Supplementary Figures and Figure Legends

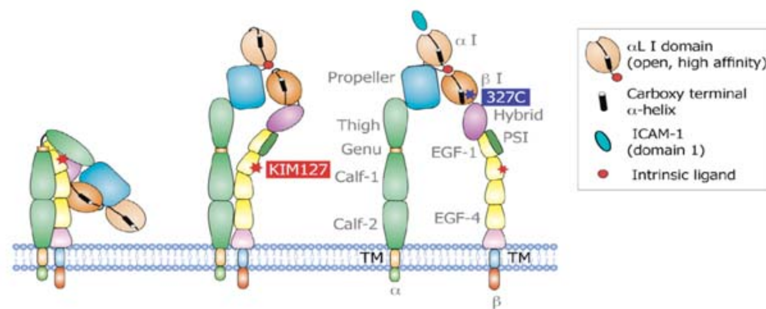

Figure S1 A scheme depicting three configurations of the LFA-1 integrin Shown is the LFA-1 integrin inactive, bent (left), extended with closed headpiece (middle) and high affinity fully activated with open headpiece (right). The asterisks depict the conformational epitopes detected by the indicated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

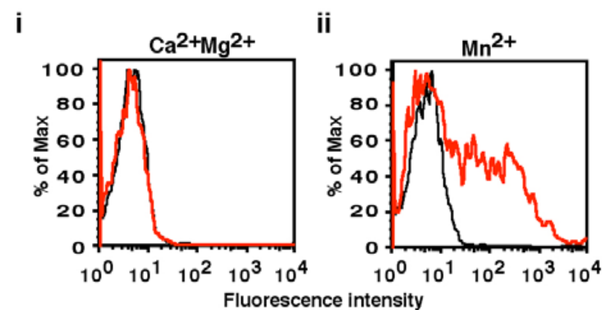

Figure S2 Soluble ICAM-1-Fc fails to bind TCR stimulated LFA-1 on PB T cell LFA-1 i. ICAM-1-Fc (0.8 μM) binding to T cells in Ca^{2+}, Mg^{2+} (1 mM each) in the absence (black) or presence (red) of OKT3 (10 μg/ml) stimulation detected by Fc staining analyzed by FACS. **ii.** ICAM-1-Fc (0.8 μM) binding to T cells suspended in either Ca^{2+}, Mg^{2+} (1 mM each, black) or in Mn^{2+} (2 mM, red), detected as in A.

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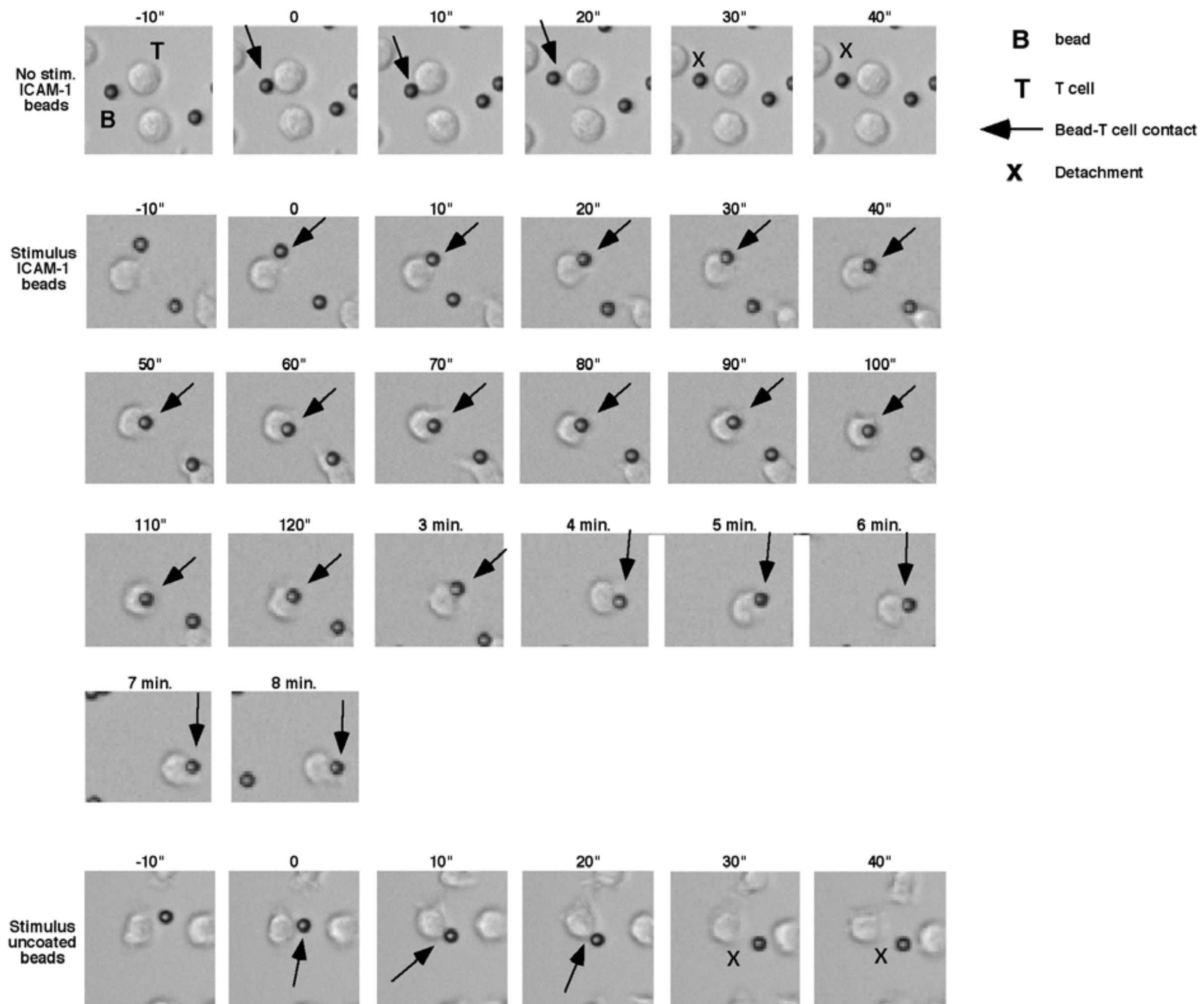

Figure S3 Real time tracking of TCR stimulated LFA-1 mediated binding of human PB T cells to ICAM-1 coated beads Successive images of a representative PB T cell (T) before and while encountering a bead (B) coated with high density ICAM-1 (600 sites/μm^2). Top row: images of a non stimulated T cell before 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.

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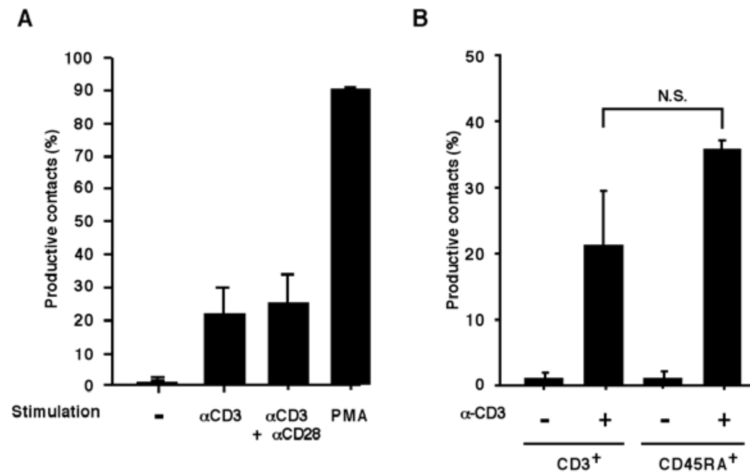

Figure S4 (A) Effect of CD28 ligation on TCR stimulated LFA-1 binding to immobile ICAM-1 The fraction of T-bead encounters yielding productive contacts (lasting > 30 sec) was determined for resting PB T cells or for T cells pretreated with either OKT3 (10 μg/ml) or both OKT3 and anti CD28 (each at 10 μg/ml) and immediately suspended with ICAM-1 coated beads (600 sites/μm^2). 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/μm^2) 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.

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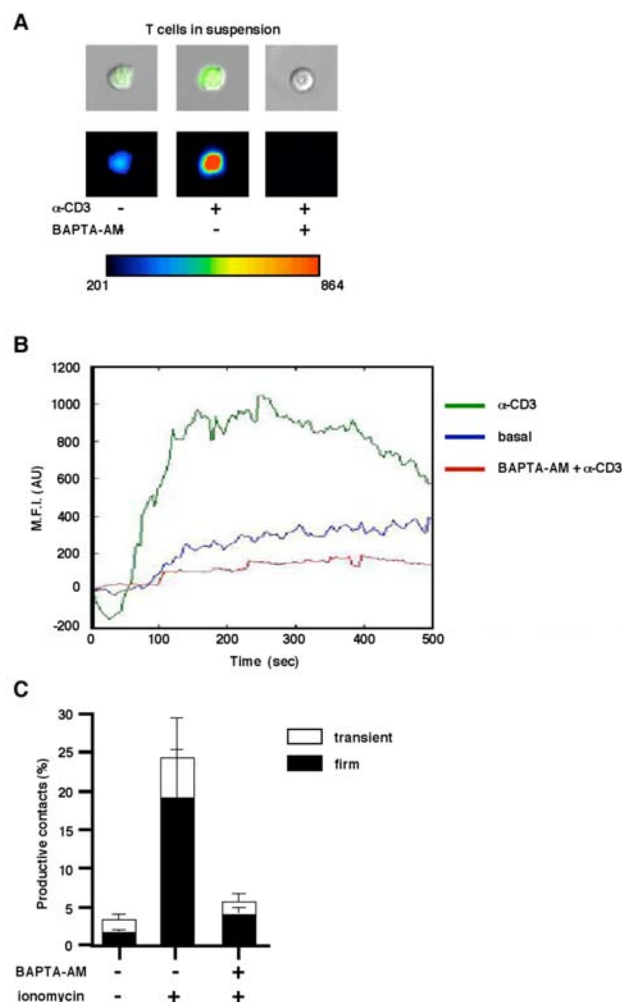

Figure S5 (A) The level of cytosolic Ca^{2+} in intact and OKT3 stimulated T cells pretreated with or without BAPTA-AM. Top row: DIC/fluorescence merge of Fluo-4 loaded T cells in suspension left intact or stimulated with the soluble CD3 ligating mAb, OKT3 (10 μg/ml) and either treated with the Ca^{2+} 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^{2+} in PB T cells** PB T cells were preloaded with Fluo-4-AM followed by the Ca^{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

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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.

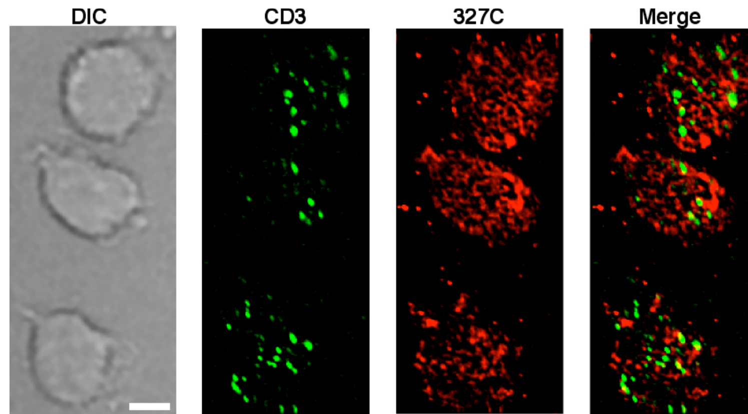

Figure S6 Ligated TCR does not colocalize with high affinity LFA-1 dots in TCR stimulated T cells spread on ICAM-1 T cells were stimulated with Alexa488-OKT3 (10 μg/ml), fixed and imaged as in 4Dii. Shown are DIC images (left), CD3/TCR (green), high affinity LFA-1 (red), and the merge of the two fluorescent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

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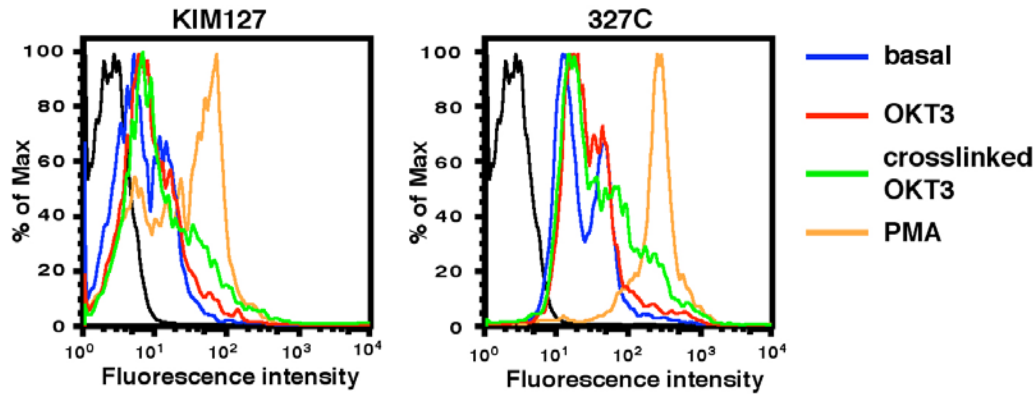

Figure S7. Extensive TCR crosslinking for prolonged time periods can conformationally activate LFA-1 in a subset of T cells

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 *µ*g/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.

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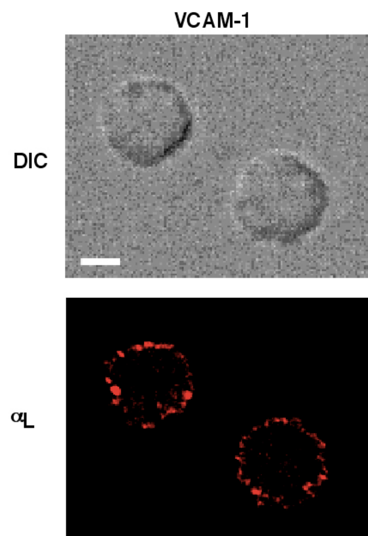

Figure S8 LFA-1 staining in T cells spread on VCAM-1 in response to stimulation by immobilized anti-CD3 mAb T cells were labeled with a trace of Alexa 568 TS2/4 (anti LFA-1, 1 μg/ml) and allowed to spread on VCAM-1 (600 sites/μm^2) coimmobilized with OKT3 (0.2 μg/ml) for 5 min before being fixed and imaged. Bar, 3 μm.

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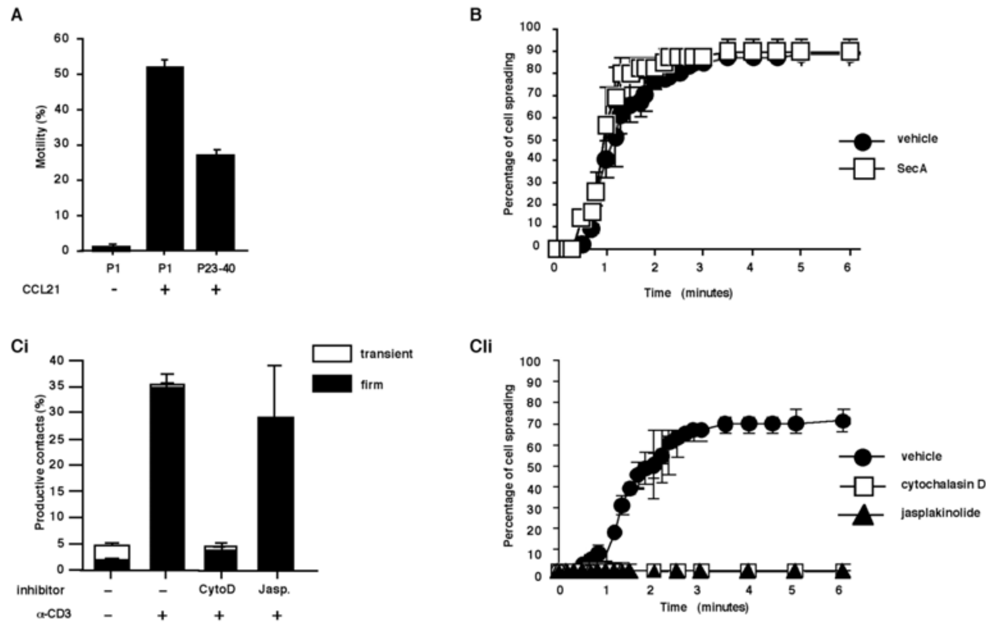

Figure S9 (A) Effect of the RhoA inhibitory peptide, RhoA23-40 on T cell motility over CCL21 The fraction of PB T cells pretreated with the cell permeable penetratin labeled RhoA 23-40 peptide or with the control penetratin peptide, P1, which could establish persistent motility over immobilized CCL21 (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/μm^2) 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/μm^2) measured for intact and OKT3 stimulated T cells. Shown is the fraction of lymphocytes arrested upon encounter of ICAM-1 under shear forces (0.5 dyn/cm^2). 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/μm^2) 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.

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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/μm^2). 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/μm^2). 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/μm^2) 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/μm^2) 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

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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.

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