carrying recycling endosomes rapidly fuse with the plasma membrane upon TCR engagement of CTLs. Moreover, VAMP8 knockdown demonstrated a critical role for VAMP8 in fusion of recycling endosomes with the plasma membrane at immune synapses. In agreement with previous studies of Vamp8 knockout mice (Loo et al., 2009; Dressel et al., 2010), VAMP8 knockdown also impaired cytotoxic granule exocytosis by human CTLs. Intriguingly, we found that VAMP8 knockdown did not diminish proximal TCR signaling, implying that VAMP8-mediated fusion plays a more specific role in promoting cytotoxic granule exocytosis. Our data suggest that VAMP8-dependent recycling endosome fusion deposits the Qa-SNARE Stx11, localized to recycling endosomes in unstimulated CTL (Halimani et al., 2014), on the plasma membrane at immune synapses for cytotoxic granule exocytosis.

CTLs survey tissues for aberrant cells and can engage and kill target cells within 2 min through regulated exocytosis of specialized secretory lysosomes (Lopez et al., 2013). Exocytosis of cytotoxic proteins needs to be stringently controlled. Remarkably, we found accumulation and exocytosis of numerous VAMP8-carrying, Rab11-positive recycling endosomes within seconds of CTL contact with anti-CD3–coated coverslips. The exocytosis of these vesicles not only outnumbered but also preceded that of perforin- and granzyme B–containing cytotoxic granules. On average, 40 or more recycling endosomes fused with the plasma membrane within 4 min of CTL immune synapse formation. By comparison, less than five fusion-like events of cytotoxic granules were observed per cell. Recycling endosomes have previously been implicated in SNARE-dependent accumulation of TCR clusters to the immune synapse (Das et al., 2004). Specifically, synaptotagmin 7 and VAMP7 mediate fusion of vesicles containing TCR and LAT, respectively (Larghi et al., 2013; Soares et al., 2013). TCR-induced signaling is impaired in VAMP7-deficient CD4+ T cells (Larghi et al., 2013). Whether these vesicles fuse with the plasma membrane or form intracellular signaling platforms is disputed. Our data suggest that VAMP8 mediates recycling endosome fusion with the plasma membrane for delivery of molecules required for cytotoxic granule exocytosis but is not crucial for T cell activation. There is quite possibly heterogeneity in the lymphocyte recycling endosome compartment, where different vesicles may contribute to intracellular signaling versus delivery of cargo to the plasma membrane. Importantly, we find that VAMP8 is required for deposition of Stx11, an effector molecule of cytotoxic granule fusion (Bryceson et al., 2007), at the immune synapse. Thus, we posit a two-step sequential process for lymphocyte cytotoxicity in which VAMP8-mediated recycling endosome fusion brings effector proteins to the plasma membrane for SNARE complex formation and subsequent cytotoxic granule exocytosis (Fig. 8). Our data suggest that recycling endosome fusion is clustered toward the center of the immune synapse, possibly reflecting involvement of the microtubule-organizing complex in both recycling endosome and cytotoxic granule exocytosis. It is possible that additional engagement of integrins at cytotoxic immune synapses would further promote polarization of the recycling endosome compartment to a focal site of exocytosis (Stinchcombe et al., 2006; Liu et al., 2009). Moreover, although our data suggest recycling endosome fusion is critical for delivery of Stx11 to the immune synapse, it is also possible that VAMP8-mediated recycling endosome fusion with the plasma membrane provides other proteins required for cytotoxic granule exocytosis. It is worth noting that Munc13-4 also resides on Rab11-positive recycling endosomes (Ménager et al., 2007). Besides mediating cytotoxic granule to plasma membrane fusion, Munc13-4 has been previously proposed to promote late endosome to lysosome fusion, facilitating cytotoxic granule maturation (Ménager et al., 2007). It will therefore be interesting to address how distinct Munc13-4 isoforms may be involved in regulating exocytosis of recycling endosomes and cytotoxic granules (Cichocki et al., 2014). In summary, our findings unravel a novel component of vesicular fusion for lymphocyte cytotoxicity, providing a new layer of control in the carefully orchestrated process of target cell killing.

Several genes required for cytotoxic granule exocytosis have been linked to development of early onset, often fatal hyperinflammatory syndromes such as HLH. Loss-of-function mutations in VAMP8 have so far not been associated with HLH. The very small coding region of VAMP8 may explain why deficiencies in VAMP8 are exceedingly rare, and patients have not been described to date. Alternatively, VAMP8 deficiency may lead to a disparate clinical presentation. Notably, one third of Vamp8 knockout mice die before the age of 1 mo (Wang et al., 2004; Kanwar et al., 2008). By comparison, Prf1 knockout mice...
are healthy until challenged by viral infection (Kägi et al., 1994), suggesting that VAMP8 mediates vital physiological functions in addition to lymphocyte cytotoxicity. Such functions may include pancreatic secretion. Thus, VAMP8 deficiency in humans may give rise to a distinct phenotype or быть embryonically lethal.

VAMP8 was originally identified as a widely expressed R-SNARE predominantly localized to the early endocytic pathway (Wong et al., 1998). Blocking studies in cell lines using antisera suggest that VAMP8 mediated homotypic fusion of early as well as late endosomes (Antonin et al., 2000b). Although VAMP8 has not been previously linked to recycling endosome fusion with the plasma membrane, several recent studies have implicated VAMP8 in secretory exocytic pathways of various cell types. In the pancreas, VAMP8 facilitates release of zymogen from acinar cells secretory granules by forming a SNARE complex with Qa-SNARE Stx4 and the Qb/Qc-SNARE SNAP-23 (Wang et al., 2004). Studies of knockout mice have revealed that VAMP8, but not VAMP2 or VAMP3, is required for secretory granule exocytosis in platelets (Ren et al., 2007). Similarly, studies of human and mouse mast cells have shown that VAMP8 mediates release of serotonin from lysosomal secretory granules (Puri and Roche, 2008; Sander et al., 2008). However, these studies did not establish the localization or trafficking of VAMP8 by high-resolution microscopy. Our data investigating the subcellular localization of endogenous, as well as ectopically expressed VAMP8, in CTLs clearly established localization of VAMP8 to a subset of Rab11a-positive recycling endosomes. Moreover, using luminal tags or pH-sensitive probes, we demonstrated that VAMP8-carrying vesicles undergo fusion with the plasma membrane at immune synapses before secretory/lysosomal granule exocytosis. In light of our novel insights into the vesicle fusion processes regulating lymphocyte cytotoxicity, dissections of VAMP8-dependent secretory granule exocytosis pathways in other cell types using high-resolution imaging are warranted. It is an intriguing possibility that VAMP8-mediated recycling endosome fusion may also represent a previously unrecognized step in the release of secretory granule contents by these other cell types, potentially revealing additional layers of complexity to their exocytic pathways.

The identity of other components of the SNARE complex mediating recycling endosome fusion in CTLs is not clear. VAMP8 can form a complex with Stx7, Stx8, and Vti1b for late endosome to lysosome fusion (Antonin et al., 2000a). In primary human CTLs, Stx4 localizes to the plasma membrane and accumulates at the immune synapse (Pattu et al., 2012), making it a strong SNARE candidate for the recycling endosome fusion. Preliminary immunoprecipitation experiments in our laboratory indicated that VAMP8 also interacts with Stx4 in CTL but failed to show any interaction with Stx7 or Stx8 (unpublished data). Additional experiments are required to verify the identity and spatiotemporal dynamics of the other SNARE complex partners, mediating recycling endosome to plasma membrane fusion in lymphocytes.

In conclusion, our findings reveal a role for VAMP8 in fusion of recycling endosomes with the plasma membrane. In CTLs, our data demonstrate that VAMP8-dependent recycling endosome fusion mediates plasma membrane delivery of proteins, i.e., Stx11, specifically required for cytotoxic granule exocytosis. Our data provide a new mechanistic view of cytotoxic lymphocyte exocytosis. In conjunction with the literature on VAMP8, our findings may warrant further high-resolution functional dissection of exocytosis in other cell types in which VAMP8 has been implicated in release of secretory granule content.

Materials and methods

Cells
Research with human material performed for this study has been approved by the local ethics committee. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by density gradient centrifugation (Lymphoprep; Axis-Shield). In some experiments, PBMCs were stimulated with 5 µg/ml of SEA at a density of 10⁶ cells/ml at 37°C for 1 h. Stimulated PBMCs were thereafter resuspended at a density of 1.5 × 10⁶ cells/ml in complete medium (AIM V medium; Invitrogen) supplemented with 10% FCS (Invitrogen) and 2% human AB sera (Sigma-Aldrich) supplemented with 100 IU/ml of recombinant human IL-2 (Biosource). After 5 d, SEA-specific CTLs were positively selected (Dynal CD8+ isolation kit; Invitrogen) and cultured further in complete medium. CTLs from day 2–3 after positive isolation were used for bead-stimulated CTLs. CD8+ T cells were isolated from PBMCs by negative magnetic separation (CD8+ negative isolation kit; Invitrogen) and stimulated with antibody-coated beads (Dynabeads Human T-Activator CD3/CD28; Invitrogen) in complete medium.

Antibodies
For Western blotting, rabbit polyclonal anti-VAMP8 (V7389; Sigma-Aldrich), anti-phospho-p42/44 MAPK (Erk1/2, Thr202/Tyr204; 9101;
Cell Signaling Technology), and anti-GAPDH (Cell Signaling Technology) antibodies were used. Secondary antibodies were HRP-conjugated donkey anti-rabbit (Invitrogen). For confocal microscopy, mouse monoclonal anti-perifin (6G9; BioLegend) and anti–granzyme B (GB11; BioLegend) as well as rabbit polyclonal anti-VAMP8 (W. Hong) antibodies were used. The VAMP8 antibody was generated using residues 1–75 of VAMP8, expressed as a fusion protein to GST (GST-endobrevin) and used to raise polyclonal antibodies against endobrevin. For TIRF microscopy and flow cytometric assays, mouse anti-human monoclonal anti-CD3 (BB11; Euroclone), mouse anti-human CD28 (CD28.2; BD), and mouse anti-human isotype IgG1 (MOPC-21; BD) antibodies were used for coating coverslips and stimulating cells. For flow cytometry stainings, fluorochrome-conjugated mouse anti-human monoclonal antibodies were used as follows: anti-CD3 (S4.1; Invitrogen), anti-CD4 (S3.5; Invitrogen), anti-CD8 (3B5; Invitrogen), and anti-CD107a (HI43; BD), and anti-FLAG (M2; GenScript).

Plasmids

The VAMP8 coding sequence was amplified from human cDNA with forward primer 5′-TATAGAATCTCATGGGGAAGAGCAGT-GAAG-3′ and reverse primer 5′-TATAGGATCCCTAGAGGACCCAC-TGACATCC-3′ and inserted in frame with C-terminal TFP and mCherry in a pC1-EGFP (Takara Bio Inc.) derived vector which has a GFP-C1-EGFP (Takara Bio Inc.) derived vector which has a green dye (QuantiTect SYBR Green PCR kit; QIAGEN) on a quantitative PCR system (Mx3000P; Agilent Technologies).

For northern blot analysis

Human CTLs were homogenized with a syringe in lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM DTT, 1 mM deoxycholate, protease inhibitors, and PMSF) (Roche) on ice. Lysates were rotated for 30 min at 4°C, and insoluble material was removed by centrifugation at 10,000 RCF. The protein concentration in lysates was determined using Bradford assay (Thermo Fisher Scientific). Proteins were separated by SDS-PAGE (NuPAGE; Invitrogen), transferred to nitrocellulose membranes (Invitrogen), and blocked by incubation with 5% skim milk powder in 20 mM Tris, 0.15 M NaCl, pH 7.4, and TBS for 2 h or overnight and blotted with specific antibodies. Blots were developed using enhanced chemiluminescence reagents (SuperSignal West Dura Chemiluminescent Substrate; Thermo Fisher Scientific) and scanned. For expression analysis, the pixel area and mean fluorescence intensity (MFI) were determined with ImageJ v1.46 (National Institutes of Health). Integrated density was calculated by multiplying band pixel area by the MFI.

Nucleofection of expression constructs and siRNA-mediated knockdown

After 2 d of bead stimulation, 5 × 10^6 CTLs were transfected with 1.5 µg of plasmid DNA (Human T cell nucleofector kit; Lonza). Cells were imaged 24–30 h after transfection. For knockdown of protein expression, 5 × 10^6 CTLs were transfected with 2 µg of siRNA (Hs_VAMP8_4; Si02656766 or Si03650325; QiAGEN) using the nucleofector kit (Lonza). After 12 h of incubation, the cells were washed and cultured in AIM V without IL-2 and used for experiments 18 h after transfection.

Knockdown efficiency was assessed by preparing whole cell lysates of 10^6 CTLs boiled in SDS loading buffer containing 4% β-mercaptoetha-
nol and 1 mM DTT. Lysates were sonicated, proteins were separated by SDS-PAGE, and expression was analyzed by Western blotting.

**SIM**

To conjugate CTLs with target cells, Raji cells were pulsed with 10 µg/ml SEA at 37°C for 30 min. The stimulation of Raji cells was performed in 96-well plates with a maximum of one million cells resuspended in 100 µl AIM V medium. The CTL- and SEA-pulsed Raji cells were washed once with AIM V and resuspended at a concentration of 2 × 10⁷ cells/ml. CTLs were mixed with target cells at a 1:1 ratio and left in suspension for 5 min at 37°C. The cell suspension was then diluted to a concentration of 4 × 10⁶ cells/ml with AIM V medium and plated onto poly-ornithine–12-mm glass coverslips and incubated at 37°C for 15 min. Cells were fixed in ice-cold 4% PFA in Dulbecco’s PBS (In-vitrogen) and stained with antibodies. The SIM setup was a prototype obtained from Carl Zeiss. Images were acquired with a 63× Plan-Apochromat (NA 1.4) with laser excitation at 488, 561, and 635 nm and then processed to obtain higher resolutions (Zen 2009; Carl Zeiss). For analysis of colocalization, Pearson’s and Mander’s coefficients were determined using the JACoP plugin of ImageJ v1.46r.

**TIRF microscopy**

The setup consisted of an inverted microscope (Optical IX70; Olympus), a solid-state laser system (85YCA10; Melles Griot) emitting at 561 nm, a TILL-TIRF condenser (T.I.L.L. Photonics), and an Acousto Optical Tunable Filter-nC (AA Opto-Electronic). The excitation/emission wavelengths were visualized with a dual-view camera splitter (Visitron Systems) to separate the red and green channels and a monochromator (Visichrome; Visitron Systems) to acquire images in epifluorescence. The setup was equipped with a camera (MicroMAX 512 BFT; Princeton Instruments) controlled by MetaMorph (Visitron Systems). A 100×/1.45 NA TIRF objective (Olympus Optical) was used. For some experiments, cells were imaged using a multiphoton confocal microscope (AIR; Nikon) with a 60×/1.49 NA Plan Apochromat objective using 488- and 561-nm lasers and a camera (iXon X3; Andor Technology). The exposure time was 100–150 ms. Experiments were performed at room temperature. Images were acquired using MetaMorph version 6.3 or NIS Elements (Nikon).

For live-cell imaging, human bead-stimulated CTLs were transfected with plasmids, washed, and resuspended in 30 µl AIM V medium and allowed to settle on anti-CD3- and anti-CD28-coated coverslips in an isotonic Ca²⁺-free modified Ringer’s solution (mM: 155 NaCl, 4.5 KC1, 5 Hepes, 2 MgCl₂, and 10 glucose; 300–310 osM). Cells were then perfused with modified Ringer’s solution containing 10 mM Ca²⁺ to optimize Ca²⁺ influx during secretion and subsequently imaged for 15–20 min. Experiments were performed at room temperature.

**Image analysis**

Images and time-lapse series were analyzed using MetaMorph 6.3, ImageJ 1.46r, or the FIJI package of ImageJ. For vesicle dwell time and fusion analysis, analysis was performed with ImageJ 1.46r. Vesicles were defined as constituting 4–6 pixels. Dwell times in the TIRF plane for exocytosis, human PBMCs were isolated from healthy donors by density gradient centrifugation (Lymphoprep). CD8⁺ T cells were isolated from PBMCs by negative magnetic selection (CD8⁻ negative isolation kit) and stimulated with antibody-coated beads (Dynabeads Human T-Activator CD3/CD28) in complete medium. The bead-stimulated CTLs were transfected with constructs encoding VAMP8-TFP, VAMP8-3×FLAG-TFP, or siRNA as indicated. CTLs were supplemented with 10 µg/ml anti-CD3 and 30 µg/ml anti-CD28 antibodies and mixed with P815 cells for the indicated time points at 37°C. Thereafter, the cells were surface stained on ice with fluorochrome-conjugated antibodies and analyzed by flow cytometry (LSR Fortessa; BD). FLAG expression was quantified on gated CD3⁺/CD8⁺/TFP⁺ CTLs, and the frequency of CD107a⁺ cells was determined among total CD3⁺/CD8⁺ CTLs with FlowJo software (v.9.7.5; Tree Star).

For assessment of intracellular Ca²⁺ mobilization, bead-stimulated CTLs were incubated with 10 µg/ml anti-CD3 or IgG control antibody in 50 µl HBSS with Ca²⁺ and Mg²⁺ (Biosource) supplemented with 1% FBS for 30 min on ice. The cells were subsequently loaded with Fluo-4 and Fura red dyes on ice and prepared for flow cytometry as previously described (Bryceson et al., 2006). The cells were preincubated for 5 min at 37°C, and then, baseline was acquired on the flow cytometer for 30 s followed by addition of 5 µg goat anti-mouse F(ab)² antibodies (Jackson ImmunoResearch Laboratories, Inc.) and further flow cytometric evaluation. CTLs were gated on forward scatter/side scatter plots. The ratio between the MFI of FL-1 and FL-3 was calculated and plotted as a function of time using FlowJo software (v.9.7.5).

**Assessment of ERK phosphorylation**

After transfection with siRNA, human bead-stimulated CTL were incubated with 10 µg/ml anti-CD3 or isotype antibody in 50 µl HBSS with Ca²⁺ and Mg²⁺ (Biosource) supplemented with 1% FBS for 30 min on ice. The cells were washed, resuspended in RPMI 1640 supplemented with 10% FBS and 5 µg goat anti-mouse F(ab)² antibodies (Jackson ImmunoResearch Laboratories, Inc.) and further flow cytometric evaluation. CTLs were gated on forward scatter/side scatter plots. The ratio between the MFI of FL-1 and FL-3 was calculated and plotted as a function of time using FlowJo software (v.9.7.5).

**Statistics**

Statistical differences in data were analyzed with paired or unpaired t test, the Mann–Whitney U, or one-way analysis of variance (ANOVA) test, as specified, and graphed using Prism v.6 (GraphPad Software).
Online supplemental material

Fig. 1 shows colocalization of endogenous and ectopically expressed VAMP8. Fig. S2 shows that VAMP8 vesicles localize with Rab11a on recycling endosomal structures. Fig. S3 shows that VAMP8-carrying vesicles are rapidly recruited to and fuse with the plasma membrane at cytotoxic lymphocyte immune synapses. Fig. S4 shows that VAMP8 is not colocalized with granzyme B–containing granules. Fig. S5 shows that VAMP8 expression is strongly down-regulated and proximal TCR signaling is normal when VAMP8 expression is down-regulated by siRNA. Video 1 shows that VAMP8 accumulates and disperses at the immune synapse upon antigen receptor engagement. Video 2 shows that VAMP8 vesicles traffic and accumulate at the immune synapse with recycling endosomes markers upon antigen receptor engagement. Video 3 shows that VAMP8-carrying recycling endosomes are rapidly recruited to and fuse with the plasma membrane at immune synapses. Video 4 shows that VAMP8 and granzyme B have different spatial and temporal characteristics. Video 5 shows that VAMP8 and granzyme B have different spatial and temporal characteristics. Video 6 shows that recycling endosome fusion is inhibited upon knockdown of endogenous VAMP8 expression. Video 7 shows that cytotoxic granule fusion is inhibited upon knockdown of endogenous VAMP8 expression. Video 8 shows deposition of Stx11 and the immune synapse upon knockdown of endogenous VAMP8 expression. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201411093/DC1.

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Author contributions: M.R. Marshall performed the majority of experiments shown in Figs. 1–7 and S1–S5, V. Pattu contributed to the TIRF microscopy and analysis, M. Halimani performed the qRT-PCR experiments and assisted in the cloning of constructs, M. Maier-Peu-Cexaux and M. R. Marshall, Y.T. Bryceson, and J. Rettig designed the experiments and wrote the manuscript.

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