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Occupancy of Lymphocyte LFA-1 by Surface-Immmobilized ICAM-1 Is Critical for TCR- but Not for Chemokine-Triggered LFA-1 Conversion to an Open Headpiece High-Affinity State

Sara W. Feigelson,* Ronit Pasvolsky,* Saso Cemerski,† Ziv Shulman,* Valentin Grabovsky,* Tal Ilani,*‡ Adi Sagiv,* Fabrice Lemaitre,‡‡ Carlo Laudanna,§ Andrey S. Shaw,‡ and Ronen Alon*

Lymphocyte arrest and spreading on ICAM-1–expressing APCs require activation of lymphocyte LFA-1 by TCR signals, but the conformational changes of this integrin during these critical processes are still elusive. Using Ab probes that distinguish between different LFA-1 conformations, we found that, unlike strong chemokine signals, potent TCR stimuli were insufficient to trigger LFA-1 extension or headpiece opening in primary human lymphocytes. Nevertheless, LFA-1 in these TCR-stimulated T cells became highly adhesive to both anchored and mobile surface-bound ICAM-1, although it failed to bind soluble ICAM-1 with measurable affinity. Rapid rearrangement of LFA-1 by immobilized ICAM-1 switched the integrin to an open headpiece conformation within numerous scattered submicron focal dots that did not readily collapse into a peripheral LFA-1 ring. Headpiece-activated LFA-1 microclusters were enriched with talin but were devoid of TCR and CD45. Notably, LFA-1 activation by TCR signals as well as subsequent T cell spreading on ICAM-1 took place independently of cytosolic Ca2+. In contrast to LFA-1–activating chemokine signals, TCR activation of LFA-1 readily took place in the absence of external shear forces. LFA-1 activation by TCR signals also did not require internal myosin II forces but depended on intact actin cytoskeleton. Our results suggest that potent TCR signals fail to trigger LFA-1 headpiece activation unless the integrin first gets stabilized by surface-bound ICAM-1 within evenly scattered actin-dependent LFA-1 focal dots, the quantal units of TCR-stimulated T cell arrest and spreading on ICAM-1. The Journal of Immunology, 2010, 185: 7394–7404.

The LFA-1 integrin is the best-studied adhesion molecule involved in lymphocyte arrest on endothelial cells as well as on various APC targets (1). Recent in vivo imaging suggests a key role for T cell LFA-1 interaction with dendritic cell ICAM-1 in long-lasting adhesions (2). LFA-1 is maintained in a low-affinity nonadhesive state in motile lymphocytes prior to their encounter with cognate Ag (3). In vitro, TCR agonists can rapidly trigger LFA-1–mediated T cell arrest and spreading on ICAM-1–bearing surfaces, processes associated with rapid segregation of the TCR ligands and ICAM-1 into central and peripheral supramolecular activation cluster (pSMAC)-like zones, respectively (4, 5).

Structural studies and epitope mapping suggest that LFA-1 exists in at least three distinct conformational states: bent, unfolded, and a high-affinity extended state. Inactive LFA-1 is compact and bent (6, 7). Constraints on LFA-1 activation can be relieved by both cytoplasmic events (inside-out activation) or by ligand binding (outside-in activation) (8). Headpiece activation involves the swinging out of the β subunit hybrid domain, which pulls on the C-terminal α helix of the β I domain (9). Use of mAbs that probe β subunit extension or β I domain opening has provided key insights into LFA-1 activation by chemokine signals (10–12). T cell spreading on ICAM-1 could be mediated by low-, intermediate-, and high-affinity LFA-1–ICAM-1 bonds (11). TCR-induced T cell spreading on ICAM-1 has traditionally been suggested to involve LFA-1 clustering within large patches rather than conformational switches of LFA-1 from low- to high-affinity states (13). Thus, it was assumed that both intermediate- and high-affinity LFA-1–ICAM-1 bonds are in situ triggered by TCR signaling. TCR signals were postulated to also drive LFA-1 release from cytoskeletal constraints, thereby enhancing macroclustering of the integrin with ICAM-1, a process commonly termed LFA-1 avidity modulation (14).

TCR activation of T cells can be induced by its ligation with anti-CD3 mAbs, widely used polyclonal TCR agonists that drive integrin-mediated T cell arrest and spreading on cognate ligands (15). Although nonphysiological ligation of the TCR with an extensively crosslinked anti-CD3 mAb can activate the LFA-1 headpiece (16),
similar effects of CD3 occupancy by isolated mAb molecules, known to induce potent TCR signaling (17–19), have not been reported. Furthermore, the redistribution of differentially activated LFA-1 triggered by these stimuli and their mechanisms of activation have not been elucidated to date. Of note, many studies on LFA-1 inside-out activation by TCR signals were performed with lymphoblasts on which both LFA-1 expression and regulation are different from that of primary T cells. We therefore induced LFA-1 activation on human freshly isolated T lymphocytes using different configurations of the polyclonal TCR agonist, the anti-CD3 mAb OKT3, and addressed whether, when, and where conformational LFA-1 switches and LFA-1 clustering events took place. Using specific mAb probes for LFA-1 extension and headpiece opening (i.e., activation) as well as an α/β 1-like allostere antagonist of LFA-1, we found that potent TCR signals on their own, in contrast to chemokine signals on their own, failed to extend or activate the LFA-1 headpiece. Interestingly, occupancy of TCR-stimulated LFA-1 by surface-immobilized ICAM-1 rather than by soluble ICAM-1 was critical for its conversion to a headpiece-activated state. This ICAM-1-driven LFA-1 activation took place within numerous submicron focal dots that remained evenly scattered underneath spread T cells rather than in the classic peripheral assemblies, pSMACs. These focal dots, rather than large focal zones of LFA-1 (20), seem to function as the critical adhesive units of TCR-stimulated T cell spreading on ICAM-1–bearing surfaces.

Materials and Methods

Informed consent was obtained from each individual studied. This study was approved by the Institutional Review Board of the Rambam Medical Center, consistent with the provisions of the Declaration of Helsinki. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Weizmann Institute of Science.

Reagents and Abs

Human ICAM-1-Fc, human VCAM-1-Fc, murine ICAM-Fc, CXCL12, and CCL21 were purchased from R&D Systems (Minneapolis, MN). IL-4 and GM-CSF were obtained from Cytolab (Rehovot, Israel). BSA (fraction V), Ca2+Mg2+-free HBSS, PMA, jasplakinolide, anti-talin (8d4), tetramethylrhodamine isothiocyanate-phallolidin, the superantigen staphylococcal enterotoxin A, polyinosinic-polycytidylic acid, and LPS were purchased from Sigma-Aldrich (St. Louis, MO). The cell-permeable Rho-inhibiting P23–40 peptide and its control PI (penetratin peptide) were synthesized as described (21). Human serum albumin (HSA, fraction V), protein α, 4-amino-5-(4-chlorophenyl)-7-[(2-butyrylpyrazolo][3,4-d]pyrimidine (PP2), blebbistatin, cytochalasin D, and bisindolylmaleimide I were purchased from Sigma-Aldrich (St. Louis, MO). Neutrolite avidin (deglycosylated neutral avidin) was purchased from Pierce (Rockford, IL). Secramine A, a gift of Dr. T. Kirchhausen (Harvard University, Cambridge, MA), was synthesized as described (22). XA413 was a gift from P. Gillespie (Roche, Nutley, NJ). The high-affinity β2 reporter 327C and the β2-blocking mAb TS1/18 were gifts from D. Staunton (ICOS, Bothell, WA). Biotinylated streptavidin-Alexa Fluor 488 or 568 secondary Abs were from Invitrogen. The high-affinity anti–LFA-1 (TS1/18 were gifts from D. Staunton (ICOS, Bothell, WA). Biotinylated neutral avidin (deglycosylated neutral avidin) was purchased from Pierce (Rockford, IL). Secramine A, a gift of Dr. T. Kirchhausen (Harvard University, Cambridge, MA), was synthesized as described (22). XA413 was a gift from P. Gillespie (Roche, Nutley, NJ). The high-affinity β2 reporter 327C and the β2-blocking mAb TS1/18 were gifts from D. Staunton (ICOS, Bothell, WA). Biotinylated streptavidin-Alexa Fluor 488 or 568 secondary Abs were from Invitrogen.

Human lymphocyte isolation

Human peripheral blood (PB) T lymphocytes were isolated from citrate-anticoagulated whole blood from healthy donors by dextran sedimentation, density separation over Ficoll-Hypaque, and nylon wool column

separation as described (24) and consisted of >90% CD3+ T lymphocytes. The resulting PB T cells (>90% CD3+ lymphocytes) were cultured in RPMI 1640/10% FCS for 15–18 h before experiments. CD45RA+ T cells were isolated using a negative cell isolation kit (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany).

Murine T cell isolation

Mice were from The Jackson Laboratory (Bar Harbor, ME). Splenocytes, obtained from spleens of 6- to 9-wk-old OT-1 transgenic mice (25) were cultured for 16 h, and CD8+ T cells were isolated using a negative cell isolation kit (MACS; Miltenyi Biotec). The K+OVA and control peptides were prepared as described (26).

PB T cell inhibitory treatments

Where indicated, lymphocytes were pretreated with the inhibitors PP2 (10 μM, 30 min), GF-109203X (5 μM, 15 min), blebbistatin (50 μM, 30 min), BAPTA-AM (25 μM, 30 min), cytochalasin D (10 μM, 20 min), jasplakinolide (0.5 μM, 60 min), or DMSO at 37°C. For inhibition of Cdcl42, T cells were pretreated with secramine A (5 μM, 15 min). Inhibitors were present in the medium throughout the experiments.

Flow cytometry

For detection of agonist-induced high-affinity LFA-1 conformations, T cells were suspended in binding medium and incubated with the β2 reporter mAbs 327C or KIM127 (10 μg/ml), either intact or biotinylated, for 5 min at 37°C. Unwashed T cells were then stimulated with the TCR-ligating mAb OKT3 (10 μg/ml), chemokines (10 nM), PMA (100 ng/ml), or Mg2+/EGTA (2 mM each) for an additional 5 min at 37°C, and stimulation was stopped by placing the cells on ice. Lymphocytes were washed, resuspended with Alexa Fluor 568- or PE-conjugated streptavidin or secondary Abs for an additional 30 min at 4°C and analyzed by FACSscan (BD Biosciences, Erembodegem, Belgium). For TCR crosslinking experiments, T cells were incubated with the TCR-ligating mAb OKT3 (10 μg/ml) for 10 min on ice, washed, and then crosslinked with goat anti-mlgG2A Abs (Jackson ImmunoResearch Laboratories) at 37°C for 5–20 min. For detection of soluble ICAM-1 binding, T cells were incubated with ICAM-1-Fc for 30 min at room temperature in binding medium. Cells were then washed twice and incubated with PE-donkey anti-human IgG (Jackson ImmunoResearch Laboratories) for 20 min at room temperature followed by FACS analysis.

Cluster analysis

T lymphocytes were either left intact or stimulated for 5 min with 10 μg/ml OKT3 at 37°C before fixation with 4% paraformaldehyde/2% sucrose and subjected to staining with the anti-αδ mAb (TS2/4/Alexa Fluor 568). Serial Z-stacked (0.2 μm/section) confocal imaging was performed with DeltaVision (Applied Precision, Issaquah, WA) with an oil 60×/1.4 Planaapo (differential interference contrast [DIC]) objective. Cell images were acquired as serial Z-stacks (0.2 μm apart) and subjected to digital deconvolution and three-dimensional reconstructions with the softWoRx software (Applied Precision). An LFA-1 cluster was defined according to size (>0.5 μm3) and fluorescence intensity (at least 2-fold greater than the mean fluorescence intensity [MFI]).

Immunostaining procedures

For immunofluorescent staining, unless otherwise indicated, cells were first fixed in PBS containing 4% (w/v) paraformaldehyde and 2% sucrose, extensively washed with PBS, and blocked with TBS (25 mM Tris [pH 7.4], 150 mM NaCl) supplemented with 2% HSA or serum. Cells were incubated with either Alexa Fluor- or biotin-labeled mAbs (45 min, 37°C) or with unlabeled mAbs followed by Alexa Fluor-conjugated secondary reagents. For double staining of intracellular molecules (e.g., talin), cells and surface proteins were first permeabilized with saponin (0.1% w/v, 5 min) and blocked with goat serum. Subsequently, cells were incubated with primary Ab, washed, and incubated with secondary Alexa Fluor-labeled Abs in the presence of 0.05% saponin. Mouse serum was added to neutralize the saponin. In some experiments, T cells were stained with a trace of Alexa Fluor-labeled TS2/4 Ab for 5 min, washed, and subjected to OKT3 stimulation as described above. The mAb was verified to not interfere with time course of lymphocyte spreading on ICAM-1, and the numbers and distribution of focal LFA-1 dots under spread T cells visualized by this method were indistinguishable from those visualized by TS2/4 labeling following fixation.
Intracellular calcium determination

Intracellular (cytosolic) calcium was monitored by the calcium-sensitive dye Fluo-4 AM (Molecular Probes/Invitrogen) using either flow cytometry or real-time fluorescent microscopy.

Flow cytometry. PB T cells were preloaded with 2 µM Fluo-4 plus 0.1% (w/v) Pluronic F-127 in HBSS (HBSS containing 2 mg/ml BSA, 10 mM HEPES [pH 7.4], 1 mM CaCl2, and 1 M MgCl2) and incubated for 15 min in the dark at 37°C. Samples were washed and resuspended in HBSS (cation-free and without BSA) and incubated for an additional 10 min at room temperature. Cells were then resuspended in HBSS/CaMg with 0.2% BSA and analyzed using the FACSscan flow cytometer (BD Biosciences) with an argon laser with a fixed output wavelength of 488 nm. Cell samples were divided into equal volumes, and the first aliquot was aspirated for 20 s to determine the baseline fluorescence of the Fluo-4-Ca2+ complex. For the stimulations, the aspiration of the baseline sample was paused after 20 s, either OKT3 (10 µg/ml) or CCL2 (10 µM) was added, and the acquisition was resumed with changes in intracellular Ca2+ concentration being recorded over a 200-s period. The samples were analyzed using FlowJo software (Tree Star, Ashland, OR). Changes in the fluorescence intensity of the Fluo-4–Ca2+ complex were measured on the fluorescence 1 channel (voltage 600), and Fluo-4-Ca2+ fluorescence was plotted as a geometric mean moving average versus time. Fluorescence intensity (MFI) per 30 s was evaluated.

Real-time fluorescent microscopy. Briefly, T lymphocytes were preloaded with Fluo-4 and Pluronic F-127 as for flow cytometry. Washed lymphocytes were incubated with either BAPTA-AM or loading buffer for 15 min, washed, and further incubated for 10 min at room temperature. Cells were then centrifuged, resuspended in HBSS, and mounted into a microslide chamber (purchased from Multichannel Systems, Martinsried, Germany) coated with 2 mg/ml HSA. The Fluo-4 signal was imaged with the FITC filter. For TCR stimulation, cells were stimulated with soluble anti-CD3 mAb (10 µg/ml) and immediately introduced into a microslide chamber. Time-lapse images were collected at 3-s intervals for 5 min. The data were analyzed using the Velocity software (Improvision/PerkinElmer, Waltham, MA).

ICAM-1 bead binding assay

To examine the encounter rate and duration between T lymphocytes and ICAM-1–coated beads, magnetic protein A beads (Dynabeads; Dynal/Invitrogen) were coated with the indicated concentrations of recombinant ICAM-1/Fc cell adhesion molecule site densities, assessed using [125I]-labeled anti–ICAM-1 (HA58), as previously described (10), and then fully blocked with saturating (100 µg/ml) concentrations of human IgG. T cells, either left intact or stimulated with agonist (PMA, 100 ng/ml; OKT3 mAb, 10 µg/ml) were injected together with the beads into microslides (ibidi, Martinsried, Germany) and assayed at 37°C. T cell–bead encounters were recorded for 15 min at six frames per minute using a 40×/0.95 NA DIC objective. Encounters were defined as any T cell contact lasting >10 s, and a productive contact was defined as any cell–bead encounter lasting >30 s. More than 95% of these productive contacts remained stable for at least 5 min.

TCR stimulation of LFA-1–mediated lymphocyte spreading

Human T cells were either left untreated or incubated with OKT3 (10 µg/ml) and immediately perfused into a flow chamber mounted on a polystyrene or a glass slide coated with human ICAM-1–Fc or human VCAM-1–Fc. T cells were preloaded on immobilized protein A (20 µg/ml) and subsequently blocked with human IgG. In other assays, protein A plates were coated with either ICAM-1–Fc, VCAM-1–Fc, or IgG control (1.5 µg/ml) and subsequently blocked with human IgG. In other assays, protein A coated with human IgG. In other assays, protein A coated with human IgG. In other assays, protein A was coated with human ICAM-1–Fc or human VCAM-1–Fc or IgG control (1.5 µg/ml) followed by rabbit anti-mouse Ab (10 µg/ml) on which OKT3 (0.2–0.4 µg/ml) was captured. CD8+ purified OT-I splenocytes were allowed to spread on 5 min. Thus, CD8+ purified OT-I splenocytes were allowed to spread on a productive contact was defined as any cell–bead encounter lasting >30 s. The site density of the ICAM-1–Fc complex was measured on the fluorescence 1 channel (voltage 600), and Fluo-4–Ca2+ fluorescence was plotted as a geometric mean moving average versus time. Fluorescence intensity (MFI) per 30 s was evaluated.

Results

TCR ligation does not trigger LFA-1 extension or headpiece opening associated with high-affinity states

To assess whether TCR signals induce inside-out conformational changes in the LFA-1 heterodimer, we exposed human resting PB T cells to saturating levels of the anti-CD3 mAb, OKT3, a prototypic TCR agonist (15, 29). T cells were coincubated with two fluorescently labeled reporter mAbs that detect conformational switches in the β2 subunit of the LFA-1. Because T cells lack β2 subunit-containing integrins other than LFA-1, these reporter mAbs are specific for LFA-1 extension and headpiece activation (10, 30). One of these mAbs, KIM127, detects an epitope on the β I domain on LFA-1 and can therefore differentiate between extended and extended LFA-1 (30). The second mAb, 327C, has been used to probe the opening of the β I domain on LFA-1, a key headpiece rearrangement that stabilizes LFA-1 at a state favorable for high-affinity ICAM-1 binding (10, 12) (Supplemental Fig. 1). Strikingly, TCR ligation by a dimeric anti-CD3 mAb, OKT3, failed to trigger either LFA-1 extension or headpiece activation induced by the opening of the headpiece β I domain (Fig. 1A) despite high flux of Ca2+ triggered by this treatment (Fig. 1B). Notably, because the mAb reporters were present throughout the assay, any transient LFA-1 conformational switches would have been detected under these conditions. In contrast, brief T cell activation by phorbol esters or prototypic chemokine signals readily triggered both β subunit extension and β I domain activation in LFA-1 (Fig. 1A). In light of the lack of LFA-1 conformational switch by this TCR stimulus, we considered that CD3 ligation by a dimingering mAb may instead patch LFA-1. Nevertheless, this CD3 ligation did not enhance any global LFA-1 macroclustering in resting human lymphocytes (Fig. 1C). Thus, in contrast to chemokine signals, TCR ligation failed on its own to trigger LFA-1 extension, headpiece opening, or macroclustering.

Fluorescence image acquisition and analysis

Fluorescence microscopy was carried out with the DeltaVision system (Applied Precision) using an oil 60×/1.4 Planap D (Nikon Instruments, Melville, NY) objective. Encounters were defined as any T cell–bead contact lasting >10 s, and a productive contact was defined as any cell–bead encounter lasting >30 s. More than 95% of these productive contacts remained stable for at least 5 min.

Statistical analysis

Real-time fluorescent microscopy. Briefly, T lymphocytes were preloaded with Fluo-4 and Pluronic F-127 as for flow cytometry. Washed lymphocytes were incubated with either BAPTA-AM or loading buffer for 15 min, washed, and further incubated for 10 min at room temperature. Cells were then centrifuged, resuspended in HBSS, and mounted into a microslide chamber (purchased from Multichannel Systems, Martinsried, Germany) coated with 2 mg/ml HSA. The Fluo-4 signal was imaged with the FITC filter. For TCR stimulation, cells were stimulated with soluble anti-CD3 mAb (10 µg/ml) and immediately introduced into a microslide chamber. Time-lapse images were collected at 3-s intervals for 5 min. The data were analyzed using the Velocity software (Improvision/PerkinElmer, Waltham, MA).

All data are reported as mean values ± SD and were analyzed by a two-tailed Student t test with equal sample variance. Analyses were performed using the statistics tool of Excel. Data sets were considered significantly different at p < 0.05.

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TCR signals increase LFA-1 affinity via an outside-in activation driven by surface-bound ICAM-1 in the absence of shear forces

In accordance with the inability of TCR signals to switch LFA-1 into a headpiece-activated state, a soluble ICAM-1 dimer failed to stably bind TCR-stimulated T cells (Supplemental Fig. 2). To detect any short-lived conformational switches of LFA-1, we next incubated OKT3-stimulated T cells with the mAb reporter of open LFA-1 headpiece (i.e., 327C) and left the reporter mAb throughout the assay together with soluble ICAM-1 dimer, at a concentration known to saturate high-affinity LFA-1. Even under these conditions, which allow the integrin reporter mAb to irreversibly bind any TCR-stimulated LFA-1 transiently switched into its high-affinity conformation by ICAM-1, the potent TCR stimulus failed to shift the LFA-1 headpiece into the open conformational state (Fig. 2A). We next reasoned that the potent TCR signals triggered by OKT3 are insufficient to conformationally switch the LFA-1 headpiece into its open state stabilizing a high-affinity conformer in the presence of soluble ICAM-1. Nevertheless, we hypothesized that this very TCR stimulus may switch the LFA-1 headpiece into its open state in the presence of surface-immobilized ICAM-1. We therefore developed a highly sensitive videomicroscopy-based assay that monitors at high temporal resolution the earliest binding of T cells to ICAM-1–coated microbeads in the presence of minimal detachment forces (Fig. 2B, Supplemental Fig. 3, Supplemental Movies 1–3). TCR stimulation of resting T cells indeed promoted significant LFA-1 binding to ICAM-1 beads (Fig. 2C). To test whether this TCR-induced LFA-1 binding to ICAM-1 beads involves high- or intermediate-affinity LFA-1–ICAM-1 bonds (11), we used an α/β I allosteric inhibitor, XVA143, which discriminates between high- and low/intermediate-LFA-1–ICAM-1 interactions (30, 32). This inhibitor competes with the binding of the intrinsic ligand on the α I domain to the β I domain (Fig. 2D), disrupts communication between the β and α headpiece domains, and thereby restricts full opening of the α I domain and stabilization of high-affinity LFA-1 binding of ICAM-1 (30). Instead, the XVA143–occupied LFA-1 remains at extended conformation with its α I domain binding ICAM-1 via low/intermediate-affinity bonds (30, 32). In contrast to allosteric α I domain inhibitors, which act as direct blockers of all LFA-1–dependent interactions (10, 16), XVA143 is unique in that it acts as a partial gain-of-function modulator that unfolds bent LFA-1 and allows it to mediate weak-intermediate strength rolling adhesions (30). Surprisingly, in contrast to its proadhesive affects on LFA-1–mediated rolling, XVA143 totally eliminated the ability of PMA-stimulated T cell LFA-1 to bind ICAM-1 (Fig. 2C). Interestingly, all adhesive contacts between TCR-stimulated LFA-1 and surface-bound ICAM-1 were also completely blocked by XVA143 at both low and high ICAM-1 site densities (Fig. 2C). Thus, when TCR-stimulated LFA-1 was locked at its extended low/intermediate-affinity state (Fig. 2D), it completely failed to bind ICAM-1–coated beads. These results suggested that TCR-stimulated LFA-1 can acquire high-affinity headpiece conformation only in the presence of surface-bound ICAM-1 (Fig. 2D). Interestingly, CD28 coligation did not further stimulate TCR-stimulated LFA-1 adhesiveness to ICAM-1–coated beads (Supplemental Fig. 4A). Furthermore, TCR-stimulated LFA-1 binding to ICAM-1 beads was observed to similar extents with both naïve and total CD3

**FIGURE 1.** TCR stimulation of resting T cells fails to induce LFA-1 extension or headpiece activation and does not trigger LFA-1 clustering. A, Agonist-mediated induction of LFA-1 extension or opening of the β I domain, critical for high-affinity LFA-1, detected by the reporter mAbs KIM127 and 327C, respectively. Fresh human PB lymphocytes were incubated with the biotin-labeled mAbs and stimulated with the CD3/TCR-ligating mAb OKT3 (10 μg/ml), PMA, or the indicated chemokines. The biotin-tagged conformation reporter mAbs were present throughout the activation period, and lymphocyte-bound mAbs were quantified by Alexa biotin-tagged conformation reporter mAbs. Thick gray and black lines denote activation period, and lymphocyte-bound mAbs were quantified by Alexa biotin-tagged conformation reporter mAbs. Thin black lines denote background staining by an isotype-matched control mAb. B, TCR versus CCL21 stimulation of Ca2+ mobilization. T cells preloaded with Fluo-4 AM were stimulated with either OKT3 or CCL21 identically as in A, and calcium flux was measured by flow cytometry. Cl DIC and fluorescent images of central focal planes of representative PB T cells (intact or pretreated with OKT3 as in A stained after fixation with anti-LFA-1 mAb TS2/4 followed by Alexa Fluor 488 secondary Abs). Scale bar, 3 μm. Cii, Average number of LFA-1 clusters per cell was analyzed as described in Materials and Methods. Experiments shown in A–C are each a representative of three.
T lymphocytes (Supplemental Fig. 4B). Notably, as ICAM-1 bead binding took place in the absence of any stirring, TCR stimulation of LFA-1 adhesiveness did not appear to require application of external forces, in sharp contrast to chemokine-mediated stimulation of LFA-1 (33). Interestingly, although CCL21 and CXCL12 induced potent extension and headpiece activation of LFA-1 (Fig. 1A), the LFA-1 on T cells stimulated by these chemokines failed to bind ICAM-1–coated beads (33). As expected, TCR-stimulated LFA-1 binding to ICAM-1–coated beads was entirely Src-dependent, as it was fully blocked by the Src inhibitor PP2 (Fig. 2E). Nevertheless, LFA-1 activation by TCR signals was insensitive to the 1,2-diacylglycerol (DAG)-dependent protein kinase C (PKC) inhibitor bisindolylmaleimide (Fig. 2E), ruling out a role for DAG-regulated PKCs in early LFA-1 activation by TCR signals.

Rapid lymphocyte spreading on ICAM-1 stimulated by TCR signals involves ICAM-1–induced high-affinity LFA-1 bonds

We next assessed the role of this LFA-1 activation during various CD3/TCR-stimulated T cell spreading on ICAM-1, a more complex process downstream to the early LFA-1–ICAM-1 binding events probed by the ICAM-1 bead assay. As expected, the saturating TCR ligation protocol used in previous sections to probe LFA-1 conformation and ICAM-1 bead binding triggered robust and rapid T cell spreading on both medium- and high-density ICAM-1 (Fig. 3A, 3B, Supplemental Movies 4, 5). Interestingly, a much higher fraction of TCR-stimulated PB T cells could spread on ICAM-1 than bind ICAM-1 beads coated at similar site densities (Figs. 2C, 3B). Thus, active spreading allowed more TCR-stimulated LFA-1 to adhere to surface-coated ICAM-1. Nevertheless, the α/β I allosteric blocker, XV A143, still abolished all TCR-stimulated T cell spreading at both low and high ICAM-1 densities (Fig. 3B). Similar to the ICAM-1 bead assay, ligation of CD28 did not result in any acceleration of CD3/TCR-stimulated T cell spreading on ICAM-1 even at subsaturating levels of TCR ligation (Fig. 3C). TCR-stimulated LFA-1–mediated spreading was entirely Src-dependent, but it did not involve DAG-dependent PKCs or PI3K signaling, as was observed for TCR-stimulated LFA-1 binding to ICAM-1 beads (Figs. 2E, 3D and data not shown). Interestingly, LFA-1–mediated spreading also did not require cytosolic-free Ca2+, as both its magnitude and kinetics were insensitive to the cell-permeable Ca2+ chelator BAPTA-AM (Fig. 3E, Supplementary Movies 6, 7).

FIGURE 2. TCR stimulation triggers high-affinity LFA-1 binding to surface-immobilized ICAM-1 prior to spreading. A, Induction of LFA-1 β I domain opening, critical for high-affinity LFA-1, detected by the reporter mAb 327C. Fresh human PB lymphocytes were incubated with the biotin-labeled mAbs and stimulated with PMA or, alternatively, incubated with soluble ICAM-1 (0.8 μg/ml, saturating the high-affinity LFA-1 subset) (12) alone or in the presence of CD3/TCR-ligating mAb OKT3 (10 μg/ml). The assay was performed identically as in Fig. 1A, and the bar graph depicts the fluorescence mean intensity obtained for each treatment with the isotype-matched control mAb staining subtracted. B, Top row, Successive images of a representative PB T cell (T) encountering a bead (B) coated with high-density ICAM-1 (600 sites/μm2) under shear-free conditions. The nonproductive T cell–bead contact lasted <30 s. Middle row, Images of an OKT3-stimulated lymphocyte encountering ICAM-1–coated bead, with binding lasting >8 min. The arrow marks the bead–T cell contact zone. Bottom images depict an OKT3-stimulated T cell encountering nonproductively an IgG-coated bead. Images are taken from Supplementary Movies 1–3. C, The fraction of T cell–bead encounters yielding productive contacts (lasting >30 s) is shown for differently treated T cells and independent beads coated with the indicated ICAM-1 densities. All data were analyzed by videomicroscopy and values are the means ± SD taken from four independent experiments. Note that all productive T cell–bead contacts were blocked in the presence of the α/β I allosteric inhibitor LFA-1 inhibitor XV A143 (1 μM); *p < 0.05 for anti-CD3 versus PMA stimulations. D, A scheme depicting full acquisition of high-affinity LFA-1 headpiece conformation by TCR inside-out signals. A partial opening of the β I domain is driven by a swing-out of the β subunit hybrid domain through the inside-out activation signal. ICAM-1 occupancy activates the α I domain and stabilizes the headpiece in a high-affinity conformation by pulling down on the α2 helix and bringing the intrinsic ligand within the α I domain to bind and open the β I domain and thereby artificially induce the opening of the β I domain detected by 327C and stabilize the extended β2 conformation detected by KIM127 (Supplemental Fig. 1). XV A143 blocks the binding of the intrinsic α I domain ligand to the β I domain and thereby abolishes high-affinity ICAM-1 binding to the α I domain. Adapted from Shulman et al. (31). E, Effects of Src and PKC blocking in T cells on TCR-stimulated LFA-1 binding to ICAM-1–coated beads. PB T cells were pretreated with the Src inhibitor PP2 or the DAG-dependent PKC inhibitor bisindolylmaleimide and then stimulated with either OKT3 (10 μg/ml, left bars) or with PMA (100 ng/ml, right bars). Cells were immediately suspended with ICAM-1–coated beads (600 sites/μm2), and the fraction of T cell–bead encounters yielding productive contacts (lasting >30 s) was determined in four fields of view as in C. Values are the means ± SD, and the experiment is a representative of three. B, bead; T, PB T cell.
Supplemental Fig. 5A). This chelator fully abolished, however, the CD3/TCR-stimulated cytosolic-free Ca^{2+} signal (Supplemental Fig. 5B), as well as LFA-1 adhesiveness triggered by ionomycin (Supplemental Fig. 5C). Thus, both TCR-stimulated LFA-1 binding to ICAM-1 and T cell spreading on ICAM-1 do not require cytosolic-free Ca^{2+} or DAG-dependent PKCs and are not facilitated by coligation of T cell CD28.

High-affinity LFA-1 is triggered within numerous scattered dots of TCR-stimulated T cells independently of myosin II contractile forces

Both intermediate- and high-affinity LFA-1–ICAM-1 bonds were suggested to promote T cell spreading on ICAM-1 (11). We next assessed whether physical proximity between the TCR stimulus and ICAM-1 could trigger numerous LFA-1–ICAM-1 interactions with low/intermediate- rather than high-affinity properties. Immobilized anti-CD3 mAb, although a weak stimulus for T cell spreading on its own, dramatically stimulated T cell spreading to both low- and high-density ICAM-1 (Fig. 4A). Nevertheless, the α/β I allosteric blocker, XV A143, which stabilized extended low- to intermediate-affinity LFA-1 conformations, eliminated all ICAM-1-dependent spreading stimulated by immobilized anti-CD3 mAb (Fig. 4A). Similarly, when fresh spleen-derived OT-I transgenic CD8+ T cells, specific for the OVA peptide 257–264 (15), were triggered by their cognate MHC–Ag complex to spread on coimmobilized ICAM-1 (Fig. 4B), this Ag-stimulated spreading was totally abrogated in the presence of XVA143 (Fig. 4B). Thus, extended intermediate-affinity LFA-1 conformers primed by TCR signals in murine T cells and artificially stabilized by the XVA143 allosteric inhibitor failed to support murine T cell spreading on ICAM-1. LFA-1 on TCR-stimulated T cells must therefore rearrange into its high-affinity ICAM-1–stabilized state to mediate effective spreading of both human and murine T cells on ICAM-1.

To gain further insights into the dynamics of TCR-triggered LFA-1 activation, we next analyzed LFA-1–mediated T cell spreading and occupancy of fluorescently tagged ICAM-1 embedded in a lipid bilayer together with a CD3-ligating mAb, both of which retain high mobility within the bilayer. Similar to T cell spreading on immobile ICAM-1 (Fig. 4A), freshly isolated human PB T cells encountering mobile ICAM-1 (Fig. 4C) underwent very rapid spreading in response to CD3 ligation (Fig. 4C). Importantly, T cell spreading was associated with highly dynamic ICAM-1 microclusters that were entirely blocked by XVA143, suggesting that lymphocyte spreading in this system is mediated by high-affinity rather than by low/intermediate-affinity LFA-1–ICAM-1 bonds (Fig. 4C). Interestingly, most LFA-1–driven ICAM-1 microclusters took place under the entire contact area of T cells with the lipid bilayer without bias toward a peripheral zone even after 10 min of spreading. Occasionally, during early phases of lymphocyte spreading, a fraction of ICAM-1 microclusters rearranged in short-lived pSMAC-like assemblies (Supplemental Movies 6–9). These results suggest that microclusters of high-affinity LFA-1–ICAM-1 bonds continuously form and disassemble under the entire ventral side of lymphocytes spread on ICAM-1, regardless of whether ICAM-1 is mobile or immobile within the adhesive contact.
FIGURE 4. Ventral focal dots enriched with LFA-1 with an open headpiece are formed at the interface of TCR-stimulated T cells spread on ICAM-1 independently of cytosolic Ca$^{2+}$. A, Time course of lymphocyte spreading on immobilized OKT3 coated alone (0.2 $\mu$g/ml) or in the presence of increasing ICAM-1 densities, in the absence or presence of XVA143 (1 $\mu$M). B, Ag-stimulated spreading of OT-I murine CD8$^+$ T cell on ICAM-1. The time course of transgenic OT-I murine CD8$^+$ T cells spread on the indicated input concentrations of the cognate MHC-peptide (K$^b$-OVA) coated alone or in the presence of a fixed coating of ICAM-1-Fc (1 $\mu$g/ml). K$^b$ complexed to an irrelevant peptide (mTRP2) was used as a control. ** p < 0.005. Right inset, Effect of intracellular Ca$^{2+}$ chelation on TCR-stimulated OT-I T cell spreading on ICAM-1. The time course is shown of vehicle- or BAPTA-AM–pretreated transgenic OT-I murine CD8$^+$ T cells spread on ICAM-1-Fc in the presence of the cognate MHC-peptide (K$^b$-OVA), coated at 1 $\mu$g/ml and at input density of 0.25 $\mu$g/ml, respectively. C, The time course of PB T cells spreading on bilayer-embedded ICAM-1 alone or with a CD3-ligating mAb in the absence or presence of XVA143. The parentheses depict the percentage of cells under which ICAM-1 clustering was detected (see Supplemental Movies 6, 8). Experiment is a representative of three. D, Images of PB T cells undergoing active spreading on an ICAM-1/anti-CD3 mAb-containing lipid bilayer, in the absence (top) or presence (bottom) of XVA143. Images correspond to the indicated time points of the videorecordings shown in Supplemental Movies 7 and 9. Fluorescent images and their corresponding DIC-fluorescence merged images are shown in the left and right panels, respectively. Ei, PB T cells spread on ICAM-1 (600 sites/$\mu$m$^2$) coimmobilized with anti-CD3 mAb (0.2 $\mu$g/ml) were fixed and stained with biotinylated 327C mAb and streptavidin-Alexa Fluor 488 (SA-488). DIC and fluorescence images of two cells within a representative field are shown in the top and middle panels, respectively. The side view z-projection images in the lower rows correspond to the upper and lower cells, respectively, and depict conformationally activated LFA-1 beneath the spread T cells. Eii, PB T cells stimulated with the soluble CD3-ligating mAb OKT3 (10 $\mu$g/ml) spread for 10 min on ICAM-1 (600 sites/$\mu$m$^2$) and stained as in Di. F, Human PB T cells pretreated with vehicle or BAPTA-AM were stimulated with OKT3 and immediately allowed to spread on ICAM-1 (600 sites/$\mu$m$^2$). T cells were fixed at time = 10 min. G, T cells spread on either ICAM-1 or VCAM-1 (600 sites/$\mu$m$^2$) each coimmobilized with OKT3 (0.2 $\mu$g/ml), fixed, and stained with Alexa Fluor 568 327C mAb. Representative lymphocytes from >25 analyzed. Gii, Three lymphocytes representative of >50 cells.
To directly label the TCR-stimulated headpiece-activated LFA-1, we next analyzed the distribution of the 327C β2 open headpiece epitope during TCR-triggered T cell spreading on ICAM-1. Consistent with the evenly scattered pattern of ICAM-1 microclusters and their tight dependence on the XVA143 blocker (Fig. 4A), headpiece-activated LFA-1 conformers were detected within numerous submicron ventral dots underneath all spread T cells (>50 dots/cell; Fig. 4E), well segregated from TCR microclusters (Supplemental Fig. 6). Both the number and distribution of these dots were insensitive to chelation of cytosolic-free Ca2+, in support of the independence of LFA-1–mediated spreading from cytosolic Ca2+ (Fig. 4F). Identical insensitivity to chelation of cytosolic-free Ca2+ was also observed in Ag-stimulated LFA-1–mediated spreading of OT-I transgenic CD8+ T cells on ICAM-1 (Fig. 4B, right inset). Furthermore, this Ag-stimulated spreading was also insensitive to inhibition of DAG-dependent PKCs (not shown), as was observed in CD3/TCR-stimulated human PB T cells (Fig. 3D). Notably, no LFA-1 headpiece activation probed by the 327C reporter of the open β1 domain could be detected in TCR-stimulated T cells spread on VCAM (Fig. 4G) or on high-density immobilized anti-CD3 mAb in the absence of ICAM-1 (Fig. 4Gii). Since under these conditions the TCRRs are assumed to undergo extensive crosslinking (34), these results suggested that the failure of LFA-1 to undergo conformational activation is not due to insufficient TCR crosslinking. Indeed, even when T cells were globally ligated with soluble OKT3 and then subjected to extensive crosslinking by secondary Abs, neither KIM127 or 327C epitopes were triggered within the time frames of our ICAM-1 bead binding and spreading assays (Figs. 2C, 3, 4H and data not shown). At prolonged incubation, however, significantly longer than these time frames, extensive crosslinking could trigger conformational LFA-1 activation in a subset of T cells (Supplemental Fig. 7), consistent with published data (16), whereas TCR ligation alone was still insufficient to trigger any detectable conformational activation (Supplemental Fig. 7). Interestingly, LFA-1 staining with TS2/4, a pan anti-αm mAb that recognizes LFA-1 irrespective of its conformational states, was enriched within the headpiece-activated LFA-1 localized to the scattered ventral dots generated by TCR-stimulated T cells spread on ICAM-1 (Fig. 5Ai). In contrast, LFA-1 stained by TS2/4 remained peripheral on TCR-stimulated T cells spread on VCAM (Supplemental Fig. 8). Thus, in the absence of ICAM-1, any TCR-prime LFA-1 fails to acquire functionally adhesive conformation defined by three criteria: β1 conformational activation (induction of the 327C epitope), susceptibility to XVA143, and microclustering within ventral focal dots. Notably, each microcluster of TCR-prime ICAM-1–rearranged LFA-1 localized within focal ventral dots was proximal to talin, visualized by intracellular staining (Fig. 5Ai), consistent with the role of this adaptor in TCR-stimulated LFA-1 adhesiveness (29, 35). Nevertheless, only a small fraction of high-affinity LFA-1–dots colocalized with F-actin (Fig. 5Aii). Instead, F-actin was mainly enriched in peripheral membrane ruffles, together with CD45 (Fig. 5Aiv).

Actomyosin forces have been recently suggested to stabilize ligand-occupied integrins at high-affinity states (36). Interestingly, inhibition of myosin II, critical for TCR-transduced contractile forces (37), lymphocyte motility (33), as well as LFA-1 deadhesion (32), did not affect rate or magnitude of TCR-triggered T cell spreading on ICAM-1 (Fig. 5B). Similarly, the main effector region of RhoA, an upstream GTPase regulator of myosin II implicated in T cell motility on CCL21 (Supplemental Fig. 9A), cell protrusion (38), and LFA-1 activation by chemokines (10–12), was dispensable for TCR-triggered spreading on ICAM-1 (Fig. 5C). Inhibition of Cdc42, a key GTPase involved in cell protrusion, also did not affect the magnitude or kinetics of CD3/TCR-stimulated T cell spreading on ICAM-1 (Supplemental Fig. 9B). T cell spreading, however, required active actin turnover as it was completely eliminated by both cytochalasin D, an actin polymerization inhibitor, and by jasplakinolide, an actin-stabilizing drug (Supplemental Fig. 9C). Surprisingly, jasplakinolide–pretreated, but not cytochalasin D–pretreated, T cells were still able to mount TCR-mediated stimulation of LFA-1 adhesiveness (Supplemental Fig. 9Ci). Thus, actin polymerization rather than RhoA- or myosin II–driven contractile forces is the major driving element in TCR-stimulated T cell spreading on ICAM-1, consistent with the suggested key role of retrograde actin flow in TCR-stimulated LFA-1–mediated T cell spreading on ICAM-1 (39) (Fig. 6). Taken together, these results suggest that focal dots of TCR-primed LFA-1 bidirectionally stabilized by ICAM-1 and F-actin are the quantal adhesive units of T cells undergoing spreading on APCs during early phases of immune synapse formation.

Discussion

LFA-1 avidity modulation by TCR signaling is a key checkpoint in T cell arrest on APCs (40). Previous findings have suggested that TCR-mediated stoppage of T cells on ICAM-1 is initiated by transient microclusters of unstable LFA-1, which is rapidly mobilized and collapses into a peripheral zone termed the pSMAC (41). These earlier studies (5, 39) alluded to the possibility that firm TCR-triggered LFA-1–mediated adhesion requires LFA-1 segregation into this large assembly. It was also largely accepted that LFA-1 within both the early microclusters and the subsequent large pSMAC must undergo various conformational transitions between bent inactive, extended intermediate-, and high-affinity states (42). Nevertheless, the molecular nature of these conformational transitions has not been discerned or spatially monitored in any of these previous studies. Furthermore, it was hypothesized that the earlier TCR-driven LFA-1–ICAM-1 microclusters may consist mainly of short-lived intermediate-affinity bonds. Using specific probes together with an α/β I allosteric inhibitor, we demonstrate that various potent TCR signals trigger a switch in LFA-1 conformation that is readily stabilized by surface-bound ICAM-1. One of the most surprising results of this study is that unlike chemokine-stimulated LFA-1 (10, 12), TCR-stimulated LFA-1 does not get extended on its own and does not acquire open headpiece conformation unless it is properly rearranged by surface-bound ICAM-1 (Fig. 6). Furthermore, we do not find any evidence that the extended low/intermediate-affinity LFA-1 state, previously suggested to promote T cell protrusions on ICAM-1 (11), is sufficient for either the early or the later adhesive assemblies underlying TCR-triggered T cell spreading on ICAM-1. Indeed, when we locked LFA-1 in an extended low-affinity LFA-1 conformation using the α/β I allosteric inhibitor XVA143 (30), we could not detect early LFA-1–dependent T cell adhesion to ICAM-1.
1–coated beads or T cell spreading on either low- or high-density ICAM-1. These findings sharply contrast with results reported in K562 cells, where XV A143 not only extended LFA-1 but stabilized extended low- or intermediate-affinity bonds with ICAM-1, which mediated LFA-1–driven rolling interactions of these cells on ICAM-1 (30). Thus, as opposed to XVA143-occupied LFA-1 on K562, XV A143-occupied LFA-1 on TCR-stimulated T cells can no longer adhere to ICAM-1 under any experimental conditions tested.

TCR-stimulated T cells generate, at their ventral side engaged with ICAM-1, numerous short-lived microclusters enriched with open headpiece LFA-1. These evenly scattered ICAM-1–LFA-1 dots were observed at both early (5–10 min) and late (15–60 min) periods of T cell spreading on either a lipid-embedded mobile form of ICAM-1 or an immobile form of ICAM-1. Based on their total elimination in the presence of the XV A143 inhibitor, we suggest that ICAM-1–stabilized high-affinity LFA-1 may favorably undergo microclustering, as previously observed in K562 cells (43). Indeed, ICAM-1–stabilized microclusters of headpiece-activated (327C-positive) LFA-1 are readily observed within evenly scattered submicron focal dots, during both early and late phases of T cell spreading on ICAM-1. These focal dots are reminiscent of

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Codistribution of talin but not F-actin with open headpiece LFA-1–enriched focal dots during T cell spreading on ICAM-1. A, OKT3-stimulated T cells spread on ICAM-1 were fixed and costained for the 327C epitope (open headpiece-activated LFA-1) as in Fig. 4 and either total LFA-1 (Ai, using the anti-αL TS2/4 mAb), the talin rod domain (Aii, using mAb 8D4, following permeabilization), F-actin (Aiii, using phalloidin, following permeabilization), or the dSMAC marker CD45 (Aiv). Images are each representative of 30 spread lymphocytes. Scale bars, 3 μm. Effects of the myosin II inhibitor blebbistatin (B) and the RhoA inhibitory P23–40 peptide (C) on the time course of lymphocyte spreading on ICAM-1 (600 sites/μm²) triggered by the TCR ligating mAb OKT3 as in Fig. 3. P1, control penetratin peptide. Values in B and C are the means ± range of two fields. The experiments shown in A–C are each representative of three.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Two proposed modalities for LFA-1 activation by GPCR and TCR signals. Inactive LFA-1 is clasped. The upper panel depicts conformational switches induced by a chemokine-GPCR signal leading to heterodimer unclasping, unbending, extension, and headpiece opening, revealing the β subunit KIM127 and 327C epitopes (represented by asterisks; see Supplemental Fig. 1). Surface ICAM-1 can further rearrange the α I domain, and allosteric outside-in activation is transmitted to the integrin tail to stabilize the unclasped heterodimer in a high-affinity state (8). This stabilization requires external (shear) forces (33). The lower panel depicts an alternative activation induced by TCR signals. The heterodimer is primed, gets weakly unclasped, but it does not undergo detectable extension or headpiece activation. The KIM127 and 327C epitopes are not exposed. Surface ICAM-1 can, however, engage the primed LFA-1 and stabilize it in a fully activated state, where the β I domain is open, exposing the 327C epitope. The α I domain is also fully stabilized by ICAM-1, since XVA143, which inhibits this stabilization, fully eliminates all adhesive LFA-1 activity. This critical LFA-1 rearrangement by ICAM-1 does not require external forces, but it may be facilitated by actin-generated forces during T cell spreading. GPCR, G protein-coupled receptor.
focal adhesions and sites of force-regulated integrin unbending (36, 44). ICAM-1 occupancy of LFA-1 may not only activate the LFA-1 headpiece but may also restrict LFA-1 mobility within microclusters (8, 43). An intriguing possibility is that immobile ICAM-1 may first trap and stabilize the small pool of extended headpiece-activated LFA-1 already expressed by resting T cells. These postulated initiators of LFA-1 nascent adhesions may then nucleate additional pools of LFA-1 molecules to focal sites enriched with ICAM-1. Our data strongly suggest that these pools cannot shift into the high-affinity states in the presence of physiological TCR signals on their own but require an ICAM-1–triggered outside-in headpiece conformational switch. In contrast, LFA-1 in the presence of ICAM-1 alone without TCR signals that alter its cytoskeletal associations prior to its occupancy by ICAM-1 and immediately thereafter would fail to properly link to the actin cytoskeleton and thus be unable to undergo this critical outside-in switch and carry out its adhesive functions. Notably, although highly adhesive, these nascent adhesions, as well as the subsequent assemblies they nucleate, rapidly turn over during TCR-triggered T cell spreading and do not readily collapse into a peripheral ring or pSMAC. Taken together, these data imply that pSMAC ring-like structures may function as terminal adhesive assemblies of T cell–APC synapse rather than as the key adhesive elements of synapse formation (4, 39, 45, 46).

The hallmark of earliest TCR signaling is a rise in cytosolic Ca2+, and this secondary messenger was proposed to link TCR signaling to LFA-1 avidity modulation by releasing LFA-1 from cytoskeletal constraints (13). Our present results highlight critical roles for ICAM-1–triggered LFA-1 conformational switching and anchorage within the cortical actin cytoskeleton rather than for LFA-1 release from this cytoskeleton in TCR-mediated LFA-1 adhesiveness. These results call into question the role of Ca2+-triggered LFA-1 release from cytoskeletal constraints (13), at least in early phases of immune synapse formation. The role of this key secondary messenger and its numerous potential targets in LFA-1 activation and redistribution in the immunological synapse has been indeed disputed (45, 47). TCR activation in lymphoblasts was shown to activate the Ca2+-dependent protease calpain, resulting in enhanced LFA-1 proteolytic cleavage of LFA-1–cytoskeletal linkages and accelerated T cell adhesion to ICAM-1 (13). This mechanism was never confirmed, however, in primary lymphocytes. Our work on these lymphocytes suggests that blocking all cytosolic Ca2+ and therefore inhibiting calpain activation by stimulated TCR does not interfere with TCR-triggered LFA-1–mediated T cell spreading on ICAM-1. Recently, TCR-triggered arrest of transgenic T blasts migrating through ICAM-1–coated filters was also shown to be partially inhibited by BAPTA-AM (47); however, in another study using T blasts, inhibition of cytosolic-free Ca2+ did not interfere with Ag-triggered arrest (45).

Our work indicates that not only does blocking all cytosolic Ca2+ not attenuate TCR stimulated LFA-1–mediated T cell arrest and spreading on ICAM-1, but it does not affect the induction, stability, or distribution of high-affinity LFA-1 focal dots. Thus, although an artificial increase in cytosolic Ca2+ triggers LFA-1–ICAM-1 adhesiveness to ICAM-1, the dramatic rise in cytosolic Ca2+ triggered by TCR ligation is not required for any of the LFA-1–ICAM-1 adhesive units we have defined as critical for T cell arrest and spreading.

In conclusion, our study suggests a previously unappreciated role for ICAM-1 in LFA-1 outside-in headpiece conformational activation event primed by TCR signaling. This conformational switch is critical for high-affinity LFA-1–ICAM-1 bond formation underlying TCR-triggered T cell arrest and spreading on ICAM-1. Notably, the focal contacts in which this outside-in conformational activation takes place form even in the absence of external forces or internal myosin II–driven contractile forces. This sharply contrasts the dependence of chemokine stimulation of LFA-1 on external forces (33). We propose that the binding of surface ICAM-1 to T cell LFA-1 has a triple role: it drives an outside-in switch of the LFA-1 into open high-affinity conformation; it drives the microclustering of ICAM-1–LFA-1 bonds (8); and it anchors LFA-1 to the cortical actin cytoskeleton (48), possibly via talin1, and thereby facilitates the outside-in switch via mechanical activation (36). LFA-1 clustering, in addition to stabilizing firm multifocal adhesions of TCR-stimulated T cells spread on ICAM-1–bearing surfaces (43), is likely to trigger outside-in Src signals (49) that further amplify the Src activation signals transduced by the TCR machinery. Our results also predict that T cells use actin-driven rather than myosin contractile forces to locally activate their ICAM-1–occupied LFA-1 with submicron microclusters. Although excluding a major role for the RhoA and Cdc42 GTPases in this actin-dependent process, our results elude to a major role for TCR-triggered Rac GTPase activities in T cell spreading on ICAM-1 (50). Our results are also consistent with recent studies that demonstrate that the arrest of T cells on ICAM-1 presented by APCs is proportional to both the magnitude of productive signals delivered by MHC–Ag complexes and the local surface density of the APC ICAM-1 (2). Future studies are necessary to delineate the active role of ICAM-1 in chemomechanical LFA-1 activation by TCR signals (51). It would also be interesting to address how ICAM-1 distribution and anchorage states on different APCs may affect the efficiency by which this key adhesive ligand translates signals from a given MHC–Ag complex to LFA-1 headpiece activation, microclustering, and macroclustering within different types of immune synapses.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figures and Figure Legends

Figure S1 A scheme depicting three configurations of the LFA-1 integrin Shown is the LFA-1 integrin inactive, bent (left), extended with closed headpiece (middle) and high affinity fully activated with open headpiece (right). The asterisks depict the conformational epitopes detected by the indicated mAbs.

Figure S2 Soluble ICAM-1-Fc fails to bind TCR stimulated LFA-1 on PB T cell

**LFA-1 i.** ICAM-1-Fc (0.8 μM) binding to T cells in Ca²⁺, Mg²⁺ (1 mM each) in the absence (black) or presence (red) of OKT3 (10 μg/ml) stimulation detected by Fc staining analyzed by FACS.  

**ii.** ICAM-1-Fc (0.8 μM) binding to T cells suspended in either Ca²⁺, Mg²⁺ (1 mM each, black) or in Mn²⁺ (2 mM, red), detected as in A.
Figure S3 Real time tracking of TCR stimulated LFA-1 mediated binding of human PB T cells to ICAM-1 coated beads  Successive images of a representative PB T cell (T) before and while encountering a bead (B) coated with high density ICAM-1 (600 sites/μm²). Top row: images of a non stimulated T cell before and during a non productive bead encounter that lasted less than 30 seconds. Middle rows: images of an OKT3 stimulated lymphocyte 10 sec before and during encounter of an ICAM-1 coated bead. Successive images 10 sec apart are shown for the first 2 minutes post encounter. Additional images show the stability of the contact which lasts > 8 mins. The arrow marks the bead-T cell contact zone. Bottom images depict an OKT3 stimulated T cell non productively encountering an IgG coated control bead. Images are taken from the Videos S1-3.
Figure S4 (A) Effect of CD28 ligation on TCR stimulated LFA-1 binding to immobile ICAM-1 The fraction of T-bead encounters yielding productive contacts (lasting > 30 sec) was determined for resting PB T cells or for T cells pretreated with either OKT3 (10 μg/ml) or both OKT3 and anti CD28 (each at 10 μg/ml) and immediately suspended with ICAM-1 coated beads (600 sites/μm²). Effect of PMA (100 ng/ml) is shown for comparison. Data was analyzed by videomicroscopy in 4 fields of view as in Fig. 2. Values are the mean ± S.D. and the data are representative of two experiments. (B) ICAM-1 bead binding to total and naïve (CD45RA⁺) PB T cells CD45RA⁺ T cells were isolated from total (CD3⁺) PB T cells as described in the materials and methods. The indicated experimental groups were each stimulated with OKT3 (10 μg/ml) and the fraction of lymphocytes generating productive encounters with ICAM-1 coated beads (600 sites/μm²) was determined in 4 fields of view. N.S. = not significant. Values are the mean ± S.D. and shown is a representative experiment of two.
Figure S5 (A) The level of cytosolic Ca\textsuperscript{2+} in intact and OKT3 stimulated T cells pretreated with or without BAPTA-AM. Top row: DIC/fluorescence merge of Fluo-4 loaded T cells in suspension left intact or stimulated with the soluble CD3 ligating mAb, OKT3 (10 μg/ml) and either treated with the Ca\textsuperscript{2+} chelator BAPTA-AM (25 μM) or a carrier; Bottom row: Spectrum analysis of the fluorescence intensity of the top images. Results are representative of 4 experiments. (B) Effect of BAPTA-AM pretreatment on OKT3 stimulated levels of cytosolic free Ca\textsuperscript{2+} in PB T cells PB T cells were preloaded with Fluo-4-AM followed by the Ca\textsuperscript{2+} chelator BAPTA-AM (25 μM) or a carrier. At t=0 loaded T cells were stimulated with OKT3 and fluorescence was monitored by real time videomicroscopy. The mean fluorescence intensity per cell (expressed in arbitrary units, A.U.) was analyzed as in Fig. 3Eii and averaged for each experimental group (n=15 in each group) at successive frames, 3 sec apart. (C) Effect of ionomycin and BAPTA-AM pretreatments on T cell adhesion to ICAM-1 under shear flow PB T cells (intact or pretreated for 15 min with ionomycin) were perfused
over medium density ICAM-1 (150 sites/μm$^2$) at a shear stress of 0.5 dyn/cm$^2$. Where indicated T cells were preloaded with BAPTA-AM (25 μM) before ionomycin loading. The fraction of perfused T cells tethered either transiently or firmly to the ICAM-1 coated substrate was determined in multiple fields. Values are the mean ± range and shown is a representative experiment of six.

Figure S6 Ligated TCR does not colocalize with high affinity LFA-1 dots in TCR stimulated T cells spread on ICAM-1 T cells were stimulated with Alexa488-OKT3 (10 μg/ml), fixed and imaged as in 4Dii. Shown are DIC images (left), CD3/TCR (green), high affinity LFA-1 (red), and the merge of the two fluorescent signals (right).
Figure S7. Extensive TCR crosslinking for prolonged time periods can conformationally activate LFA-1 in a subset of T cells

Induction of LFA-1 extension or opening of the β I domain, critical for high affinity LFA-1, detected by the reporter mAbs KIM127 and 327C, respectively. Fresh human PB lymphocytes were incubated with the biotin labeled mAbs and either left intact (basal, blue), stimulated either with the CD3/TCR ligating mAb, OKT3 (10 μg/ml) for 20 min. at 37ºC (OKT3 alone, red) or incubated with the CD3/TCR ligating mAb, OKT3 (10 μg/ml) for 10 min. on ice followed by washing and crosslinking with goat anti mouse IgG2a secondary antibodies (20 μg/ml) for 20 min (crosslinked OKT3, green). PMA stimulation is shown for comparison (PMA, orange). The biotin tagged conformation reporter mAbs were present throughout the activation period and lymphocyte-bound mAbs were quantified by PE-conjugated streptavidin. Thin black lines denote background staining by an isotype matched control mAb.
Figure S8 LFA-1 staining in T cells spread on VCAM-1 in response to stimulation by immobilized anti-CD3 mAb T cells were labeled with a trace of Alexa 568 TS2/4 (anti LFA-1, 1 μg/ml) and allowed to spread on VCAM-1 (600 sites/μm²) coimmobilized with OKT3 (0.2 μg/ml) for 5 min before being fixed and imaged. Bar, 3 μm.
Figure S9 (A) Effect of the RhoA inhibitory peptide, RhoA23-40 on T cell motility over CCL21 The fraction of PB T cells pretreated with the cell permeable penetratin labeled RhoA 23-40 peptide or with the control penetratin peptide, P1, which could establish persistent motility over immobilized CCL21 (2 μg/ml). Locomotion was defined as a cell displacement of at least 3 cell diameters during the 15 min assay period. Results are the mean ± S.D. of three fields of view and shown is a representative experiment of two. (B) Sequestering Cdc42 from the membrane does not interfere with TCR stimulated PB T cell spreading on ICAM-1 Effects of the Cdc42 sequestering compound Secramine A (SecA) on the time course of lymphocyte spreading on ICAM-1 (600 sites/μm²) triggered by the TCR ligating mAb, OKT3 as in Fig. 3. Values in x-y are the mean ± range of two fields. (Ci) Stabilized F-actin is permissive for TCR-stimulated LFA-1 adhesiveness to ICAM-1 Differential effects of the F-actin stabilizing drug, jasplakinolide (jasp), and of cytochalasin D (cyto D) on LFA-1 adhesiveness to ICAM-1 (600 sites/μm²) measured for intact and OKT3 stimulated T cells. Shown is the fraction of lymphocytes arrested upon encountering of ICAM-1 under shear forces (0.5 dyn/cm²). All data were analyzed by videomicroscopy and values are the mean ± S.D. taken from four fields of view. (Cii) Inhibition of actin abrogates TCR stimulated lymphocyte spreading on ICAM-1 Effects of the indicated actin inhibitors on the time course of lymphocyte spreading on ICAM-1 (600 sites/μm²) triggered by the TCR ligating mAb, OKT3 as in Fig. 3. Values in x-y are the mean ± range of two fields. The experiments shown in B and Cii are representative of three.
Supplementary Movie Legends

Movie S1:
A time lapse movie of a representative non stimulated PB T cell (T) before and while encountering a bead (B) coated with high density ICAM-1 (600 sites/μm²). Note that all bead encounters last less than 40 seconds. 1 movie sec equals 30 sec. Bar, 10 μm.

Movie S2:
A time lapse movie of a representative agonist stimulated PB T cell (T) before and while encountering a bead (B) coated with high density ICAM-1 (600 sites/μm²). Note the stability of the contact which lasts > 8 mins. 1 movie sec equals 30 sec. Bar, 10 μm.

Movie S3:
A time lapse movie of a representative agonist stimulated PB T cell (T) before and while encountering an IgG coated control bead (B). Note that all bead encounters last less than 40 seconds. 1 movie sec equals 30 sec. Bar, 10 μm.

Movie S4:
A time lapse movie depicting representative PB T cells suspended with control mAb and settled on an ICAM-1 coated surface (600 sites/μm²) and immediately videorecorded. 1 movie sec equals 20 sec.

Movie S5:
A time lapse movie depicting representative PB T cells suspended with α-CD3 (10 μg/ml) and settled on an ICAM-1 coated surface (600 sites/μm²) and immediately videorecorded. 1 movie sec equals 20 sec. The time points at which individual cells underwent spreading are indicated beside each lymphocyte in the still images depicted in Fig. 3A.

Movie S6:
A time lapse movie of PB T cells settled on lipid bilayers containing ICAM-1 and α-CD3 mAb. Images were taken 7 sec apart. Right panel depict DIC images. Left panel depicts ICAM-1-Cy5 fluorescent images. Note that numerous dynamic scattered
clusters of ICAM-1, and only a few early and short-lived ICAM-1 enriched pSMAC assemblies, can be seen underneath the T cell as it spreads on the ICAM-1/CD3 mAb planar bilayer.

**Movie S7:**
A time lapse movie of a single representative PB T cell settled on lipid bilayers containing ICAM-1 and α-CD3 mAb. Images were taken 7 sec apart. Left panel depicts ICAM-1-Cy5 fluorescent images. Right panel depict merged images of the DIC and ICAM-1-Cy5. Note that numerous scattered clusters of ICAM-1 can be seen underneath the T cell as it spreads on the ICAM-1/CD3 mAb planar bilayer.

**Movie S8:**
A time lapse movie of PB T cells pretreated with XVA143 (1 µM) settled on lipid bilayers containing ICAM-1 and α-CD3 mAb. Images were taken 7 sec apart. Right panel depict DIC images. Left panel depict ICAM-1-Cy5 fluorescence images.

**Movie S9:**
A time lapse movie of a single representative PB T cell pretreated with XVA143 (1 µM) settled on lipid bilayers containing with ICAM-1 and α-CD3 mAb. Images were taken 7 sec apart. Left panel depicts ICAM-1-Cy5 fluorescent images. Right panel depicts merged images of the DIC and ICAM-1-Cy5.