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CUTTING EDGE

Cutting Edge: Cytotoxic Granule Polarization and Cytolysis Can Occur without Central Supramolecular Activation Cluster Formation in CD8$^+$ Effector T Cells[1]

James P. O'Keefe[*‡] *and Thomas F. Gajewski*[2*†‡§]

***The functional implication of molecular segregation within the immunological synapse remains uncertain. We recently reported that effector but not naive TCR transgenic murine CD8$^+$ T cells formed immunological synapses containing a central supramolecular activation cluster (cSMAC), suggesting that execution of effector functions such as cytolytic activity might be facilitated by the cSMAC structure. We have now explored this hypothesis using two approaches. First, by simultaneously imaging cSMAC formation and mobilization of cytotoxic granules to the synapse, we observed no correlation between the presence of a cSMAC and granule reorientation. Second, we took advantage of the observation that CD28 costimulation markedly enhances cSMAC formation. Granule polarization to the contact site was indistinguishable with B7-1$^+$ and B7-1$^-$ target cells, and cytolytic activity against B7-1$^+$ or B7-1$^-$ targets was similar and granule-dependent. Together, our results indicate that the formation of a cSMAC is not required for cytolytic activity in CD8$^+$ effector T cells. The Journal of Immunology,* 2005, 175: 5581–5585.**

CD8$^+$ T cells play a critical role in the clearance of viral infections and the eradication of tumors. Upon encountering a target cell with the corresponding Ag specificity, a CD8$^+$ effector T cell engages the target cell. The TCR and other coreceptors then become localized, facilitating the formation of signaling complexes with intracellular proteins and the initiation of signal transduction cascades, thus driving execution of effector functions. For CD8$^+$ T cells, a critical effector function is the directional release of lytic granules into the target cell, resulting in eventual target cell death.

It has been appreciated that, following initial TCR-dependent T cell stimulation, a subset of molecules is repositioned to the T cell/Ag-expressing cell (AEC)[3] contact site, or immunological synapse (1–3). Further molecule subpartitioning can occur at the immunological synapse, resulting in what have been termed the central and the peripheral supramolecular activation cluster (cSMAC and pSMAC, respectively). Protein kinase C (PKC)-θ is a novel member of the PKC family that is the only PKC isoform to be recruited to the immunological synapse (4), and has been used as a marker for the cSMAC structure (1, 5). Talin, a β-actin binding protein that is associated with integrin signaling complexes (6), has been used as a marker for the pSMAC (1, 3).

Although striking images of cSMAC/pSMAC segregation have been observed in some T cell/AEC model systems, the physiological function of this architecture remains elusive. It has been proposed that immune synapse formation is necessary for agonist-driven signaling (2, 7); however, recent observations have called into question this hypothesis (8, 9). We have recently observed using a CD8$^+$ TCR Tg system that naive T cells become activated, produce IL-2, proliferate, and differentiate into effector cells, all in the absence of detectable cSMAC/pSMAC segregation (5). In as much as effector cells but not naive cells were capable of forming a cSMAC in this model, the question of whether cSMAC formation in effector cells could be linked to other functions specific to effector, but not naive, CD8$^+$ T cells, such as directed cytolytic activity, was raised. This hypothesis was supported by observations of Griffiths and colleagues (3) who reported secretion of cytotoxic granule contents in the vicinity of the cSMAC. In this study, we examined this hypothesis directly. Contrary to expectations, we found no evidence that generation of a cSMAC is required for granule reorientation or cytolytic activity by CD8$^+$ effector T cells.

Materials and Methods

T cell purification and differentiation

All of the mice were housed in the University of Chicago Animal Facility under specific pathogen-free conditions. Animals were used in agreement with the University of Chicago Institutional Animal Care and Use Committee according to the National Institutes of Health guidelines for animal use. 129S6/SvEvTac-*Rag2^{tm1Fwa}* were serially intercrossed with 2C TCR transgenic mice on the C57BL/6J background to obtain B6;129 2C/RAG2$^{-/-}$ mice (herein referred to as 2C/RAG2$^{-/-}$) (10). Effector CD8$^+$ T cells were generated as described previously by Cham et al. (11). Briefly, naive T cells were purified by negative selection from the spleens of 2C/RAG2$^{-/-}$ mice, and effector cells

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[3] Abbreviations used in this paper: AEC, Ag-expressing cell; cSMAC, central supramolecular activation cluster; pSMAC, peripheral supramolecular activation cluster; PKC, protein kinase C; CMAC, 7-amino-4-chloromethylcoumarin; MTOC, microtubule organizing center.

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were generated by coculture of naive cells in vitro with mitomycin C-treated P815.B7-1 cells for two 4-day stimulations.

Immunofluorescence Abs and reagents

The following reagents were used: polyclonal rabbit anti-PKCθ and goat anti-talin (Santa Cruz Biotechnology); anti-tubulin mAb (DM1A, Neomarker; Lab Vision); anti-Granzyme A (7.1; generous gift from Markus Simon, Max-Planck-Institute, Freiburg, Germany); and Lysotracker Red DND-99 (Molecular Probes). Secondary Abs conjugated to Texas Red and FITC were all obtained from Jackson ImmunoResearch Laboratories.

Immunofluorescence

To distinguish target cells from T cells, target cells were loaded with the vital dye 7-amino-4-chloromethylcoumarin (CMAC) Cell-Tracker Blue (Molecular Probes) as previously described (12). Targets (1.5×10^5) were mixed with an equal number of Ficoll-Hypaque-purified CD8$^+$ effector T cells in DMEM with 10% FCS, centrifuged at 5000 rpm for 30 s, and incubated for 5 min at 37°C. Supernatants were aspirated, and conjugates were then gently resuspended in serum-free DMEM and plated onto poly-L-lysine (Sigma-Aldrich)-coated slides for two additional minutes before fixation. Slides were fixed in 3% (w/v) paraformaldehyde in PBS for 15 min. Samples were permeabilized in 0.3% (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 10 min, and then rinsed in PBS and blocked in DMEM containing 10% FCS for 5 min. All subsequent Ab incubations were performed in calcium-/magnesium-free Dulbecco's PBS containing 2% FCS. Primary and secondary Abs were applied sequentially for 60 min at room temperature and washed five times with Dulbecco's following each incubation. After fluorochrome labeling, specimens were mounted in Mowiol 4–88 (Hoechst Celanese), with 10% 1,4-diazobicyclo[2.2.2]octane (Sigma-Aldrich) added as an antifade. Samples were analyzed using a Zeiss Axiovert100 microscope with a 63× oil objective lens. Image capture and deconvolution analysis (where appropriate) was performed using SlideBook software (Intelligent Imaging Innovations). Thirty-two conjugates were scored per condition, and experiments were repeated at least three times. Standard Student's *t* tests were performed when appropriate.

Chromium release assay

Chromium release assays were performed as previously described (13). Briefly, ^{51}Cr-labeled targets (2×10^3) were plated with effector CD8$^+$ T cells at the indicated E:T ratio in a 96-well V-bottom plate (ICN Biomedicals) either in the presence or absence of CTLA4Ig fusion protein (50 μg/ml). After 4 h of incubation at 37°C, 50 μl of supernatant was transferred to a LumaPlate-96 (PerkinElmer Life Sciences) and allowed to dry overnight. Plates were then counted using a TopCount-NXT plate reader (PerkinElmer Life Sciences).

Results and Discussion

Granzyme A polarization does not correlate with PKCθ focusing

In light of recent observations that cSMAC formation could be seen in effector but not naive CD8$^+$ TCR Tg T cells (5), we examined the hypothesis that generation of a cSMAC might be linked to granule-mediated cytolytic activity, which is a function conducted only by effector cells.

Using primed 2C TCR Tg CD8$^+$ effector cells and L^d-expressing P815 cell targets, we first examined the percentage of conjugates that generate a cSMAC and the percentage of conjugates that mobilize cytotoxic granules toward the T cell/target cell interface. Granules were visualized by intracellular staining for granzyme A. As shown in Fig. 1*A*, PKCθ and talin staining successfully distinguished cSMAC/pSMAC segregation in T cell-target cell conjugates. Unconjugated effector CD8$^+$ T cells displayed discrete, punctate staining of intracellular vesicles containing granzyme A in a nonpolarized pattern (Fig. 1*B*) (14). Effector CD8$^+$ T cells conjugated to P815 cells polarized granzyme A toward the T cell/target cell contact site (Fig. 1*B*). However, when parallel sets of conjugates were scored for the percentage that formed a cSMAC and the percentage that mobilized cytotoxic granules, there was no statistical association. Whereas 10.6 ± 5.6% of effector cell/target cell conjugates displayed focused PKCθ, 82.1 ± 6.9% mobilized cytotoxic granules toward the interface (Fig. 1*D*) ($p = 0.0001$).

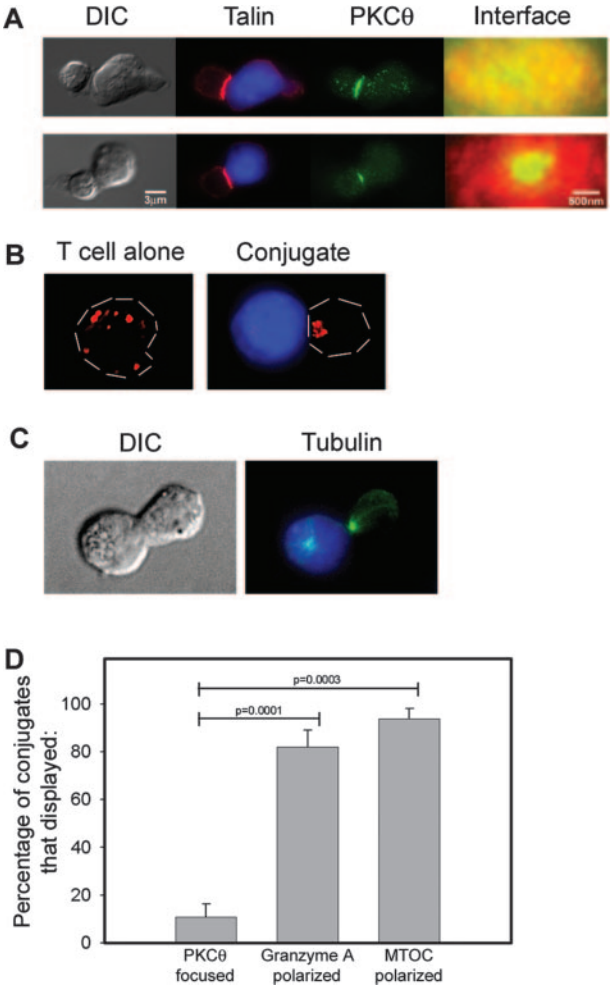

FIGURE 1. Granzyme A and MTOC polarization do not correlate with PKCθ focusing. Effector CD8$^+$ T cells were cocultured for 8 min with CMAC-labeled P815 (blue), fixed, stained for indicated molecules (talin in red and PKCθ in green), and imaged by immunofluorescence microscopy. *A*, Representative image of a conjugate displaying recruited PKCθ (*top panels*) or focused PKCθ (*bottom panels*) with the immunological synapse delineated by talin (red). Size bars for the different sized images are indicated in white. Representative images of either a T cell alone or a conjugate displaying granzyme A (red). *C*, Representative image of an effector CD8$^+$ T cell-P815 conjugate displaying tubulin. *D*, The percentage of conjugates that displayed focused PKCθ, the percentage of conjugates polarized granzyme A, and the percentage of conjugates that polarized the MTOC was calculated. The mean, SD, and unpaired *t* test of three independent experiments were calculated.

Microtubule organizing center (MTOC) polarization has been shown to precede granule reorientation and is thought to be required for this process (15, 16). The MTOC was readily imaged using intracellular staining with anti-tubulin (Fig. 1*C*). As was seen with cytotoxic granules, MTOC reorientation toward the interface also occurred in 93.8 ± 4.4% of conjugates, a substantial greater percentage than that which formed a cSMAC (Fig. 1*D*) ($p = 0.0003$). These data indicate that the most CD8$^+$ effector cells reorient both the MTOC and granzyme A-containing granules toward the T cell/P815 cell interface, even though only a minority generates a detectable cSMAC structure.

Simultaneous imaging of granule polarization and cSMAC generation

Although scoring independent sets of conjugates for PKCθ focusing and granule reorientation provided a statistical indication that

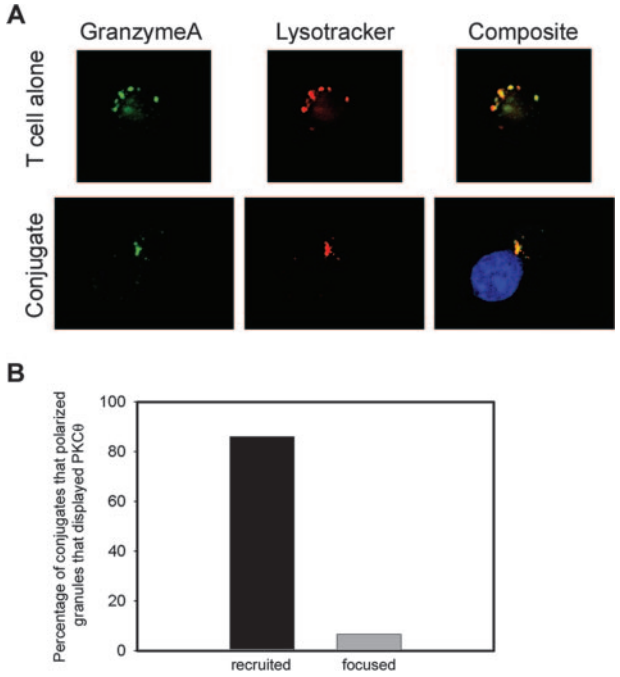

FIGURE 2. PKCθ focusing is not required for the polarization of granules in effector CD8$^+$ T cells. *A*, Effector CD8$^+$ T cells were labeled with Lysotracker Red DND-99. Representative images of either T cell alone or conjugate displaying granzyme A (green) and Lysotracker (red). *B*, The percentages of conjugates that polarized granules that recruited PKCθ and the percentage of those that focused PKCθ to a cSMAC were calculated.

cSMAC formation was not required for granule movement, it was desirable to image both events simultaneously in the same T cells. Therefore Lysotracker Red DND-99 (LysoTracker), a cell-permeant dye, which accumulates and fluoresces in acidic compartments in live cells (17), was used as an alternative stain to identify granules. As shown in Fig. 2*A*, both anti-granzyme A mAb and Lysotracker labeled the same intracellular vesicles.

Granule staining by Lysotracker was readily detectable in the CD8$^+$ effector T cells. Furthermore, simultaneous detection of granule reorientation and PKCθ localization was assayable (Fig. 2*B*). The majority (91.9 $\pm$ 2.3%) of conjugated CD8$^+$ T effector T cells with P815 target cells polarized granules toward the contact site. Of these conjugates, PKCθ was recruited to the immunological synapse in a diffuse pattern in the vast majority of cases; however, focused PKCθ was detected in fewer than 10% of conjugates (Fig. 2*C*). Thus, granule polarization toward the target cell clearly could occur without a detectable cSMAC.

B7-1 costimulation augments cSMAC formation but does not augment granule polarization nor cytolytic activity

We and others (5, 18) have recently observed that B7-1 expression by the target cell increases the fraction of T cells that generate a cSMAC (Fig. 3*A*). This augmentation was decreased to the level observed with P815 cells in the presence of the B7-1-blocking fusion protein (Fig. 3*A*). By coculturing T cells with either Lysotracker-labeled P815 or P815.B7-1 targets, we could

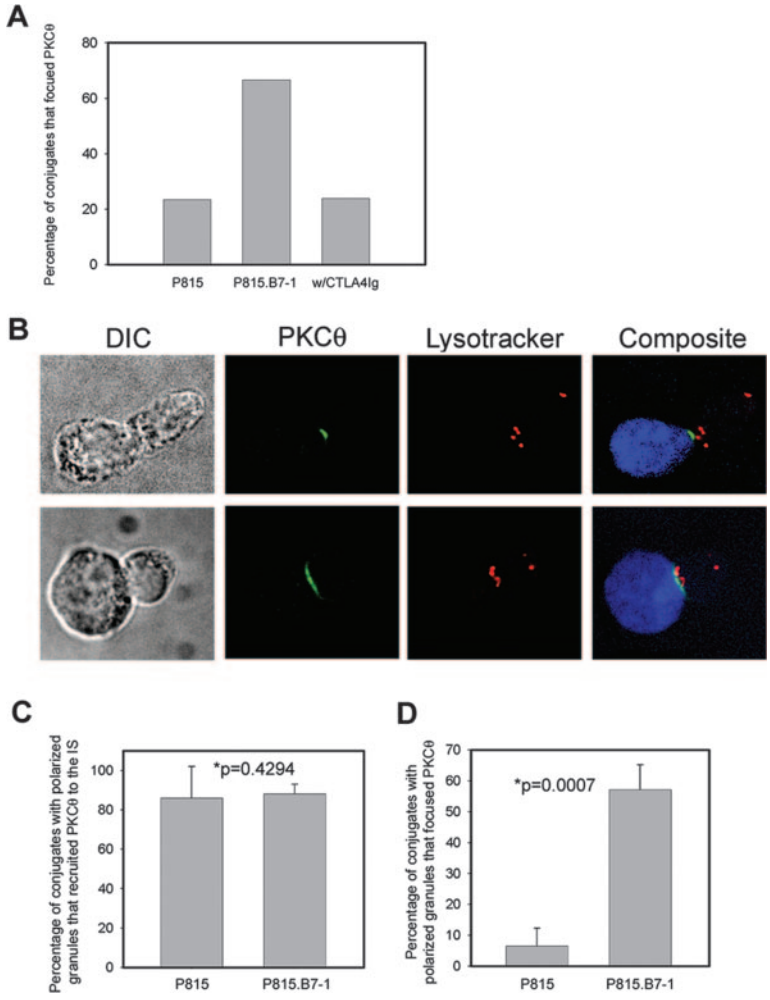

FIGURE 3. B7-1 costimulation increases PKCθ focusing but not granule polarization. *A*, Effector CD8$^+$ T cells were cocultured for 8 min with either CMAC-labeled P815 or P815.B7-1 cells in the presence or absence of CTLA4Ig and scored for PKCθ localization and focusing. The percentage of conjugates that localized PKCθ that focused PKCθ was calculated. *B*, Representative images of conjugates displaying polarized granules and either evenly distributed PKCθ (*bottom panels*) or focused PKCθ (*top panels*) are shown. *C*, The percentage of conjugates that polarized granules that recruited PKCθ. Of the percentage of conjugated polarized granules and recruited PKCθ, the percentage that focused PKCθ was calculated. The mean, SD, and unpaired *t* test of three independent experiments were calculated.

regulate the degree of cSMAC formation and examine correlation with granule reorientation and cytolysis. Representative images of simultaneous granule reorientation and PKCθ localization are depicted in Fig. 3*B*.

A comparable percentage of effector CD8$^+$ T cells conjugated to either P815 or P815.B7-1 cells displayed polarized granules (in excess of 90% in both cases), and a similar percentage of conjugates with polarized granules recruited PKCθ to the immunological synapse irrespective of the presence or absence of B7-1 (Fig. 3*C*). However, only a small fraction of conjugates with polarized granules displayed focused PKCθ in the absence of B7-1 (Fig. 3*C*). The lack of cSMAC formation in T cells that readily polarized lytic granules toward the T cell/target cell interface strongly suggests that granule polarization can occur independently of cSMAC formation in effector CD8$^+$ T cells.

Granule polarization toward the immunological synapse has been observed in mutant effector CD8$^+$ T cells incapable of granule secretion (19). Therefore, it was formally possible that there could be discordance between the repositioning of lytic granules to the immunological synapse and the actual exocytosis of these granules into the target cell to induce target cell death. We were unable to visualize directly the granule exocytosis event in our experimental system. Therefore, to address whether the presence of B7-1 influenced the cytolytic potential of effector CD8$^+$ T cells, a 4-h ^{51}Cr-release assay was performed using targets either expressing or lacking B7-1. The presence of B7-1 on target cells did not significantly alter the percentage-specific lysis (Fig. 4), as has been reported previously (20). In addition, kinetic analyses revealed that lysis was not accelerated in the presence of B7-1 (data not shown). Thus, despite markedly increasing the formation of a cSMAC by CD8$^+$ effector cells, B7-1 expression had no impact on cytolytic activity.

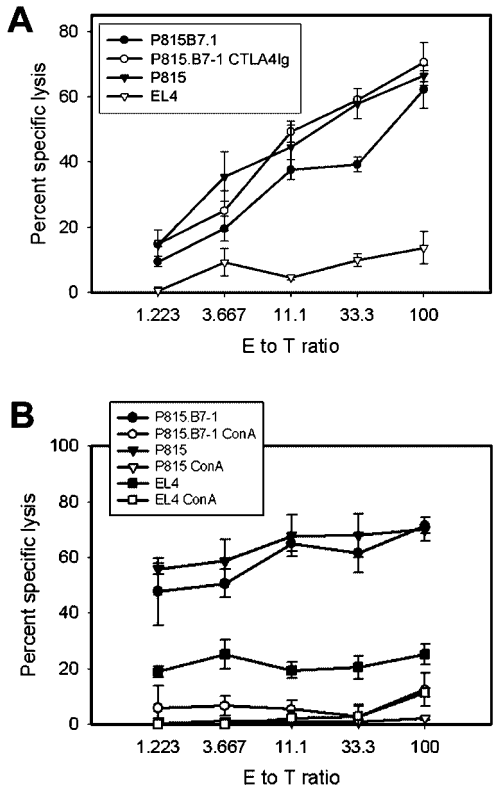

FIGURE 4. B7-1 ligation does not augment cytolytic activity in CD8$^+$ T cells. *A*, A ^{51}Cr-release assay was performed using effector CD8$^+$ T cells along with the following target cells: EL4, P815, P815.B7-1, or P815.B7-1 in the presence of CTLA4Ig fusion protein. *B*, A ^{51}Cr-release assay was performed using effector CD8$^+$ T cells along with the following target cells in the presence or absence of 100 nM ConcanamycinA: EL4, P815, or P815.B7-1.

Stinchcombe et al. (3) demonstrated the possibility of the formation of a cSMAC displaying discrete signaling and secretion domains at the contact site between a target cell and an effector CD8$^+$ T cell. Recruitment of molecules to the immunological synapse clearly occurs following the engagement of an AEC. However, the importance of further segregation into discrete SMACs is debatable. Although the formation of a cSMAC is observable in effector CD8$^+$ T cells, it does not appear to be absolutely required for the polarization of granules nor the subsequent execution of cytolytic effector function.

Our results are supported by other work assessing the number of TCR/peptide-MHC complexes required for cytolysis to occur. Faroudi et al. (21) demonstrated that CD8$^+$ T cells can reorient the MTOC and granules at peptide concentrations that yielded only marginally detectable levels of phosphotyrosine or CD2 enrichment at the CTL-target contact. Furthermore, Purbhoo et al. (9) demonstrated that cytotoxicity could occur at very low peptide-MHC densities, such that three peptide-MHC complexes were sufficient to get target cell killing. This correlates with too few TCRs to detect pSMAC/cSMAC segregation. However, we were careful to note that at higher peptide-MHC densities, stable immunological synapse formation was detectable, and the morphological changes observed in the target cell at these higher peptide-MHC densities were more indicative of perforin-mediated cytotoxicity (9).

The functional significance of the immunological synapse remains elusive. cSMAC/pSMAC segregation appears dispensable for T cell activation (5, 8) and for the execution of T cell function (5, 22). One hypothesis is that the immunological synapse plays a role in the down-regulation and attenuation of T cell signaling (8). Alternatively, the observation that CD28 costimulation potentiates cSMAC formation suggests that the generation of a cSMAC may be reflective of a modulated TCR signal. Additional work will be necessary to distinguish these possibilities.

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Disclosures

The authors have no financial conflict of interest.

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