Report

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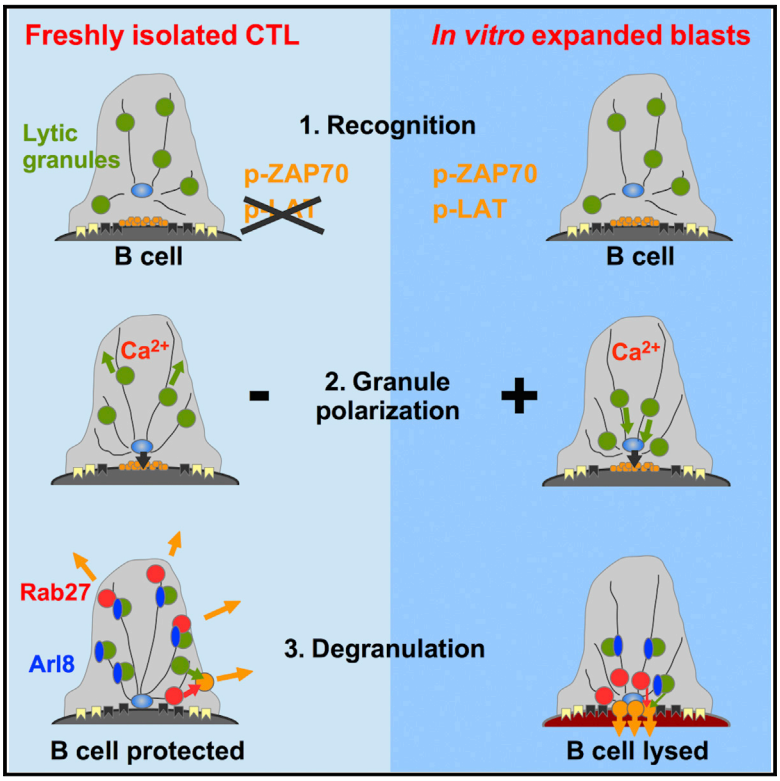
Human Cytotoxic T Lymphocytes Form Dysfunctional Immune Synapses with B Cells Characterized by Non-Polarized Lytic Granule Release

Graphical Abstract



Highlights

- B cells instruct cytotoxic T cells (CTL) to degranulate in a non-polarized fashion
- B cells resist killing by freshly isolated CTLs but not by in vitro expanded blasts
- Non-lytic degranulation is associated with defective activation of LAT
- Granule dispersion and exocytosis are mediated by the GTPases Arl8 and Rab27a

Authors

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In Brief

Kabanova et al. provide evidence that resting human B cells escape killing by cytotoxic T cells (CTLs) by inducing non-polarized exocytosis of their lytic granules. Non-polarized degranulation is associated with an altered formation of the immune synapse and may represent a mechanism that allows B cell malignancies to evade CTLs.

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Human Cytotoxic T Lymphocytes Form Dysfunctional Immune Synapses with B Cells Characterized by Non-Polarized Lytic Granule Release

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SUMMARY

Suppression of the cytotoxic T cell (CTL) immune response has been proposed as one mechanism for immune evasion in cancer. In this study, we have explored the underlying basis for CTL suppression in the context of B cell malignancies. We document that human B cells have an intrinsic ability to resist killing by freshly isolated cytotoxic T cells (CTLs), but are susceptible to lysis by IL-2 activated CTL blasts and CTLs isolated from immunotherapy-treated patients with chronic lymphocytic leukemia (CLL). Impaired killing was associated with the formation of dysfunctional non-lytic immune synapses characterized by the presence of defective linker for activation of T cells (LAT) signaling and non-polarized release of the lytic granules transported by ADP-ribosylation factor-like protein 8 (Arl8). We propose that non-lytic degranulation of CTLs are a key regulatory mechanism of evasion through which B cells may interfere with the formation of functional immune synapses by CTLs.

INTRODUCTION

A global dysfunction of cytotoxic T cells (CTLs) has been observed in many types of tumors and persisting infections that have been generally associated with a so-called "immune exhaustion." Dysfunction involves an up-regulation of inhibitory receptors, reduced cytokine production, and impaired activity of CTLs (Speiser et al., 2014). Nevertheless, a strong rationale behind therapeutic approaches aiming to reactivate latent CTLs comes from studies in which anti-tumor immunity can be restored in melanoma patients by blocking the inhibitory checkpoint receptors (Pardoll, 2012). Thus, a better understanding of the exhaustion mechanisms could further contribute to the spectrum of therapies aimed at reversing functional anergy of CTLs in various pathological settings.

CTL cytotoxicity depends on the recognition of cognate targets by the antigen-specific T cell receptor (TCR) followed by a directed release of the contents of specialized secretory lysosomes called 'lytic granules' toward the target. This degranulation process depends on the formation of the lytic immune synapse (IS) between the effector CTL and the target resulting in the translocation of the CTL's microtubule organizing center (MTOC) towards the synaptic interface. MTOC docking beneath the IS that depends on protein kinase C (PKC) isoforms (Liu et al., 2013) ensures dynein- and Ca^{2+}-dependent targeted delivery and exocytosis of the granule content directly into the synaptic cleft, which results in target cell lysis by concerted action of granzymes and perforin (Stinchcombe et al., 2006).

Not much is known about regulation of lytic granule exocytosis in CTLs, especially with respect to their dysfunction in tumor pathogenesis. Chronic lymphocytic leukemia (CLL) is the most common B cell neoplasia and is characterized by the accumulation of mature long-lived B cells in the blood and lymphoid tissues and is due, at least in part, to an impairment of cytotoxic immunity. While very efficient in presenting tumor-associated antigens (Trojan et al., 2000), CLL B cells have been shown to resist being killed by autologous CTLs (Krackhardt et al., 2002). Here, we addressed the mechanisms of leukemic B cell resistance by analyzing the molecular features of immune synapses formed between effector CTLs and B cells that were freshly isolated from CLL patients and healthy donors. We observed that resting B cells, even from healthy donors, were intrinsically resistant to cytotoxic lysis and that this was due to their ability to induce dysfunctional degranulation in CTLs.

RESULTS

Resting B Cells Resist Being Killed by Inducing Non-Lytic Degranulation in Freshly Isolated Effector CTLs

To acquire insight into molecular mechanisms underlying the functional impairment of effector CTLs, we first assessed the cytotoxic potential of CTLs freshly isolated from peripheral blood of CLL patients who had never received any treatment. These patients showed an elevated percentage of $CD28^-$ $CD8^+$ CTLs belonging

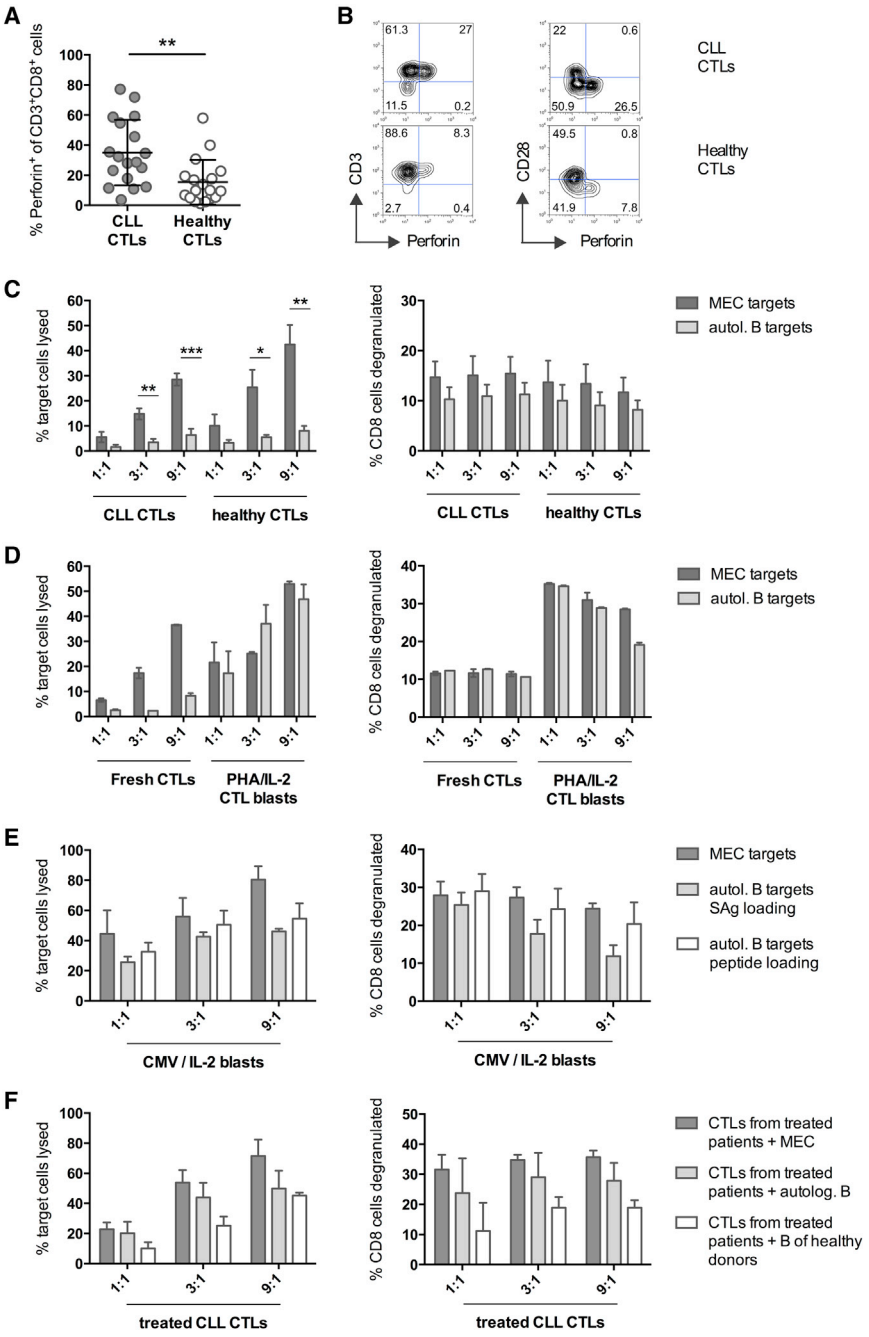

Figure 1. Effector CTLs from Untreated CLL Patients and Healthy Donors but Not CTL Blasts or CTLs from Immunotherapy-Treated Patients Fail to Lyse Donor B Cells Despite Regular Degranulation

(A) Immunophenotypic analysis of CTLs isolated from peripheral blood of CLL patients (n = 18) and healthy donors (n = 18). Each dot represents a single patient.

(B) Flow cytometry profile of the total $CD8^+$ population from a CLL patient and a healthy donor.

(C) Flow cytometry analysis of cytotoxicity and degranulation for CTLs freshly isolated from CLL patients (n = 5) and healthy donors (n = 5).

(D) Flow cytometry analysis of cytotoxicity and degranulation for freshly isolated CTLs and CTL blasts expanded from the same donor by PHA/IL-2 stimulation.

(E) Flow cytometry analysis of cytotoxicity and degranulation for CTL blasts expanded from healthy donors (n = 3) by CMV peptide/IL-2.

(F) Flow cytometry analysis of cytotoxicity and degranulation for CTLs freshly isolated from immunotherapy-treated CLL patients (n = 3).

For assays CTL blasts were incubated with indicated targets at 1:1, 3:1, and 9:1 CTL-to-target ratio for 4 hr. Shown are mean values ± SD; *p < 0.01, **p < 0.01, ***p < 0.001.

to the effector perforin-rich subset (Figures 1A and 1B). CLL CTLs appeared to maintain functional competence given that they were able to kill control targets such as the CLL-derived B cell line MEC loaded with superantigen (SAg) to promote polyclonal TCR recognition (Figure 1C; left panel). Cytotoxic lysis of MEC cells correlated with CTL degranulation that was assessed as surface translocation of the lysosome-associated membrane protein 1 (LAMP1) that associates with the CTL surface during granule exocytosis (Figure 1C; right panel) (Betts et al., 2003). By contrast, CTLs showed greatly reduced levels of cytotoxicity when conjugated with SAg-loaded autologous CLL B cells while degranulating to a similar extent (Figure 1C; Figure S1). Strikingly, CTLs freshly isolated from healthy donors were able to kill susceptible MEC targets, while their cytotoxicity against autologous B cells was inhibited despite regular degranulation (Figure 1C). Hence, our findings suggested that non-lytic degranulation of freshly isolated CTLs could be induced specifically in the presence of donor B cells, irrespective of malignancy status.

The phenotype of CTLs can be altered upon in vitro expansion in the presence of interleukin-2 (IL-2) (Hackmann et al., 2013; Zippelius et al., 2004). We therefore expanded CTLs in vitro by IL-2 and phytohaemagglutinin (PHA) stimulation and evaluated their cytotoxic potential relative to freshly purified CTLs from the same donor. Although we could not directly compare cytotoxicity of unstimulated CTLs and CTL blasts as a result of differences in the percentage of perforin-positive CTLs (average 23.0% ± 12.3% and 60.8% ± 9.2% in total pool, respectively), we could nonetheless compare how their cytotoxic behavior changed in respect to different targets. At variance with their freshly purified counterparts, expanded CTL blasts acquired the potential to kill SAg-loaded donor B cells (Figure 1D). Likewise, CMV-specific CTL blasts could kill donor B cells pulsed with either SAg or CMV peptide (Figure 1E). In addition, we observed that CTLs freshly isolated from patients

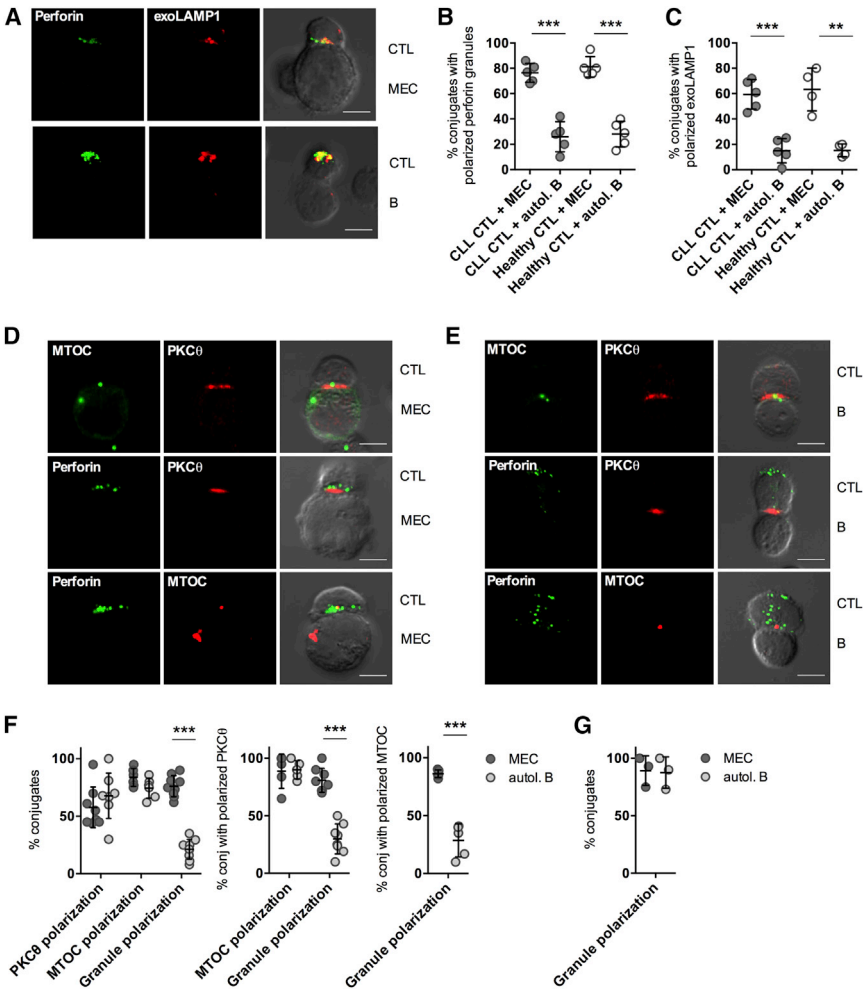

Figure 2. Non-Lytic Degranulation of CTLs Is Associated with a Selective Perturbation of Late CTL Polarization Events

(A) Immunofluorescence images of lytic and non-lytic CTL synapses (40 min) stained with antibodies against perforin and exocytosed LAMP1 (exo-LAMP1).

(B and C) Quantitative analysis of lytic granule and exoLAMP1 polarization in perforin-positive CTLs from CLL patients (n = 5) and healthy donors (n = 4 or 5).

(D and E) Immunofluorescence images of (D) lytic and (E) non-lytic CTL synapses (30 min) stained with antibodies against PKCθ, γ-tubulin, and perforin.

(F) Analysis of PKCθ/MTOC/lytic granule polarization in perforin-positive CTL synapses (n = 5–9 donors). Values are expressed as the percentage of conjugates among total conjugates (left panel), conjugates with polarized PKCθ (middle panel), and conjugates with a polarized MTOC (right panel).

(G) Analysis of granule polarization in synapses of CTL blasts (n = 3 donors). Measurements were taken from 30 conjugates for each donor and condition; each dot represents mean value for a single donor. Images are representative of 9 independent experiments. Scale bar, 5 μm. Shown are mean values ± SD; $^{**}p < 0.01$, $^{***}p < 0.001$.

treated with a combination of B-cell-targeting antibodies and chemotherapy were also able to kill autologous B cells (Figure 1F; see the Supplemental Experimental Procedures for details on therapy regimens). We associated this enhancement in cytotoxicity with a phenotypical change occurring in CTLs, since the same cells were also able to kill allogeneic B cells of healthy donors (Figure 1F). Our findings therefore imply that an alteration of the phenotype induced in CTLs by IL-2 activation in vitro or immunotherapy in vivo dramatically enhances the ability of CTLs to kill donor B cells.

B Cells Fail to Induce Polarized Degranulation of Lytic Granules Directed to the IS

Imaging analysis of synapses formed by freshly isolated CTLs revealed that, while MEC targets promoted the formation of lytic IS with perforin-positive granules both polarized and exocytosed in the direction of synaptic cleft, resistant B cell targets failed to induce polarization of the granules despite inducing degranulation (Figure 2A). Quantitative analysis confirmed that the majority of effector CTLs from both CLL and healthy donors engaged with MEC targets were able to concentrate lytic granules at the IS and release them locally, while they had a dramatically impaired ability to translocate granules toward the IS with B cells. (Figures 2B and 2C).

To evaluate whether a defect in granule polarization could indeed be responsible for inhibited CTL function, we assessed if the Fas/FasL axis, a second major mechanism of intrinsic CTL cytotoxicity (Trapani and Smyth, 2002), was implicated in killing by freshly isolated CTLs. We observed that neither FasL-blocking antibody nor CTL pre-treatment with brefeldin, which blocks the exocytosis of FasL-containing vesicles (Li et al., 1998), inhibited cytotoxicity against MEC cells (Figures S2A and S2B). Additionally, in a panel of B cell lines, we found that the Burkitt lymphoma-derived line BJAB, resistant to being killed and able to induce non-polarized CTL degranulation, exhibited similar levels of Fas expression and sensitivity to anti-Fas-antibody-induced cell death as compared to MEC cells (Figure S2C–S2G). This finding suggests that in our system the Fas/FasL pathway contributes minimally to the cytotoxic potential of freshly isolated CTLs.

We further compared different phases of IS maturation for lytic and non-lytic IS and observed that, in CTLs conjugated with MEC cells, PKCθ accumulated at the IS leading to MTOC and lytic granule polarization (Figure 2D). In CTLs conjugated with resistant B cells, PKCθ and MTOC translocated toward the IS normally, but this did not lead to polarization of lytic granules (Figures 2E and 2F;left panel). Consistently, a similar difference in granule polarization was observed for CTL conjugates with either PKCθ or MTOC already recruited to the synapse (Figure 2F, middle and right panels). Importantly, the acquisition of cytotoxic potential against donor B cells by in vitro expanded CTL blasts correlated with the ability of CTLs to polarize their lytic granules at the IS (Figure 2G),

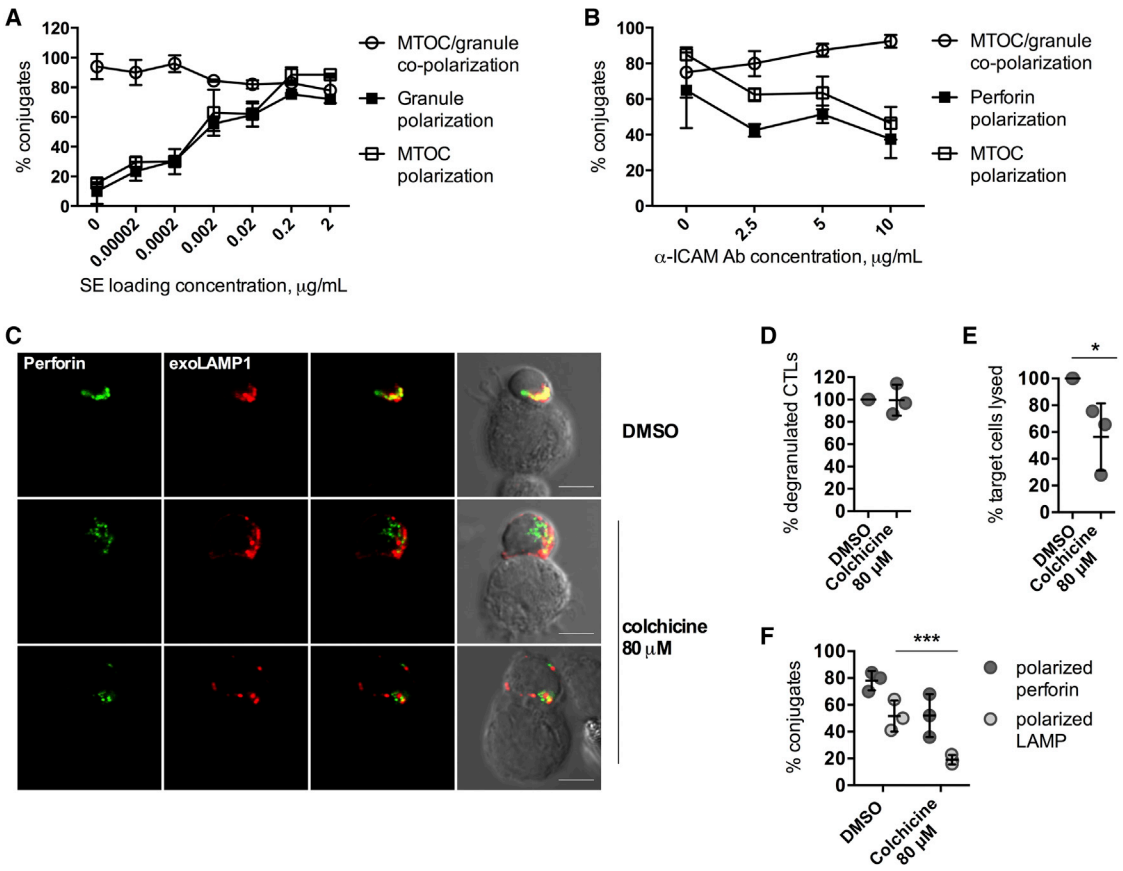

Figure 3. Non-Lytic Degranulation Can Be Induced in Functional Lytic Synapses by Microtubule Disruption but Not by Lowering the Number of Engaged TCRs or Withdrawal of LFA-1 Costimulation

(A and B) Quantitative analysis of MTOC/granule polarization in perforin-positive CTLs incubated for 30 min with MEC cells (A) pulsed with different SAg concentrations or (B) at different concentrations of blocking ICAM-1-specific antibody.

(C) Immunofluorescence images of synapses (40 min, stained for perforin and exo-LAMP1) between MEC cells and CTLs pre-treated with 80 μM colchicine or carrier for 30 min prior to conjugation. Scale bar, 5 μm.

(D and E) Flow cytometry analysis of degranulation (D) and cytotoxicity (E) on control and colchicine-treated CTLs incubated with MEC targets for 4 hr. Quantitative analysis of lytic granule and exoLAMP1 polarization in control and colchicine-treated perforin-positive CTLs incubated with MEC cells for 40 min (30 conjugates per donor and condition) is shown (E).

Each dot represents mean values for three donors (A and B) or a single donor (D–F). Shown are mean values ± SD; *$p < 0.05$, ***$p < 0.001$.

thus suggesting that the observed differences in killing cytotoxic potential were primarily defined by CTL ability to control granule movement.

Finally, we analyzed F-actin polarization in CTLs engaged with B cells and MEC targets, as it has been proposed that CLL $CD4^+$ and $CD8^+$ T cells have an intrinsic defect in actin polymerization at the IS (Ramsay et al., 2008). Our analysis indicated that non-lytic synapses had a decreased capacity to polymerize actin at the IS (Figures S3A and S3B). This finding therefore suggests that an impaired F-actin accumulation at the IS could be potentially linked to a selective defect in late polarization events characteristic of non-lytic CTL synapses.

Non-Lytic CTL Degranulation Does Not Depend on the Number of Engaged TCRs or LFA Costimulation

TCR triggering with low-affinity ligands can lead to an impairment in lytic granule polarization and exocytosis (Jenkins et al., 2009), thus potentially implicating the strength of TCR triggering in the control of granule movement. Considering that we used a mixture of SAgs for target cell pulsing, the affinity of TCR binding to HLA-DR/SAg complexes displayed by MEC and B cells was comparable, although it could be argued that the defect in CTL polarization occurred as a result of different numbers of TCRs engaged (average mean fluorescence intensity [MFI] HLA-DR 279.7 ± 50.5 for MEC and 107.2 ± 25.5 for B cells). To test this hypothesis, we pulsed MEC cells with progressively lower concentrations of the SAg mixture and observed that decreasing HLA-DR/SAg led to a decline in the number of CTL conjugates able to polarize MTOC and perforin granules. However, the granules did not fail to follow the MTOC at any SAg concentration (Figure 3A). Hence, factors other than the number of engaged TCRs are likely to be responsible for the inhibition of granule polarization in non-lytic IS.

Lymphocyte function-associated antigen-1 (LFA-1) interaction with intracellular adhesion molecule (ICAM-1) expressed

on antigen-presenting cells (APCs) has been shown to facilitate CTL killing by stabilizing the nascent IS (Anikeeva et al., 2005). We observed that, although donor B cells expressed less ICAM-1 compared to MEC cells, the resistant BJAB cells had comparably high ICAM-1 levels (Figure S3C), suggesting that an enhanced LFA-1 costimulation was not sufficient to promote lytic granule polarization. Moreover, the relatively lower ICAM-1 levels on donor B cells were nonetheless sufficient to promote significant LFA-1 accumulation at the IS (Figures S3D and S3E), concomitant with recruitment of phosphorylated Vav-1 and talin, which are typically activated following LFA-1 triggering (Figures S3F–S3H) (Nolz et al., 2007). Additionally, an ICAM-1 blockade affected the efficiency of CTL conjugation as expected but did not provoke uncoupling between the MTOC and granule movement in synapses that were still formed (Figure 3B). Taken together, all three lines of evidence indicated that a decreased LFA-1 polarization in non-lytic synapses was not responsible for defective granule polarization.

Non-Lytic Degranulation Can Be Induced in Functional Lytic Synapses by Microtubule Disruption

To evaluate whether interference with granule translocation could lead to a non-lytic CTL degranulation, we analyzed synapses of CTLs pre-treated with the microtubule-disrupting agent colchicine. Interestingly, although the treatment induced a complete collapse of the microtubule cytoskeleton, colchicine-treated CTLs were still able to degranulate when engaged with MEC cells (Figures 3C and 3D). Cytotoxicity of such CTLs decreased significantly (Figure 3E), which correlated with randomization in their degranulation pattern (Figure 3F). This finding indicated that non-polarized granule exocytosis could be one of the principal factors responsible for CTL dysfunction.

Non-Lytic Degranulation of Freshly Isolated CTLs Is Associated with a Selective Defect in LAT Activation

The strength of TCR-induced signaling and kinetics of Ca^{2+} influx could have an important and non-redundant function in the regulation of lytic granule polarization (Beal et al., 2009; Jenkins et al., 2009). In view of this, we evaluated both parameters in freshly isolated enriched CTL effectors (Figure S4) and CTL blasts engaged with either MEC or donor B cells. Thus, we explored one case of non-lytic (unstimulated effector CTLs + B cells) and three cases of lytic (unstimulated effector CTLs + MEC cells and CTL blasts + B cells/MEC cells) degranulation. We did not observe significant differences in the overall profile of Ca^{2+} elevation between the different combinations, although Ca^{2+} influx was significantly accelerated in CTL blasts (60.0 ± 4.0 s after CTL-target contact to influx) compared to freshly isolated CTLs (176.3 ± 18.2 s) (Figure 4A). Therefore, at variance with the altered granule polarization in $CD4^+$ CTL blasts associated with diminished Ca^{2+} influx (Beal et al., 2009), no obvious defect in Ca^{2+} signaling characterized non-lytic CTL degranulation.

Assessing the strength of TCR-induced signaling in the same experimental setup showed that the magnitude of ZAP-70 and Erk2 phosphorylation were similar between non-lytic and lytic synapses, while activation of the linker for activation of T cells (LAT) and extracellular-signal-regulated kinase 1 (Erk1) were significantly decreased in freshly isolated effector CTLs engaged with resistant B cell targets (Figures 4B and 4C). However, treatment of CTLs with the Erk inhibitor PD98059 did not affect either granule polarization or cytotoxic potential of freshly isolated CTLs (Figures S5A and S5B), ruling out a defective Erk1 activation in the non-lytic degranulation phenotype. Furthermore, protein kinase Akt was activated in CTLs engaged with MEC cells but became poorly activated in B cell synapses with both unstimulated CTLs and CTL blasts (Figures 4B and 4C). Considering that CTL blasts showed strong cytotoxicity against B cells (Figures 1D and 1E) and that the Akt inhibitor wortmannin did not affect granule polarization in CTL synapses (Figure S5C), our data indicated that Akt signaling is dispensable for cytotoxic killing.

Finally, using imaging analysis of protein phosphorylation at the IS we confirmed that ZAP-70 was equally activated in both lytic and non-lytic synapses, while LAT activation was selectively blocked in the latter case (Figures 4D and 4E). Therefore, our findings provide evidence that non-lytic degranulation of freshly isolated CTLs is associated with a defect in LAT activation, but not Akt or Erk1 signaling, and that the acquisition of killing capacity by CTL blasts is accompanied by intact LAT signaling.

Both Lytic and Non-Lytic Degranulation Is Dependent on a Rab7/Rab27a-Positive Compartment

Rab27a, a member of the Rab subfamily of GTPases, which is dispensable for lytic granule transport to the IS, defines a late endosomal compartment that promotes lytic granule maturation and exocytosis (Ménager et al., 2007). Confocal imaging of Rab27a in freshly isolated CTLs showed that the Rab27a-positive compartment extensively colocalized with another late endosomal marker, Rab7 (Figure 5A). However, neither Rab27a nor Rab7 showed a significant colocalization with lytic granules as compared to the granule marker LAMP1 (Figure 5B–5D), although occasional fusion events could be detected between Rab27a/Rab7 and lytic granules in CTLs engaged in synapses (Figure 5B and 5C). Rab27a depletion by siRNA nucleofection led to a dramatic impairment in the ability of freshly isolated CTLs to degranulate with both MEC and B cells (Figure 5E and 5F) thus indicating that Rab27a, together with Rab7, defines a vesicular compartment which is essential for both lytic and non-lytic degranulation of CTLs.

The Arl8 Adaptor Is Necessary for Microtubule-Based Migration of Lytic Granules and Degranulation

The ADP-ribosylation factor-like protein 8 (Arl8), identified in the lysosomal proteome, has been implicated in kinesin-dependent plus-end directed granule movement toward the cell periphery (Nylandsted et al., 2011; Rosa-Ferreira and Munro, 2011). We analyzed its possible implication in lytic granule movement in CTLs and observed that Arl8-specific staining largely overlapped with perforin-positive granules in resting CTLs (Figure 5D–5G). Importantly, it also colocalized with lytic granules in CTLs engaged in synapses (Figure 5G), suggesting that kinesin-binding Arl8 could be implicated in lytic granule transport.

Of the two Arl8 isoforms, Arl8a and Arl8b, which are 91% identical and phenocopy each other, the latter seemed to be the predominant isoform expressed in CTLs, similar to dendritic and NK cells (Garg et al., 2011; Tuli et al., 2013) (Figure 5H). Consistent with the high sequence homology between the two genes, we

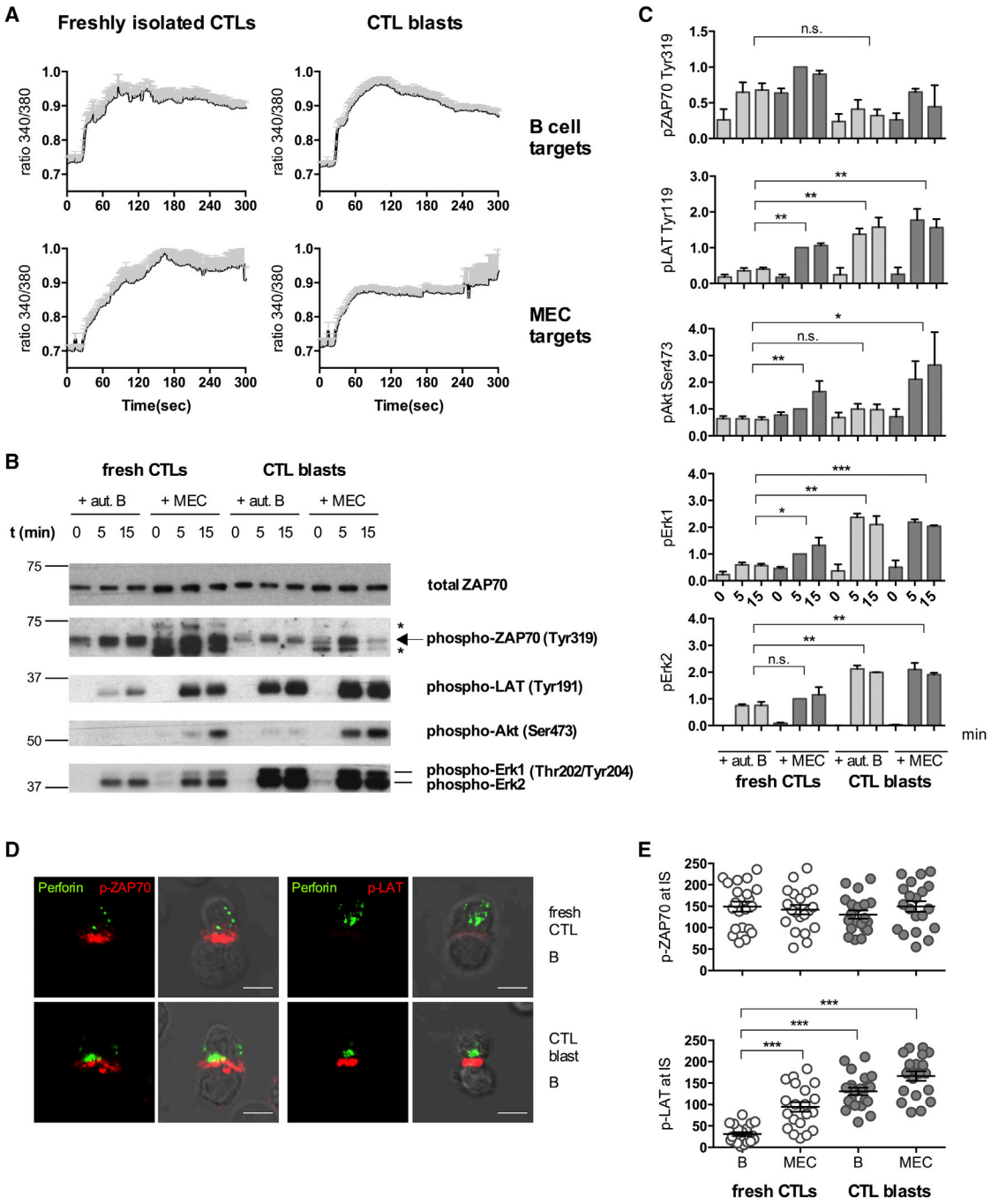

Figure 4. Non-Lytic Degranulation of Freshly Isolated CTLs Is Associated with a Selective Defect in LAT Activation which Is Rescued in In Vitro Expanded CTL Blasts

(A) Time-dependent changes in intracellular Ca^{2+} concentration in freshly isolated CTLs and CTL blasts induced by interaction with targets (n = 2 donors). The curves represent mean values ± SD for 20 CTLs.

(B) Immunoblots of non-lytic and lytic CTL synapses. *Non-specific bands.

(C) Quantification of immunoblots (n = 2 or 3 donors).

(D and E) Immunofluorescence images (D) and quantitative analysis (E) of ZAP-70/LAT phosphorylation in perforin-positive CTL synapses. Values are expressed as a difference in average fluorescence intensity of two synaptic membrane regions versus two distal membrane regions; 20 conjugates were analyzed for each donor (n = 2) and condition.

Immunoblots and images are representative of three and two independent experiments for freshly isolated CTLs and CTL blasts, respectively. Scale bar, 5 μm. Shown are mean values ± SD; *p < 0.05, **p < 0.01, ***p < 0.001.

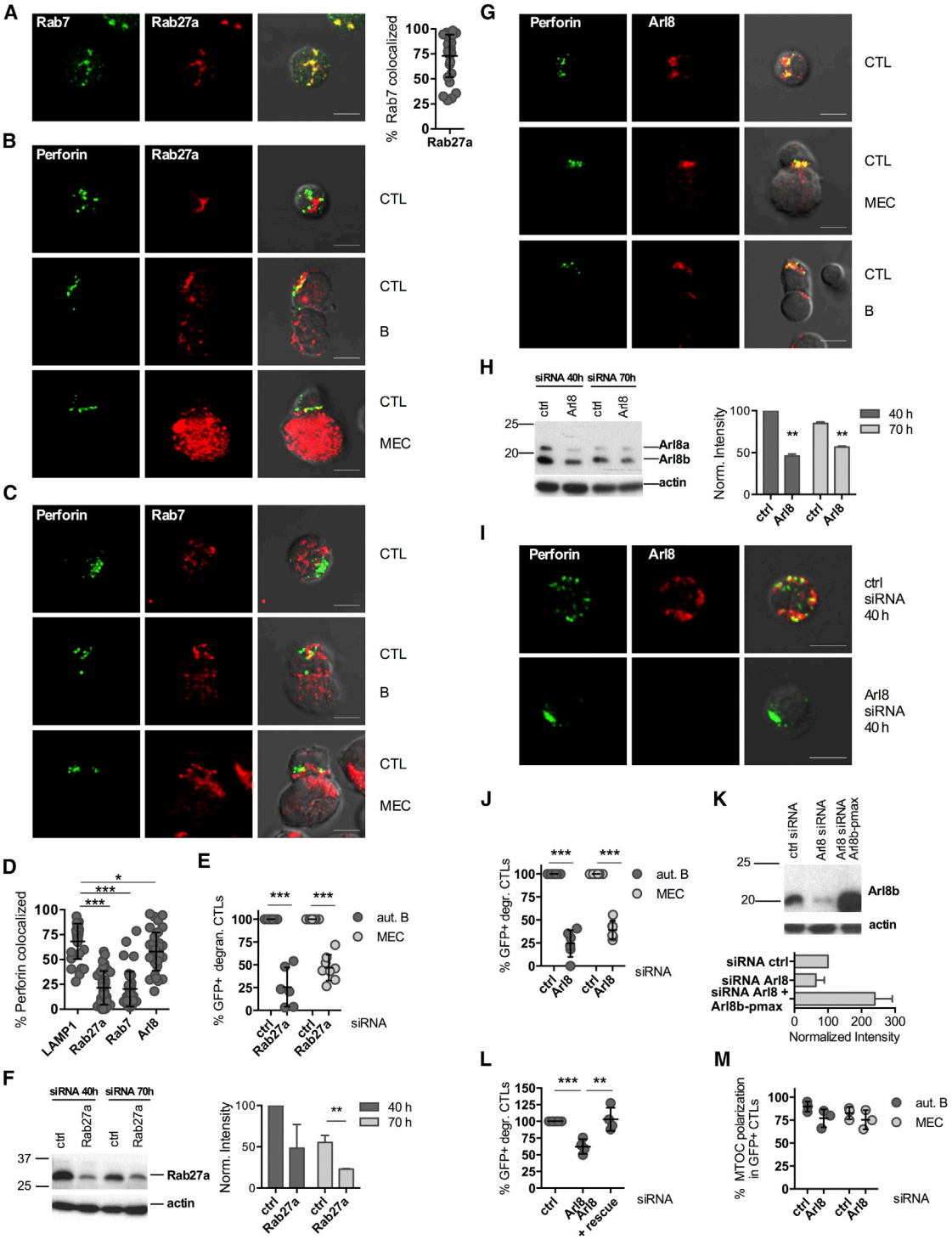

Figure 5. Non-Lytic Degranulation Is Dependent on a Rab7/Rab27a-Positive Compartment and Arl8-Assisted Lytic Granule Translocation

(A) Immunofluorescence images and colocalization analysis of Rab7/Rab27-positive compartments in freshly isolated CTLs.

(B and C) Immunofluorescence images of CTLs, either as such or conjugated with targets for 40 min, stained with antibodies against perforin and Rab27a/Rab7 (n = 3 donors).

(D) Co-localization analysis between lytic granules, LAMP1-, Rab27a-, Rab7- and Arl8-positive compartments in CTLs. Quantitative measurements were taken for 15-20 CTLs (n = 2 donors).

(E) Flow cytometry analysis of degranulation on pmaxGFP/siRNA co-trasfected CTLs analyzed at 40/70 hr post-nucleofection for B cell/MEC conjugates, respectively.

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were only able to assess the impact of global Arl8 silencing in our system since the Arl8b-specific siRNA pool knocked down both Arl8a and Arl8b (Figure 5H). First, Arl8 depletion resulted in the perinuclear clustering of lytic granules (Figure 5I), suggesting that Arl8 favors lytic granule positioning in the proximity of the plasma membrane. Furthermore, Arl8 silencing led to a profound impairment in the ability of CTLs to degranulate both with donor B cells and MEC cells (Figure 5J), while the effect of siRNA treatment was reversed by co-transfecting CTLs with a vector-expressing Arl8b (Figure 5K and 5L). Given that it has been observed that Arl8b silencing negatively affects MTOC recruitment at the synapse in NK cells (Tuli et al., 2013), we analyzed MTOC polarization in Arl8-silenced CTLs. We did not observe a decrease in MTOC translocation toward the IS (Figure 5M), indicating that the phenotype of Arl8 deficiency was associated with inhibited granule movement but not MTOC migration. Collectively, our observations indicate that Arl8 is essential for both non-lytic and lytic degranulation of CTLs.

DISCUSSION

Here, we illustrate a peculiarity of the interaction between resting human B cells and effector CTLs, resulting in a dysfunctional degranulation and unexpectedly shared by both leukemic and healthy cells. We found that B cells, which are intrinsically susceptible to cytotoxic lysis can instruct CTLs to release lytic granules in a non-polarized fashion and thus protect themselves from being killed. This may be relevant in the context of B cell malignancies. We attempted to dissect the underlying molecular mechanism and found that an impaired convergence of lytic granules toward the IS was associated with a selective blockade of the TCR-signaling cascade at the level of LAT. This impaired convergence was achieved through Arl8-assisted lytic granule movement toward the cell periphery where the granules mature by fusing with Rab27a- andRab7-positive late endosomes and release their contents diffusely rather than into the synaptic cleft.

The phenotype of non-lytic degranulation, in which ZAP-70 activation and Ca^{2+} influx proceed without further spread of the signaling onto LAT, contrasts with a situation in which engagement of murine CTL blasts with low-affinity ligands causes a dramatic defect in the activation of lymphocyte-specific protein tyrosine kinase (Lck) thus leading to a profound block in both lytic granule polarization and degranulation (Jenkins et al., 2009). Coupled with this observation, our results imply an important bifurcation of TCR signaling in CTLs. We propose that the signaling upstream of ZAP-70 controls CTL activation generally while signaling downstream of ZAP-70, as exemplified here by LAT, finely tunes the pattern of CTL polarization.

We demonstrate that LAT signaling is intact in CTL blasts, which are cytotoxic to otherwise resistant B cells. Our observation might also explain why non-lytic degranulation could have been overlooked in the traditional experimental setups based on the usage of CTL blasts. In vitro expansion boosts effector functions of tumor-specific CTLs that are isolated from melanoma patients (Zippelius et al., 2004) and can even restore cytotoxicity in CTLs and NK cells from patients with genetic mutations (Bryceson et al., 2007; Hackmann et al., 2013). In this regard, it is interesting to note, according to our data, a similar phenotypical change occurs in CTLs from leukemic patients in response to a combined chemo-immunotherapy treatment. Collectively, our work underscores the importance of studying freshly isolated CTLs and the function of LAT that could provide important insights into the mechanisms regulating lytic granule transport.

Our results identify the kinesin-binding Arl8 as one of the factors controlling lytic granule positioning. Importantly, while Arl8 appears to promote the plus-end directed movement of lytic granules during non-lytic degranulation, it might also play a role in the assembly of a conventional lytic synapse of CTLs. In support of our findings, it has been demonstrated that the terminal transport of granules toward the synaptic interface is kinesin-1-dependent (Kurowska et al., 2012), thus potentially implicating Arl8 in this process.

We also show that lytic granule maturation mediated by Rab27a is implicated in non-lytic degranulation of CTLs. Rab7, which extensively colocalized with the Rab27-positive compartment, has already been implicated in regulation of lytic granule polarization based on the observation that an overexpression of the dynein-binding effector of Rab7 and RILP in human CTL blasts has been shown to promote granule translocation in the minus-end direction and clustering around the MTOC (Daniele et al., 2011; Stinchcombe et al., 2006). We and others, however, failed to detect an association between Rab7 and lytic granules by immunofluorescence analysis (Ménager et al., 2007), which implies that the identity of trafficking regulators for lytic granules is yet to be confirmed.

Further studies are needed to understand if the mechanism of non-lytic CTL degranulation might play a role in tumor growth and could represent a mechanism of immune evasion. Interestingly, though, our findings underscore the role of B cells in immune regulation of cytotoxic response since B cells have long been suggested to play a role in the induction of tolerance in naive $CD8^+$ T cells (Bennett et al., 1998; Overstreet et al.,

(F) Immunoblots of CTLs nucleofected with siRNAs and analyzed at 40/70 hr post-treatment (left); quantification of n = 3 immunoblots (right).
(G) Images of CTLs (n = 5 donors), either as such or conjugated with targets for 40 min and stained with antibodies against perforin and Arl8.
(H) Immunoblots of CTLs nucleofected with siRNAs (left); quantification of n = 3 immunoblots (right).
(I) Images of CTLs nucleofected with siRNA (n = 3 donors) stained with antibodies against perforin and Arl8.
(J) Flow cytometry analysis of degranulation on pmaxGFP/siRNA co-trasfected CTLs analyzed at 40/70 hr post-nucleofection for B cell and MEC conjugates, respectively.
(K and L) Rescue experiment was performed by co-transfecting CTLs (n = 2 donors) with siRNAs and Arl8b-pmax vectors. Shown are (K) immunoblots with quantification and (L) flow cytometry analysis of degranulation on 40 hr post-treatment CTLs engaged with donor B cells. In graphs (E), (J), and (L), each dot represents the replicate value for a single donor (n = 3 or 4).
(M) Quantitative analysis of MTOC polarization in synapses (30 per donor, n = 3) of GFP-positive CTLs co-transfected with pmaxGFP/siRNA at 40 and 70 hr post-nucleofection for B cell and MEC conjugates, respectively. Scale bar, 5 μm; shown are mean values ± SD; *p < 0.05, **p < 0.01, ***p < 0.001.

2010; Qin et al., 1998). In turn, our work illustrates a molecular mechanism by which antigen-presenting B cells can directly affect functioning of effector CTLs. In this regard, major questions that are still unanswered include which stimuli are responsible for the induction of non-lytic degranulation and what impact this regulatory process may have on tumor pathogenicity in vivo.

EXPERIMENTAL PROCEDURES

Patients and Healthy Controls

Peripheral blood samples of CLL patients (18 untreated and three treated as indicated in the Supplemental Experimental Procedures) and healthy donors were analyzed after we received their signed informed consent according to institutional guidelines.

Lymphocyte Isolation and Culture

CTLs and B cells were purified by negative selection using RosetteSep cocktails (StemCell) to purity > 95% for CTLs and >90% for B cells. Primary lymphocytes were cultured in bicarbonate-free RPMI-1640 with 20 mM HEPES (RPMI-HEPES), 7.5% iron-enriched HyClone BCS (ThermoScientific), and 2 mM L-glutamine and 50 IU/mL penicillin. For the assays with freshly isolated CTLs, we chose donors with at least 15% of perforin-positive cells among total $CD8^+$ population (average 23.0% ± 12.3%).

Culturing of B cell lines, reagents, generation of CTL blasts and colchicine treatment of CTLs, flow cytometry assays of cytotoxicity and CTL degranulation, Ca^{2+} flux measurements, and antibodies used for flow cytometry are detailed in the Supplemental Experimental Procedures and Table S1.

Immunofluorescence Microscopy

APCs (1.25×10^5) were loaded with SAg mixture and 2.5 μM Cell-Tracker-Blue (Invitrogen), then washed in RPMI-HEPES and incubated with CTLs (1.25×10^5) in 20 μl of RPMI-HEPES at 37°C bath for the indicated time. 15 min before the end of conjugation cells were gently re-suspended and transferred onto slides- then fixed with 4% formaldehyde and stained as indicated in Table S2. Confocal microscopy on 0.9-μm-thick sections was performed on a LSM700 (CarlZeiss) using a 63× objective with Zen-2009 software and processed with ImageJ. For the quantitative analysis CTL conjugates were scored as having polarized granules/exo-LAMP1 vesicles if the large majority (>90%) of granules/vesicles were localized at the IS. Colocalization analysis was performed by calculating Manders coefficient with the JACoP plugin.

Immunoblotting

Equal amount of cell lysates (10–20 μg of lysates for phospho-blotting prepared as indicated in the Supplemental Experimental Procedures or 10^6 cells for Rab27/Arl8 blotting) were immunoblotted using primary antibodies (Table S3).

CTL Nucleofection with siRNAs

Freshly isolated CTLs ($5–10 \times 10^6$) were nucleofected with 200 nM iBONi siRNA pools (Table S4; Riboxx GmbH) and 2 μg pmax-GFP vector using a Human T Cell Nucleofector kit (Lonza, program V-024). siRNA rescue experiment is detailed in the Supplemental Experimental Procedures.

Statistics

Mean values, SD values, and the p values associated with two-tailed unpaired Student's test were calculated using the GraphPad software.

SUPPLEMENTAL INFORMATION

The Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.084.

AUTHOR CONTRIBUTIONS

A.K. designed and performed research, analyzed data, and wrote the manuscript; F.S. and A.G. performed research; V.C., A.G., and G.C. contributed vital reagents; M.B. contributed vital reagents and reviewed the manuscript; C.T.B. designed, researched, and wrote the manuscript.

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