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Cutting Edge: Cell Surface Linker for Activation of T Cells Is Recruited to Microclusters and Is Active in Signaling

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A controversy has recently emerged regarding the location of the cellular pool of the adapter linker for activation of T cells (LAT) in TCR signaling. The phosphorylated tyrosines on LAT nucleate various signaling complexes that regulate T cell activation (1). An interesting controversy has recently emerged regarding the issue of how LAT complexes are recruited and triggered upon TCR engagement. LAT is localized at the plasma membrane and also in intracellular vesicles in resting and stimulated cells (2, 3). The question of which LAT pool is recruited to TCR-rich microclusters, is phosphorylated, and participates in TCR signaling has been the subject of debate (discussed in Refs. 4, 5). There are two different points of view regarding the origin of this pool. Superresolution photoactivated localization microscopy (PALM) data from two laboratories argue for a model in which LAT is preclustered at the plasma membrane (6, 7). Lillemeier et al. (6) also used electron microscopy of ripped membrane sheets to study LAT clustering. They proposed that TCR and LAT are segregated into “protein islands” before TCR triggering, which following signaling, appose each other without mixing. Sherman et al. (7) showed much smaller clusters of LAT that modestly increase in size upon TCR activation. Furthermore, they observed mixing of LAT with TCR and ZAP70 at discrete sites upon TCR engagement. Thus, although the exact sizes of LAT clusters as well as the fashion in which they mix with the TCR differ in these two studies, both groups argue that TCR triggering targets through LAT recruitment from the plasma membrane.

A very different view has been proposed in two other recent studies. Purbhoo et al. (8) demonstrated that a substantial fraction of LAT was present in subsynaptic vesicles. Using dynamic imaging in activated T cells, they observed interaction of LAT vesicles with SLP-76 microclusters coincident with LAT phosphorylation (8). In a second study, Williamson et al. (9) used PALM to confirm the preclustering of LAT at the cell membrane in resting cells. However, they concluded from their photon-counting experiments that in activated cells, clustered LAT is located and phosphorylated in intracellular vesicles. These two studies concluded that the active pool of LAT is recruited from subsynaptic vesicles rather than from the large number of LAT molecules located at the plasma membrane.

The source of LAT that participates in TCR signaling is thus currently in question. It is important to resolve this controversy, as each model has far-reaching implications for how signaling molecules become activated upon TCR triggering. Distinguishing between these models is central to understanding T cell biology. In this study we provide evidence that cell surface LAT is efficiently and rapidly recruited to microclusters. Furthermore, we demonstrate that the surface pool of LAT is phosphorylated and can thus propagate signals downstream of the TCR.

Materials and Methods

Reagents

Human anti-CD3e Abs (BD Pharmingen) were used to coat coverslips. The following Abs were used: rabbit anti-CD4 (Sigma-Aldrich), mouse anti–β-actin (Sigma-Aldrich), mouse anti-LAT (Upstate Biotechnology), rabbit anti-

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Abbreviations used in this article: LAT, linker for activation of T cells; NHS, N-hydroxysuccinimide; PALM, photoactivated localization microscopy; TIRF, total internal reflection fluorescence; YFP, yellow fluorescent protein.
pLAT (Invitrogen), mouse antipolysphosphatase C-γ1, mouse SLP-76 (Ab Solutions), mouse anticalnexin (BD Biosciences), mouse anti-GAPDH (Gentauro), and mouse anti-pY 4G10 (Millipore), and rabbit pY (271). Alexa 488–, Alexa 647–, or allopoxycyanin-conjugated anti-CD4 (clones OKT4 and SK3; ebioscience) were used for labeling of CD4. Indo-1 AM was from Invitrogen.

Cell culture and generation of stable cell lines

Jurkat E6.1 and LAT-deficient JCam2.5 cells have been described previously (3). For stable cell lines, Jurkat cells were retrovirally transduced with CD4-LAT or CD4-LAT41 constructs cloned in a viral plasmid (pMSCVneo). Following transduction, cells were cultured in medium containing 1.8 mg/ml G418 for 2 wk, and were then sorted for CD4 expression.

Confocal microscopy and image processing

Spreading assays were performed as described previously (7). For gentle fixation of cells without permeabilization, cells were fixed with 0.25% paraformaldehyde for 30 min. Images from fixed cells were collected with a Zeiss 510 LSCM, using a ×63, 1.4 numerical aperture objective (Carl Zeiss). Images from live cells were collected with a Nikon Ti-E inverted microscope, using a ×60, 1.49 numerical aperture objective (Nikon Instruments) in total internal reflection fluorescence (TIRF) mode with an iXon DU88 EM-CCD camera (Andor). Imaris 7.2.3 (Birplase/Andor) was used for image processing. Pearson correlation coefficients were calculated from single slices masked on individual cells with thresholds determined with the cluster selection tool. Adobe Photoshop and Illustrator (Adobe Systems) were used to prepare figures.

Cell surface Ab labeling and biotinylation

For Ab labeling of surface LAT, cells were incubated with labeled anti-CD4 for 30 min at 4°C. After three washes in cold buffer, labeled cells were transferred into warm imaging buffer and dropped immediately onto coverslips. For surface Ab removal, labeled cells were resuspended in medium at pH 2.5 for 1 min and immediately washed in buffer at neutral pH. Washed cells were resuspended in imaging buffer and processed for imaging. For cell surface biotinylation, cells were cooled to 4°C and biotinylated for 30 min at 4°C with the cell impermeant sulfon N-hydroxysuccinimide (NHS) ester polyethyleneglycol-4 biotin at 0.2 mg/ml (Thermo Fisher Scientific). After quenching any remaining reagent, cells were resuspended in RPMI 1640 and stimulated immediately with 10 μg/ml OKT3 for 1 min at 37°C, lysed, and biotinylated proteins were isolated on NeutrAvidin agarose beads (Thermo Fisher Scientific). Proteins were eluted with sample buffer containing DTT (100 mM) and analyzed by immunoblotting. For depletion of cell surface–labeled proteins, lysates were subjected to 3 serial purification on NeutrAvidin agarose beads.

Results and Discussion

Characterization of the CD4-LAT chimera

To investigate the origin of the active pool of LAT, we needed to distinguish between surface and intracellular LAT. Previously, the fluorescent lipids DiI and DiD have been used to label intracellular vesicles in a nonspecific manner and to claim colocalization with intracellular LAT (8). To specifically follow LAT from the cell surface, approaches such as surface Ab labeling or cell surface biotinylation would allow one to distinguish between surface and intracellular pools of LAT. This has proved very difficult, as LAT is a transmembrane protein with a very short predicted extracellular domain too small to generate an Ab response or to biotinylate. Thus, we attempted to produce a LAT molecule tagged on the extracellular surface. Our effort to generate a peptide tag coupled to LAT that could be biotinylated from the extracellular surface (as used in Ref. 9) was unsuccessful, as all versions were mislocalized in the cell, unsuccessfully labeled from the outside of the cell, or resulted in cells that were not viable (data not shown). Therefore, we used a CD4-LAT chimera we previously generated (10) in which the extracellular domain of CD4 was fused to the transmembrane and cytosolic regions of LAT (Fig. 1A). We generated stable cell lines expressing CD4-LAT in LAT-deficient JCam2.5 cells. By using an Ab to CD4, we detected expression of CD4 on the cell surface of our stable cell line. In contrast, no CD4 expression was detected on the surface of untransduced JCam2.5 cells (Supplemental Fig. 1A). The CD4-LAT chimera retained the ability to interact with signaling proteins SLP-76 and phospholipase C-γ1 upon T cell activation. Kinetics of LAT and SLP-76 phosphorylation upon CD3 activation in cells expressing CD4-LAT were similar to those of endogenous proteins in wild-type E6.1 Jurkat cells. However, no phosphorylation of LAT was detected upon incubation with CD4 Ab, indicating that crosslinking of CD4 did not lead to T cell activation in a nonspecific manner (Supplemental Fig. 1B, 1C).

Previously the CD4-LAT chimera has been shown to cluster with endogenous LAT at the plasma membrane of RBL cells (10). To assess whether the cellular distribution of CD4-LAT was similar to endogenous LAT in Jurkat cells, cells expressing CD4-LAT were transfected with LAT-yellow fluorescent protein (YFP), which colocalizes with endogenous LAT (11). Images taken at the coverslip and higher up in the cell showed that the cellular distribution of CD4-LAT and LAT-YFP were identical (Fig. 1B). Finally, CD4-LAT expression reconstituted TCR-induced cytosolic calcium flux in LAT-deficient JCam2.5 cells, indicating that this molecule retains LAT function (Fig. 1C). As a control, we also generated a stable cell line expressing CD4-
LAT41, a mutant construct in which the cytosolic tail of LAT is truncated and thus lacks any phosphorylation sites. Although efficient cell surface staining of CD4-LAT41 was achieved, the CD4-LAT41 truncation mutant did not reconstitute calcium flux, confirming that CD4-LAT function is dependent on its cytosolic tail (Supplemental Fig. 1D–F). Taken together, these results demonstrate that the CD4-LAT chimeric molecule behaves similar to wild-type LAT and could be used to elucidate the contribution of plasma membrane LAT.

**LAT at the cell surface is recruited to microclusters**

We next sought to determine whether LAT molecules from the cell surface or internal pools are recruited to microclusters at the initial stages of signaling. To label the cell surface pool of LAT, we incubated cells stably expressing CD4-LAT with fluorescently labeled anti-CD4 on ice, which ensures exclusive labeling of the pool of LAT located at the plasma membrane. Following labeling and removal of excess Ab in cold buffer, cells were resuspended in 37˚C buffer, immediately dropped onto coverslips coated with stimulatory Ab, and fixed after 2 min activation. Labeling of the cells with Ab did not interfere with cellular activation as demonstrated by the formation of phosphotyrosine-positive microclusters (Fig. 2A). Interestingly, CD4-LAT labeled at the cell surface was recruited to phosphotyrosine-containing microclusters, indicating that LAT from the surface of the cell is efficiently recruited to microclusters following T cell activation. In contrast, the CD4-LAT41 truncation mutant was not recruited to the activation clusters, even though efficient labeling of the construct at the cell surface was achieved (Fig. 2A, lower panel). These data indicate that incorporation of surface LAT into microclusters is dependent on the cytosolic signal-transducing region of LAT. Additionally, in control cells dropped onto nonstimulatory coverslips, CD4-LAT was dispersed throughout the plasma membrane, indicating that labeling of cells with the CD4 Ab does not cause the nonspecific aggregation of CD4-LAT (Supplemental Fig. 1G).

Next, we evaluated whether endocytic pools of LAT were recruited to the activation-induced pY microclusters. To efficiently label surface and endocytotic LAT pools, we incubated CD4 Ab with cells expressing CD4-LAT at 37˚C for 1 h (Fig. 2B, top panel, z-stack). Labeled cells were then dropped onto stimulatory coverslips and efficient recruitment of LAT to pY-positive microclusters was observed (Fig. 2B, top panel). To specifically evaluate the contribution of the endocytic pool, cell surface Ab was removed by washing the cells in low pH medium, leaving only internal LAT labeled. Following surface Ab removal, we observed exclusive labeling of the LAT endocytic pool (Fig. 2B, lower panel, z-stack). Such cells failed to exhibit CD4 labeling at the microclusters (Fig. 2B, lower panel). Importantly, the low pH rinse did not inhibit formation of microclusters as detected by antiphosphotyrosine, indicating that activation of TCR-induced signaling pathways remained intact. Identical results were obtained with a second fluorescently labeled CD4 Ab (Supplemental Fig. 1H).

We also performed live cell imaging on cells that were surface-labeled with fluorescent anti-CD4. We used TIRF microscopy for dynamic imaging to enable selective visualization of the cell surface region. By using this technique, we observed recruitment of CD4-LAT within seconds of cellular contact with the coverslip (Fig. 2C, Supplemental Video 1).

Following cluster formation, the clusters dissipated rapidly, reminiscent of the pattern of LAT-YFP clusters (12). Epifluorescent images at the end of the video show that none of the surface-labeled LAT is in the endocytic compartment at this time (Supplemental Fig. 1J). Live cell TIRF imaging in cells transfected with Grb2-YFP, which localizes to activation clusters, showed that surface-labeled CD4-LAT was strongly colocalized with Grb2-YFP clusters (Supplemental Video 2).

To definitively confirm that LAT visualized at the clusters was at the cell membrane, cells expressing CD4-LAT were gently fixed and then stained with anti-CD4 without membrane

**FIGURE 2.** Cell surface LAT is recruited to microclusters. (A) JCam2.5 cells stably expressing CD4-LAT or CD4-LAT41 were labeled with anti-CD4-Alexa 488 (clone OKT4) at 4˚C, dropped onto stimulatory coverslips at 37˚C, and fixed after 2 min. After fixation, cells were permeabilized and immunostained for phosphotyrosine. Confocal slices 0.7 μm apart were collected through the entire cell. (B) JCam2.5 cells stably expressing CD4-LAT were labeled with anti-CD4-Alexa 488 at 37˚C and imaged as above. Lower panel. Cells labeled at 37˚C were stripped of surface Ab by low pH rinse. Following washing in buffer at neutral pH, cells were dropped onto stimulatory coverslips and imaged. (C) JCam2.5 cells stably expressing CD4-LAT were labeled with anti-CD4-Alexa 488 at 4˚C, dropped onto stimulatory coverslips, and imaged live by TIRF microscopy. Still images 84 s apart are shown from Supplemental Video 1. (D) JCam2.5 cells stably expressing CD4-LAT or CD4-LAT41 were transiently transfected with Grb2-YFP, dropped onto stimulatory coverslips at 37˚C, fixed after 2 min, and immunostained for CD4 without permeabilization. Pearson correlation coefficient values (±SE) are indicated to the right of the images. Original magnification ×700.
permeabilization to evaluate whether CD4-LAT was accessible from the extracellular surface. Our gentle fixation conditions did not cause permeabilization of the membrane as confirmed by staining for β-tubulin (data not shown). Grb2-YFP was transiently transfected into cells as a cluster marker. Following CD4 immunostaining, strong colocalization of CD4 with Grb2-YFP was observed (Fig. 2D). In control cells expressing the CD4-LAT41 truncation mutant, no CD4 recruitment to clusters was observed. Thus, the pool of LAT that is recruited to microclusters within seconds of T cell activation and is critical for T cell signaling is recruited from the cell surface and not from endocytic pools of LAT.

LAT at the cell surface is phosphorylated

The authors of the two previous studies that proposed recruitment of LAT from vesicles suggested that vesicular LAT is phosphorylated in trans by plasma membrane–associated ZAP70 (8, 9). However, these studies focused on cells that had been activated for 10 min, whereas peak LAT phosphorylation occurs between 1 and 2 min after TCR engagement (Supplemental Fig. 1C and Ref. 13). Therefore, we focused our investigation on LAT recruitment and activation at 2 min after TCR engagement. To assess the activation state of plasma membrane LAT molecules that are recruited to microclusters, we surface-labeled LAT with fluorescent anti-CD4, dropped the cells onto stimulatory coverslips, fixed them after 2 min, and immunostained for phosphorylated LAT. We observed efficient recruitment of surface-labeled CD4-LAT to pLAT191-labeled microclusters (Fig. 3A). In contrast, the CD4-LAT41 truncation construct was not recruited to microclusters and no pLAT191 clusters were detected. Although this observation strongly argues that surface LAT is recruited to and phosphorylated at microclusters, because the cells were permeabilized to enable immunostaining there was still a formal possibility that pLAT191 labeling occurred on a vesicular pool of LAT.

To directly investigate whether cell surface LAT is phosphorylated, we employed the membrane-impermeable biotinylation reagent sulfo-NHS-biotin, which allowed us to directly label plasma membrane proteins and specifically isolate this fraction using NeutrAvidin agarose. In control experiments we observed that 99% of the sulfo-NHS-biotin–treated cells were viable, and efficient biotinylation and purification of the CD4-LAT chimera in the avidin-bound fraction was achieved (data not shown). Importantly, 0.1% SDS was included in the lysis buffer to exclude coimmunoprecipitation of associated proteins. Furthermore, CD4-LAT was not detected when avidin purification was carried out in control nonbiotinylated cells. Following biotinylation, cells were activated to evaluate the presence of the active pool of LAT in the avidin-bound, cell surface–labeled fraction. As shown in Fig. 3B, phosphorylated LAT is strongly detected in the isolated surface-biotinylated plasma membrane proteins only..

**FIGURE 3.** Cell surface LAT is phosphorylated. (A) JCam2.5 cells stably expressing CD4-LAT or CD4-LAT41 were labeled with anti-CD4-Alexa 488 (clone OKT4) at 4°C, dropped onto stimulatory coverslips at 37°C, and fixed after 2 min. After fixation, cells were permeabilized and immunostained for pLAT191. Confocal slices 0.7 μm apart were collected through the entire cell. Pearson correlation coefficient values (± SE) are indicated to the right of the images. Original magnification ×700. (B) JCam2.5 cells stably expressing CD4-LAT were either unlabeled (no biotin) or labeled with sulfo-NHS-biotin at 4°C. After quenching and washing, cells were stimulated for 1 min at 37°C, lysed, and biotinylated proteins were isolated on NeutrAvidin agarose beads. Whole cell lysates (Lysate) and affinity-purified samples (AffP: neutravidin-agarose) were then immunoblotted as indicated. (C) Lysates from unlabeled or biotinylated cells were subjected to three rounds of serial purification on NeutrAvidin agarose beads. Input and postpurification lysates (Post 3× AffP) were immunoblotted as indicated. (D) Three models for LAT recruitment to signaling microclusters. LAT can move laterally within the plasma membrane to be recruited to microclusters. In this model activated ZAP70 phosphorylates LAT at the plasma membrane (1). LAT in microclusters could be recruited from vesicles that originate from a recycling pool from the plasma membrane (2) or directly from the Golgi apparatus (3). In models 2 and 3, ZAP70 phosphorylates vesicular LAT in trans. In this study, we provide evidence for model 1 at early time points after activation. At the right, a microcluster is internalized and directed toward degradation pathways.
from activated cells. Because only plasma membrane proteins exposed to the extracellular solution are expected to be labeled, these results strongly suggest that phosphorylated LAT is at the plasma membrane in activated T cells. To verify that sulfo-NHS-biotin does not label intracellular sites in intact cells, we assayed for the biotinylation of GAPDH, an abundant cytosolic protein, and calnexin, an integral membrane protein localized in intracellular membranes (14). No anti-GAPDH or anticalnexin immunoreactivity was associated with the avidin-bound fraction (Fig. 3B, bottom panels).

To determine the relative percentage of phosphorylated LAT in the cell lysate that comes from the cell membrane and intracellular pools, lysates were subjected to serial purification using NeutrAvidin agarose to efficiently deplete biotinylated cell surface proteins (Supplemental Fig. 1J). Depleted lysates were evaluated for the presence of phosphorylated and total LAT. LAT levels were decreased to 40% in depleted lysates, indicating that ~60% of LAT is at the cell surface. Strikingly, only 10% of phosphorylated LAT remained in the lysate after biotinylation and depletion of surface proteins, indicating that 90% of phosphorylated LAT at 2 min after TCR activation originates from the cell surface (Fig. 3C). Thus, these experiments provide an independent method of demonstrating that the LAT localized at the plasma membrane of a T cell is efficiently phosphorylated and can propagate downstream signals. These data further suggest that vesicular LAT does not play a major role at early times after T cell activation.

Recent efforts have focused on discovering how LAT is activated upon TCR engagement, and specifically, the pool of LAT that contributes to T cell activation has come under discussion. Three trafficking routes can be envisioned for movement of LAT into sites of T cell activation: 1) Proteins could diffuse or be transported laterally in the plasma membrane and be recruited to the microclusters; 2) LAT molecules from the plasma membrane could move into endocytic vesicles that then traffic to sites of activation; and 3) LAT vesicles may be directly trafficked from the Golgi apparatus (Fig. 3D and discussed in Ref. 15). Our data clearly demonstrate that LAT at the cell surface prior to activation is rapidly recruited to microclusters and phosphorylated. These results are consistent with superresolution PALM data from two studies that showed recruitment of LAT into clusters at the T cell plasma membrane (6, 7). Importantly, our studies focus on early kinetics at the peak of LAT phosphorylation. It is possible that an intracellular pool of LAT may be important at later time points. Thus, the cell may first use the direct plasma membrane to microcluster route for the initial rapid response to TCR stimulation and later rely on intracellular vesicles to replenish these sites of activation with LAT. In addition to shedding light on the way plasma membrane proteins are trafficked to sites of activation, a full understanding of LAT dynamics and accumulation at microclusters will provide insights into mechanisms involved in signal generation and persistence, and clarify the physiology of the immune synapse.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

Supplemental Figure 1

A. z stack

B. CD4-LAT pY merge z stack

C. CD4 labeling at 37ºC (clone SK3)

D. CD4-LAT pY merge z stack

E. CD4-LAT pY merge z stack

F. CD4-LAT pY merge z stack

G. CD4 labeling at 4ºC (CD45 coated coverslips)

H. CD4 labeling at 4ºC (clone SK3)

I. Epifluorescence Image

J. Epifluorescence Image
**Supplemental Legends**

**Movie 1 (time series in Figure 2C):** JCam2.5 cells stably expressing CD4-LAT were labeled with anti-CD4 antibody conjugated to Alexa 488 for 30 minutes at 4°C, dropped onto anti-CD3 coated coverslips and imaged dynamically by TIRF microscopy. Playback rates are 20X faster than real time.

**Movie 2:** JCam2.5 cells stably expressing CD4-LAT were transiently transfected with Grb2-YFP. Cells were labeled with anti-CD4 antibody conjugated to Alexa 488 for 30 minutes at 4°C, dropped onto anti-CD3 coated coverslips and imaged dynamically by TIRF microscopy. Playback rates are 20X faster than real time.

**Supplemental Figure S1:**

**A.** Histogram showing cell surface labeling using anti-CD4-APC to label untransduced JCam2.5 cells and JCam2.5 cells stably expressing CD4-LAT. **B.** Indicated cells were stimulated with 10μg/ml OKT3 for 2 minutes following which cell lysates were prepared. LAT was immunoprecipitated from lysates. Immunoprecipitates were blotted for LAT, PLC-γ1 or SLP-76 as indicated. **C.** Cells were stimulated with 10μg/ml OKT3 for the indicated time points. Whole cell lysates were prepared and immunoblotted for the indicated proteins. Lower panels: JCam2.5 cells stably expressing CD4-LAT were stimulated with 10μg/ml OKT3 or 10μg/ml OKT4. Cell lysates were prepared after 2 minutes and immunoblotted for pLAT191 and β-actin. **D.** Schematic of CD4-LAT41 chimeric construct used in the study. **E.** Histogram showing cell surface labeling using anti-CD4-APC to label untransduced JCam2.5 cells and JCam2.5 cells stably expressing CD4-LAT41. **F.** JCam2.5 cells or cells stably transduced with CD4-LAT or CD4-LAT41 were stimulated with 10μg/ml OKT3 and assayed for TCR-induced intracellular calcium flux. **G.** JCam2.5 cells stably expressing CD4-LAT were labeled with anti-CD4 antibody conjugated to Alexa 488 for 30 minutes at 4°C. After extensive washing to remove excess antibody, cells were dropped onto anti-CD45 coated coverslips at 37°C and fixed after 2 minutes. Post-fixation, cells were permeabilized and immunostained for phosphotyrosine. Confocal slices 0.7μm apart were
collected through the entire cell. **H.** JCam2.5 cells stably expressing CD4-LAT were labeled with anti-CD4 antibody (clone SK3) conjugated to Alexa 488 for 30 minutes at 37°C and imaged as above. Lower panel: Cells labeled at 37°C for 1 hour were stripped of surface antibody by acid wash at pH 2.5. Following washing in buffer at neutral pH, cells were dropped onto stimulatory coverslips and imaged as above. **I.** Epifluorescence image of cell at the end of Movie 1 and Figure 2C shows no endocytic LAT. **J.** JCam2.5 cells stably expressing CD4-LAT were either unlabeled (no biotin) or labeled with sulfo-NHS-biotin at 4°C (4°C label). After quenching and washing, cells were stimulated for 1 minute at 37°C, lysed, and lysates were subjected to 3 rounds of serial purification on neutravidin-agarose beads. Input and post purification lysates (Post 3X AffP) were immunoblotted with streptavidin.