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*J Immunol* 2006; 177:7707-7714; doi: 10.4049/jimmunol.177.11.7707

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Talin1 Regulates TCR-Mediated LFA-1 Function

William T. N. Simonson,* Santos J. Franco,2* and Anna Huttenlocher 3†

The leukocyte integrin LFA-1 plays a critical role in T cell trafficking and T cell adhesion to APCs. It is known that integrin-mediated adhesion is regulated by changes in integrin ligand-binding affinity and valency through inside-out signaling. However, the molecular mechanisms involved in TCR-mediated LFA-1 regulation are not well understood. In this study, we show that the cytoskeletal protein talin1 is required for TCR-mediated activation of LFA-1 through regulation of LFA-1 affinity and clustering. Depletion of talin1 from human T cells by small interfering RNAs impairs TCR-induced adhesion to ICAM-1 and T cell-APC conjugation. TCR-induced LFA-1 polarization, but not actin polarization, is defective in talin1-deficient T cells. Although LFA-1 affinity is also reduced in talin1-deficient T cells, rescue of LFA-1 affinity alone is not sufficient to restore LFA-1 adhesive function. Together, our findings indicate that TCR-induced up-regulation of LFA-1-dependent adhesiveness and resulting T cell-APC conjugation require talin1. The Journal of Immunology, 2006, 177: 7707–7714.

Lymphocytes require dynamic regulation of adhesive contacts for appropriate migration through tissues and activation by Ag. An important mediator of these functions is the integrin LFA-1, an \( \alpha_4\beta_2 \) heterodimeric cell surface receptor that binds ICAM cell surface glycoproteins. LFA-1 is required for arrest of rolling lymphocytes on endothelium, extravasation into lymphatic or inflamed tissue, and interactions with APCs (1). The importance of LFA-1 for T cell function is suggested in vivo by the substantial defects in adaptive immunity observed in \( \beta_2 \) subunit-deficient mice (2, 3).

In resting T lymphocytes, LFA-1 is maintained in a low-affinity conformation and is distributed evenly around the plasma membrane. Upon stimulation by chemokine or TCR engagement, LFA-1 adopts an extended, open conformation with higher affinity for ligand. It also clusters on the surface of the T cell, thereby assuming the high valency state. In the context of Ag recognition, ligation of TCR and costimulatory molecules conveys a stop signal to migrating T cells, resulting in clustered, high-affinity LFA-1 at the point of contact with the APC (4). LFA-1 clustering at the T cell-APC interface is maintained and reinforced by the subjacent reorganization and polarization of the actin cytoskeleton and associated structural proteins. Thus, activation and polarization of LFA-1 and structural proteins promote a stable contact with the APC that is required for productive T cell activation.

The relative contribution of these two modes of LFA-1 regulation, affinity and valency, to stable adhesions and the formation of T cell-APC conjugation is a subject of continued debate (5, 6). What is clear, however, is the importance of the actin cytoskeleton to LFA-1 function. Disruption of the actin cytoskeleton in T cells abolishes TCR-induced binding to ICAM-coated surfaces, most likely by impairing the reinforcement of integrin-mediated contacts (7). Several proteins, including talin1, link LFA-1 to the actin cytoskeleton. Talin1 is a 235-kDa protein composed of a 45-kDa N-terminal head domain and 190-kDa C-terminal rod domain (8).

Talin1 also plays a vital role in regulating integrin function: talin1-deficient fibroblast-like cells exhibit defective \( \beta_1 \) and \( \beta_3 \) integrin affinity and clustering into focal adhesions (11–13).

Talin1 has also been implicated in the regulation of LFA-1 function. Talin1 associates with LFA-1 in neutrophils (14), and regulates chemokine-induced T cell adhesion and migration (15, 16).

We therefore sought to determine whether talin1 regulates LFA-1 function following TCR engagement. In this study, we demonstrate that depletion of talin1 from Jurkat T cells and human peripheral blood T (PBT) cells by small interfering RNA (siRNA) results in a substantial reduction in adhesiveness to ICAM-1 following TCR stimulation, as well as reduced superantigen-induced adhesion of Jurkat cells to B cells. We also show that both LFA-1 affinity and clustering are impaired in talin1-deficient T cells, but that rescue of affinity alone is not sufficient to rescue T cell adhesion to immobilized ICAM-1 or APCs. Together, our results indicate a novel mechanism in which talin1 regulates LFA-1 and recruits it to the site of TCR ligation, thereby stabilizing the nascent contacts between T cells and APCs.

4 Abbreviations used in this paper: PBT, peripheral blood T; siRNA, small interfering RNA; EGFP, enhanced GFP; PKC, protein kinase C; RT, room temperature; SEE, staphylococcal enterotoxin E; TRITC, tetramethylrhodamine isothiocyanate.

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Received for publication April 28, 2006. Accepted for publication September 19, 2006.

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Materials and Methods

Reagents

Abs used in this study were: mouse anti-CD11a clone TSI1/22, mouse anti-CD18 clone TSI1/18 (Pierce-Endogen), FITC mouse anti-CD11a clone G252.2 (BD Biosciences), rabbit anti-p42/44 ERK (BioSource International), mouse anti-CD3 clone OKT3 (eBioscience), and mouse anti-talin1 clone 8d4 (Sigma-Aldrich); the 32C7 Ab was a gift from D. Staunton (ICB, Cambridge, MA) and has been described previously. Rabbit anti-actin antibody was obtained from Santa Cruz Biotechnology, Inc. Tetramethylrhodamine isothiocyanate (TRITC) goat anti-rabbit IgG and FITC anti-mouse IgG from The Jackson Laboratory, human rICAM-1Fc fusion protein from R&D Systems, and recombinant staphylococcal enterotoxin E (SEE) superantigen from Toxin Technology. Stealth siRNA oligos targeting talin1 (5’-GAAGCCGUUCUCAUUUUAGCGACAG and its complement) and control siRNA oligos (5’-GAUUCUAGAUUUCUAGCGGAC and its complement) were obtained from Invitrogen Life Technologies.

Enhanced GFP (EGFP)-talin1 has been described previously (19). EGFP-talin1 head was cloned by excising the talin1 head cDNA (encoding aa 1–433) from EcoRI/SalI digestion from the pB6 R vector (a gift from R. Hynes, Massachusetts Institute of Technology, Cambridge, MA) and cloning it into pEGFP-C1 (BD Clontech) that had been frame shifted by cutting with Bg/II, blunting, and religating. EGFP-talin1 rod was cloned by removing the talin1 rod cDNA (encoding aa 434-2541) from the pB6 Ω 7 vector (R. Hynes) and cloning it into the same frame-shifted pEGFP-C1 vector.

Cell culture and transfection

Human Jurkat E6.1 T cells and Daudi B cells were obtained from American Type Culture Collection and grown in complete RPMI 1640, as recommended. Human PBT cells were purified from heparin-anticoagulated blood from consenting healthy donors using centrifugation over Lymphoprep solution (Axis-Shield), followed by panning to remove monocytes. The University of Wisconsin Human Subjects Committee approved all procedures involving PBT cells. PBT cells were activated with PHA (Sigma-Aldrich) for 24 h, then cultured in complete RPMI 1640 with 25 IU/ml IL-2 (Chiron) for 5–6 days before transfection. Resulting cells were >97% CD3 positive.

Cells were transfected with 2 μg of siRNA and 10 μg of DNA using the Amaxa Nucleofector device and reagents per the manufacturer’s instructions. When EGFP fusion constructs were introduced, cells to be used in conjugation assays were sorted 24 h posttransfection for GFP signal on a FACSDiVa cell sorter (BD Biosciences). Cells were used 48 h posttransfection, when talin1 knockdown was maximal. Cell viability at 48 h posttransfection was 80% for Jurkat T cells and 40% for PBT cells.

Immunoblotting

Immunoblotting for talin1 and EGFP was performed, as described (19).

Adhesion assays

ICAM-1Fc-coated plates were prepared by coating wells of a 96-well high protein-binding plate (Greiner Bioscience) with 2,4, 1.38, 0.77, or 0.44 μg/ml ICAM-1Fc in PBS for 2 h at room temperature (RT) (corresponding to a site density of 500, 400, 300, or 200 sites/μm²). Wells were then blocked with 1% BSA for 1 h at RT. To quantify ICAM-1Fc site density, we labeled ICAM-1Fc with an Alexa 488 labeling kit (Invitrogen Life Technologies), per the manufacturer’s instructions. Plates were coated with a range of concentrations of labeled ICAM-1Fc, as above, washed, blocked, and read on a fluorescence plate reader. Fluorescence intensity was compared against a standard curve of labeled ICAM-1Fc to characterize the relationship between ICAM-1Fc-coating concentration and site density.

Adhesion assays were then performed essentially as described (20). Briefly, cells were labeled with 0.5 μg/ml calcein-AM (Invitrogen Life Technologies) for 15 min at 37°C, then washed and stimulated with 20 ng/ml IL-2 (Sigma-Aldrich) or 5 μg/ml anti-CD3 Ab OKT3 before being added to ICAM-1Fc-coated wells (1 × 10⁵ cells/well). In some experiments, cells were also stimulated with 1 mM MnCl₂. Cells were allowed to adhere for 30 min at 37°C/5% CO₂, and then wells were washed gently twice (Jurkat cells) or four times (PBT cells) with 200 μl of PBS with or without 1 mM MnCl₂. Fluorescence intensity of each well was detected with a Fluorescence Microscopy Plus fluorescence plate reader (Tecan) before and after washing to determine the percentage of adherent cells. Background fluorescence resulting from spontaneous calcein-AM release into the medium was determined from medium from which labeled cells had been removed by centrifugation and filtration, and was subtracted from each prewash reading.

Cell spreading

Jurkat T cells, transfected 48 h previously with siRNA, were labeled with calcein-AM and stimulated, as above. Cells were then allowed to settle on and adhere to ICAM-1Fc glass coverslips for 30 min at 37°C. Coverslips were prepared by coating with 0.01% polylysine (Sigma-Aldrich) for 10 min, then with 10 μg/ml ICAM-1Fc for 2 h at RT, and were then blocked with 1% BSA. Adhered cells were then fixed by the addition of 3% paraformaldehyde and mounted on microscope slides. Cells were visualized with a Nikon TE300 epifluorescence microscope using a x40 objective. Cell spreading was determined by quantifying the fluorescent area of each individual cell, and then subtracting the mean area of unstimulated control siRNA-transfected cells. The difference was then divided by the mean area of unstimulated control siRNA-transfected cells.

T cell-APC conjugation assay

Conjugation assays were performed essentially as described (21). Daudi B cells were labeled with PKH26 and incubated for 60 min in the presence or absence of 1 μg/ml SEE superantigen. A total of 1 × 10⁵ Jurkat T cells labeled with EGFP fusion constructs or calcein-AM was then centrifuged for 3 min at 200 × g at 4°C with an equal number of Daudi B cells. Cell mixtures were incubated at 37°C for the indicated time, then vortexed vigorously, fixed with an equal volume of 6% paraformaldehyde, and analyzed on a flow cytometer. In all experiments, some Jurkat T cells were preincubated with 10 μg/ml anti-CD18 TSI1/18 Ab to block LFA-1 function. The efficiency of LFA-1-dependent conjugation was determined by dividing the number of two-color conjugation events by the number of conjugates plus single T cell events, then subtracting the percentage of T cells forming conjugates in the presence of LFA-1-blocking Ab.

Flow cytometry

LFA-1 surface expression on siRNA-transfected T cells was determined as described (15). To examine LFA-1 affinity induction in talin1-deficient T cells, we stimulated and stained T cells essentially as described (22). The 32C7 reporter Ab was labeled with an Alexa 647 labeling kit (Invitrogen Life Technologies) per the manufacturer’s instructions. PBT or Jurkat cells, transfected with siRNA 48 h previously, were suspended in binding buffer (Dulbecco’s PBS with 0.9 mM CaCl₂, 0.49 mM MgCl₂, and 0.5% BSA) and stimulated with 1 mM MnCl₂, 20 ng/ml PMA, or 5 μg/ml anti-CD3 mAb (PBT cells only) in the presence of 10 μg/ml reporter Ab for 10 min at 37°C. Cells were then washed with cold PBS, fixed in 3% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Fluorescence microscopy

PBT cells were conjugated to anti-CD3-coated latex beads (6 μm diameter; Polysciences), as described (23), then stained with FITC anti-CD11a or TRITC phallloidin. For each treatment, at least 50 conjugates consisting of a single PBT and a single bead were visualized with a Nikon TE300 epifluorescence microscope using a x100 objective (NA = 1.4) and scored for LFA-1 or F-actin polarization toward the bead-cell interface by a blinded investigator. Images were captured using a charge-coupled device camera (Hamamatsu Photonics) and manipulated with MetaMorph software (Universal Imaging).

To examine EGFP fusion protein localization in T cells conjugated to APCs, Jurkat cells transfected with talin1 siRNA plus an EGFP fusion construct were incubated with Daudi B cells loaded with SEE and labeled with 7-amino-4-chloromethylcoumarin CellTracker Blue (Invitrogen Life Technologies). Jurkat-Daudi conjugates were then adhered to polylysine-coated coverslips, fixed, stained with rabbit anti-Fc, and TRITC goat anti-mouse IgG, and visualized, as described above.

Results

Talin1 knockdown inhibits LFA-1-mediated adhesion of T cells

To define the role of talin1 in regulating LFA-1 function following TCR engagement, we used RNA interference to reduce talin1 expression in both Jurkat cells and human PBT cells. Talin1 protein expression was reliably reduced to ~30% of control levels (Fig. 1A). To determine whether talin1 is required for TCR-mediated adhesion to ICAM-1, we examined the ability of Jurkat and PBT cells to adhere to ICAM-1Fc-coated plates following stimulation.
FIGURE 1. Talin1 is required for T cell adhesion and spreading on ICAM-1Fc. A, Immunoblot of lysates from Jurkat and PBT cells transfected 48 h previously with control or talin1 siRNA. Blot was probed for talin1 (top) and ERK1/2 as a loading control (bottom). B and C, Jurkat (B) or PBT cells (C) were left unstimulated or stimulated either with PMA or CD3 cross-linking and allowed to adhere to plates coated with the indicated density of ICAM-1Fc for 30 min. Data shown are means ± SEM of three independent experiments. *p < 0.05; **p < 0.001 compared with siControl cells. D, Quantification of T cell spreading on ICAM-1Fc-coated glass coverslips. Jurkat T cells were stimulated as indicated, then allowed to adhere to coverslips for 30 min. The percentage increase in cell area was determined by subtracting the mean unstimulated control cell area from the stimulated cell areas, then dividing by the mean unstimulated control cell area. **p < 0.001 compared with siControl cells. E, Representative images of siRNA-transfected Jurkat T cells stimulated and allowed to adhere to ICAM-1Fc-coated glass coverslips.

by CD3 cross-linking (to simulate TCR ligation) or with the phorbol ester PMA, which activates integrins while bypassing TCR-proximal signaling. Talin1 knockdown substantially impaired both PMA- and anti-CD3-induced adhesion to ICAM-1Fc in Jurkat and PBT cells (Fig. 1, B and C), indicating that talin1 is required for LFA-1-mediated adhesion to ICAM-1Fc-coated surfaces. Preincubation of stimulated cells with an LFA-1 function-blocking Ab completely blocked adhesion, indicating that adhesion in this assay is specifically mediated by LFA-1 (data not shown). The reduced adhesiveness of talin1 knockdown Jurkat T cells was accompanied by a reduced ability to spread on ICAM-1Fc-coated coverslips. When stimulated by CD3 cross-linking or PMA, control Jurkat T cells spread on ICAM-1Fc and displayed membrane projections, including filopodia-like protrusions. In contrast, the talin1-deficient T cells showed reduced spreading and absence of membrane projections (Fig. 1, D and E). These findings indicate that talin1 is required for TCR-mediated LFA-1-dependent adhesion to ICAM-1.

Talin1 is required for LFA-1 affinity regulation, but not surface expression

Previous studies have demonstrated that talin1 may be required for normal surface expression of β2 integrins on fibroblasts (24). To determine whether the reduced LFA-1-mediated adhesion was due to decreased surface expression of LFA-1, we analyzed expression of the two LFA-1 subunits, αL (CD11a) and β2 (CD18), on siRNA-transfected Jurkat and PBT cells by flow cytometry. We did not observe any difference in cell surface expression of either subunit in talin1-deficient Jurkat cells (Fig. 2A) or PBT cells (Fig. 2B). These findings indicate that the reduced adhesion of talin1-deficient T cells is independent of changes in cell surface expression of LFA-1.

Substantial evidence supports a critical role for talin1 in regulating β2 and β3 integrin affinity (13). Thus, reduced talin1 expression may inhibit LFA-1-mediated adhesion by impairing LFA-1 affinity up-regulation in response to TCR stimulation. To determine the ability of talin1-deficient T cells to regulate LFA-1 affinity, we used an activation reporter Ab, 327C, that recognizes an LFA-1 neoepitope generated upon the transition to the high-affinity conformation (22). This neoepitope is exposed following T cell treatment with stimuli, including chemokines, phorbol ester, and CD3 cross-linking, that result in generalized activation of the T cell and inside-out integrin activation. The high-affinity conformation can also be induced in the absence of intact intracellular signaling pathways by treatment with manganese ions. The 327C Ab revealed reduced LFA-1 affinity in both unstimulated and PMA-stimulated talin1-deficient Jurkat cells compared with control cells, indicating that talin1 is required for both basal LFA-1 affinity and LFA-1 affinity up-regulation following cell stimulation (Fig. 2, C and E). We did not observe any increase in LFA-1 affinity as measured by 327C binding in Jurkat cells following CD3 cross-linking (data not shown); whereas CD3 cross-linking is likely to up-regulate LFA-1 affinity in Jurkat cells, the effect is most likely below the detection limit of this assay. We observed no difference between talin1-deficient and control cells stimulated with MnCl2, supporting the conclusions that talin1-deficient T cells maintain normal surface expression of functional LFA-1. Talin1 knockdown in PBT cells did not impair LFA-1 affinity to the same extent as in Jurkat cells, but did significantly reduce LFA-1 affinity following PMA stimulation in PBT cells (Fig. 2, D and F). However, we observed no difference in LFA-1 affinity between control and talin1 knockdown PBT cells stimulated by CD3 cross-linking. This may be a result of residual talin1 expression in talin siRNA-transfected PBT cells at a level sufficient to permit moderate LFA-1 affinity increases. Collectively, these data indicate that talin1 is required for efficient inside-out LFA-1 affinity regulation. However, the relatively modest effect of talin1 knockdown on LFA-1 affinity raises the possibility that talin1 knockdown is exerting its effect on LFA-1 function by other mechanisms.

Induction of high-affinity LFA-1 does not rescue adhesion of talin1 knockdown T cells

To determine the extent to which impaired LFA-1 affinity regulation contributes to the adhesion phenotype of talin1-deficient T cells, we attempted to rescue their adhesion to ICAM-1Fc-coated plates by induction of the high-affinity LFA-1 conformation with MnCl2. Although MnCl2 treatment increased the adhesiveness of all treatment groups, the disparity between control and talin1-deficient cells persisted in both Jurkat and PBT cells, suggesting that
impaired affinity induction is not the major cause of reduced LFA-1 function in the context of TCR ligation (Fig. 3).

**Talin1 is required for TCR-mediated LFA-1 clustering**

The inability to rescue LFA-1-dependent adhesion of talin1-deficient T cells with MnCl₂ suggests that impaired integrin-cytoskeletal interactions is an important defect resulting from talin1 knockdown. Accordingly, a recent study demonstrated that talin1 is required for LFA-1 clustering into focal zones in migrating T lymphocytes (16). We used an established LFA-1 clustering assay to determine the role of talin1 in this process. siRNA-treated PBT cells were incubated with anti-CD3-coated latex beads to simulate Ag recognition and induce the recruitment of immunological synapse-associated molecules, including F-actin and LFA-1, to the bead-cell interface. We found that LFA-1 clustering at the bead-cell interface was significantly reduced in talin1-deficient T cells (Fig. 4). In contrast, F-actin polarization toward the beads was unaffected by the absence of talin1.
Talin1 is required for LFA-1-mediated T cell conjugation to APCs

LFA-1-ICAM interactions are required for durable T cell-APC conjugation. To determine whether talin1 is required for Ag-induced T cell-APC interactions, a flow cytometry-based conjugation assay was performed using siRNA-treated Jurkat cells and superantigen-loaded Daudi B cells. The majority of T cell-APC conjugation is mediated by LFA-1 because LFA-1 function-blocking Abs reduced conjugation at 30 s by 90% (data not shown). However, after 10 min, LFA-1 function-blocking Abs reduced conjugation efficiency by only 60%, suggesting that LFA-1-independent mechanisms make a larger contribution to T cell-APC interactions at later time points. In this study, we assayed the role of talin1 in LFA-1-dependent conjugation, as described in Materials and Methods. Conjugation of talin1-deficient T cells to APCs was significantly reduced at all time points up to 5 min compared with control cells (Fig. 5A). The effect of talin1 knockdown was more pronounced at earlier time points, suggesting that the role of talin1 in regulating LFA-1 function is most critical immediately following T cell-APC contact, and becomes more redundant as the contact matures. To further address the requirement for integrin affinity in T cell-APC interactions, talin1-deficient T cells were treated with MnCl$_2$ in the presence of APC and conjugation efficiency was assayed. High-affinity LFA-1 enhanced conjugation efficiency, but did not rescue conjugation in talin1-deficient cells to control levels (Fig. 5B). Together, the findings indicate that talin1 is required for LFA-1 function during T cell conjugation to APCs, and that this requirement is not fulfilled by LFA-1 affinity up-regulation alone.

Talin1 targeting to the T cell-APC contact site requires the talin1 rod domain

Previous studies have demonstrated that talin1 targets to the T cell-APC contact site (18). To identify the determinants of talin1 targeting and to further characterize the role of talin1 during T cell conjugation, Jurkat cells were transfected with control or talin1 siRNA plus cDNAs encoding EGFP fused to the N terminus of mouse full-length talin1, talin1 head domain, or talin1 rod domain. Western blot confirmed expression of the talin1 constructs (Fig. 6A). To examine the role of each talin1 domain in determining talin1 localization, the Jurkat cells with talin1 siRNA and EGFP fusion constructs were incubated with superantigen-loaded Daudi B cells. As expected, the subcellular localization of EGFP alone was diffuse in the cytoplasm of the T cell, whereas EGFP-talin1 was localized to the contact zone between the T cell and APC (Fig. 6B). EGFP-talin1 rod was even more strongly associated with the T cell-APC contact zone, whereas EGFP-talin1 head was primarily diffuse throughout the T cell, but was also weakly associated with the plasma membrane. Together, the findings indicate that the primary determinants of talin1 localization to the T cell-APC contact site are in its rod domain. Interestingly, overexpression of EGFP-talin1 and EGFP-talin1 rod domain was associated with a modest increase in LFA-1 surface expression, whereas overexpression of EGFP alone or EGFP-talin1 head domain did not alter LFA-1 expression (Fig. 6C).

Talin1 head domain, but not rod domain rescues LFA-1 affinity

Previous studies have demonstrated that the talin1 head domain is required to regulate integrin affinity. To determine the region of talin1 required for regulating LFA-1 affinity, transfected cells were stimulated and stained with the affinity reporter Ab 327C. Both full-length talin1 and talin1 head domain fused to EGFP rescued LFA-1 affinity in talin1-knockdown Jurkat cells (Fig. 7A). Talin1 head domain expression did not increase LFA-1 affinity as much as full-length talin1 expression, most likely because of inefficient targeting of the talin1 head domain to the T cell plasma membrane.
T cell conjugation to APCs requires the talin1 head and rod domain

To determine the regions of talin1 required to rescue T cell-APC conjugation, the flow cytometry-based conjugation assay was performed. Introduction of full-length EGFP-talin1 fully rescued conjugation of talin1-deficient Jurkat cells to superantigen-loaded Daudi B cells (Fig. 7B). In contrast, the talin1 head domain or rod domain alone was not sufficient to rescue conjugation formation. The data provide further evidence that LFA-1 affinity modulation alone by the talin1 head domain is not sufficient to rescue the LFA-1 adhesive deficit observed in talin1-deficient T cells. In addition, the fact that the rod domain did not rescue conjugation efficiency indicates that up-regulation of LFA-1 surface expression is not sufficient to mediate the increased conjugation efficiency observed in cells overexpressing full-length EGFP-talin1. Together, these findings suggest that both the talin1 head and rod domains, through effects on integrin affinity and cytoskeletal coupling, respectively, are required for efficient T cell-APC conjugation.

Discussion

Integrins, a family of cell surface adhesion molecules, are critical mediators of cellular adhesion to extracellular matrix and to other cells. One example of major importance to the generation of immune responses is the leukocyte integrin LFA-1. On T lymphocytes, LFA-1 (via its binding to ICAM cell surface glycoproteins) is required for adhesion to endothelium, migration through tissues, and adhesion to APCs. Recent studies have shown that deficient or inappropriate LFA-1-mediated adhesion has significant pathological consequences (3, 25). To prevent this, T lymphocytes feature several mechanisms to rapidly modulate LFA-1 function in a tightly regulated fashion. The vital role of LFA-1 in mediating immune responses has inspired a number of studies investigating these regulatory mechanisms, and has been exploited for therapeutic purposes with the development of an LFA-1-blocking Ab for the treatment of autoimmune conditions (26). In this study, we identify talin1 as an essential component of the pathway up-regulating LFA-1 function following TCR-mediated T cell activation. Talin1, a cytoskeleton-associated protein and the first protein shown to bind the cytoplasmic domains of integrins (27), has been implicated in the function of all integrins, including LFA-1. Previous studies have shown that talin1 is required for chemokine-induced LFA-1-dependent adhesion and migration (15, 16); however, this is the first study to demonstrate that talin1 is required for TCR-induced adhesion of T cells to ICAM-1 and for T cell-APC conjugation.
Recently, imaging of intact, living lymphatic tissue has provided important insights into the character of T cell-APC interactions. When APCs bear cognate Ag, an initial period of transient interactions is followed by the formation of relatively stable conjugates (28). These conjugates permit the activation of T cell signaling networks that result in T cell activation, and are maintained by LFA-1 engagement of ICAM molecules on the APC surface. We find that talin1 is most important in establishing stable T cell-APC conjugation following brief contacts, suggesting that it is involved in the rapid up-regulation of LFA-1 function following TCR engagement.

Cells have two principal means of regulating integrin function: affinity and clustering. It is clear that talin1 is a critical regulator of integrin affinity. Binding of the talin1 N-terminal four-point-one, ezrin, radixin, moesin domain to the β integrin subunit cytoplasmic tail is thought to up-regulate integrin affinity by forcing apart the α and β subunit cytoplasmic domains, precipitating a change in the integrin extracellular domain to an open, extended conformation (17). Although we also demonstrate that talin1 is required for LFA-1 affinity regulation, this role of talin1 appears not to be sufficient for T cell conjugation to APC, because rescue of LFA-1 affinity with the talin1 head domain was not able to rescue conjugation to APCs. This conclusion is further supported by persistently reduced LFA-1-dependent adhesion and conjugation in talin1-deficient cells despite artificial integrin activation. These findings clearly do not rule out a role for affinity in LFA-1-mediated adhesiveness; however, they do suggest that LFA-1 clustering may also be impaired in talin1-deficient T cells.

Although previous studies have shown that talin1 is important for integrin clustering into focal adhesions of fibroblastic cells and the focal zone of migrating T cells (11, 16), it has been difficult to separate the role of talin1 in regulating integrin affinity from its potential role in regulating integrin localization. Experiments showing abnormal integrin localization in talin1-deficient cells are confined by the presence of integrin ligand, and therefore may only reflect reduced retention of integrins at ligand-containing surfaces due to their reduced affinity for ligand. Indeed, varying ligand density or artificial induction of high-affinity integrin with divalent cations both change the pattern of integrin localization in adherent cells (29, 30). The use of beads coated with a stimulating Ab allowed us to circumvent the variable of integrin affinity, and to show that talin1-deficient T cells are unable to efficiently cluster LFA-1 at the site of an activating stimulus.

In contrast to LFA-1 clustering, actin polarization to the site of TCR stimulation occurred normally in talin1-deficient T cells, suggesting that localized actin polymerization may precede recruitment of talin1 and LFA-1 to sites of TCR stimulation. This result suggests a model in which talin1 is recruited via its actin-binding activity to polymerized actin at the T cell-APC contact zone, where it promotes LFA-1 clustering, possibly by direct recruitment of LFA-1 to the T cell-APC interface. Alternatively, the primary role of talin may be to couple LFA-1 molecules at the contact zone to the actin cytoskeleton, thereby stabilizing their localization. Our experiments cannot distinguish between these modes of action, but we expect that talin1 plays both roles to some extent. Our conclusion that actin cytoskeletal polarization precedes and is required for effective LFA-1 valency regulation is supported by a number of studies describing proteins that regulate LFA-1 clustering toward APCs. Some proteins, including ADAP/SLAP-130, SKAP-55, and RapL, are required for LFA-1 clustering, but not actin cytoskeleton polarization (17, 31, 32); in contrast, proteins that are required for actin cytoskeleton polarization, such as Vav1 and the Tec kinases Rik and Itk, are inevitably also required for LFA-1 clustering (33, 34).

To further test this hypothesis, we investigated the localization and function of several talin1 mutants fused to EGFP. Full-length wild-type talin1 and talin1 C-terminal rod domain both localized strongly to the T cell-APC contact zone. In contrast, a construct expressing only the talin1 N-terminal head domain containing the primary integrin-binding site did not target to the T cell-APC contact zone and only weakly localized to the T cell membrane, despite its ability to up-regulate LFA-1 affinity. Together, these results suggest that the C-terminal rod domain of talin1 is required for its localization to the T cell-APC contact zone, whereas its ability to bind LFA-1 through the head domain is dispensable. These results are in accord with several previous studies investigating the determinants of talin1 targeting to focal contacts (10, 35). An intriguing possibility is that talin1 is targeted to the T cell-APC contact zone entirely by the actin-binding capacity of its C-terminal I/LWEQ module. Our laboratory has recently demonstrated that this module is both necessary and sufficient for talin1 targeting to focal contacts (36); however, it remains unclear as to whether actin binding is sufficient to explain this activity. This question may be resolved by further detailed analysis of the C-terminal actin-binding domain.

Although the talin1 rod domain is required for talin1 targeting to the T cell-APC contact zone, our model predicts that talin1 also requires integrin-binding activity in the head domain to bind integrins, activate them, and link them to the actin cytoskeleton. Two integrin-binding domains have been identified in talin1, one in the rod domain and one in the head domain. The rod domain site has lower affinity for integrin cytoplasmic domains and has no integrin-activating activity (9); therefore, we expected that the head domain site would provide the more critical integrin-binding activity. When talin1 knockdown T cells were transfected with EGFP-talin1 fusion constructs, only the full-length wild-type EGFP-talin1 was able to rescue conjugation to APCs to control levels; the talin1 head domain and talin1 rod domain were unable to rescue. Therefore, talin1 localization to the T cell-APC contact zone is not sufficient for its promotion of LFA-1-dependent T cell-APC adhesion; this activity requires both localization driven by the rod domain and integrin binding through the head domain.

The question remains as to what mechanisms underlie recruitment of LFA-1 to talin1 at the T cell-APC contact zone following TCR engagement. Talin1 may be constitutively associated with LFA-1, or the two molecules may be modified during T cell activation in a way that promotes their association. Two candidates for this modification are alterations in talin1 conformation induced by phosphatidylinositol 3,4-bisphosphate leading to unmasking of the talin1 four-point-one, ezrin, radixin, moesin domain (37), and the cleavage of talin1 between its head and rod domains by calpain 2, leading to increased affinity of the two fragments for integrin β subunit cytoplasmic tails by cooperative binding (38). The latter possibility is supported by several studies demonstrating reduced LFA-1 clustering and LFA-1-dependent adhesion of T cells treated with pharmacologic inhibitors of calpain.

In summary, our findings indicate that talin1 is a critical adaptor linking TCR signaling to LFA-1 activation and adhesion. This property requires both the integrin-binding determinants of talin1 and its proper localization to mediate efficient T cell-APC conjugation. Future studies will address the role of talin1 and its LFA-1 and cytoskeletal connections to the formation of the immunological synapse in Ag-specific T cells. A challenge for future investigation will also be to determine the functional consequences of these interactions to T cell activation in vivo.
Acknowledgments

We are indebted to D. Staunton for his provision of the 327C Ab, and to Kathy Schell and the University of Wisconsin flow cytometry core facility for flow cytometry assistance. We thank Vanessa Ott for her critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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