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LFA-1-Mediated T Cell Costimulation through Increased Localization of TCR/Class II Complexes to the Central Supramolecular Activation Cluster and Exclusion of CD45 from the Immunological Synapse[1]

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LFA-1-Mediated T Cell Costimulation through Increased Localization of TCR/Class II Complexes to the Central Supramolecular Activation Cluster and Exclusion of CD45 from the Immunological Synapse[1]

Beth Graf,* Timothy Bushnell,† and Jim Miller[2]*‡

T cell activation is associated with a dramatic reorganization of cell surface proteins and associated signaling components into discrete subdomains within the immunological synapse in T cell:APC conjugates. However, the signals that direct the localization of these proteins and the functional significance of this organization have not been established. In this study, we have used wild-type and LFA-1-deficient, DO11.10 TCR transgenic T cells to examine the role of LFA-1 in the formation of the immunological synapse. We found that coengagement of LFA-1 is not required for the formation of the central supramolecular activation cluster (cSMAC) region, but does increase the accumulation of TCR/class II complexes within the cSMAC. In addition, LFA-1 is required for the recruitment and localization of talin into the peripheral supramolecular activation cluster region and exclusion of CD45 from the synapse. The ability of LFA-1 to increase the amount of TCR engaged during synapse formation and segregate the phosphatase, CD45, from the synapse suggests that LFA-1 might enhance proximal TCR signaling. To test this, we combined flow cytometry-based cell adhesion and calcium-signaling assays and found that coengagement of LFA-1 significantly increased the magnitude of the intracellular calcium response following Ag presentation. These data support the idea that in addition to its important role on regulating T cell:APC adhesion, coengagement of LFA-1 can enhance T cell signaling, and suggest that this may be accomplished in part through the organization of proteins within the immunological synapse. ***The Journal of Immunology,*** **2007, 179: 1616–1624.**

The integrin, LFA-1, is a well-established intercellular adhesion molecule that plays a key role in several stages of T cell activation and effector function (1). LFA-1 is the primary adhesion molecule that mediates T cell homing to peripheral lymphoid organs and extravasation into sites of tissue inflammation. In addition, LFA-1 mediates adhesion to APC and so plays an important role in T cell activation. Adhesion mediated through LFA-1 is highly regulated during T cell activation. Both chemokine receptor and TCR signaling lead to the rapid induction of LFA-1-mediated adhesion, which is mediated primarily through a conformational change resulting in increased ligand affinity, as well as LFA-1 clustering and an increase in avidity (2–5). Inactivation of LFA-1 is also necessary for effective T cell homing and activation, indicating that the dynamic regulation of integrin-mediated adhesion is a critical component in regulating immune responses (6).

LFA-1 has also been suggested to play a role in providing T cell costimulation, although it has been difficult to distinguish between direct effects of LFA-1 engagement on T cell signaling and indirect effects mediated through intercellular adhesion (7–12). Functionally, LFA-1 has been shown to enhance IL-2 expression leading to the induction of T cell proliferation (7–19), and to modulate effector T cell differentiation, most notably inhibiting Th2 differentiation (20–26). These results raise the possibility that LFA-1 may transduce specific costimulatory signals that can impact both on T cell activation and differentiation. Cross-linking of LFA-1 has been shown to activate phospholipase C (27–29), PI3K and JNK (30), Pyk2 (31), DNAM (CD226) (32), cytohesion and ERK (33, 34), and JAB1 (34, 35). But whether these signals are induced during T cell Ag recognition in the context of APC and how these signals might be integrated with TCR-derived signaling are not yet clear.

One potential site of signal integration between the TCR and costimulatory receptors is within the spatial organization of cell surface receptors and associated signaling components within the immunological synapse (36–39). LFA-1 is the major known component of the peripheral supramolecular activation cluster (pSMAC)[3] of the synapse. The pSMAC also contains two LFA-1-associated proteins that regulate LFA-1 adhesion: talin, an actin-binding protein, and RAPL, a Rap1 effector (40, 41). The pSMAC surrounds a central region, the central supramolecular activation cluster (cSMAC), that is enriched for cell surface proteins (TCR and CD28) and associated signaling proteins (protein kinase Cθ (PKCθ)) that mediate T cell activation (40, 42–44). In CD8 T cells, the cSMAC also contains a secretory domain for the docking

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[3] Abbreviations used in this paper: pSMAC, peripheral supramolecular activation cluster; CD18KO, CD18 knockout; cSMAC, central supramolecular activation cluster; PKCθ, protein kinase Cθ; WT, wild type.

and release of cytolytic granules (45). Several proteins are excluded from the immunological synapse, notably CD43 and CD45. CD43 and a number of other proteins are actively transported to the opposite pole of the synapse through association with ezrin-radixin-moesin cytoskeletal elements (46–48). In contrast, CD45 is polarized toward the APC, but largely excluded from the region of T cell:APC contact defined by the immunological synapse (46, 49, 50).

Although the movement and organization of proteins within the immunological synapse have been well described, the functional significance of the synapse remains controversial. Initially, it was proposed that TCR signaling occurred within the cSMAC region; however, recent data indicate that the cSMAC is a site for TCR down-regulation (51). Instead, sustained TCR signaling is thought to take place within microclusters that form at the periphery of the synapse and only transduce signals while being transported through the pSMAC enroute to the cSMAC (52–54). Whether signals from other receptors, such as CD28 or LFA-1, are also transduced dynamically following initial engagement or within their steady-state sites of localization is not clear. In this study, we have addressed the role of LFA-1 in the generation and organization of the immunological synapse. We show that LFA-1-mediated formation of the pSMAC region can enhance the accumulation of TCR/class II complexes within the cSMAC region and is critical for the exclusion of CD45 from the synapse. Coengagement of LFA-1 also results in a significant increase in intracellular calcium responses. Together these data suggest that, in addition to its effects on cell adhesion, LFA-1 may influence TCR signaling through the organization of proteins within the immunological synapse.

Materials and Methods

Cells and Abs

Stably transfected 6132 Pro cells expressing class II alone (ProAd), or in combination with ICAM-1 (ProAd-ICAM) or B7-1 (ProAd-B7), have been described elsewhere (14, 26, 55). The A20 B cell line that expresses MHC class II, ICAM-1, ICAM-2, CD80, CD86, and CD48 was obtained from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 50 mM 2-ME, 0.1 mM nonessential amino acids, 2 mM HEPES buffer, and 40 μg/ml gentamicin (Invitrogen Life Technologies). This medium was supplemented, where necessary, with 200 μg/ml G418 and/or 250 μg/ml xanthine, 15 μg/ml hypoxanthine, and 6 μg/ml mycophenolic acid. Mice lacking expression of LFA-1, CD18 knockout (CD18KO), were obtained from A. Beaudet (Baylor College of Medicine, Houston, TX) (56) and backcrossed onto a BALB/c background for >10 generations. Lymph node and splenic CD4-positive T cells specific for OVA 323–339 in the context of I-Ad were isolated from wild-type (WT) and CD18KO, DO11.10 TCR transgenic mice, as described (14). CD4-positive T cells were stimulated at a 1:50 ratio with irradiated BALB/c splenocytes, 0.2 mg/ml whole OVA protein, and 5 U/ml human rIL-2 (R&D Systems); or 0.2 μg/ml OVA peptide and 20 U/ml human rIL-2. T cells were then rested for 7–21 days before use in experiments. Rabbit Abs to the cytosolic tail of class II α-chain were generated, as described (57). Abs to CD45 (I3/2.3) or talin (C-20) were obtained from Southern Biotechnology Associates or Santa Cruz Biotechnology, respectively. All animal procedures were reviewed and approved by the University of Rochester Committee on Animal Resources.

Cell conjugation and immunofluorescence microscopy

APC were preincubated with 2.0 μg/ml OVA peptide for 2 h at 37°C, mixed with T cells at a 1:1 ratio, and centrifuged for 30 s at a relative centrifugal force of 2000 $\times$ *g* at room temperature in a microfuge. Where noted, APC were labeled with CMAC Cell Tracker Blue (Molecular Probes) during peptide loading. The cell pellets were incubated for 5–10 min at 37°C. T cell:APC cell pellets were resuspended in medium and plated on poly(L-lysine) (Sigma-Aldrich)-coated coverslips for 3 min at 37°C. The cells were fixed in 3% (w/v) paraformaldehyde, permeabilized in 0.3% (v/v) Triton X-100 (Sigma-Aldrich), and blocked in block solution (DMEM plus 10% FCS, 0.1% sodium azide). Primary and secondary Abs were applied sequentially in block solution, and the slides were washed, incubated overnight in PBS, and mounted in 90% glycerol containing 1,4-diazabicyclo[2,2,2]octane (Sigma-Aldrich) and Mowial 4-88 (Calbiochem). Samples were analyzed on a Zeiss Axiovert controlled by SlideBook software (Intelligent Imaging Innovations). Nearest-Neighbor deconvolution, digital analysis, and three-dimensional rendering were accomplished using SlideBook software. The efficiency of T cell:APC conjugate formation was determined under the microscope by assessing the percentage of APC that were associated with a T cell. The relative concentration of class II or CD45 in different regions of the immunological synapse was determined on a deconvolved midplane section through the T cell:APC conjugate that bisected the cSMAC region. Masks were drawn across the cSMAC, across the synapse, excluding the cSMAC region, or around the periphery of the cell outside on the synapse. The ratio of the mean fluorescence intensity of these regions we calculated as described in the figure legends.

Calcium signaling

A20 cells were preincubated with 2.0 μg/ml OVA peptide for 2 h at 37°C and labeled with Alexa 488 or with Alexa 633 (Molecular Probes). T cells were loaded with 1 μM Indo-1AM (Molecular Probes) for 30 min at 37°C. T cells and APC were mixed at a 1:1 ratio in solution and run on an LSRII flow cytometer to establish a baseline for intracellular calcium levels in the T cells. The T cell:APC mixture was then centrifuged for 15 s at a relative centrifugal force of 2000 $\times$ *g* at room temperature in a microfuge. The cell pellets were resuspended in warm medium and run on the flow cytometer at 37°C for 5–10 min. T cell:APC conjugates were identified by dual Indo-1/Alexa 488 (or Alexa 633) fluorescence, and intracellular calcium levels were determined by ratiometric analysis of Indo-1-Blue to Indo-1-Violet fluorescence using FlowJo software (Tree Star).

Results

LFA-1 enhances the accumulation of TCR/class II complexes in the cSMAC

To determine whether LFA-1 was required for the formation and/or organization of the immunological synapse, we used both ICAM-positive and ICAM-negative transfected cells to determine whether LFA-1 was sufficient and CD18KO T cells to determine whether LFA-1 was necessary. In all experiments, we have used previously activated WT or CD18KO T cells, as outlined in *Materials and Methods*. We have no evidence to suggest that these cells differ significantly, except for the expression of LFA-1. Both WT and CD18KO T cells up-regulate T cell activation markers, and we have not detected a significant difference in CD69, CD25, CD4, or TCR in the activated WT and CD18KO T cells that are used in these experiments (data not shown). CD18KO T cells do have a defect in IL-2 secretion, which is clearly evident during activation of both naive and previously activated T cells. Therefore, T cell-priming cultures are supplemented with exogenous IL-2, which results in similar expansion during priming of WT and CD18KO T cells. Both WT and CD18KO T cells differentiate into similar numbers of Th1 (IFN-γ-secreting) cells. As expected from previously published results (20–24), there are more Th2 (IL-4-producing) cells in the CD18KO T cells, but these represent only a small percentage of the total cells (less that 10%). T cells were restimulated with 2.0 μg/ml OVA peptide, because this represents the dose of Ag that elicits maximum IL-2 secretion from previously activated WT and CD18KO T cells.

As expected, LFA-1/ICAM interactions significantly enhanced T cell:APC adhesion, as evidenced by an increased number of T cell:APC conjugates when WT, but not CD18KO, T cells interacted with ICAM-positive APC (ProAd-ICAM and A20) (Fig. 1*A*). The conjugates that were detected in the absence of LFA-1/ICAM interactions were evaluated to assess the role of LFA-1 in immunological synapse formation. We have found that despite the dramatic defect in adhesion, the same number of CD18KO and WT T cells will up-regulate CD69 and enter the cell cycle (B. Graf and J. Miller, unpublished observations). These data suggest that

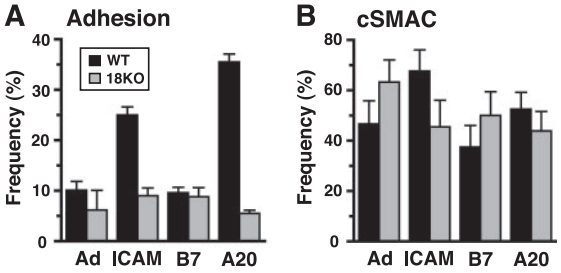

FIGURE 1. The cSMAC is formed in the absence of LFA-1/ICAM interactions. Previously activated WT (■) and CD18KO (▨) T cells were mixed with an equal number of Ag-pulsed ProAd (Ad), ProAd-ICAM (ICAM), ProAd-B7 (B7), and A20 APC; copelleted to initiate cell:cell contact; plated on poly(L-lysine)-coated coverslips; and stained for MHC class II. *A*, The frequency and SD of T cell:APC adhesion, represented as the percentage of APC that had formed a conjugate with a T cell. The differences in adhesion between WT and CD18KO T cells with ICAM-negative APC (ProAd and ProAd-B7) are not statistically significant ($p > 0.05$), whereas the differences in adhesion between WT and CD18KO T cells with ICAM-positive APC (ProAd-ICAM and A20) are highly significant ($p < 0.001$). *B*, The frequency and SD of conjugates containing a cSMAC region, identified by the clustering of MHC class II molecules in the T cell:APC contact region. None of the differences in frequency between WT and CD18KO T cells with either ICAM-positive or ICAM-negative APC are statistically significant ($p > 0.05$). The number of conjugates analyzed ranged from 28 to 32, except for CD18KO T cells with ProAd-ICAM ($n = 22$), CD18KO T cells with A20 ($n = 41$), and WT T cells with A20 ($n = 57$).

the conjugates that are detected in CD18KO T cells are representative of the total population and not a small subset of T cells that can bypass LFA-1 for adhesion and subsequent T cell activation.

T cell:APC conjugates were stained with anti-talin, as a marker for the pSMAC, and with an Ab to the cytosolic tail domain of MHC class II, to identify clusters of class II molecules in APC that are juxtaposed to the TCR in the cSMAC (40). A clear, discernable cSMAC region was readily detected in both WT and CD18KO T cell conjugates with both ICAM-positive and ICAM-negative APC

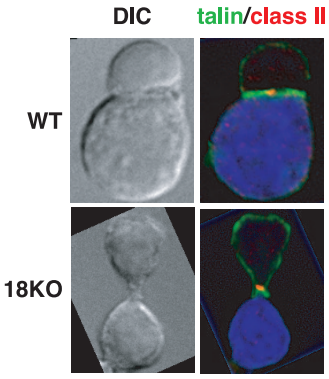

FIGURE 2. TCR signaling is not sufficient to form extensive T cell:APC interactions. WT and CD18KO (18KO) T cell conjugates with Ag-pulsed ProAd-ICAM APC were stained for talin (green) and class II (red). Differential interference contrast (DIC) and fluorescent images are shown. Both WT and CD18KO T cells have formed a cSMAC, as indicated by the clustering of class II molecules in the APC. In the presence of LFA-1/ICAM-1 interactions (WT T cells with ProAd-ICAM APC), all of the conjugates analyzed formed a large cell:cell contact area, as illustrated in the example shown. In contrast, in T cell conjugates in which only TCR was engaged (CD18KO T cells with ProAd-ICAM APC and WT or CD18KO T cells with ProAd APC), about half of the conjugates displayed a reduced contact region with a distended membrane tether extending from the T cell to the APC, as illustrated in the example shown.

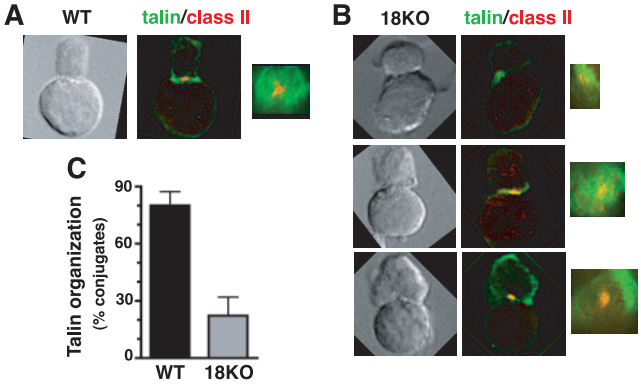

FIGURE 3. LFA-1 is required to organize talin into a pSMAC structure. *A* and *B*, WT (*A*) and CD18KO (*B*) T cell conjugates with Ag-pulsed A20 APC were stained for talin (green) and class II (red). The images are all orientated so that the T cell is the top cell in the conjugate. Differential interference contrast images are shown on the *left*. Note that the conjugates between A20 and either WT or CD18KO T cells exhibit a similar morphology, including a large cell:cell contact area. Midsection, deconvolved images, and three-dimensional projections of the immunofluorescent staining are shown in the *middle* and *right*, respectively. *C*, The frequency and SD of conjugates displaying an organized pSMAC structure. An organized pSMAC structure is based on the three-dimensional projections and is defined as a central localization of class II staining surrounded by a region of talin staining, with little or no colocalization of class II and talin. Note that in CD18KO T cells, class II was often not centralized (*top conjugate* in *B*) and talin was typically not excluded from the region of class II staining (*top* and *middle conjugates* in *B*). In the few CD18KO T cells that were scored as displaying an organized pSMAC structure in *C*, even though class II was centrally localized and did not colocalize with talin, talin was not efficiently recruited to the synapse and/or not well distributed across the pSMAC region (see *bottom conjugate* in *B*). The different frequencies of pSMAC formation between WT and CD18KO T cells were highly significant ($p < 0.001$, $n = 30$ for WT and $n = 18$ for CD18KO T cells).

(for examples, see images in Figs. 2 and 3). However, in the absence of LFA-1/ICAM interactions, many of the T cell conjugates with the fibroblast transfectants were distended and the area of cell:cell contact was reduced, leaving only the region around the cSMAC still tethered between the cells (Fig. 2). This membrane tether was most likely created by shear pressure when the conjugates were resuspended before transfer to slides, and suggests that in the T cell:fibroblast conjugates that form in the absence of LFA-1/ICAM interactions the major site of adhesion is at or near the cSMAC. This was not seen in CD18KO T cell conjugates with A20 cells; WT and CD18KO T cells formed similar contact regions with A20 cells (see images in Fig. 3, *A* and *B*). These data indicate that A20 cells express additional T cell-interacting proteins, which are not sufficient to promote adhesion (Fig. 1*A*), but can stabilize the area of cell:cell contact (Fig. 3*B*).

Interestingly, when the frequency of conjugates that had formed a cSMAC was enumerated, there was no significant difference whether LFA-1 was engaged or not (Fig. 1*B*). This was true even for the T cell:fibroblast conjugates, where many of the conjugates did not form extensive cell:cell contacts. Similar frequencies of cSMAC formation were also seen with Pro cell transfectants that do and do not express B7-1. These data are consistent with the model that TCR-induced activation of the actin cytoskeleton drives the formation of the cSMAC (58, 59). Although there are some differences in the literature as to whether coengagement of LFA-1 and/or CD28 can enhance synapse formation (42, 60–63), it is clear from all the studies, including the present one, that neither LFA-1 nor CD28 is essential to the formation of the cSMAC region.

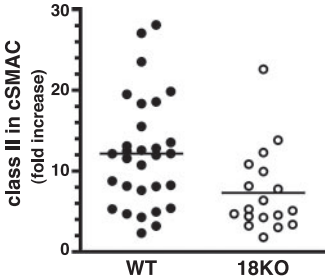

FIGURE 4. LFA-1 enhances the amount of class II that is recruited to the cSMAC. WT and CD18KO T cell conjugates with Ag-pulsed A20 cells were stained for class II. The relative increase in class II concentration (determined by fluorescence intensity) within the cSMAC, compared with the APC cell surface that was not in contact with the T cell, is shown for individual conjugates with WT and CD18KO T cells. The horizontal bar represents the mean. The difference in class II accumulation in WT and CD18KO T cell conjugates was statistically significant ($p < 0.01$). The number of conjugates analyzed for WT T cells was 30 and for CD18KO T cells was 18.

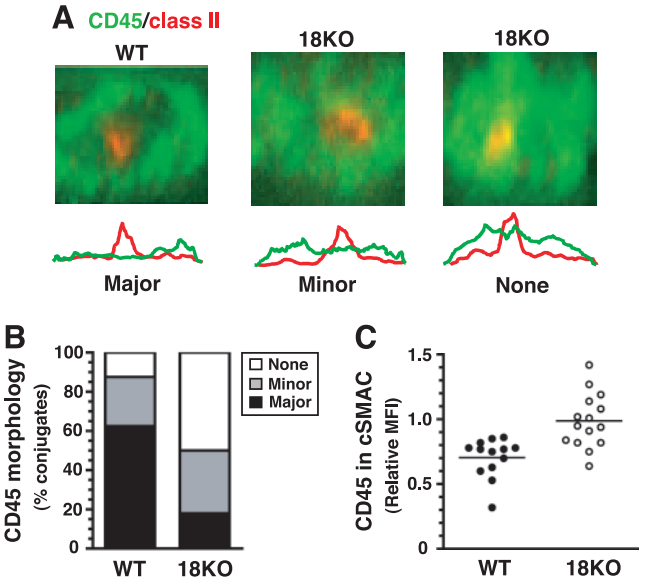

FIGURE 5. LFA-1 is required to exclude CD45 from the pSMAC. WT and CD18KO T cell conjugates with Ag-pulsed A20 cells were stained for CD45 (green) and class II (red). *A*, Representative interface projections of the immunological synapse from WT (*left*) and CD18KO (*center* and *right*) T cells illustrate the three relative distributions of CD45 that were detected. Major, CD45 was excluded from most of the synapse. Minor, CD45 was localized within the synapse, but largely excluded from the cSMAC. None, CD45 was present across the synapse and significantly colocalized with TCR/class II complexes in the cSMAC. The fluorescence intensity profile of a midplane section through the center of the cSMAC is shown below each micrograph. *B*, The percentage of WT and CD18KO T cell conjugates displaying these distributions of CD45 is shown. The increased frequency of major CD45 exclusion and the increased frequency of any CD45 exclusion (major + minor) seen in WT compared with CD18KO T cells are highly significant ($p = 0.001$, $n = 32$ for WT and $n = 34$ for CD18KO T cells). *C*, The relative fluorescence intensity of CD45 within the cSMAC compared with the remainder of the T cell surface is shown for individual conjugates with WT and CD18KO T cells. The horizontal bar represents the mean. The difference in CD45 exclusion from the cSMAC in WT and CD18KO T cell conjugates was statistically significant ($p < 0.001$). The number of conjugates analyzed for WT T cells was 15 and for CD18KO T cells was 13.

Although LFA-1 was not required for the formation of the cSMAC, LFA-1/ICAM interactions did increase the relative concentration of class II in the cSMAC (Fig. 4). Because the area of cell:cell contact was similar in WT and CD18KO T cell conjugates with A20 APC, we restricted these and subsequent analysis of the immunological synapse structure to T cell:A20 conjugates. When the amount of class II within the cSMAC region was assessed, a significant increase in class II accumulation in WT T cells compared with CD18KO T cells was detected (Fig. 4). Notably, there is an increase in the frequency of WT T cell:A20 conjugates that had a greater than 10-fold increase in class II concentration in the cSMAC (60%) compared with CD18KO T cell:A20 conjugates (22%). This effect on class II accumulation appears to be specific for LFA-1, because other potential costimulatory molecules expressed in A20 cells, including B7 and CD48, are available for interaction with CD18KO T cells, but are not sufficient to drive class II accumulation in the cSMAC. Thus, although LFA-1 was not required for the formation of the cSMAC, LFA-1/ICAM interactions can increase the relative concentration of class II in the cSMAC. Although recent data indicate that the pool of cSMAC-associated TCR is not actively signaling (51), the relative amount of class II accumulation can reflect the amount of TCR that was engaged during synapse formation (64, 65). This increase in TCR engagement in the presence of LFA-1 may contribute to the ability of LFA-1 to promote T cell activation.

LFA-1 is required to organize talin into a pSMAC structure

The molecular events that drive the formation of the pSMAC are not well understood. Talin is normally colocalized with LFA-1 within the pSMAC region (40). LFA-1 binds directly to talin, and talin binding is thought to play a role in LFA-1 activation. In addition, talin binds to actin and has the potential to be recruited to the immunological synapse in the absence of LFA-1 and possibly drive the organization of the pSMAC. To address this possibility, we assessed the localization of talin in the presence and absence of LFA-1. As we have previously shown (55), LFA-1 coengagement increased the amount of talin that was recruited to the immunological synapse. Nevertheless, detectable levels of talin were recruited in the absence of LFA-1/ICAM interactions. When the organization of talin was evaluated in three-dimensional reconstructions, the talin recruited to the synapse in the absence of LFA-1 was not organized into a pSMAC structure (Fig. 3). In WT T cells there is a central localization of class II staining surrounded by a region of talin staining, with little or no colocalization of class II and talin. In contrast, in CD18KO T cells, class II was often not centralized and talin was typically not excluded from the region of class II staining. In the few CD18KO T cells that were scored as displaying an organized pSMAC structure (Fig. 3*C*), even though class II was centrally localized and did not colocalize with talin, talin was not efficiently recruited to the synapse and/or not well distributed across the pSMAC region (for an example, see bottom conjugate in Fig. 3*B*). Thus, although other cellular adhesion molecules can stabilize an area of T cell:APC contact around the cSMAC region, LFA-1 is required for the efficient recruitment and organization of talin. Whether LFA-1 itself drives the organization of the pSMAC region or whether some other regulatory protein, such as RAPL is required, is not clear.

LFA-1 is required for the spatial segregation of TCR and CD45

T cell signaling is regulated both positively and negatively by the phosphatase, CD45 (66). CD45 dephosphorylates Y505 in Lck, which is an essential step in Lck activation, yet CD45 also inhibits Lck by removing the activating phosphate at Y394. In T cell:APC

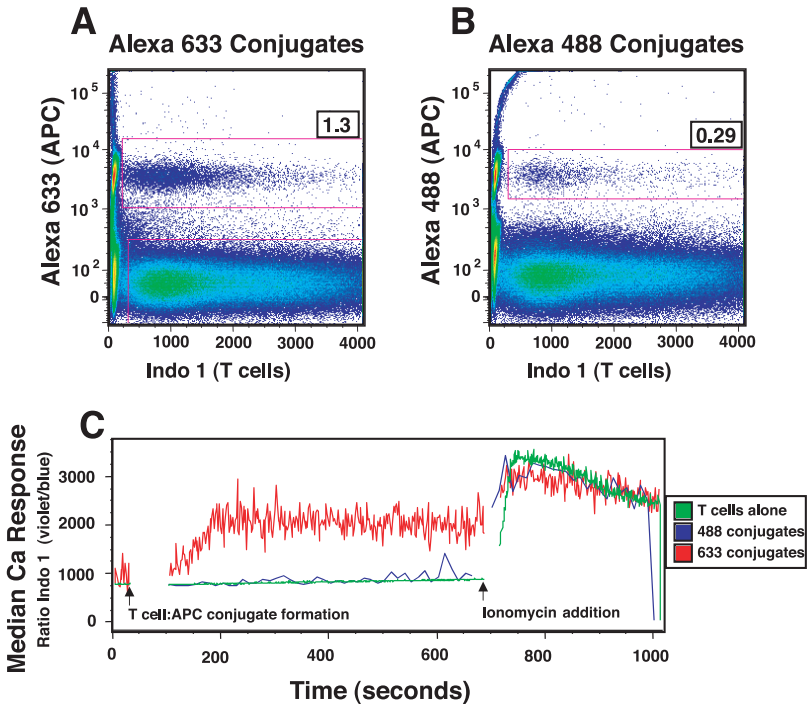

FIGURE 6. Measurement of T cell calcium responses in T cell:APC conjugates. In vitro primed, resting DO11.10 T cells were loaded with Indo-1AM and mixed with Ag-pulsed, Alexa 633-labeled A20 cells. The cells were analyzed by flow cytometry to establish a baseline of Indo-1 fluorescence, and then briefly pelleted to induce T cell:APC conjugate formation (arrow on *left* in *C*). The cells were resuspended, rapidly returned to the flow cytometer, and maintained at 37°C. T cell:APC conjugates were gated based on coincident Indo-1 and Alexa 633 fluorescence (*A*). The percentage of T cell:APC conjugates is shown as an *inset*. The change in the ratio of violet to blue fluorescence of Indo-1, indicative of an increase in intracellular calcium, was monitored over time (shown in seconds in *C*). The data are represented as the median of the ratio of calcium-bound (violet) to calcium-free (blue) Indo-1 in the T cell:APC and T cells-alone populations. Note the rapid rise in intracellular calcium in T cell:APC conjugates (red line in *C*), but not in T cells that have not formed conjugates with APC (green line in *C*). To assure that the calcium response that was detected was mediated only by T cell:APC conjugates that formed at the initiation of the assay, and not by new conjugates formed during the analysis, additional Ag-pulsed A20 cells, labeled with a different dye (Alexa 488), were added after the T cell:APC conjugates were resuspended, just before the sample was returned to the flow cytometer. Note that some T cell:APC conjugates do form with APC while in suspension (*B*), but these T cells do not flux calcium (blue line in *C*). After 10 min, ionomycin was added (arrow on *right* in *C*), to assure that all T cells were effectively loaded with Indo-1.

conjugates, CD45 is polarized toward the APC, but largely excluded from the immunological synapse (46, 49, 50). Similar numbers of WT and CD18KO T cell conjugates with A20 cells polarized CD45 toward the APC (67 and 55%, respectively; not significantly different), indicating that LFA-1 was not required for CD45 recruitment. Therefore, we asked whether LFA-1 was required to control the distribution of CD45 within the immunological synapse (Fig. 5). Consistent with previous studies (49, 50) in most WT T cell:A20 conjugates, CD45 was largely excluded from the area of cell:cell contact within the immunological synapse and segregated from the cSMAC (Major, *left image* in Fig. 5*A*). In contrast, in the majority of the CD18KO T cell:A20 conjugates, there was no separation of CD45 and TCR/class II complexes and CD45 was localized across the immunological synapse, including the cSMAC region (None, *right image* in Fig. 5*A*). An intermediate phenotype was seen in some of the WT and CD18KO T cell:APC conjugates, where CD45 was present within the immunological synapse, but restricted to the pSMAC region with little overlap with TCR/class II complexes in the cSMAC (Minor, *center image* in Fig. 5*A*). This difference between WT and CD18KO T cells was stable, and when conjugates were analyzed after 60 min of interaction similar differences in CD45 exclusion were detected (75% of WT T cells demonstrated major exclusion and 67% of CD18KO T cell exhibited no CD45 exclusion). As discussed above, CD18KO T cells did form large contact areas with A20 cells that include what is normally the pSMAC region of the immunological synapse. The failure to exclude CD45 from this region indicates that the adhesion molecules that are stabilizing this contact area in CD18KO T cells are not sufficient to modulate CD45 localization. Rather, LFA-1/ICAM interactions are required to mediate CD45 exclusion from the immunological synapse.

The magnitude of TCR signaling is reduced in T cell:APC conjugates that form in the absence of LFA-1

The ability of LFA-1 to increase the accumulation of class II in the cSMAC and exclude CD45 from the synapse would predict that LFA-1 would enhance proximal T cell signaling. To test this possibility, we combined flow cytometric adhesion and calcium flux assays to monitor the level of calcium signaling on individual T cell:APC conjugates (Fig. 6). Therefore, Indo-1-loaded T cells were mixed with Alexa 633-labeled A20 cells, briefly spun together to initiate cell:cell contact, resuspended, and analyzed by flow cytometry. T cell:APC conjugates can be identified by double immunofluorescence, allowing for the monitoring of calcium flux selectively in the subpopulation of T cells that formed conjugates with APC (Fig. 6*A*). By focusing only on the calcium response in the subset of T cells that have formed conjugates with A20 cells, we can control for the adhesion defect in CD18KO T cells. However, in the flow cytometry-based calcium flux assay, we are monitoring calcium signaling in different T cell:APC conjugates over time. If LFA-1 contributes to the duration of T cell:APC interactions, it is possible that any observed difference between WT and CD18KO T cells could reflect a difference in the dynamics of T cell:APC interaction, rather than a difference in levels of sustained

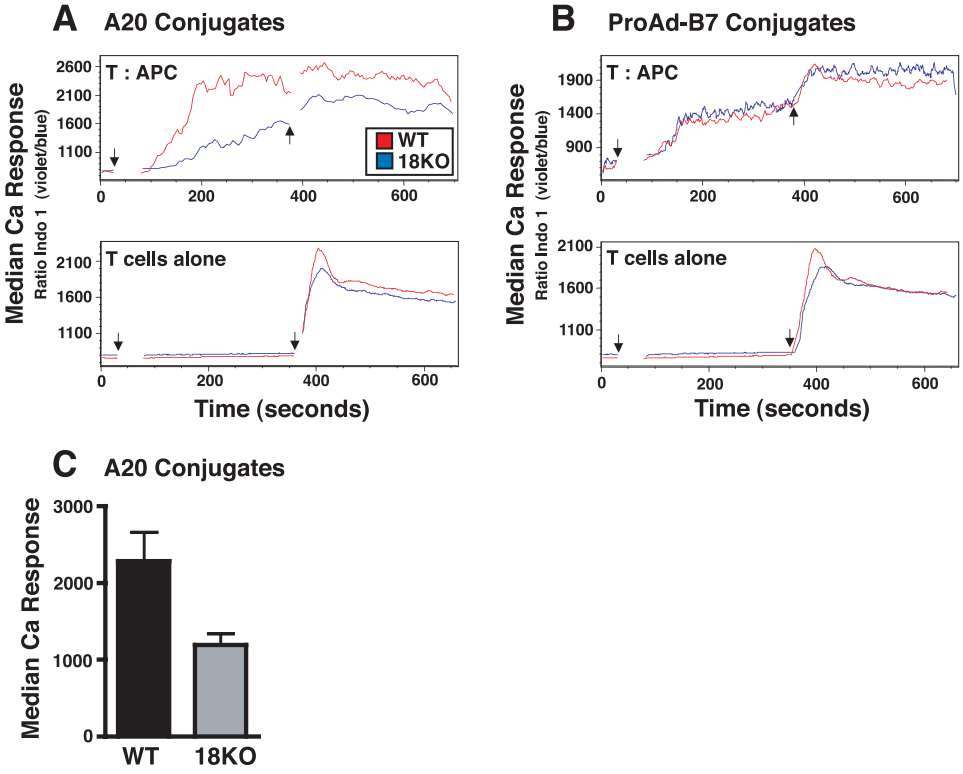

FIGURE 7. LFA-1/ICAM interactions enhance calcium signaling in T cell:APC conjugates. *A* and *B*, WT and CD18KO T cells were analyzed for T cell calcium responses, as described in Fig. 6. Calcium responses were initiated by pelleting T cells and APC (arrow on *left*) and monitored for 5 min before the addition of ionomycin (arrow at *right*). *A*, WT T cell:A20 conjugates (red line) demonstrated a significantly higher calcium response than CD18KO T cell:A20 conjugates (blue line). *B*, To control for possible intrinsic defects in CD18KO T cells, calcium responses were also measured in conjugates with ProAd-B7 cells that do not express ICAM. Both WT (red line) and CD18KO (blue line) T cells gave identical calcium responses. T cells that do not form conjugates (T cells alone) do not flux calcium. The data are represented as the median of the ratio of calcium-bound (violet) to calcium-free (blue) Indo-1. *C*, The mean and SD of the median calcium response from WT and CD18KO T cell conjugates with A20 cells ($p < 0.005$, $n = 4$).

signaling. To control for this possibility, we determined whether any of the T cells fluxing calcium had initiated APC interactions during the course of the analysis. T cells were copelleted with Alexa 633-labeled A20 cells to initiate T cell:APC conjugate formation and T cell signaling and then resuspended, as described above. Before the cells were returned to the flow cytometer, additional Ag-pulsed A20 cells that were labeled with a different dye (Alexa 488) were added. We could then measure conjugate formation and calcium signaling in T cells that formed conjugates during the initial interaction with APC (Alexa 633-labeled A20 cells) and those T cells that might interact with APC in suspension (Alexa 488-labeled A20 cells) and initiate signaling during the analysis. A small number of T cells did interact with the Alexa 488-labeled A20 cells (Fig. 6*B*); however, the T cells in these conjugates did not flux calcium (Fig. 6*C*). It is interesting to speculate that T cells in suspension may not polarize and in the absence of polarization may be unable to form functional interactions with APC (67). However, for the purposes of this experiment, the important result is that the small number of T cells that do initiate interactions with APC once the sample is resuspended do not contribute to the measured calcium response.

When this assay was used to compare the calcium response in WT and CD18KO T cells, we found that the magnitude of the calcium response is significantly reduced in the absence of LFA-1/ICAM interactions (Fig. 7, *A* and *C*). To assure that this difference reflected the absence of LFA-1 during T cell activation, WT and CD18KO T cells were stimulated with Ag presented by ProAd-B7 APC that do not express ICAM (Fig. 7*B*). In the absence of any LFA-1 ligand on the APC, WT and CD18KO T cells displayed an equivalent calcium response. Thus, the enhanced calcium response in WT T cells stimulated with A20 B cells as APC most likely reflects a direct effect of LFA-1/ICAM interactions on the magnitude of proximal T cell signaling events. Taken together, these data indicate that in addition to its impact on T cell:APC conjugate formation, LFA-1 can modulate the strength of TCR signaling, possibly through the organization of proteins within the immunological synapse.

Discussion

The formation of the immunological synapse in T cell:APC conjugates has received considerable recent attention (36–39). The elegant spatial and kinetic rearrangement of cell surface proteins and associated signaling components has important implications in the regulation of T cell activation. Despite this interest, the functional consequences of the specific localization of proteins within the immunological synapse are not well understood. In this study, we have shown that coengagement of LFA-1 can increase the accumulation of class II juxtaposed to the TCR in the cSMAC. This apparent increase in the efficiency of TCR engagement on a single cell level in the presence of LFA-1 may contribute to the decreased Ag sensitivity in the absence of LFA-1. In addition, we show that LFA-1 is important for the exclusion of the phosphatase, CD45, from the immunological synapse. In the absence of LFA-1, CD45 remains distributed across the immunological synapse. Because CD45 can inactivate Lck, and possibly other components in the TCR signaling pathway, the failure to separate CD45 from the site of TCR and Lck activation may limit both the magnitude and duration of TCR signaling in the absence of LFA-1. These results indicate that LFA-1 may enhance T cell activation through the organization of proteins within the immunological synapse. Interestingly, recent data also indicate that CD28 may function in part through its specific localization within the synapse (43, 44, 68). CD28 is required to target PKCθ to the cSMAC region of the immunological synapse, and this may enhance PKCθ-mediated activation of NF-κB and up-regulation of IL-2 expression. Thus, a major contribution of costimulation to T cell signaling may be reflected not just in the appropriate recruitment and activation of signaling intermediates, but also in their positioning and targeting within the immunological synapse.

The role of LFA-1 as a costimulatory molecule has been controversial, largely due to the difficulties in distinguishing between LFA-1-mediated adhesion and signaling. Clearly, LFA-1 plays a key role in T cell:APC adhesion, limiting traditional biochemical and molecular analyses of T cell signaling in the presence and absence of LFA-1. To control for this, many studies have used Ab or soluble ligand-mediated cross-linking of LFA-1 and identified several candidate signaling intermediates downstream of LFA-1, most notably JAB1 and ERK (34, 35). However, it is not clear whether these pathways are induced during T cell:APC interactions, and, if so, how they would be integrated with TCR-derived signals. As an alternative, we have relied on single cell assays to evaluate signaling in the T cell:APC conjugates that do form in the absence of LFA-1 and show that LFA-1 engagement can enhance the magnitude of Ag-induced calcium responses. Previous studies had also indicated that cross-linking of LFA-1 with Ab (27) or with Chinese hamster ovary cells expressing high levels of ICAM-1 (29) could result in enhanced calcium responses. In the later study, single cell imaging was performed; however, in the Chinese hamster ovary transfectants, LFA-1/ICAM-1 did not form pSMAC structures, so cells expressing low levels of ICAM-1 were compared with cells expressing higher levels of ICAM-1. Although increased LFA-1 engagement resulted in enhanced calcium responses (29), it was not clear whether this reflected some direct signaling through LFA-1 or modulation of TCR-derived signals. In our studies, we have found that the ability of LFA-1 to enhance T cell calcium signaling correlates with increased class II accumulation in the cSMAC and exclusion of CD45 from the synapse. These results raise a third possibility for LFA-1 function: that in addition to its important role in T cell adhesion and its possible role in direct signaling, LFA-1 may modulate T cell activation through the organization of proteins within the immunological synapse.

During immunological synapse formation, TCR engagement initiates at the perimeter of the T cell:APC interaction and then TCR moves across the surface of the APC in an actin-dependent manner, relocalizing TCR/class II complexes into the cSMAC region. At the same time, LFA-1/ICAM interactions, initiated in the center of the contact region, move out to the perimeter and form the pSMAC (58). LFA-1 could facilitate this initial class II capture by the TCR, either by increasing the contact area between the T cell and APC, allowing the TCR to scan a larger surface area, or by contributing to the actin-dependent movement of TCR/class II complexes to the cSMAC (60). Alternatively, LFA-1 could stabilize the immunological synapse and restrict class II diffusion from the cSMAC region. Fluorescence-recovery-after-photobleaching experiments have indicated that in the presence of LFA-1, class II within the cSMAC does not freely exchange with class II outside of the cSMAC (58). Given the fast off rate for TCR (especially with a low-affinity TCR, such as DO11.10 (69)), TCR binding alone may be insufficient to retain class II within the cSMAC and LFA-1 in the pSMAC region may limit class II diffusion away from the cSMAC. Thus, LFA-ICAM interactions may facilitate the recruitment of class II into the cSMAC and/or restrict the diffusion of TCR and/or class II out of the cSMAC.

There are two pools of class II that are recruited into the cSMAC: a small pool of antigenic peptide-bound class II and a larger pool of class II molecules, which are thought to contain partial agonists for the TCR (61, 64, 70). Both pools of class II-peptide complexes appear to be required for T cell activation, but because they have different affinities for the TCR, the role of LFA-1 in accumulation of these pools of class II may differ. One possibility is that LFA-1 primarily facilitates the recruitment of the low-affinity, cross-reactive peptide/class II complexes, which may be more important in signaling at limiting doses of agonist peptide. At higher Ag concentrations, sufficient agonist peptide/class II complexes may be recruited to allow for T cell activation even in the absence of LFA-1.

Both CD45 and CD43 are excluded from the immunological synapse. CD43 relocalization to the distal pole complex on the opposite end of the T cell from the synapse is an active process mediated by interaction of the cytosolic tail of CD43 with ezrin-radixin-moesin cytoskeletal proteins (47, 48). In contrast, the mechanism of CD45 exclusion from the immunological synapse is not understood. One early model was that molecular size accounted for the distribution of proteins (71–74). Small receptor ligand pairs (TCR/MHC, CD28/B7) would cluster in the central region, intermediate size pairs (LFA-1/ICAM) in the peripheral region, and large molecules (CD43, CD45) would be excluded. Although it has become clear that the regulation of protein localization within the immunological synapse is more complex, the ability of LFA-1 to exclude CD45 may still be passively mediated through molecular crowding. LFA-1 is actively recruited to the immunological synapse and bound to a cell surface ligand on the APC. In contrast, CD45 has no known ligand on the APC to hold it at the synapse, and the large lumenal domain of CD45 may simply be restricted from diffusing into the tight ring of adhesion mediated by LFA-1/ICAM interactions. Alternatively, LFA-1-mediated actin polymerization may generate a cytoskeletal fence that restricts the diffusion of CD45 into the immunological synapse because of it large cytosolic phosphatase domain or indirectly through the interaction of the cytosolic tail of CD45 with fodrin (75) or with SKAP55 (76).

It has recently been suggested that TCR signaling does not take place in the cSMAC region. Rather, TCR continuously forms microclusters at the outside edges of the pSMAC (52–54). These microclusters then move across the pSMAC, where they accumulate in the cSMAC. Analysis of proximal signaling intermediates indicates that TCR signaling only takes place within the microclusters as they move through the pSMAC. CD45 is polarized toward the APC, but largely excluded from the immunological synapse. Thus, CD45 is at high concentration at the site of TCR microcluster formation, possibly enhancing association of TCR/CD4 with activated Lck (dephosphorylated at Y505). Microclusters then move through the pSMAC, which in the presence of LFA-1 is a region of very low CD45. Interestingly, it has recently been shown that CD45 is further excluded from these microcluster regions (54). Based on our data, we proposed that in the absence of LFA-1, CD45 remains at a high concentration within the pSMAC region, so that microclusters cannot fully exclude CD45. This results in an inactivation of Lck (dephosphorylation of Y394) and a reduction of TCR signaling within the microclusters. This may account for the reduced calcium signaling in the absence of LFA-1. Interestingly, this same mechanism may account for the reduced accumulation of class II in the cSMAC in the absence of LFA-1. Addition of the Lck inhibitor PP2 disrupts microclusters (53). Thus, in the absence of LFA-1, CD45-mediated inactivation of Lck may reduce the number or size of individual microclusters, reducing the number of TCR and associated class II complexes that accumulate at the cSMAC region.

In the last few years, there is growing interest in targeting LFA-1 to regulate immunopathology (77, 78). Acute blockade of LFA-1/ICAM interactions can prolong transplants, often inducing tolerance, and can alleviate autoimmune disease (79–81). Studies done in LFA-1- or ICAM-deficient animals also demonstrate immune effects, including reduced autoimmune diabetes, spontaneous skin lesions, and reduced antitumor responses (56, 82–86). Although the effect of LFA-1 antagonism is clear, the immunological basis is still

not fully understood. In many cases, the primary target of LFA-1 antagonists in vivo is not clear, in part because of potential defects in multiple leukocyte populations (56, 85–87). Furthermore, in those cases in which the primary effect is on T cells, it is not clear whether this is mediated through inhibition of LFA-1's role in T cell activation, T cell effector generation, or homing of effector T cell to tissue sites (88–92). Nevertheless, LFA-1 antagonists are being brought to the clinic with increasing frequency. A better understanding of how LFA-1 regulates different events during the generation and maintenance of a T cell immune response may aid the development of more effective treatment regimes with existing LFA-1 antagonists and may help focus the development of the next generation of small molecule inhibitor within this pathway.

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Disclosures

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