The coreceptor CD2 uses plasma membrane microdomains to transduce signals in T cells

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The interaction between a T cell and an antigen-presenting cell (APC) can trigger a signaling response that leads to T cell activation. Prior studies have shown that ligation of the T cell receptor (TCR) triggers a signaling cascade that proceeds through the coalescence of TCR and various signaling molecules (e.g., the kinase Lck and adaptor protein LAT [linker for T cell activation]) into microdomains on the plasma membrane. In this study, we investigated another ligand–receptor interaction (CD58–CD2) that facilies T cell activation using a model system consisting of Jurkat T cells interacting with a planar lipid bilayer that mimics an APC. We show that the binding of CD58 to CD2, in the absence of TCR activation, also induces signaling through the actin-dependent coalescence of signaling molecules (including TCR-ζ chain, Lck, and LAT) into microdomains. When simultaneously activated, TCR and CD2 initially colocalize in small microdomains but then partition into separate zones; this spatial segregation may enable the two receptors to enhance signaling synergistically. Our results show that two structurally distinct receptors both induce a rapid spatial reorganization of molecules in the plasma membrane, suggesting a model for how local increases in the concentration of signaling molecules can trigger T cell signaling.

Introduction

Biochemical and genetic studies have revealed numerous signal molecules involved in T cell activation. Signaling is initiated by T cell receptor (TCR) recognition of antigenic peptide displayed by antigen-presenting cells (APCs). This interaction leads to the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) domains in TCR (particularly TCR-ζ chain) by Lck, a Src family kinase, and the subsequent recruitment and activation of the tyrosine kinase ZAP70 (Weiss and Littman, 1994). The major target of tyrosine phosphorylation by ZAP70 is LAT (linker for T cell activation), an adaptor protein that recruits several signaling molecules that initiate a variety of downstream events, including actin polymerization, calcium elevation, Ras activation, and transcriptional changes (Finco et al., 1998; Samelson, 2002).

Imaging studies also have revealed intriguing spatial reorganization of molecules during T cell signaling. A hallmark study was the discovery of the immunological synapse, a bullseye pattern with the ring of adhesion molecules surrounding TCR at the center of contact between APC (or membrane bilayer that mimics an APC) and the T cell (Dustin et al., 1998; Monks et al., 1998; Grakoui et al., 1999). More recent studies revealed submicrometer-sized structures within plasma membranes of activated T cells, where membrane-associated signaling molecules are organized into distinct compartments or membrane microdomains (Bunnell et al., 2002; Campi et al., 2005; Douglass and Vale, 2005; Yokosuka et al., 2005). Actin polymerization appears to be important in the initial formation of the microdomains but is not essential for their continued stability. It has been suggested that these microdomains are primarily held together by networks of protein interactions (Douglass and Vale, 2005), although lipid rafts (phase-partitioned lipids) could play important roles as well (Harder, 2004; Suzuki et al., 2007). Other membrane proteins in the T cell as well as in other immune cells also have been reported to segregate into various domain structures (Dustin et al., 1998; Bromley et al., 2001; Davis and Dustin, 2004; Kaizuka et al., 2007). How these spatial organizations are linked to signaling events of T cells,

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Abbreviations used in this paper: APC, antigen-presenting cell; cSMAC, central SMAC; CTB, cholera toxin subunit B; GPI, glycosylphosphatidylinositol; ITAM, immunoreceptor tyrosine-based activation motif; SMAC, supramolecular activation cluster; TCR, T cell receptor; TIRF, total internal reflection fluorescence.

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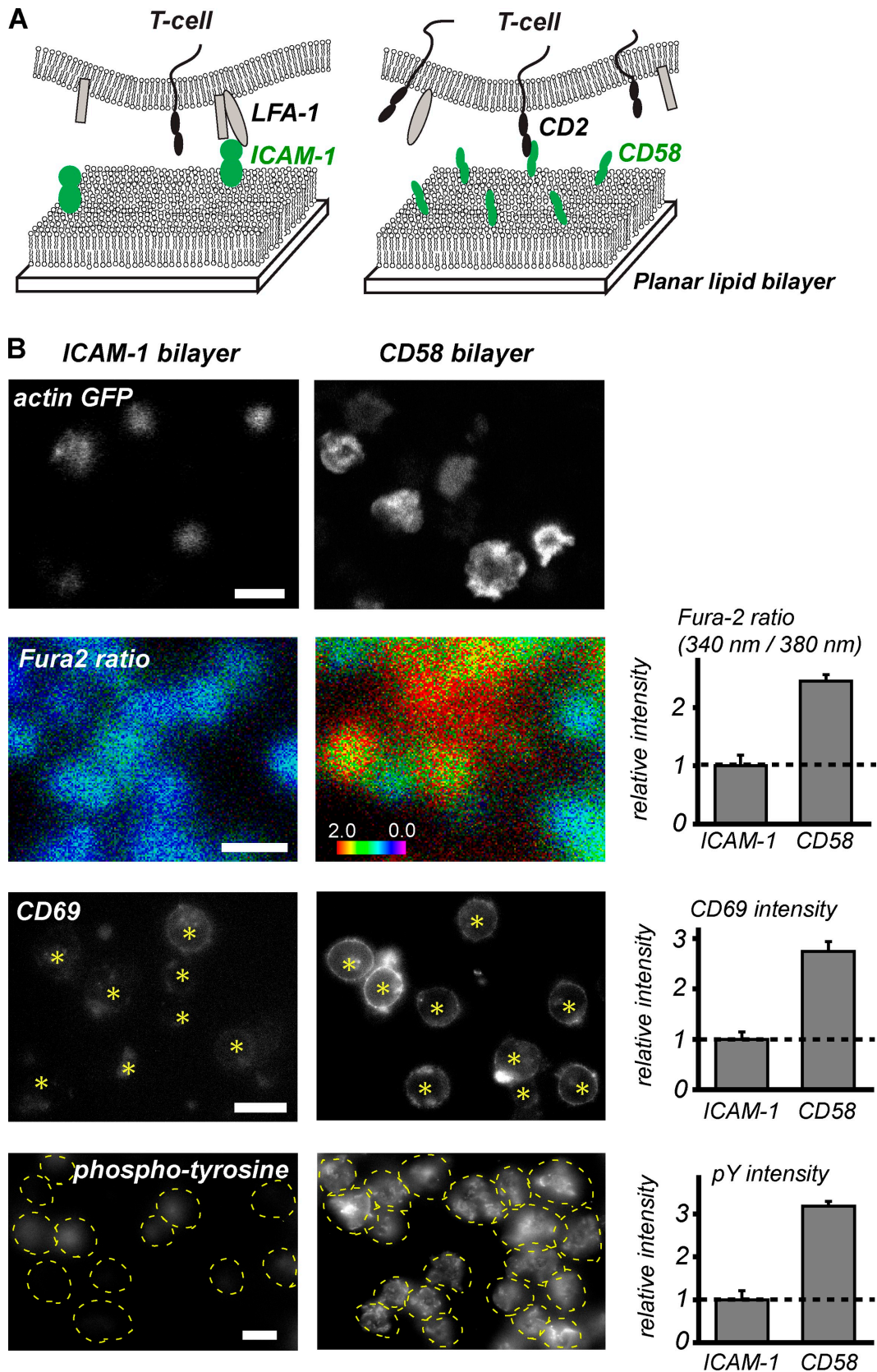www.jcb.org/cgi/doi/10.1083/jcb.200809136

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Figure 1. **CD2–CD58 interaction at cell–bilayer interface mediates signaling in the absence of direct TCR stimulation.** (A) A schematic of the experimental system is shown. Jurkat T cells interacted with planar lipid bilayers containing GPI-linked CD58 molecules (the ligand for CD2) supported on a glass substrate. (B) Signaling events were examined for T cells interacting with bilayers containing GPI-linked CD58 (40 molecules/µm^2) or ICAM-1 (an adhesion molecule used as a control; 10 molecules/µm^2). Similar results were obtained over a wide range of ligand densities in the bilayer (Fig. S1 A). Actin-GFP cells, imaged by spinning-disk confocal microscopy, spread on CD58 but not ICAM-1 bilayers. Intracellular calcium (measured by Fura-2 emission ratio

however, remains poorly understood. Thus, investigations of mechanisms and functions that are common or distinct within different membrane microdomains will be important for immune cell biology.

Besides TCR–peptide–MHC complex, there are various other proteins that interact between the T cell and APC (Springer et al., 1987; van der Merwe and Davis, 2003). One of the most abundant is CD2, a transmembrane protein in T cells, which binds to its ligand CD58 (or CD48 for rodent), a glycosylphosphatidylinositol (GPI)-anchored protein on APC (Dustin et al., 1987; Davis et al., 1998). It was proposed that the CD2–CD58 interaction acts to enhance APC–T cell adhesion and thus promotes T cell activation at lower antigen concentrations by maintaining cell–cell contact (Bachmann et al., 1999). In addition to a role in adhesion, CD2 was also suggested to be involved in signaling, mainly based upon data showing that polyvalent engagement of CD2 (e.g., anti-CD2 antibodies) can induce signaling events in T cells (Siliciano et al., 1985; Springer et al., 1987; Kanner et al., 1992). However, none of these prior studies demonstrated that signaling can be induced by membrane-bound CD58, the natural ligand for CD2. Animal studies also have not clearly identified a function for CD2 because mice bearing a CD2 knockout only exhibit very partial defects in immune responses (Killeen et al., 1992). However, such results might be explained by adaptation or functional redundancy among other coreceptors in the T cell that act in a similar manner to CD58–CD2 (Green et al., 2000). Thus, the biochemistry and biology of CD2 and other coreceptors remain much less well understood than TCR.

In this study, we have analyzed how CD58–CD2 might affect T cell signaling using an artificial membrane bilayer system (a simplified activation system that mimics APC). Planar lipid bilayer systems combined with high resolution surface imaging have been widely used for studies of the spatial organization of TCR signaling by peptide–MHC (Grakoui et al., 1999; Campi et al., 2005; Yokosuka et al., 2005; Varma et al., 2006) or anti-TCR antibodies (Kaizuka et al., 2007). Ligands for coreceptors also have been incorporated in planar bilayers, where they enhance T cell polarization and proliferation (Dustin et al., 1998; Grakoui et al., 1999; Bromley et al., 2001), but their effects on T cell signaling were not investigated directly in these earlier studies. In this study, we have asked whether CD58 incorporated into planar lipid bilayers will induce T cell signaling and whether such a signaling system bears similarities to what is known for TCR. We show that CD58–CD2 ligation causes a similar signaling response that has been described for TCR signaling. Despite the very different structures of CD2 and TCR, we also find that both receptor systems induce a similar condensation of a similar set of downstream signaling molecules (e.g., TCR-ζ chain, Lck, and LAT) into microdomains. A comparison of the similarities and differences of the spatial and temporal patterns of CD2, TCR, and downstream kinases/adaptor proteins leads us to propose a model for how these receptors trigger the initial signaling response and might synergistically promote T cell activation.

Results

CD2–CD58 interaction at a cell–bilayer interface mediates signaling in the absence of TCR stimulatory molecules

Previous studies have shown that anti-CD2 antibodies (Siliciano et al., 1985) or biotinylated CD58-Fc cross-linked with avidin (Kanner et al., 1992) will stimulate signaling responses in T cells. However, these prior studies have not established whether GPI-anchored CD58 diffusing freely in a membrane (the natural ligand for CD2) will also induce signaling independent of other molecules on APC. To investigate this question, we used an assay system in which Jurkat T cells interact with planar lipid bilayers containing the native GPI-linked CD58 (Fig. 1 A). In our assay, the concentration of CD58 molecules in the bilayer was adjusted to ∼40 molecules/μm^2, which is comparable to or slightly lower than estimates of CD58 concentrations in B cells (∼100–200 molecules/μm^2; Selvaraj et al., 1987a,b). In our experiments, we compared the responses of cells that were placed on bilayers containing CD58 to those placed on the adhesion molecule ICAM-1 (∼10 molecules/μm^2). Jurkat cells adhere to ICAM-1–containing bilayers and form immunological synapses upon TCR activation (Kaizuka et al., 2007) but exhibit minimal signaling responses in the absence of TCR activation (Dustin et al., 1997; Yokosuka et al., 2005; Varma et al., 2006).

Jurkat cells exhibit several characteristic signaling responses when stimulated with anti-TCR antibodies, including activation of actin polymerization and cell spreading (Kaizuka et al., 2007), increase in intracellular calcium and tyrosine phosphorylation (Weiss and Littman, 1994), and increased transcription of the surface protein CD69 by activation of the Ras pathway (D'Ambrosio et al., 1994). We examined all of these responses for Jurkat cells interacting with CD58-containing bilayers. Cells interacting with CD58 bilayers also spread rapidly (<5 min) by extending a large actin-filled lamellae where actin filaments exhibited prominent retrograde flow (Fig. 1 B and Video 1). In contrast, cells on ICAM-1 bilayers did not spread and form symmetric lamellae (Fig. 1 B), although Jurkat cells adhered to the ICAM-1 bilayer as confirmed by reflection interference contrast microscopy (not depicted) and can crawl on bilayers containing high concentrations of ICAM-1 by partially polarizing their actin (Dustin and Springer, 1988). As measured by Fura-2 fluorescence, we also found a rapid (10–60 s) rise in cytosolic calcium (Fig. 1 B and Video 2) as well as calcium oscillations (Fig. S1 B and Video 3) after Jurkat cells contacted a CD58 bilayer. A rise in intracellular calcium also was observed in cells in calcium-free buffer, indicating that the initial calcium release was from an internal pool, most likely the endoplasmic

[340 nm/380 nm] at 2 min of cell contact; see Materials and methods), cell surface expression of CD69 (fixed cell staining with an FITC-labeled anti-CD69 antibody at 3 h), and tyrosine phosphorylation (immunofluorescence with an antiphosphotyrosine antibody at 2 min) were elevated to a greater extent on CD58 compared with ICAM-1 bilayers. Asterisks and dashed lines indicate single cells. More than 50 cells were measured in all assays, and the data are representative for more than three independent experiments. Data are quantified as relative intensity to control experiment (ICAM-1 bilayer). pY, phosphotyrosine. Error bars indicate SEM (*n* > 50). Bars, 10 μm.

reticulum. The increase in calcium was constant over a 10-fold range of CD58 in the bilayer (10–100 molecules/μm^2; Fig. S1 A). We did not observe a rise in calcium when Jurkat cells were adhered to bilayers with a 10-fold range of ICAM-1 (2.5–25 molecules/μm^2; Fig. S1 A). By immunostaining, we observed increases in phosphotyrosine staining and the surface expression of CD69 (Fig. 1 B) for cells on CD58 bilayers compared with ICAM-1 bilayers. These results demonstrate, for the first time, that CD2–CD58 interaction at a membrane–membrane interface will elicit downstream signaling events in the absence of TCR stimulation and thus has additional functions beyond simply promoting cell adhesion.

We also wished to explore whether CD2-mediated signaling can occur in native T cells. To test this, primary mouse T cells (AND TCR transgenic T cell) were adhered to bilayers containing either fluorescently labeled GPI-linked CD48 (mouse ligand for CD2) or mouse ICAM-1 molecules (Fig. S2). On CD48 bilayers, cytosolic calcium increased significantly compared with native T cells interacting with ICAM-1 bilayers (Fig. S2 A). These results demonstrate that membrane-bound ligand (CD58 or CD48) activates CD2-mediated signaling in both primary cells and Jurkat cells in the absence of TCR activation.

CD2–CD58 interaction enhances T cell signaling additively with TCR

The aforementioned results show that CD2–CD58 interaction without TCR stimulation induces downstream signaling. However, CD2–CD58 and MHC–TCR would be expected to operate together at the interface of a native T cell interacting with an APC. To investigate the effects of TCR–CD2 costimulation separate from other membrane molecule interactions, we prepared planar lipid bilayers containing both CD58 and anti-TCR antibodies (Fig. 2 A). We previously showed that membrane-bound anti-TCR antibody is a potent stimulatory ligand for TCR in a Jurkat T cell–bilayer system (Kaizuka et al., 2007). In this study, we compared cell responses to dual-ligand bilayer and to bilayers with only one stimulant, either anti-TCR or CD58. In this assay, TCR signaling (calcium and CD69 elevation) was maximally stimulated and constant over an ∼8-fold range of anti-TCR density on the bilayer (Fig. 2, B and C), which is consistent with the fact that TCR signaling saturates in relatively small numbers of TCR stimulatory molecules (Irvine et al., 2002; Purbhoo et al., 2004). In contrast, cells on CD58/anti-TCR bilayers exhibited a much more pronounced elevation of intracellular calcium and CD69 compared with cells on anti-TCR alone. This effect was observed at several time points of stimulation with the bilayer (Fig. S3 A). Inhibition of the CD2–CD58 interaction by anti-CD58 antibodies abolished this enhancement of signaling by dual activation (Fig. S3 B). Our results indicate that the CD2–CD58 interaction can activate the early T cell signaling response beyond what can be achieved by TCR activation alone.

CD2-mediated signaling involves molecules in a TCR signaling pathway: Lck, TCR-ζ, ZAP70, or LAT

To further explore the molecular mechanism of CD2–CD58-mediated signaling, we investigated the involvement of various
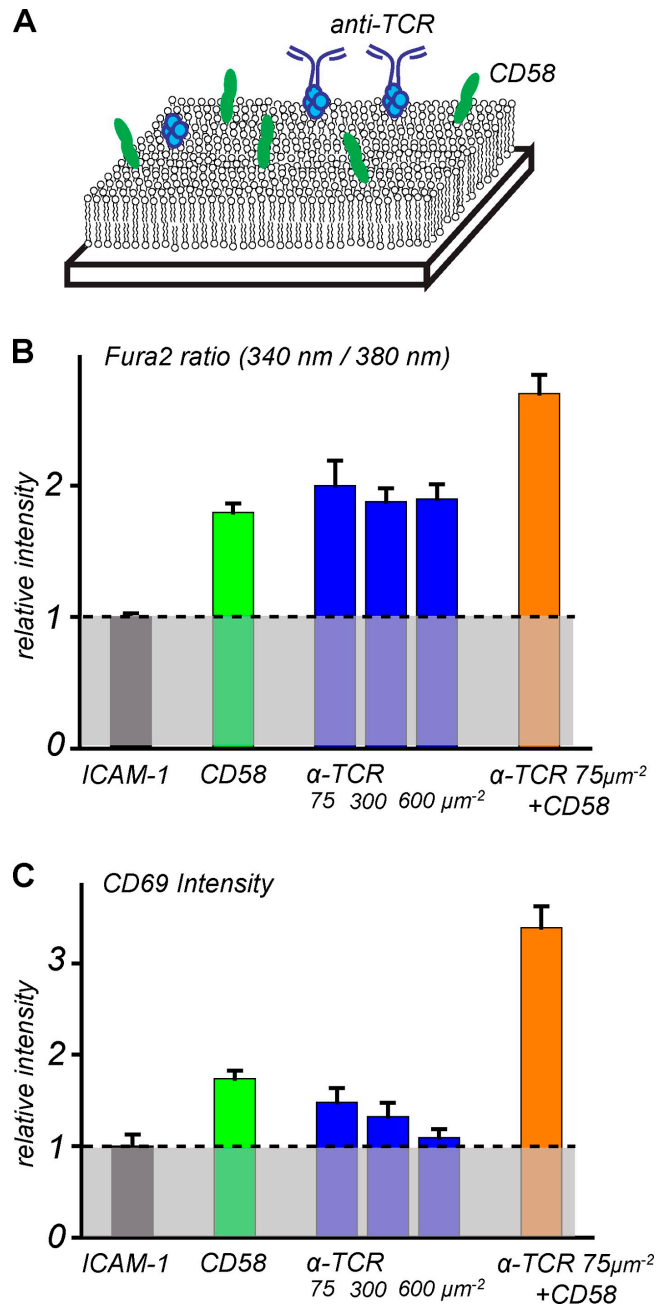
Figure 2. **CD2–CD58 interaction enhances T cell signaling.** (A) To study TCR–CD2 dual-signaling system, bilayers were prepared containing GPI-linked CD58 and anti-TCR antibody linked to lipid via biotin-streptavidin conjugate (monobiotinylated antibody and biotinylated lipid were connected by streptavidin). (B and C) Calcium elevation (Fura-2 emission ratio, 340 nm/380 nm; B) and CD69 surface expression level (C) were measured in Jurkat cell interacting bilayers containing different amounts of anti-TCR (75, 300, and 600 molecules/μm^2), CD58 (40 molecules/μm^2), both CD58 and anti-TCR (40 and 75 molecules/μm^2, respectively), and ICAM-1 (10 molecules/μm^2) after 3 h of cell–bilayer contact. Representative data for three independent experiments are shown. Measurements and quantifications were performed as described in Fig. 1 (data shown are mean ± SEM, $n > 50$).

signaling molecules in the pathway by using chemical inhibitors or mutant cell lines (Fig. S4 B). T cell signaling is strongly inhibited by the Src kinase inhibitor PP2, which inhibits Lck and related kinases such as Fyn. Using cytoplasmic calcium

increase as a readout of signaling, we found that PP2 strongly inhibited CD58–CD2 signaling as well. A similar defect was also observed in Lck-negative cell line (J.CaM1; unpublished data). A major substrate for Lck is the ITAM domains in the subunits of TCR. Interestingly, although signaling was induced in the absence of TCR stimulation, we found that TCR is required for CD58–CD2-mediated signaling, as suggested previously (Bockenstedt et al., 1988). In a cell line that expresses low levels of TCR β subunit (J.RT3-T3.5), we found a significant defect in Ca^{2+} increase elicited by CD58. This defect could be partially rescued by transfection and stable expression of TCR β subunit gene. Interestingly, we also found that transfection of TCR-ζ chain in J.RT3-T3.5 cells could partially rescue CD2–CD58 signaling (Fig. S4 C). TCR-ζ chain has intracellular ITAM domains that are phosphorylated by Lck, but it is not involved in TCR–MHC interaction. The levels of rescue are partial in the aggregate cell population, perhaps because expression levels of transfected molecules varied between cells. These results demonstrate that TCR-ζ (without the ligand-binding components of TCR–CD3 complex) is required for CD2–CD58-mediated signaling. A ZAP70-negative line (P116), an LAT-deficient cell line (J.CaM2), and an SLP76-deficient cell line (J14) also all showed significant reductions in their calcium response compared with normal Jurkat cells on CD58 bilayers (Fig. S4 B). These defects are not a result of poor CD2 expression among these mutant cell lines, as these cell lines show similar CD2 levels as wild-type cells (Fig. S4 A).

Collectively, our results show that the well-characterized pathway leading to calcium increase after TCR activation (Lck phosphorylation of ITAM motifs in TCR, particularly the ζ chain, subsequent recruitment/activation of ZAP70 that phosphorylates LAT, and PLC-γ1 recruitment to phospho-LAT through SLP76) appears to be activated downstream of CD58–CD2 ligation as well. Additionally, actin filaments appear to be involved in CD2–CD58 signaling, as has been shown for TCR signaling (Valitutti et al., 1995; Varma et al., 2006) because depolymerization of actin filaments with latrunculin B significantly inhibited CD2-mediated signaling. These results are largely consistent with previous studies using soluble anti-CD2 antibodies to elicit signaling, which reported the involvement of TCR, Lck, LAT, and ZAP70 in signaling events (Bockenstedt et al., 1988; Martelli et al., 2000). Using high resolution imaging, we revealed how these signaling molecules and actin are involved in the CD2-mediated signaling system, as will be presented in the following sections.

CD2–CD58 interaction organizes signaling membrane microdomains

Our prior experiments with fluorescence microscopy showed that CD2 forms microdomains with Lck and LAT on the plasma membrane after activating Jurkat cells with glass-bound anti-TCR antibodies in the absence of any CD2 ligand (Douglass and Vale, 2005). Thus, we next wanted to investigate whether a similar clustering of CD2 occurs when Jurkat cells interact with CD58-containing planar lipid bilayers without TCR activation. Previous studies showed formation of CD2–CD58-enriched adhesive zones at the membrane interface (Dustin, 1997; Depoil

et al., 2005). In our high resolution imaging studies, we found that fluorescently labeled CD58 molecules were gathered into submicrometer-sized microdomains at the actin-rich leading edge of the Jurkat cells (Fig. 3 and Video 4). After their formation, these CD2–CD58 microdomains moved toward the cell center by actin retrograde flow, similar to movements of TCR microdomains described previously (Fig. 3 D; Krummel et al., 2000; Yokosuka et al., 2005; Varma et al., 2006; Kaizuka et al., 2007). The rates of CD58 transport (0.086 ± 0.004 μm/s, mean ± SEM, $n = 103$) were comparable but slightly lower compared with what we have reported previously for TCR and integrins in Jurkat cells (0.14 μm/s and 0.13 μm/s, respectively; Kaizuka et al., 2007), suggesting that slippage in the coupling to retrograde moving actin filaments might occur more often for CD2. As a result of this actin-based transport, CD58–CD2 assembled in the central region of the cell (Fig. 3 C; Dustin et al., 1996; Dustin, 1997), thus forming a central supramolecular activation cluster (SMAC [cSMAC])–like structure as described for TCR (Grakoui et al., 1999). We also observed that formation of new microdomains and transport of preformed microdomains were abolished by actin depolymerization with 100 nM latrunculin B, which confirmed that both processes are actin dependent (Video 5).

We next sought to determine whether CD2–CD58 microdomains are enriched with other signaling molecules, as has been described for TCR microdomains (Campi et al., 2005; Douglass and Vale, 2005; Yokosuka et al., 2005; Varma et al., 2006). We found that GFP-tagged wild-type Lck kinase distributed uniformly in plasma membrane (Fig. S5 A). However, only a subset of Lck may be activated, a process that involves dephosphorylation of tyrosine 505 by membrane-anchored phosphatase CD45, which relieves intermolecular autoinhibitory SH2 interactions and also allows autophosphorylation of Y394 and full activation of the kinase (Hermiston et al., 2003). To examine the localization of active Lck, we transfected Jurkat cells with an Lck mutant (LckY505F-GFP; mimicking dephosphorylated residue 505). Strikingly, LckY505F-GFP localized selectively to the CD2 microdomains (Fig. 4 A). These results suggest that relieving of SH2 autoinhibition allows Lck to interact with proteins within the CD2 microdomains. Similarly, by immunostaining with an antibody that specifically recognizes the active, autophosphorylated form of Src family kinases, we also found that active Src kinases (mainly Lck and Fyn in T cells) were enriched in CD2 microdomains (Fig. 4 B). We also observed an increase in staining by antiphospho-Src antibody of primary mouse T cells (AND T cells) interacting with CD48 bilayers (mouse homologue of human CD58; Fig. S2 B). In contrast, we found that the phosphatase CD45 is excluded from the CD2 microdomains (3.0 ± 0.3–fold higher intensity per unit area outside vs. inside of microdomains; mean ± SEM, $n = 12$ cells; Fig. 4 C), as was found in prior studies with TCR microdomains (Douglass and Vale, 2005; Varma et al., 2006). Consistent with an enrichment of active kinases and exclusion of an abundant phosphatase, the CD2 microdomains were highly enriched for phosphotyrosine antibody staining (Fig. 4 D).

We next examined the localization of two critical downstream targets of kinase phosphorylation: the ζ chain of TCR
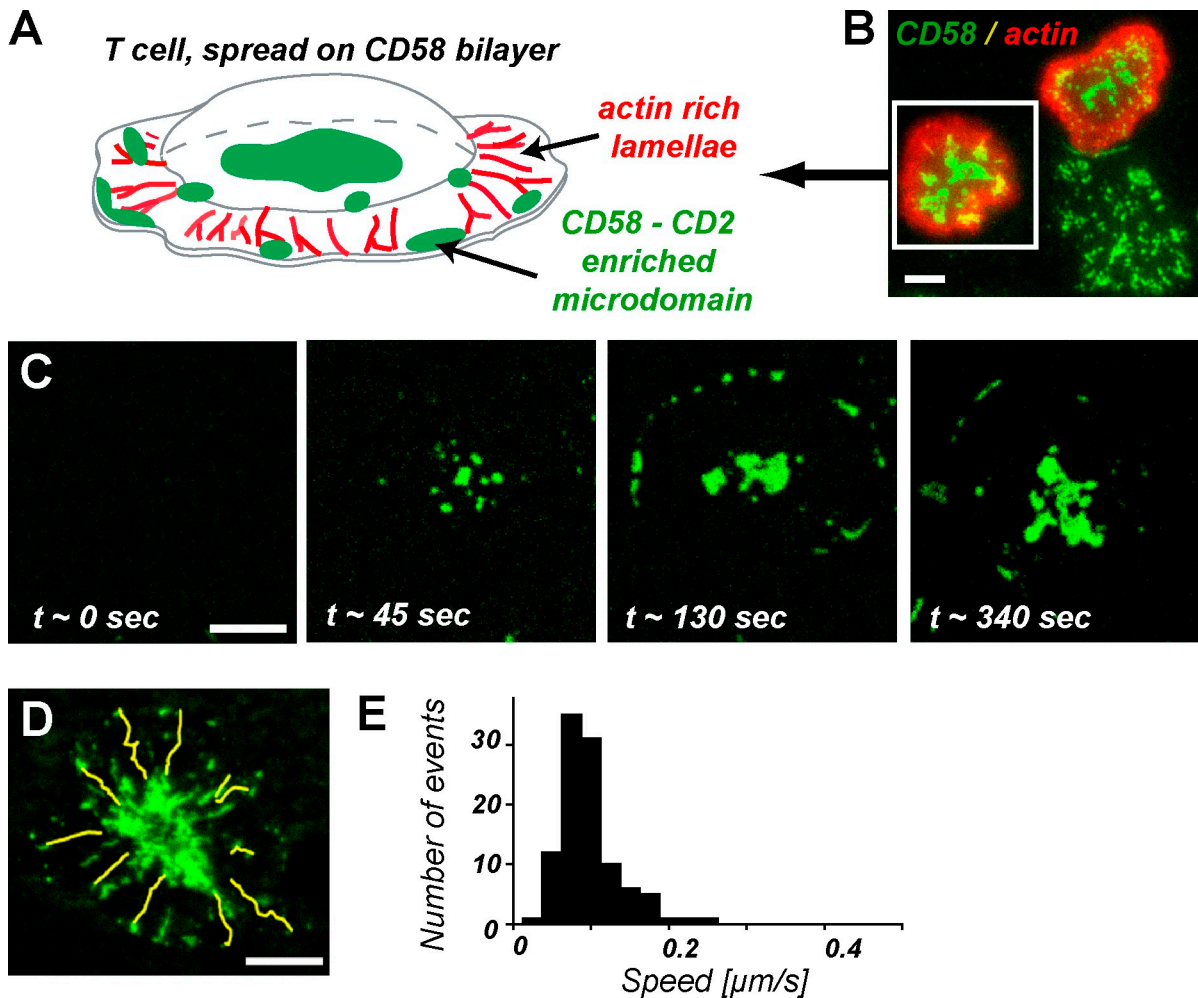
Figure 3. **CD2–CD58 interaction is organized in membrane microdomains that are generated and transported by actin filaments.** (A) A schematic of a Jurkat T cell spreading on CD58 bilayer forming CD2–CD58-enriched microdomains in the actin-rich lamellae, corresponding to the boxed region in B. (B) Spinning-disk confocal fluorescence microscopy of Jurkat cells expressing actin-GFP (red) that spread on CD58 (green)-containing bilayer imaged at the cell–bilayer interface. (C) Dynamics of CD58 microdomains. The $t = 0$ s time was defined as the time at which the T cell had just adhered to the bilayer and some accumulation of CD58 under the cell was observed. But before the cell began to spread ($t = \sim$45 s), microdomains were formed at the initial cell–bilayer contact under cortical actin filaments. After the cell spreads, new microdomains were continuously formed at cell periphery where actin filaments are polymerizing ($t = \sim$130 s). Microdomains were transported to the cell center and formed a larger central cluster (cSMAC-like structure; $t = \sim$340 s). (D) Trajectories of individual CD2–CD58 microdomains transported from the cell periphery to the cell center are shown. (E) A histogram of the speeds of CD2–CD58 microdomain transport is shown. Bars, 5 µm.

and LAT. GFP-tagged ζ chain distributed throughout the plasma membrane (Fig. S5 B). However, using an antibody specific for the phosphorylated form of the ζ chain, we found that phospho-ζ was localized selectively to the CD2 microdomains, although the amount of phospho-ζ was small and only detected by total internal reflection fluorescence (TIRF) microscopy (Fig. 4 E). Using GFP-tagged wild-type LAT, we also observed LAT accumulation in CD58–CD2 microdomain (Fig. 4 F), as previously described for cells stimulated with glass-absorbed anti-TCR antibodies (Douglass and Vale, 2005). We also confirmed that these molecular rearrangements did not occur when T cells interact with ICAM-1 bilayer (Fig. S5 E).

Thus, CD2–CD58 interaction results in organization of critical proximal signaling molecules, including activated tyrosine kinase, phosphorylated TCR, and LAT, into microdomains. Signaling, as defined by the intensity of phosphotyrosine staining, was most active at newly generated microdomains at the

cell periphery. In contrast, phosphotyrosine staining was reduced at the cell center, where microdomains aggregated into a cSMAC-like structure (Fig. 4 G). However, the relative reduction in phosphotyrosine staining in the CD2 cSMAC was not as pronounced as observed for TCR cSMAC (Lee et al., 2003; Campi et al., 2005; unpublished data). Rapid signal deactivation in TCR cSMAC may be partially facilitated by CD45, which is excluded from early TCR microdomains but localizes with TCR in cSMAC (Varma et al., 2006). However, in contrast, CD45 was excluded from CD2 microdomains from their genesis until their formation of cSMAC-like structures (Fig. S5 D).

CD2-mediated signaling requires its cytoplasmic domain

We next investigated the structural requirements in the CD2 molecule that allow it to form microdomains and signal. To test the role of the CD2 cytoplasmic domain, we transfected wild-type
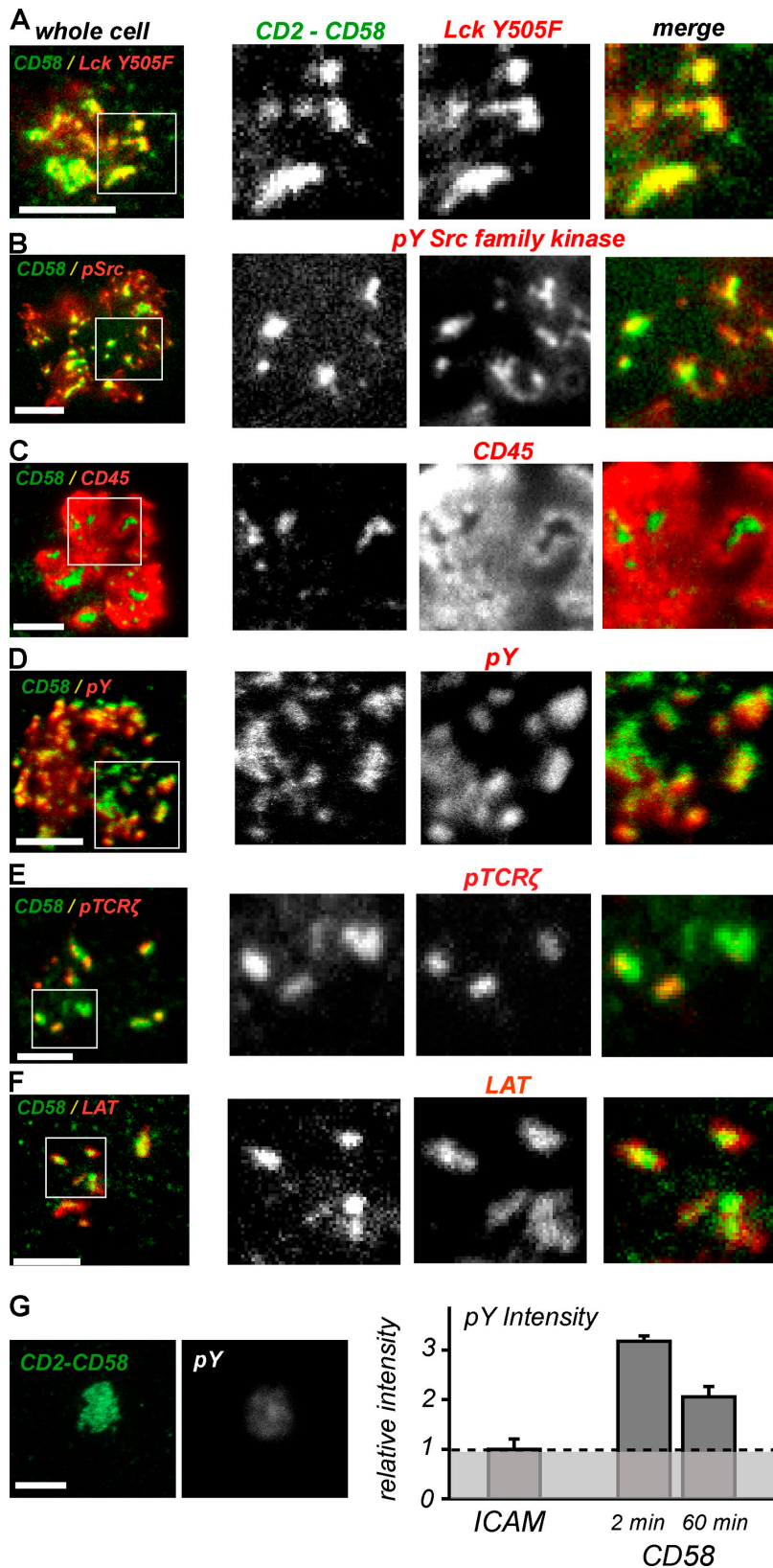
Figure 4. **CD2–CD58 microdomains organize signaling molecules.** Dual-color images of various signal molecules (red) and CD2–CD58 microdomains (green) at the cell–bilayer interface by TIRF microscopy. (A–F) ~2 min after interaction with CD58 lipid bilayers, live cell imaging was performed with the GFP-tagged mutant Lck (LckY505F; A) or GFP-LAT (F), and the remainder are immunofluorescence images using antibodies against phosphotyrosine (pY) of activated Src family kinases (including autophosphorylated tyrosine 394 in Lck; B), CD45 (C), phosphotyrosine (D), and phosphorylated ITAM domain of TCR-ζ (E). The white boxes show regions that are magnified in the panels on the right. The intensity ratio of CD45 (outside and inside of microdomains) is ~3.0 ± 0.3 (mean ± SEM, $n = 12$). The same molecular patterning was observed by widefield and confocal microscopy as well. (G) Immunostaining of phosphotyrosine at 60 min of cell–CD58 bilayer contact shows concentration of signal in a cSMAC-like cluster and an overall reduction in fluorescence intensity compared with a 2-min time point (quantitation as described in Fig. 1). Data shown are mean ± SEM ($n > 20$). Bars, 5 µm.

CD2 (CD2WT) and a truncated CD2 lacking its cytoplasmic domain (CD2TM) into J.CaM2 cells, which express very low levels of CD2 and LAT (Finco et al., 1998; Roose et al., 2003). In the control CD2WT-transfected J.CaM2 cells, CD2 micro-domains formed and were enriched in LckY505F, transfected LAT, and phosphotyrosine (Fig. 5, C and E; and Fig. S5 C). Interestingly, CD2TM-transfected J.CaM2 cells also adhered to CD58-containing bilayers and formed CD58–CD2 clusters.
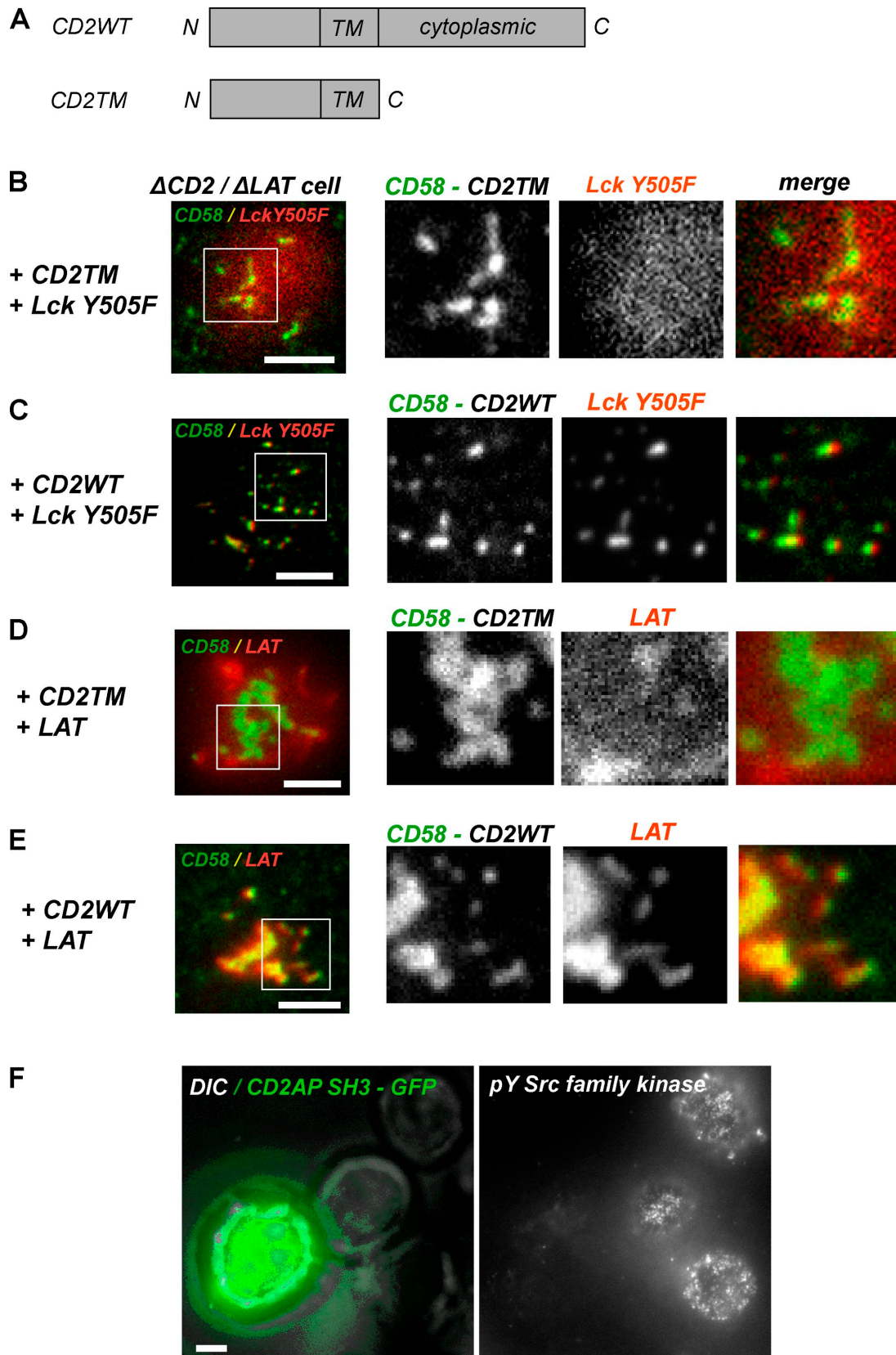
Figure 5. **CD2-mediated signaling requires its cytoplasmic domain.** (A) A schematic of two CD2 constructs tested for localization and recruitment of signaling proteins is shown. One is the control wild-type protein (CD2WT), and the second is a truncated CD2 molecule that lacks the entire cytoplasmic domain (CD2TM). Both constructs were tagged with mCherry at the C terminus and transfected into a J.CaM2 cell line, which is deficient in both CD2 and LAT. N, N terminus; C, C terminus, TM, transmembrane domain. (B–E) J.CaM2 cells were cotransfected with either CD2WT-mCherry or CD2TM-mCherry and either GFP-LckY505F or GFP-LAT and were imaged by TIRF microscopy ~2 min after contact with a CD58-containing bilayer. The white boxes show regions

This result indicates that CD2's extracellular domain is sufficient for the self-association of CD58–CD2 complexes into microdomains, which is consistent with earlier results (Dustin et al., 1998). However, these CD2TM microdomains did not recruit activated Lck (LckY505F-GFP; Fig. 5 B) or LAT (although some weak LAT clustering was occasionally observed; Fig. 5 D). Accordingly, tyrosine phosphorylation in CD2TM microdomains was minimal (Fig. S5 C). A role for the cytoplasmic domain is also suggested by an experiment showing a reduction of phosphotyrosine staining after transfection with SH3 domains of CD2AP (CD2-associated protein), which bind to polyproline domains in the CD2 cytoplasmic region and probably generates a dominant-negative effect (Fig. 5 F; Dustin et al., 1998). Thus, we conclude that CD2's cytoplasmic domain is dispensable for microdomain formation but is required for recruitment and activation of downstream signaling molecules. A particularly important role of the cytoplasmic domain of CD2 might be in the activation of autoinhibited Lck, as described in the Discussion.

Spatial-temporal patterning of CD2 and TCR during costimulation

Our results show that CD2–CD58 induces microdomain formation, and work from others has shown that TCR also induces microdomains during its signaling response (Campi et al., 2005; Yokosuka et al., 2005; Varma et al., 2006). This raises the question of what happens during costimulation of both TCR and CD2. Do these molecules colocalize or spatially segregate, and how is the localization of the downstream signaling molecules affected? To address these questions, we performed live cell and immunofluorescence imaging of Jurkat T cells interacting with bilayers containing both CD58 and anti-TCR antibody. In this dual stimulatory system, the stimulated TCR and CD2 colocalized in newly generated microdomains at the early stage of cell–bilayer contact (Fig. 6 A). However, ~5 min after the initial contact, TCR and CD2 began to separate, with TCR concentrating in the center and CD2 accumulating peripheral to this TCR-enriched cSMAC zone (Fig. 6 B). The separation of TCR and CD2 was only obvious when TCR cSMAC was generated (Fig. 6 B). A small amount of TCR was contained in CD2-rich zone, and vice versa, but the separation of TCR and CD2 into distinct zones near the center was very different from their colocalization in peripheral microdomains.

We next localized other signaling molecules and tyrosine phosphorylation in Jurkat cells exposed to the dual CD58/anti-TCR bilayers. Strong tyrosine phosphorylation was observed in the peripheral microdomains, as was seen previously (Fig. 4 D; Campi et al., 2005; Yokosuka et al., 2005). After CD2 and TCR separated near the cell center, the level of phosphorylation was significantly reduced in TCR cSMAC, as shown previously (Lee et al., 2002), but there was still substantial phosphotyrosine staining in the surrounding CD2 zone (Fig. 6 B). Phosphorylated

Lck (pY394) also was enriched in the CD2 zone (Fig. S5 F), and, interestingly, even phosphorylated TCR-ζ chain preferentially colocalized with CD2 and not with the bulk TCR in the cSMAC (Fig. 6 C). We also found that mutant Lck (LckY505F-GFP) and LAT-GFP preferentially localized in the CD2 zone over the cSMAC (Fig. 6 C). Thus, the peripheral CD2 zone appears to be a region that enhances signaling compared with TCR-enriched cSMAC.

Discussion

Using an artificial bilayer system, we show for the first time that the native GPI-anchored form of CD58 can trigger a downstream signaling cascade through the CD2 receptor. Interestingly, this signaling process is accompanied by a dramatic, actin-dependent reorganization of CD2 and downstream signaling molecules into microdomains, as was previously reported for signaling through TCR. We also show that when CD2 and TCR are stimulated together, they cocluster at the periphery but then segregate from one another near the cell center. Thus, the formation and retrograde transport of membrane microdomains appears to be a common mechanism in both CD2 and TCR signaling pathways and may play a crucial role in determining the biochemical environment in which signaling takes place.

Common themes of CD2–CD58 and TCR signaling

CD2 is most widely cited as an adhesion molecule, and its effects on T cell activation have been suggested to involve prolongation of the close apposition between APC and the T cell. However, previous studies also have shown that antibody-induced cross-linking of CD2 elicits signaling responses in T cells (Siliciano et al., 1985; Bockenstedt et al., 1988), although such effects of artificial cross-linking might be nonphysiological. Our study shows that CD58 in its native state (attached to a membrane via a GPI anchor) can elicit a strong signaling response in Jurkat T cells. Thus, it is likely that CD58–CD2 complexes perform an important role in signaling in addition to promoting adhesion between the T cell and APC.

Several proximal TCR signaling events, including elevation of tyrosine phosphorylation, intracellular calcium, CD69 expression, and actin polymerization, also occur after CD58–CD2 ligation, which is in agreement with prior studies with anti-CD2 antibodies (Siliciano et al., 1985; Bockenstedt et al., 1988). Many of the well-characterized proteins in TCR signaling cascade are also involved in CD2 signaling, including Lck, ZAP70, and LAT. Interestingly, the ITAM domains of TCR (particularly the ζ chain) are required for CD2 signaling and serve as substrates for downstream signaling, even though the α/β chains are not involved in ligand interactions and are not

that are magnified in the panels on the right. (F) Normal Jurkat cells GFP-tagged SH3 domains of CD2AP interacting with CD58 bilayers were fixed and stained with an antibody against phosphotyrosine (pY) of active Src kinase. The merge of differential interference contrast image and GFP fluorescence (left) shows that the cell overexpressing the dominant-negative GFP-tagged SH3 domain has little phosph-Src staining compared with the untransfected cells (immunofluorescence image on the right). Bars, 5 μm.

Figure 6. Spatial-temporal patterning of CD2, TCR, and signaling molecules. Triple-color imaging of Jurkat cells interacting with bilayer containing fluorescent CD58 and anti-TCR antibody. (A and B) Patterning of CD2–CD58 (green) and TCR (blue) microdomains shows their initial colocalization at 2 min (A) and then separation at 15 min (B) after the initial cell–bilayer contact. Immunofluorescence images of phosphotyrosine (pY) corresponding with these two microdomain patterns are shown at right. (C–E) Localization of signal molecules (active Lck [Y505F]; C), phospho–TCR-ζ (D), and LAT (E) is shown in red in the merged images (separate CD2–CD58 [green] and TCR [blue] microdomains are shown on the left); imaged after 15 min of cell–bilayer contact. Bars, 5 μm.
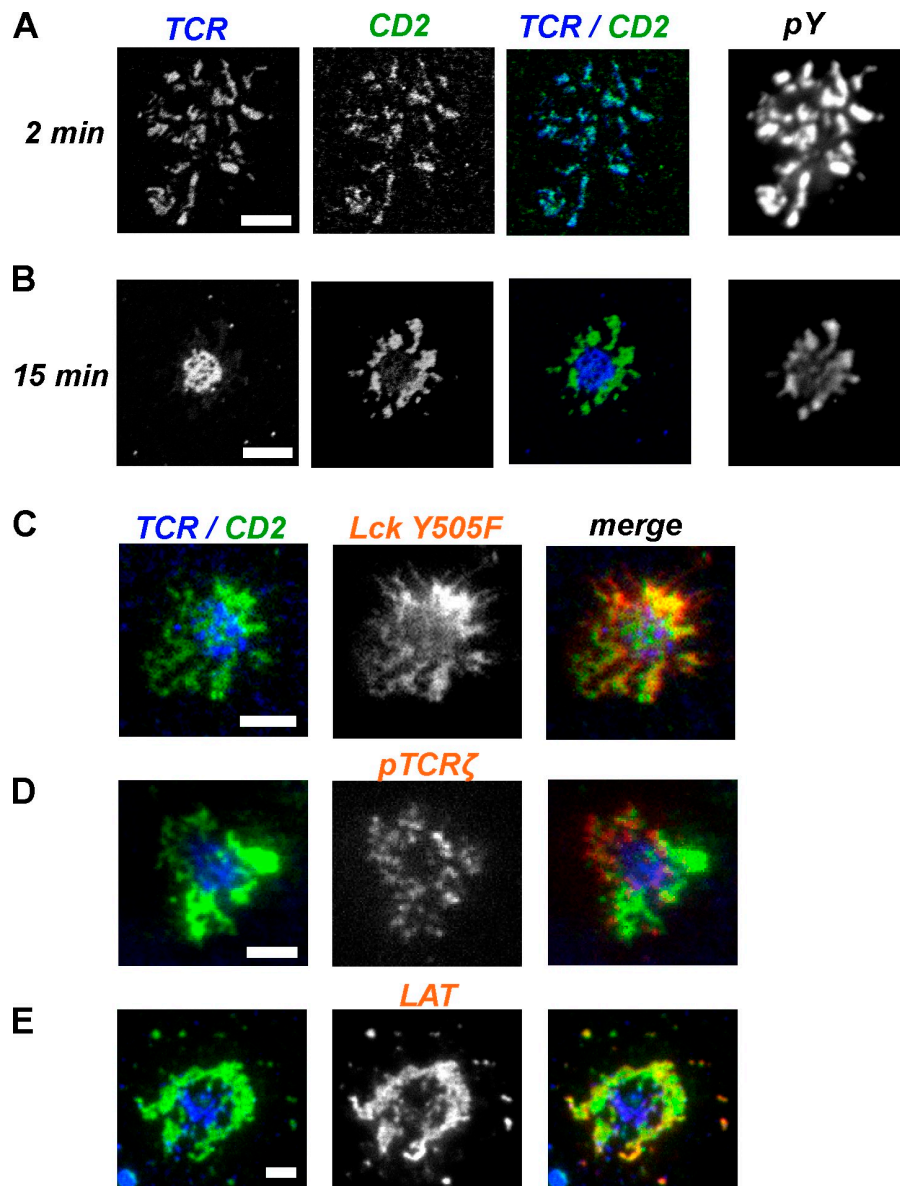
even required for this process (see subsequent Discussion). Uniformly distributed TCR-ζ could serve as a substrate for Lck and then an adaptor for ZAP70. Thus, the concentration of phosphorylated ITAM domains generated upon CD2 ligation is lower than that for TCR–CD3 complexes clustered by direct TCR stimulation (unpublished data). We speculate that such variance between TCR and CD2 signaling systems may result in a quantitative difference in calcium flux (Fig. S3 A) or a more pronounced difference in integrin activation. Signaling through TCR stimulation activates the integrin LFA-1, which then binds to its ligand ICAM-1 and forms an adhesion zone (peripheral SMAC) surrounding the cSMAC (Grakoui et al., 1999). However, in the absence of TCR stimulation, a peripheral SMAC does not form in T cells interacting with a bilayer containing CD58 and ICAM-1 (Dustin et al., 1998; unpublished data).

Another common theme of TCR and CD2 signaling is the formation of microdomains at the actin-rich cell periphery, which are enriched with several critical signaling molecules.

As postulated earlier (Douglass and Vale, 2005), we believe that these microdomains are primarily held together by a network of protein–protein interactions and become resistant to perturbation by actin-depolymerizing drugs. However, clusters of lipids/cholesterol (lipid "rafts") also may have roles in T cell signaling (Harder, 2004). Consistent with this idea, we have found that the binding of Jurkat cells to planar bilayers containing bound cholera toxin subunit B (CTB; which binds and clusters the ganglioside lipid GM_1) results in an increase in intracellular calcium level, albeit to a lower extent than that induced by lipid-bound CD58 (unpublished data). A similar rise in intracellular calcium was noted previously with soluble CTB (Gouy et al., 1994). But unlike CD2 or TCR-mediated microdomains, CTB-GM_1 clusters did not recruit signal molecules (e.g., LAT and activated Lck; unpublished data).

We propose that an interaction between the extracellular domains of CD2 is important for microdomain formation based upon our finding that truncation of the cytoplasmic domain still
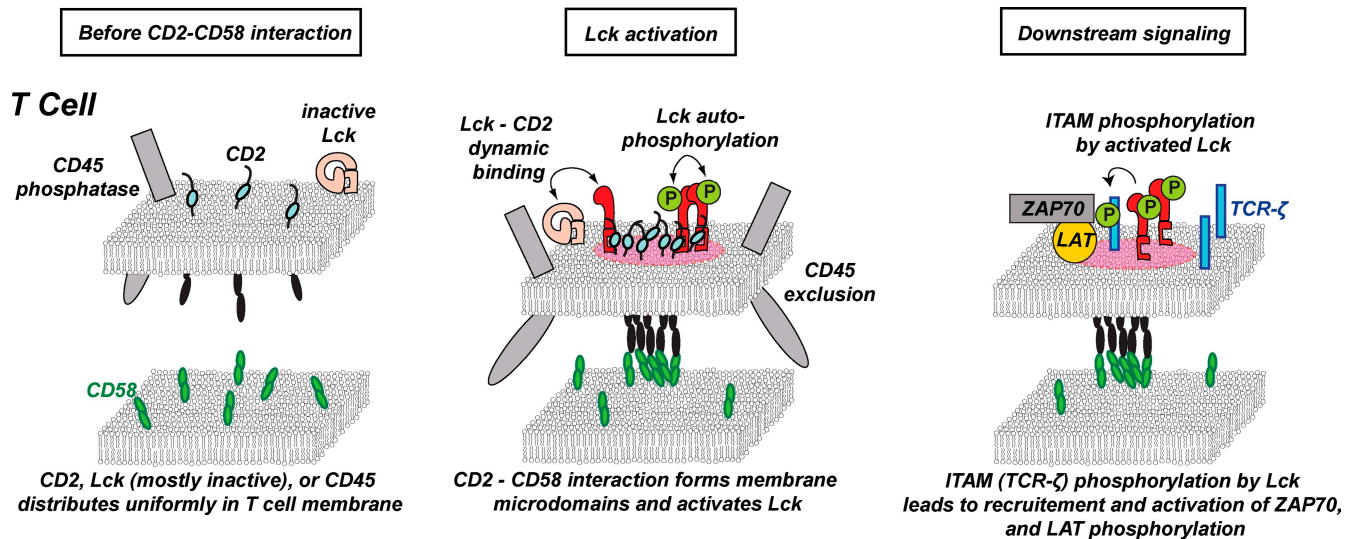
| Before CD2-CD58 interaction | Lck activation | Downstream signaling |

T Cell

CD45 phosphatase — CD2 — inactive Lck

Lck - CD2 dynamic binding — Lck auto-phosphorylation — CD45 exclusion

ITAM phosphorylation by activated Lck — ZAP70 — LAT — TCR-ζ

CD58

CD2, Lck (mostly inactive), or CD45 distributes uniformly in T cell membrane

CD2 - CD58 interaction forms membrane microdomains and activates Lck

ITAM (TCR-ζ) phosphorylation by Lck leads to recruitement and activation of ZAP70, and LAT phosphorylation

Figure 7. **Model of CD2–CD58 microdomain-mediated signaling.** Uniformly diffused molecules in plasma membrane of T cells (left) are spatially organized upon CD2–CD58 interaction (middle and right). CD2–CD58 interaction generates microdomains mediated by intermolecular interaction between CD2 extracellular domains. Cytoplasmic domains of CD2 within highly concentrated microdomains, particularly poly proline regions, can activate Lck by competitively binding to autoinhibitory SH3 domain in Lck (inactive, closed form). The active, open form of Lck molecules can trans-autophosphorylate other Lck molecules at tyrosine 394, resulting in further enhancement of Lck kinase activity. The membrane-anchored phosphatase CD45 is excluded from CD2 microdomains, which prevents Lck deactivation through CD45-mediated dephosphorylation of tyrosine 394. CD45 outside of the microdomains plays a role in dephosphorylating phosphotyrosine (P) 505 in Lck, which inhibits kinase activity via interaction with autoinhibitory SH2 domain. A subset of Lck, opened by CD45 dephosphorylation at Y505, can then diffuse into CD2 microdomains, leading to further activation. Active Lck molecules in microdomains can phosphorylate ITAM domains in TCR and facilitate subsequent downstream signaling involving LAT and other molecules. Although not shown, similar models may apply to the activation of related Src-like kinases such as Fyn. Although microdomains act to concentrate proteins, these molecular interactions are dynamic, and proteins can diffuse into and dissociate from these domains, as shown previously (Douglass and Vale, 2005).

allows CD58-mediated microdomain formation to occur. This result is consistent with a previous study showing that CD2 molecules expose a linker domain between its two extracellular Ig domains upon CD58 ligation and that the exposed linker domain may promote intermolecular interactions (Li et al., 1996). A dynamic actin network at the leading edge is also required for microdomain formation because latrunculin completely blocks the formation of new microdomains. However, the mechanism by which actin participates in the formation of TCR and CD2 microdomains is not understood and remains an important topic for future investigation, although some candidate molecules for actin–receptor linkage are suggested in past studies (Hutchings et al., 2003; Tavano et al., 2006).

After TCR (Kaizuka et al., 2007) and CD2 microdomains are formed, they are both transported centripetally by attaching to the retrogradely moving actin network, resulting in their concentration toward the cell center. Previous work has shown that the peripheral TCR microdomains are more heavily stained for phosphotyrosine than the central cSMAC (Campi et al., 2005; Mossman et al., 2005; Yokosuka et al., 2005), and we find a similar result for CD58–CD2 signaling as well. Thus, for both TCR and CD2 signaling, the initial clustering of receptors and intracellular activators appears to be associated with strong signaling, whereas their transport and accumulation toward the cell center may be involved in down-regulation (Lee et al., 2002, 2003). However, in the single ligand systems, TCR and CD2 microdomains also exhibited differences in their morphology and dynamics. Notably, the size of TCR microdomains does not change significantly from their genesis at the leading edge until their accumulation in the cSMAC (Varma et al., 2006; Kaizuka

et al., 2007). In contrast, CD2 microdomains vary in size, and they often coalesce or dissociate into several pieces (Fig. 3).

A model for signaling by CD2 and TCR

Why do CD2 and TCR both use clustering and microdomain formation in their signaling processes? We propose that a common link may be the requirement of microdomain formation for the activation of Lck, the likely linch-pin kinase for both signaling cascades (Fig. 7; Danielian et al., 1989; Weiss and Littman, 1994). In contrast to receptor tyrosine kinases, both CD2 and TCR lack cytoplasmic kinase domains and must activate Lck, a lipid-modified protein diffusing in the inner leaflet of the membrane. Lck is normally autoinhibited by two intramolecular interactions: one between an SH3 domain and an internal linker domain and a second between an SH2 domain that can bind to a C-terminal phosphorylated tyrosine (Y505; Hubbard, 1999). These inhibitory interactions can be relieved by competitive binding of the SH3 domain to other proteins and by the dephosphorylation of Y505, which releases the intramolecular SH2 interaction. In addition, autophosphorylation of the kinase activation loop (Y394) can further stimulate kinase activity. However, the precise mechanism of how Lck is activated in cells is still poorly understood. We propose that an important role of TCR and CD2 microdomains is in concentrating activators of Lck and excluding inhibitors. The proline-rich cytoplasmic domain of CD2 might be a direct Lck activator (by binding the autoinhibitory SH3 domain in Lck; Bell et al., 1996), but high local concentrations may be required to compete with this tethered intramolecular interaction. A similar model could apply to activation of Fyn (Holdorf et al., 1999). High local concentrations

of Lck in the microdomain could also stimulate autophosphorylation of the activation loop and thus cooperatively enhance activation. In addition, the phosphatase CD45 is excluded from microdomains, thus creating a sanctuary that might help in maintaining active Lck by shielding the phosphorylated activation loop from contact with the most abundant membrane phosphatase. However, CD45 outside of the microdomains might play an important role in the initial dephosphorylation of Y505 in Lck. A subset of Lck population with dephosphorylated Y505 can then diffuse into and become activated by CD2 in the microdomains. In this model, substrates of activated Lck (e.g., TCR-ζ) might have to be transiently trapped by the microdomains to become activated and further propagate the signal. Thus, the balance of kinase, phosphatase, and autoinhibitory reactions inside of compared with outside of microdomains may play an important role in shifting equilibrium to favor downstream signaling in T cells (summarized in Fig. 7). Such signal transduction mechanisms may be also relevant for functions of other T cell coreceptors and potentially for activating membrane receptors that do not have catalytic domains in other systems as well. Thus, further studies on the molecular mechanisms of microdomain nucleation and dynamics will be important for understanding Src kinase activation in T cells and perhaps other signaling systems.

Coordination of TCR and CD2 signaling

CD2–CD58 and MHC–TCR signaling would be expected to operate together in a native APC–T cell interface. Might these two pathways function synergistically to enhance T cell activation? Challenging an important role for CD2 in T cell signaling, prior work has shown that CD2 knockout mice exhibit a fairly normal immune response (Killeen et al., 1992). However, other coreceptors may perform functions similar to CD2. Consistent with this idea of functional redundancy, CD2–CD28 double-deficient mouse T cells showed more severe defects in proliferation and activation (Green et al., 2000). Interestingly, CD28–CD80 interactions have also recently been shown to segregate from TCR–peptide–MHC interactions in the cSMAC (Yokosuka et al., 2008).

Our results suggest that CD2 signaling could have an important role in enhancing T cell activation. Specifically, combined CD58–CD2 and TCR ligation results in enhanced cytosolic calcium and CD69 expression, and we find stronger phosphotyrosine staining in the CD58–CD2 zone that lies peripheral to TCR-enriched, phosphotyrosine-depleted cSMAC. Many immune functions require long-term sustained T cell activation possibly mediated through continuous TCR recycling and stimulation (Huppa et al., 2003). The CD58–CD2 domains may contribute to such a sustained signaling, and the spatial separation of the CD2 from TCR after their retrograde flow toward the center may be particularly important for this sustained signaling mechanism. However, we do not have direct evidence that the peripheral ring of CD58–CD2 is responsible for the enhanced T cell signaling response, and this remains an important question for the future. In addition, the concentrations of CD58 and anti-TCR in our bilayer system may differ considerably from native APC (which also activates through peptide–MHC). Further work on the physiological roles of CD58–CD2 signaling in T cell activation will require investigations with primary T cells and APCs and ideally new strategies using animal models.

Materials and methods

Cells and reagents

Jurkat T cell (E6.1) and all mutant lines (Lck-deficient line [J.CaM1], TCR β subunit–deficient line [J.RT3-T3.5], J.RT3-T3.5 line with stable expression of TCR β [PF2.4], LAT and CD2-deficient line [J.CaM2], ZAP70-deficient line [P116], and SLP76-deficient line [J14]) were obtained from A. Weiss (University of California, San Francisco, San Francisco, CA). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. 2×10^7 cells in 400 µl RPMI 1640 were transfected by electroporation at 250 V/975 µF with 30–50 µg DNA. Transfected cells were analyzed 24 h later. AND TCR transgenic T cell is obtained, cultured, and activated as previously described (Varma et al., 2006). Plasmids for Lck, LAT, CD2, TCR-ζ, and CD2AP were provided by M. Davis (Stanford University, Palo Alto, CA), A. Weiss, B. Seed (Harvard University, Cambridge, MA), M. Krummel (University of California, San Francisco), and A. Shaw (Washington University, Saint Louis, MO), respectively, and wild-type or mutant genes are fused to EGFP or mCherry at their C terminus. Actin-GFP was obtained from Clontech Laboratories, Inc. All lipids were purchased from Avanti Polar Lipids, Inc. Other reagents used in experiments are anti-CD3ε monoclonal antibody Hit3a (BD), antiphospho-Src (Y416) antibody (Cell Signaling Technology), anti-CD45 antibody (BD), antiphospho–TCR-ζ antibody (BD), anti-CD69 antibody (BD), antiphosphotyrosine 4G10 (Millipore), anti-CD2 antibody (BD), anti–β-actin antibody (Sigma-Aldrich), fluorescent secondary antibodies (Alexa Fluor 633–labeled goat anti–mouse IgG and goat anti–rabbit IgG antibodies), Fura-2, fluorescent streptavidin, CTB (Invitrogen), and latrunculin B (BIOMOL International L.P.).

Preparation of planar lipid bilayers

Human CD58-GPI, ICAM-1–GPI, mouse CD48-GPI, and ICAM-GPI were purified, fluorescently labeled with Alexa Fluor 488 or 647 dyes (Invitrogen), and incorporated into liposomes of dioleoylphosphocholine as previously described (Dustin et al., 1998). Liposomes containing CD58, ICAM-1, and biotin-PE (cap-biotinyl-phosphatidylethanolamine) were mixed with dioleoylphosphocholine liposomes to adjust to final concentrations of $\sim$10–100 molecules/µm^2 (CD58), $\sim$2.5–25 molecules/µm^2 (ICAM-1), and $\sim$150–1,200 molecules/µm^2 (biotin-PE). Liposomes were deposited on a glass surface cleaned by piranha solution (a mixture of sulfuric acid and hydrogen peroxide), and a single fluid planar bilayer was created on the substrate under imaging buffer (Hepes buffer saline containing 10 mM glucose, 1 mM CaCl$_2$, and 0.5 mM MgCl$_2$). Ligand densities in bilayers were measured by serial dilution of fluorescent ligand to bilayer density, whose single molecules were observable and then directly counted by single molecule imaging. From the relative intensities, we estimated that the density of CD58 under the cell is 1.6–4.0-fold higher than in the surrounding bilayers. This translates to 64–160 molecules/µm^2 underneath the cell, which is similar to cellular densities of CD58 ($\sim$150 molecules/µm^2). Anti-CD3ε antibody was monobiotinylated following the procedure of Carrasco et al. (2004). Fluorescent streptavidin and monobiotinylated anti-CD3ε antibody were reacted sequentially and conjugated with biotinylated lipids in a planar bilayer (Kaizuka et al., 2007), assuming two sites of biotins in lipid bilayers were occupied by one streptavidin.

Microscopy and image analysis

All cell incubation and live cell imaging were performed at 37°C. Single cell level imaging was performed either by TIRF microscopy with a microscope (TE2000U; Nikon) equipped with an electron-multiplying charge-coupled device camera (iXon; Andor) and/or spinning-disk confocal microscopy with an inverted microscope (TE2000U) equipped with a 100× NA 1.49 Apochromat TIRF objective lens (Nikon), a Yokogawa spinning-disk confocal scan head (Solamere Technology Group), and a camera (Cascade II; Photometrics). Both microscopes were run with µManager software (http://www.micro-manager.org). Calcium signaling assay was performed on cells preincubated with 10 µM Fura-2 for 30 min, and Fura-2 emission ratio (340 nm/380 nm excitation) was imaged by a microscope (TE2000U) equipped with wavelength switcher (Lambda DG4; Sutter Instrument Co.) and a camera (Cascade II). Actin-GFP (Fig. 1 B) was imaged with a point-scanning confocal microscope (LSM510; Carl Zeiss, Inc.). Immunofluorescence was performed by antibody staining against fixed (with 3%

paraformaldehyde for 15 min) and permeabilized (with 0.1% Triton X-100 for 2 min) cells interacting with lipid bilayers. Optimal staining was obtained by using antibody solutions at 1:200 dilutions in cell–bilayer samples (all primary and secondary antibodies except for antiphospho–TCR-ζ [used at 1:50]). Stained cells were imaged by TIRF microscope as described for live cell imaging except for antiphosphotyrosine by widefield fluorescence microscope (200M; Carl Zeiss, Inc.; equipped with MicroMAX [Princeton Instrument] or Orca II ER charge-coupled device [Hamamatsu Photonics]) when estimating tyrosine phosphorylation in the whole cell. Fluorescence intensities of phosphotyrosine were quantified after the subtraction of background intensity. Live cells stained with FITC-labeled anti-CD69 antibody at 4°C and fixed cells stained with antiphosphotyrosine antibody and fluorescent secondary antibody were imaged by widefield microscope, and fluorescence intensities of images were quantified after the subtraction of background intensity. Requirement of LAT in CD2-mediated signaling was tested by using CD2-LAT–deficient J.CaM2 cell line transfected with wild-type CD2 molecules (Fig. S4 B and Fig. S5 C). Trajectories and speed of CD2 microdomain transportation were measured and analyzed as previously described (Kaizuka et al., 2007).

Online supplemental material
Fig. S1 shows CD58 dependence and calcium oscillation in a Jurkat T cell. Fig. S2 demonstrates CD2-mediated signaling in primary T cells. In Fig. S3, additive signaling by TCR and CD2 dual activation is further investigated. In Fig. S4, molecular requirements for CD2-mediated signaling are examined by calcium assay. Fig. S5 shows various control experiments for molecular patterning and signaling by CD2 microdomains. Videos show calcium flux (Video 2) and oscillation (Video 3), actin retrograde flow (Video 1) and microdomain transport (Video 4), and their disruption by latrunculin (Video 5). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200809136/DC1.

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