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Contact-Dependent T Cell Activation and T Cell Stopping Require Talin1

Sarah A. Wernimont,* Andrew J. Wiemer,‡,§ David A. Bennin,‡,§ Susan J. Monkle,§ Thomas Ludwig,‖ David R. Critchley,§ and Anna Huttenlocher‡,§

T cell–APC contact initiates T cell activation and is maintained by the integrin LFA-1. Talin1, an LFA-1 regulator, localizes to the immune synapse (IS) with unknown roles in T cell activation. In this study, we show that talin1-deficient T cells have defects in contact-dependent T cell stopping and proliferation. Although talin1-deficient T cells did not form stable interactions with APCs, transient contacts were sufficient to induce signaling. In contrast to prior models, LFA-1 polarized to T cell–APC contacts in talin1-deficient T cells, but vinculin and F-actin polarization at the IS was impaired. These results indicate that T cell proliferation requires sustained, talin1-mediated T cell–APC interactions and that talin1 is necessary for F-actin polarization and the stability of the IS. The Journal of Immunology, 2011, 187: 000–000.

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ative T cells migrate within lymph nodes and scan APCs for specific protein–MHC combinations. Once found, triggering of the TCR by MHC-presented cognate Ag activates intracellular signaling pathways, ultimately leading to T cell proliferation and cytokine production. At a molecular level, TCR triggering contributes to the formation of the immune synapse (IS), which is comprised of TCR signaling microclusters, adhesive molecules such as the integrin LFA-1, and polarized F-actin (1). The interaction between T cells and APCs is a central event in the activation of T cells; however, the length of interactions between T cells and APCs required to induce T cell activation remains controversial. For instance, some in vitro studies suggest that long-lived interactions from 6–24 h are required to induce full CD4+ T cell proliferation (2–5), whereas other studies show that transient interactions are sufficient to induce T cell activation (6, 7). In vivo experiments investigating T cell–APC interactions are also divided, indicating that the type of activating condition influences the stability of the interaction. Tolerizing conditions seem to promote transient interactions, whereas priming conditions seem to favor stable longer-lasting interactions with contacts maintained for hours during at least one phase of activation (8, 9).

The T cell integrin LFA-1 (αLβ2) is required to maintain T cell adhesion to APCs expressing ICAM-1. CD4+ T cells lacking LFA-1 fail to stably conjugate with APCs (10), and CD8+ T cells fail to form stable interactions with ICAM-1–deficient dendritic cells (11). However, the relative importance of these stable interactions in terms of immune response generation differs. For instance, CD4+ T cells from LFA-1 knockout mice fail to proliferate normally in response to Ag (12), whereas CD8+ T cells are able to proliferate following ICAM-1–deficient DC stimulation but fail to develop memory responses (11).

LFA-1 is regulated both by affinity and avidity (the degree of clustering) and localizes to the IS in T cell–APC conjugates (13). Following TCR stimulation, phosphorylation of the proximal scaffolding proteins linker of activated T cells and Src homology 2 domain-containing leukocyte protein of 76 kD contribute to the formation of signaling complexes that lead to Rap (a Ras-related small GTPase) activation and F-actin polarization, both of which contribute to integrin activation (14). A number of positive regulators of LFA-1 activation have been identified including talin, RapL, ADAP, SKAP55, and MST1 (15). RapL and talin are thought to contribute to full T cell integrin activation through direct binding of the αL and β2 subunits, respectively. Moreover, Kindlin-III has recently been shown to modulate LFA-1 activation (16). The relative importance of these integrin-binding proteins in T cell activation remains unexplored.

Although the cytoskeletal linker talin was among the first identified IS components (17), its exact role in T cell biology is unclear. Talin is composed of an N-terminal 4.1, ezrin, radixin, moesin domain, which can regulate integrin affinity, a C-terminal rod domain that contains a large number of vinculin binding sites, and a C-terminal IL/WEQ domain, which binds actin (18). In addition to regulating β2 integrins (15), talin can also regulate the activity of β1 and β3 integrins (19). Previous work has shown that talin is required for T cell–APC interactions through the regulation of both LFA-1 clustering and affinity (20, 21). Although talin

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S.A.W. designed, performed, and analyzed experiments and wrote the manuscript; A.J.W. performed experiments; D.A.B. provided experimental support and helped with mouse colony maintenance; S.J.M. and D.R.C. generated the Talin1+/loxPloxP mice; T.L. provided the Rosa26-CreERT2 mice; and A.H. designed and analyzed experiments and cowrote the manuscript.

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Abbreviations used in this article: CD4-Cre, Cre recombinase under control of the CD4 promoter; CH, calponin homology; GFP-PH-AKT, GFP-tagged pleckstrin homology-AKT; IS, immune synapse; Lifeact-Ruby, mRuby-tagged Lifeact; 4-OHT, 4-hydroxytamoxifen; PH, pleckstrin homology; PKC-θ, protein kinase Cθ; Utr, utrophin; UtCH-GFP, GFP-tagged calponin homology domain from utrophin; WA, WASP-family verprolin-homologous protein 2.

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is a known component of the IS and is required for T cell–APC interactions, prior studies relied on Jurkat T cell lymphoma lines and superantigen-mediated conjugation, which do not allow for studies of T cell activation and proliferation. Additionally, these systems may not provide accurate models of T cell activation, because Jurkat signaling downstream of the TCR is distinctly different from primary T cells (22), and superantigen-mediated conjugation bypasses proximal signaling (23).

In addition to LFA-1, formation of T cell–APC conjugates requires the polarization of the actin cytoskeleton and its stabilization at the IS (24). Actin polarization at the IS is dependent on WASP-family verprolin-homologous protein 2 (WAVE2) regulation of Arp2/3 actin nucleating complex (25), and, once polarized, actin filaments are stabilized by HS-1 (26). Previous studies, using Jurkat superantigen-mediated conjugates, have shown that actin polarization precedes LFA-1 clustering (20, 21). It has been proposed that following TCR engagement, WAVE2-dependent actin polymerization contributes to vinculin- and talin-mediated integrin clustering and activation through the formation of a WAVE2–Arp2/3–vinculin complex (21). Notably, models proposing that actin polarization precedes LFA-1 polarization in T cells contrasts to findings from talin1-deficient NK cells, which suggest that talin1 is required for normal actin polarization at cell–cell contact sites (27).

To better understand how talin regulates LFA-1 activity, T cell–APC interactions, and CD4+ T cell activation, we used conditional talin1 knockout mice to specifically delete talin1 in T cells. We generated (30).

**Cell culture.** Single-cell suspensions were made from spleen of control and knockout mice between 6 and 8 wk of age, and cells were expanded for 7–10 d with OVA23–290 (Anspec, Fremont, CA) and IL-2 (Chiron, Emeryville, CA). These OVA peptide expanded cells were used on days 7–11 for experiments. Alternatively, CD4+ T cells were isolated from cell suspension by negative selection and AutoMACS sorting (Miltenyi Biotec, Auburn, CA). Isolated CD4+ cells were resuspended in RPMI-1640 complete supplemented with IL-2 and stimulated on plates coated with 1 µg/mL anti-CD3 (2C11) (BioLegend, San Diego, CA) and 1 µg/mL anti-CD28 (eBioscience) in RPMI supplemented with IL-2 (Chiron). These plate-activated cells were used on days 7–11 following isolation for in vitro assays. The LB27.4 B cell line was purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 complete media.

**Retroviral transduction.**

Phoenix viral packaging cells were transiently transfected with DNA for mCherry vector alone, GFP-tagged pleckstrin homology–AKT (GFP-PH-AKT), m-Ruby–tagged Lifeact (Lifeact-Ruby), or GFP-tagged calponin homology domain from utrophin (Urch-GFP). Viral superantigen-mediated T cell activation, and CD4+ T cell proliferation, we used conditional talin1 knockout mice to specifically delete talin1 in T cells. We generated (30).

**Materials and Methods**

**Mice**

Talin1LoxP/LoxP mice were generated as previously described (28). Mice were fully backcrossed for six generations and crossed with mice expressing the OTII TCR transgene that recognizes OVA peptide 223–230 (The Jackson Laboratory, Bar Harbor, ME) and Cre recombinase under control of the CD4 promoter (CD4-Cre) mice (Taconic Farms, Hudson, NY). To obtain cells for retroviral transduction, Talin1LoxP/LoxP,OTII mice were crossed with Rosa26-CREERT2 mice (29). Genotyping was done as previously described (28). All experiments used Talin1LoxP/LoxP,CD4-Cre or Talin1LoxP/LoxP,CD4-Cre/OTII mice for talin1-deficient T cells and Talin1LoxP/LoxP,CD4-Cre/OTII mice for control T cells. The Institutional Animal Care and Use Committee at the University of Wisconsin approved all experimental protocols involving the use of mice.

**Reagents**

**Abs for flow cytometry.** PE anti-CD4, allophycocyanin anti-CD4, PE anti-CD3, FITC anti-CD8, PE anti-CD62L, FITC anti-CD44, allophycocyanin anti-CD69, FITC anti-CD25, PE anti-Foxp3, allophycocyanin anti-CD25, PE anti-β2, PE anti-β1, FITC anti-α4, PE anti-α4, PE IFN-γ, FITC IL-2, PE IL-10, and FITC IL-4 were all from eBioscience (San Diego, CA).

**Abs for immunoblotting/imunofluorescence.** Talin (8d4), vinculin, and actin were from Sigma-Aldrich (St. Louis, MO). β-tubulin, ZAP70, p-ZAP70, AKT, p-AKT, and WAVE2 were from Cell Signaling Technology (Danvers, MA). Nore1 was from Abcam (Cambridge, MA). p-ERK, p-p42/44, and vinculin were from Santa Cruz Biotechnology (Santa Cruz, CA). p-p38, p-ERK, and JNK were from Invitrogen (Carlsbad, CA). P24/Arc (Arp2/3) was from Millipore. RIAM Ab was previously described (30).

**In vitro proliferation**

In vitro proliferation assay was performed essentially as described (31). OVA peptide-expanded T cells were stained with 0.25 µM CFSE (Invitrogen) and 2.5 µM PKH26 (Sigma-Aldrich), according to the manufacturer’s instructions. Six million control and talin1-deficient cells were mixed together and injected i.v. into recipient mice (note: dye were switched to control for dye effects within each experiment). One hour after injection, mice were sacrificed, and splenocytes and spleen were removed and stained with Abs to CD4. Cells were analyzed on an FACS-Calibur (BD Biosciences, San Jose, CA) and the ratio of CD4+ talin1-deficient to control cells determined.

**Homing experiments**

Naive splenocytes from control and Talin1LoxP/LoxP,CD4-Cre mice were stained with 2.5 µM CFSE (Invitrogen) and 2.5 µM PKH26 (Sigma-Aldrich), according to the manufacturer’s instructions. Six million control and talin1-deficient cells were mixed together and injected i.v. into recipient mice (note: dye were switched to control for dye effects within each experiment). One hour after injection, mice were sacrificed, and cervical and inguinal lymph nodes and spleen were removed and stained with Abs to CD4. Cells were analyzed on an FACS-Calibur (BD Biosciences, San Jose, CA) and the ratio of CD4+ talin1-deficient to control cells determined.

**In vivo proliferation**

In vivo proliferation was performed essentially as described (12). Briefly, CD4+ cells from wild-type and knockout mice were isolated by CD4+ negative selection and stained with 2.5 µM CFSE (Invitrogen). Five million cells were injected i.v. into age-sex-matched recipient mice. Eighteen hours later, 25 µg LPS (Sigma-Aldrich) or 25 µg LPS and 50 µg OVA (Sigma-Aldrich) was injected i.p. Seventy-two hours later, mice were sacrificed and splenocytes isolated and stained for CD4. The degree of CFSE dye dilution was determined for CD4+ T cells on an FACS-Calibur (BD Biosciences). The proliferative index was determined using the ModFit 3.2.1 (Verity, Topsham, ME) analysis program.

**Th1/Th2 cytokine production**

Th1/Th2 cytokine profiling was done as previously described (31). Briefly, OVA peptide-expanded T cells were restimulated on days 7–10 following isolation on 24-well plates coated with 1 µg/mL anti-CD3 (2C11) (BioLegend) along with 2 µg/mL soluble anti-CD28 (eBioscience) in the
presence of brefeldin A (eBioscience). Four hours following restimulation, cells were stained with anti-CD4 and fixed with 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA). Intracellular staining was performed. The percentage of CD4+ T cells producing cytokines was determined by flow cytometry using an FACS Calibur (BD Bioscience) and analyzed using FlowJo (Tree Star, Ashland, OR).

Immunoblotting
For immunoblotting following activation, 60 × 10^6 OVA peptide-expanded control and talin-deficient cells were resuspended in PBS (Mediatech, Manassas, VA) and coated on ice for 10 min with biotinylated anti-CD3 (eBioscience) prior to cross-linking with streptavidin (The Jackson Laboratory) and coated on ice for 10 min. Alternatively, cells were left unstimulated. Cells were lysed in 50 mM Tris (pH 7.6), 0.15 M NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40 containing 0.2 mM PMSF, 1 μg/ml peptatin, 1 μg/ml aproteinin, 1 μg/ml leupeptin, and 1 μM sodium orthovanadate on ice and cleared by centrifugation. Protein concentration was determined by a bicinchoninic acid protein assay kit (ThermoScientific, Waltham, MA), and equal concentrations of protein were added to SDS sample buffer, boiled, and run on a 10% acrylamide gel. Proteins were transferred to a nitrocellulose membrane and stained. Blots were imaged with an Odyssey infrared imaging system (LicoR Biotechnologies, Lincoln, NE).

Calcium flux
Calcium flux was performed on OVA peptide-expanded cells as previously described (31).

Adhesion assays
Adhesion assays were performed as previously described (31).

T cell–APC conjugation assays
T cell conjugation to LB27.4 B cells was performed as described previously (31).

Live imaging
Live imaging of T cell–APC interactions was done using methods adapted from previous studies (32). LB27.4 APCs were loaded with 2.5 μM CMAC (Invitrogen) according to the manufacturer’s directions and pulsed with 2.5 μg/ml OVA peptide for 30 min prior to adhesion to the bottom of a polyl-lysine-coated (Sigma-Aldrich) glass-bottom plate (in some cases, LB27.4 APCs were labeled with 2.5 μM PKH-26 [Sigma-Aldrich]). Meanwhile, OVA peptide-expanded T cells resuspended in HBSS (Mediatech) supplemented with 1 mM HEPES (Mediatech), 10% FBS (HyClone, Walmsley, MA), and 0.25% low-melt agarose (Fisher, Waltham, MA), were plated on APC, and overlayed with 1 ml mineral oil (Sigma-Aldrich). Cells were maintained at 37˚C for the duration of acquisition. Confocal images of mCherry, GFP-PH-AKT, LifeAct-Ruby, and UtR-CH-GFP localization were obtained using a laser scanning confocal microscope (Olympus, Center Valley, PA) with a 60× Plan Apo/1.45 oil immersion objective with 3 × 2 slice 1.8 μm apart and one image every 30 s. Conjugation of non-transduced OVA peptide-expanded T cells was measured using an epifluorescent microscope (Nikon, Melville, NY) and a CoolSnap ES2 camera (Photometrics, Tucson, AZ). One bright field image and fluorescent image was acquired every 45 s for 45 min of total imaging. Images were acquired using MetaMorph Imaging software (MDS Analytical Technologies, Downingtown, PA). Duration of conjugation was calculated as the time from initial T cell–APC contact to the T cell leaving APC.

Immunofluorescence
LB27.4 B cells were stained with 1 μM CMAC (Invitrogen) according to the manufacturer’s directions and pulsed with 2.5 μg/ml OVA peptide for 30 min at 37˚C. Equal numbers of T cells and B cells in RPMI were combined, centrifuged, and incubated at 37˚C for 30 min prior to resuspension in PBS and pulse vortexing. Cells were allowed to adhere to polyl-lysine-coated (Sigma-Aldrich) coverslips for 5 min prior to fixing with 4% paraformaldehyde (Electron Microscopy Services) for 15 min. Cells were permeabilized with 0.1% Triton X-100 and blocked in goat serum. Cells were stained with primary Abs as indicated along with FITC/tetrachlorohydroxamic isothiocyanate-conjugated anti-rat or FITC-conjugated anti-rabbit secondary Abs (The Jackson Laboratory). Images were acquired on a laser scanning confocal microscope (Olympus) using a 60× Plan Apo/1.45 oil immersion objective with a 1× or 10× zoom factor and captured into Fluoview software (FV10-ASW version 1.07; Olympus).

Statistical analyses
Statistical analyses were performed using Prism 4 software (GraphPad, La Jolla, CA). Two-tailed paired t test for single comparisons or one-way ANOVA for multiple comparisons followed by Tukey posttest on continuous variable data, which was normally distributed and had equal variance, were used.

Results

Talin1Loxp/Loxp:CD4-Cre mice develop CD4+ T cells that have impaired lymph node homing and trafficking
Talin1 is the only talin isoform expressed in T cells (33), and germline deletion of talin1 is embryonic lethal (34). To eliminate talin specifically from T cells, a Cre recombinase-mediated conditional knockout system was used to excise the talin1 gene from T cell genomic DNA. Talin1 was deleted during the double-positive stage (CD4+, CD8+) of development by expression of CD4-Cre. T cells from Talin1Loxp/Loxp:CD4-Cre mice have a 97% reduction in talin1 expression compared with control (Fig. 1A). As controls, we used nonflowed littersmates that express CD4-Cre. Although fibroblasts from talin1 knockout mice showed upregulation of the closely related talin2 (35), talin2 expression was not observed in control or talin1-deficient T cells (Supplemental Fig. 1A).

T cells in Talin1Loxp/Loxp:CD4-Cre mice developed in the thymus, and we observed similar proportions of CD4 and CD8 single- and double-positive cells in the knockout relative to the wild-type control mice (Fig. 1B). Although the total number of CD4+ cells was relatively similar between wild-type and Talin1Loxp/Loxp:CD4-Cre mice, we found that Talin1Loxp/Loxp:CD4-Cre mice had significantly fewer CD4+ T cells in peripheral lymph nodes and more CD4+ T cells in the blood and spleen (Fig. 1C–F). We also found that 1 h after injection into recipient mice, talin1-deficient CD4+ cells failed to traffic to lymph nodes (Fig. 1G), similar to findings in LFA-1 knockout mice (12, 36). Additionally, Talin1Loxp/Loxp:CD4-Cre mice had a severe impairment in regulatory T cell development, with few CD4Foxp3CD25+ cells found in the spleen of mice (Supplemental Fig. 1B), indicating that both talin1 and β2 integrin are necessary for the normal development of regulatory T cells (37).

The few CD4+ cells that were found in the lymph nodes of Talin1Loxp/Loxp:CD4-Cre mice tended to have a more activated phenotype, with increased CD25 and CD69 expression and an increased percentage of memory cells with more CD44hiCD62Llow cells, although the total number was similar to control mice (Supplemental Fig. 1C, 1D). Talin1Loxp/Loxp:CD4-Cre mice in contrast to littermate controls developed prolapsed rectums at 8–10 wk of age that may have been exacerbated by a helicobacter infection. For the current study, cells were isolated from Talin1Loxp/Loxp:CD4-Cre and littermate Talin1Loxp/Loxp:CD4-Cre mice controls between 6 and 8 wk of age, prior to onset of rectal prolapase. Because of the impaired T cell trafficking and the increased percentage of activated T cells in the lymph nodes of Talin1Loxp/Loxp:CD4-Cre mice, most of the studies were done on OVA peptide-expanded splenic T cells. Due to potential developmental differences between Talin1Loxp/Loxp:CD4-Cre and Talin1−/−:CD4-Cre mice, key findings were repeated in T cells from Talin1Loxp/Loxp:CD4−/− CreER12 mice that were treated in vitro with 4-OH tamoxifen to deplete talin1 (Supplemental Fig. 2).

Talin1 is required for contact-dependent CD4+ T cell proliferation
Previous work showed that the integrin LFA-1 is required for CD4+ T cell proliferation in response to Ag-loaded APCs (12), and we hypothesized that talin would also be required. To test the effect of talin on Ag-induced T cell proliferation, we crossed
Talin1LoxpLoxp:CD4-Cre mice with mice expressing the OTII transgenic TCR, which recognizes OVA peptide 223–230. These OVA peptide-expanded talin1-deficient CD4+ cells had a slight increase in α6, expression compared with control but had no difference in α4, β1, or β2 integrin expression (Supplemental Fig. 1). We then used CFSE dye dilution to assess the ability of CD4+ T cells to proliferate in response to OVA peptide-loaded irradiated splenocytes. In this assay, each round of cell division resulted in a 50% dilution in CFSE, which was detected by flow cytometry and quantified as the proliferative index. Although we found that control cells had a dose-dependent increase in proliferation in response to increasing concentrations of Ag, talin1-deficient T cells had impaired proliferation at all concentrations of OVA peptide (Fig. 2A, 2B). Additionally, we found that the LFA-1 function-blocking Ab impaired control T cell proliferation at low, but not high, concentrations of OVA peptide but did not affect proliferation of talin1-deficient cells (Supplemental Fig. 1F).

We next tested whether this proliferation defect also occurred in T cells in vivo. CFSE-labeled naïve CD4+ T cells were injected i.v. into wild-type recipient mice 24 h before stimulation with LPS in the presence or absence of OVA. Seventy-two hours following stimulation, we found no CFSE+ cells in the lymph nodes of mice injected with Talin1LoxpLoxp:CD4-Cre:OTII cells. Due to the homing defect of talin1-deficient cells, we analyzed the degree of dye dilution in splenic CD4+ T cells by flow cytometry in control and talin1-deficient T cells. We found a significant decrease in the rate of dye dilution in talin1-deficient cells compared with controls, suggesting that talin1 is required for T cell proliferation in vivo (Fig. 2C, 2D).

**Talin1 is required for contact-dependent CD4+ T cell cytokine production**

To determine if talin was required for cytokine production following stimulation, CD4+ T cells from Talin1LoxpLoxp:CD4-Cre:OTII mice and Talin1LoxpLoxp:CD4-Cre:OTII mice were activated with OVA peptide, and 7 d after isolation, cells were restimulated with plate-bound CD3 and soluble CD28 in the presence of brefeldin A. Cells were then stained with Abs to IFN-γ, IL-4, IL-2, and IL-10. As expected, both control and talin1-deficient T cells exhibited Th1 skewing with increased production of IFN-γ and IL-2. However, there were 5-fold fewer talin1-deficient cells producing cytokine compared with control cells (Fig. 2E–G), indicating that talin is required for optimal Th1 cytokine production.

**Talin1 is not required for contact-independent CD4+ T cell proliferation**

Given the observed defects in contact-dependent proliferation, we next wanted to determine if CD4+ T cells from Talin1LoxpLoxp, CD4-Cre:OTII mice were capable of proliferating in response to contact-independent stimulation. To test this, we stimulated control and talin1-deficient T cells with anti-CD3/CD28–coated beads, which induce TCR signaling and costimulation and also PMA/ionomycin, which activates protein kinase C (PKC-0) and induces calcium signaling downstream of proximal TCR stimulation, respectively. Although there were slight decreases in proliferation for talin1-deficient T cells compared with control cells following both stimulations, these differences were not statistically significant (Fig. 3A, 3B). These findings suggest that talin1-deficient cells have a specific defect in contact-dependent proliferation.

**TCR induced signaling is intact in talin1-deficient cells**

We were intrigued by our findings of a specific defect in contact-dependent proliferation, so we sought to better understand the differences between the contact-dependent and -independent induced proliferation. First, we looked at T cell signaling in response to TCR cross-linking. Following TCR cross-linking, control and talin1-deficient T cells showed a similar pattern of phosphorylation...
of ZAP70, linker of activated T cells, AKT, ERK, and p38 MAPK (Fig. 3C, 3D). Additionally, using the calcium indicator dye Indo-1, we found no difference in the ratio of bound to unbound calcium following TCR cross-linking, indicating that TCR signaling was similar in control and talin1-deficient cells (Fig. 3E).

Together, these findings show that the impaired proliferation observed in talin1-deficient cells is not due to a global defect in TCR signal transduction.

Talin1-deficient CD4⁺ T cells have impaired T cell–APC contacts

Previous reports using small interfering RNA-mediated depletion of talin have shown that talin is required for Jurkat T cell adhesion to ICAM-1 and conjugation to APCs induced by superantigen (20, 21). To determine if talin was necessary for primary T cell adhesion and conjugation, we tested the ability of talin1-deficient cells to adhere to ICAM-1 following TCR cross-linking or PMA stimulation. Following both treatments, control T cells showed a 3- to 4-fold increase in adhesiveness, whereas no increase was observed in talin1-deficient cells (Fig. 4A). These results indicate that talin is required for two-dimensional T cell adhesion and inside-out integrin signaling induced by TCR stimulation. To determine if the defects were due to impaired integrin affinity modulation, we treated cells with MnCl₂, which increases LFA-1 affinity for ligand. Whereas control cells had a 3-fold increase in adhesion to ICAM-1 following MnCl₂ treatment, talin1-deficient cells did not show any statistically significant increase in adhesion (Fig. 4A).

We also tested the ability of talin1-deficient T cells to adhere to APCs by a flow cytometry-based assay. We found that whereas control cells had a dose-dependent increase in Ag-dependent conjugation to the B cell lymphoma line LB27.4, talin1-deficient cells did not adhere to APCs beyond baseline levels. Additionally, this defect in conjugation was not rescued with MnCl₂, suggesting that increasing integrin affinity alone is not sufficient to rescue adhesion or conjugation (Fig. 4B).

Talin1-deficient T cells do not form stable interactions with APCs

To further understand the nature of T cell–APC interactions in talin1-deficient cells, we examined T cell–APC contacts using live three-dimensional imaging. Because talin1-deficient cells cannot adhere to a two-dimensional surface, we used a three-dimensional system comprised of low-melt agarose in the presence of serum to examine T cell–APC interactions (32). Talin1-deficient T cells displayed normal random motility in this three-dimensional environment. In the absence of OVA peptide, both control and talin1-deficient T cells showed only transient interactions with APCs and maintained a polarized morphology while migrating along APCs (Fig. 4C, Supplemental Videos 1, 2). The average time of contact between T cells and B cells was ∼5 min without OVA peptide. In the presence of OVA peptide, control T cells established longer-lasting interactions with APCs, with average contact times of ∼30 min in a 45-min video (Fig. 4D, 4E, Supplemental Video 3). Moreover, control T cells arrested migration and displayed a loss of uropod formation and cell polarization.
during these stable interactions with APCs. In contrast, talin1-deficient T cells in the presence of OVA peptide behaved more like T cells interacting with APCs without Ag (Fig. 4C, Supplemental Video 4); there were many contacts with an average duration of <5 min. Moreover, talin1-deficient T cells maintained a polarized morphology while contacting APCs and did not arrest migration (Fig. 4D,4E). These findings indicate that stable T cell–APC interactions and Ag-induced T cell stopping require talin1.

Transient contacts are sufficient to induce signaling in talin1-deficient cells
To determine if the transient T cell–APC contacts observed in talin1-deficient T cells were sufficient to induce T cell signaling, we used live imaging of a fluorescent reporter of PI3K activation in T cells contacting APCs. The PH domain of AKT, which binds to phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate and is a marker of PI3K activation, has previously been shown to polarize to the site of T cell–APC contacts (4, 38). We were unable to transfect T cells from Talin1Loxp/Loxp:CD4-Cre:OTII mice with retrovirus, consistent with the reported role of integrin activation in viral infection (39).

To work around this, we retrovirally transduced T cells from Talin1Loxp/Loxp:Rosa26-CreERT2:OTII mice with constructs for mCherry and GFP-PH-AKT. Following an initial expansion, talin1 was depleted from T cells using treatment with 250 nM 4-OHT. This treatment resulted in >95% reduction in talin1 protein expression in T cells (Fig. 5A). T cells from Talin1Loxp/Loxp:Rosa26-CreERT2:OTII mice treated with 4-OHT had similar defects in T cell–APC contact and contact-dependent proliferation as T cells from Talin1Loxp/Loxp:CD4-Cre:OTII mice (Supplemental Fig. 2).

We expressed mCherry and GFP-PH-AKT to similar levels in control and 4-OHT–treated cells (Fig. 5A) and assessed localization of GFP-PH-AKT in control and 4-OHT–treated T cells before and after stimulation with OVA peptide. Whereas we saw no polarization of GFP-PH-AKT to the site of T cell–APC contact in the absence of OVA peptide, there was robust polarization of GFP-PH-AKT to the T cell–APC contact site in control cells in the presence of OVA peptide (Fig. 5B, Supplemental Videos 5, 6). Additionally, although the 4-OHT–treated T cells from Talin1Loxp/Loxp:Rosa26-CreERT2:OTII cells did not arrest upon contact with Ag-loaded APCs, GFP-PH-AKT polarized to the T cell–APC contact site
in the presence, but not absence, of OVA peptide (Supplemental Videos 7, 8). This suggests that transient contacts are sufficient to induce signaling in talin1-deficient cells, although they are not able to induce T cell arrest.

To further explore the capacity of talin1-deficient T cells to signal in response to Ag-loaded APCs, we allowed T cells to interact with APCs in the presence or absence of OVA peptide and then fixed and stained conjugates on poly-L-lysine–coated cover slips. This assay only assessed the limited number of conjugates that formed; however, we found that PKC-θ and phosphorylated Zap70 polarized to the site of T cell–APC contact in both control and talin1-deficient cells (Fig. 6A, 6B). Surprisingly, LFA-1 also polarized to the contact site, suggesting that talin is not required for LFA-1 clustering at the synapse. Additionally, we noted that RapL, which directly binds αL integrin and regulates LFA-1 clustering (40), and its regulator, RIAM, were localized to the IS in talin1-deficient T cells (Supplemental Fig. 3A, 3B). Despite the ability of transient contacts to induce signaling in talin1-deficient cells, promoting cell–cell contact using round-bottomed wells was not sufficient to rescue T cell proliferation in talin1-deficient T cells (Fig. 6C, 6D).

### Talin is required for F-actin polarization to the synapse

Because LFA-1 polarized to T cell–APC contacts in talin1-deficient T cells, we sought to better understand the organization of the IS in talin1-deficient cells. T cell–APC conjugates were fixed on coverslips and stained with anti–LFA-1 and rhodamine phalloidin. LFA-1 and F-actin strongly polarized to the IS in talin1-deficient T cells (Fig. 6C, 6D).
contacting APC with Ag, whereas F-actin polarized to the IS in <5% of conjugates formed by talin1-deficient T cells (Fig. 7A). We also found that LFA-1 was able to establish a ringlike structure, corresponding to the peripheral supramolecular activation cluster, at the IS in some talin1-deficient T cell conjugates (Supplemental Fig. 3B).

To further investigate the defect in actin polarization, we used immunofluorescence to stain for components of the actin polymerizing machinery, including Arp2/3 and WA VE2. We found that both Arp2/3 and the F-actin regulator WA VE-2 were localized at the IS in both control and talin1-deficient T cells following conjugation in the presence of OV A peptide, supporting the idea that talin is not necessary for the polarization of key actin regulatory proteins to the IS (Fig. 7). Moreover, HS-1, which has been implicated in stabilizing F-actin at the IS (26), also polarized in talin1-deficient cells (Supplemental Fig. 4). In contrast, we found that vinculin failed to polarize to the IS in talin1-deficient T cells compared with control T cells in the presence of Ag (Fig. 7D).

Taken together, our results suggest that WA VE2 and Arp2/3 are recruited to the site of T cell–APC contact in the absence of talin1, but that talin1 is necessary for the polarization of vinculin and F-actin to the IS.

**Talin1 is necessary for the polarization of stabilized F-actin to T cell–APC contacts**

To better understand F-actin dynamics in talin1-deficient T cells contacting APC, we used live imaging to examine two different

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**FIGURE 5.** Live imaging of signaling in Talin1-deficient cells. OVA peptide-expanded Talin1^{Loxp/Loxp}: Rosa26-CreERT2:OTII cells were retrovirally transduced with mCherry vector and GFP-PH-AKT. Prior to analysis, cells were treated with 4-OH tamoxifen or vehicle to induce genomic deletion of talin1. A, Immunoblotting shows >97% loss of talin with tamoxifen treatment. B, Ratio-metric images of PH-AKT signaling relative to mCherry. Asterisk depicts APC. Blots and images are representative of 3 independent experiments including 15 conjugation events. Scale bars, 10 μm.

**FIGURE 6.** Transient contacts signal normally, but forcing contact cannot rescue proliferation in talin1-deficient cells. A and B, OVA peptide-expanded cells were conjugated with OVA peptide-loaded LB7.4 cells and fixed on coverslips and stained with the indicated Abs. Graphs in right panels are line scan through conjugate: x-axis corresponds to fluorescent intensity, whereas y-axis corresponds to micrometer position through conjugate. Blue line represents APC, green line represents LFA-1, and red line represents indicated Ab. Scale bars, 10 μm. Images are representative of three independent experiments and at least 30 conjugates. C, Bright field images of an equal number of unstimulated cells in round- and flat-bottom plates. Scale bars, 100 μm. D, Bar graph depicting proliferative index of OVA peptide-expanded T cells in response to OVA peptide in round-bottom plates. Data are mean ± SE from three independent experiments. *p < 0.05.
fluorescent probes that bind F-actin. Lifeact-Ruby (41) binds to all F-actin, whereas UtrCH-GFP binds to stabilized F-actin (42–44). We had previously reported that UtrCH-GFP specifically localizes to the uropod of migrating neutrophils (42–44). Similarly, we found that UtrCH-GFP localizes to the uropod in motile T cells in three dimensions, whereas Lifeact-Ruby localized to the cell cortex in both the leading edge and uropod. In the absence of Ag, both control and 4-OHT–treated T cells isolated from Talin1Loxp/Loxp: Rosa26-CreERT2:OTII mice retained UtrCH-GFP in the uropod during transient T cell–APC contacts (Fig. 8, Supplemental Videos 9, 10). In the presence of OVA peptide, control T cells lost their uropod upon contact with APCs, and UtrCH-GFP established a more uniform distribution around the cell cortex with some enhancement at the T cell–APC contact site. Although Lifeact-Ruby was distributed along the cell cortex, we found that occasionally, Lifeact-Ruby was enriched at peripheral regions of T cell–APC contact sites, highlighting an area of active F-actin dynamics (Fig. 8, Supplemental Video 11). In contrast, talin1-deficient T cells retained UtrCH-GFP in the uropod during T cell–APC contacts in the presence of Ag, indicating that stabilized F-actin failed to polarize to the synapse without talin (Fig. 8, Supplemental Video 12). Lifeact-Ruby was enriched at the site of transient T cell–APC contacts in talin1-deficient T cells, suggesting that dynamic F-actin can polarize to the contact site between T cells and APC in the absence of talin. Taken together, our findings indicate that talin1 is necessary for the stabilization of F-actin at the IS.

**Discussion**

Since the identification of talin as an IS component (17), there has been considerable interest in understanding its role in LFA-1 function and T cell activation. Using mice with a specific depletion of talin1 in T cells, we now demonstrate for the first time, to our knowledge, that talin is critical for maintenance of T cell–APC contacts, contact-mediated T cell proliferation, and polarization of stable F-actin to the IS. We found no evidence of defects in TCR signaling contributing to these phenotypes because protein phosphorylation following TCR cross-linking was unaltered, and phospho-ZAP70 and PKC-ζ localized to the IS of talin1-deficient T cells. Additionally, using live imaging, we observed accumulation of GFP-PH-AKT at the site of T cell–APC contact in the absence of talin1-dependent T cell arrest. Based on our findings, we propose that the current model of LFA-1 activation in T cells should be revised to indicate that talin is dispensable for LFA-1 polarization to the synapse but is required to polarize stabilized F-actin and mediate full T cell arrest.

The defects that we observed in talin1-deficient T cell lymph node homing, APC interactions, and proliferation are similar to those previously reported for CD4+ T cells isolated from LFA-1 knockout mice (12, 45). These shared phenotypes suggest that the defects observed in Talin1Loxp/Loxp.CD4-Cre T cells may primarily be due to defects in LFA-1 activation. The capacity of LFA-1 to bind its ligand ICAM-1 is regulated by changes in affinity and clustering following TCR signaling (13). We have previously shown that talin modulates both components of LFA-1 function.
In this study, we show that talin1-deficient T cells fail to adhere to ICAM-1 and APCs but retain the ability to cluster LFA-1 at T cell–APC contacts. Despite having this ability to cluster LFA-1, talin1-deficient T cells failed to fully polarize F-actin to the IS. These findings differed from previous reports suggesting that talin is required for LFA-1 clustering, but not F-actin polarization, during Jurkat superantigen-mediated conjugation (20, 21). These differences are likely due to the types of stimulation and cell types used: Jurkat T cells treated with superantigen versus Ag-induced primary T cell conjugation. Superantigen-mediated conjugation has previously been shown to bypass proximal TCR signaling (23), and Jurkat T cell signaling is different from primary T cells (22). The use of talin knockout mice and Ag-specific primary T cells represents an advance toward better understanding the role of talin1 in T cell adhesion and activation.

Although there are clear defects in LFA-1 function in the absence of talin, we found that LFA-1 is still clustered at the IS in talin1-deficient T cells. In other integrin-mediated adhesions, talin1, RapL, and kindlins are thought to work in concert to regulate integrin activity (19). Although RapL, is not required for T cell adhesion to ICAM-1 (46), both talin1 and kindlin-III have been independently shown to be important for LFA-1–dependent adhesion of T cells (16, 20). In this work, we show that RapL and the associated integrin regulatory protein RIAM are localized to the site of T cell–APC contact in talin1-deficient T cells, suggesting that RapL and/or RIAM may potentially be sufficient to cluster LFA-1. However, despite the clustering of LFA-1 at the IS, LFA-1 is not functioning normally in talin1-deficient T cells because the cells have impaired adhesion to both APCs and ICAM-1–coated plates. Together, these findings indicate that although talin1 is dispensable for LFA-1 polarization, it is necessary for LFA-1–mediated adhesive function.

Based on previous studies, it was surprising to find impaired F-actin polarization at the site of T cell–APC contacts in talin1-deficient T cells. Previous work has reported that F-actin polarization to the IS is required for T cell–APC interactions and full T cell proliferation because disruption of F-actin with inhibitors following conjugate formation impairs T cell activation (47, 48). To better understand the types of F-actin found at the IS, we used two probes of F-actin: Lifeact, which binds to all F-actin present in the cell, and the Utr CH domain of Utr, which binds specifically to stable F-actin. Because Utr-CH binds to stable F-actin populations, localization of Lifeact alone corresponds to areas of dynamic F-actin (44). To our knowledge, these probes have not previously been used to characterize the dynamics of F-actin at T cell–APC contact sites.

Live imaging of F-actin dynamics using Lifeact-Ruby and UtrCH-GFP showed that stable F-actin, specifically, failed to polarize to the T cell–APC contact site in talin1-deficient cells. We found that migrating talin1-deficient T cells maintained UtrCH-GFP, a marker of stabilized F-actin, at the uropod without any evidence of its redistribution following transient T cell–APC contacts, whereas Lifeact-Ruby was enriched at T cell–APC contact sites during transient contacts. This combined with the observation that Arp2/3 and WAVE2 are at the IS in talin1-deficient T cells suggests that actin polymerization at the IS is intact in talin1-deficient cells but that talin is required for F-actin stabilization.

This failure to polarize F-actin in talin1-deficient cells may be due to a requirement for talin to directly bind actin at the IS and provide the link to polarized integrins, or it may be due to an inability to recruit vinculin to the IS in the absence of talin1. Supporting the latter hypothesis, we showed that there was a defect in vinculin polymerization to the IS in talin1-deficient T cells even though vinculin can bind Arp2/3, which polarized normally to the
actions for T cell proliferation have been challenged with the proliferation (10, 51). However, the requirement of these inter
actions is highlighted by the recent findings that T cells lacking leukocyte-t-actin, an actin bundling protein that mediates F-actin accumulation at the IS, are not efficiently activated (50).

One area of controversy is the relative importance of forming stable T cell–APC interactions for T cell activation. Whereas some in vitro studies show that T cell arrest is required for T cell pro-
liferation and activation (2, 3), others show that transient inter-
actions are sufficient for T cell activation (6, 7). In this article, we demonstrate that talin1 is necessary for T cell arrest and forma-
tion of stable interactions between T cells and APCs. The failure of talin1-deficient T cells to arrest and develop stable contacts with APCs in combination with the findings of impaired contact-dependent proliferation supports the hypothesis that T cell arrest is required for T cell proliferation and that talin is a critical regulator of this process. We suspect that talin mediates a connection be-
tween F-actin and LFA-1 that is required for complete arrest of T cells on APCs because simply promoting cell–contact is not sufficient to restore T cell proliferation in talin1-deficient T cells.

In vivo studies investigating the importance of T cell–APC interactions indicate that T cells form stable, long-lasting interactions with APCs during priming conditions, which contribute to proliferation (10, 51). However, the requirement of these interactions for T cell proliferation have been challenged with the finding that CD8 T cells fail to form stable interactions with ICAM-1–deficient APCs in vivo but are still able to proliferate (11). Although we show that talin is required for in vivo CD4 T cell proliferation in response to OVA, the question of T cell arrest during in vivo priming is hard to address with this system because we cannot determine whether impaired proliferation in talin1-deficient T cells is due to impaired T cell–APC interactions or due to impaired T cell trafficking to lymph nodes. However, our work supports the idea that talin-mediated T cell arrest is required for full CD4 T cell proliferation and activation.

In this study, we provide evidence that talin1 is required for the formation of sustained T cell–APC interactions and T cell contact-
dependent proliferation. In addition to its role in regulating LFA-1 function in T cells, it also plays a critical role in maintaining F-
actin polarization to the IS. Although talin-dependent T cell–APC interactions seem critical in generating a number of T cell–APC responses, future challenges will be to address how talin may support the differentiation of T cells following antigenic challenge and how this differentiation may affect outcomes in response to infection and autoimmunity in vivo.

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