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Tec Kinases Regulate TCR-Mediated Recruitment of Signaling Molecules and Integrin-Dependent Cell Adhesion[1]

Lisa D. Finkelstein,* Yoji Shimizu,† and Pamela L. Schwartzberg[2]*

T cells deficient in the Tec kinases Itk or Itk and Rlk exhibit defective TCR-stimulated proliferation, IL-2 production, and activation of phospholipase C-γ. Evidence also implicates Tec kinases in actin cytoskeleton regulation, which is necessary for cell adhesion and formation of the immune synapse in T lymphocytes. In this study we show that Tec kinases are required for TCR-mediated up-regulation of adhesion via the LFA-1 integrin. We also demonstrate that the defect in adhesion is associated with defective clustering of LFA-1 and talin at the site of interaction of *Rlk*$^{-/-}$*Itk*$^{-/-}$ and *Itk*$^{-/-}$ T cells with anti-TCR-coated beads. Defective recruitment of Vav1, protein kinase C*θ*, and Pyk2 was also observed in *Rlk*$^{-/-}$*Itk*$^{-/-}$ and *Itk*$^{-/-}$ T cells. Stimulation with ICAM-2 in conjunction with anti-TCR-coated beads enhanced polarization of Vav1, protein kinase C*θ*, and Pyk2 in wild-type cells, demonstrating a role for integrins in potentiating the recruitment of signaling molecules in T cells. Increased recruitment of signaling molecules was most pronounced under conditions of low TCR stimulation. Under these suboptimal TCR stimulation conditions, ICAM-2 could also enhance the recruitment of signaling molecules in *Itk*$^{-/-}$, but not *Rlk*$^{-/-}$*Itk*$^{-/-}$ T cells. Thus, Tec kinases play key roles in regulating TCR-mediated polarization of integrins and signaling molecules to the site of TCR stimulation as well as the up-regulation of integrin adhesion. *The Journal of Immunology,* **2005, 175: 5923–5930.**

Engagement of the TCR complex by peptide/MHC results in rapid activation of intracellular tyrosine kinases and tyrosine phosphorylation of signaling molecules. Subsequently, the actin cytoskeleton becomes polarized, and signaling proteins accumulate at the contact site between the T cell and APC, where they arrange into a complex known as the immunological synapse. Although the role of the synapse is unclear, one potential function is to help maintain contact between the T cell and APC through the recruitment and stabilization of cell adhesion molecules at the site of interaction, thereby permitting the prolonged signaling required for effective T cell responses (1).

Among the molecules that accumulate at the immune synapse are integrins, adhesion receptors that are important for cell-cell contact as well as interactions with extracellular matrix proteins (2). In T cells, the β_2 integrin LFA-1 ($\alpha_L\beta_2$) is crucial for providing contact with APCs, whereas β_1 integrins establish connections to the extracellular matrix. Integrins are expressed on resting T cells in a low adhesive state to allow for lymphocyte circulation. In response to a stimulus, integrins undergo rapid qualitative changes that enhance their adhesiveness, an actin cytoskeleton-dependent process known as inside-out signaling. Adhesion can be up-regulated by a variety of cell surface signals, including engagement of the TCR/CD3 complex.

Genetic and biochemical data have demonstrated that proximal TCR signaling events are required for activation of inside-out signaling; Jurkat T cells deficient in the tyrosine kinase ZAP-70, the adaptor linker for activation of T cells, or the enzyme phospholipase C-γ (PLC-γ1)[3] (3) exhibit defective TCR-triggered activation of β_1 integrins (3, 4). Pharmacological studies argue that protein kinase C (PKC) and other diacylglycerol-regulated proteins, PI3K and Ca^{2+}, are important for triggering integrin up-regulation (2). In addition, several downstream signaling molecules have been implicated in the regulation of TCR-mediated inside-out signaling, including Vav1, a guanine nucleotide exchange factor for Rho family GTPases that regulate the actin cytoskeleton; the adaptor protein adhesion and degranulation promoting adaptor protein (ADAP) and its binding partner SKAP-55; the small GTPase Rap1 and its effectors regulator for cell adhesion and polarization enriched in lymphoid tissues (RAPL) and Rap1-GTP-interacting protein (RIAM); and the calcium-sensitive protease calpain (5–12). Nonetheless, how and whether these signaling molecules interact or cooperate in inside-out signaling and how they are regulated remain poorly defined. Recent data suggest that the Tec family of kinases also participates in these pathways; the expression of mutant forms of the Tec kinase Itk in Jurkat T cells can block TCR activation of β_1 integrins (13). Furthermore, Itk-deficient T cells show decreased conjugate formation with Ag-loaded APCs (14).

The Tec kinases are a family of nonreceptor tyrosine kinases that are unique in that most family members possess a pleckstrin homology domain that interacts with products of PI3K, allowing for membrane recruitment and activation (15). In naive T cells, the primary Tec kinases are Itk and Rlk, with Tec expressed at lower levels (16). Although proximal TCR signaling events, such as tyrosine phosphorylation of CD3ζ, ZAP-70, and linker for activation of T cells, are intact in *Itk*$^{-/-}$ or *Rlk*$^{-/-}$*Itk*$^{-/-}$ T cells, mutations affecting the Tec kinases result in defective TCR-induced PLC-γ activation, Ca^{2+} mobilization, and ERK activation (17, 18). In addition, Itk has recently been shown to regulate TCR-mediated

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[3] Abbreviations used in this paper: PLC-γ, phospholipase C-γ; PKC, protein kinase C; SFRPMI, serum-free RPMI 1640; WT, wild type; ADAP, adhesion and degranulation promoting adaptor protein; DOCK, downstream of Crk.

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actin polymerization and polarization as well as the localized activation of Cdc42 at the contact site between a T cell and an APC (13, 14, 19, 20). These data suggest that the Tec kinases are critical for TCR-driven actin cytoskeleton reorganization and, moreover, are centrally positioned in the signaling pathways that regulate integrin adhesion.

To explore the role of Tec kinases in cellular functions controlled by cytoskeletal reorganization, we examined integrin-mediated adhesion and recruitment of signaling complexes in Itk- and Rlk/Itk-deficient T cells. We show in this study that Tec kinases are critical for TCR-induced up-regulation of β_2 integrin adhesion. Adhesion defects in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ cells correlate with an inability to recruit LFA-1 and talin as well as Vav1, PKCθ, and Pyk2 to the site of TCR stimulation. In addition, we demonstrate a novel role for LFA-1 in enhancing the recruitment of signaling molecules to the site of TCR stimulation. Our studies also provide evidence of multiple similarities between $Itk^{-/-}$ and $Vav1^{-/-}$ T cells, particularly with respect to TCR-stimulated actin polarization, LFA-1 and PKCθ recruitment, Pyk2 tyrosine phosphorylation, and the up-regulation of integrin adhesion.

Materials and Methods

Mice and Abs

$Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ mice were previously described (17). Abs and reagents were from the following sources: anti-phosphotyrosine mAb 4G10 (Upstate Biotechnologies); goat anti-Pyk2, talin, and LFA-1; rabbit anti-PKCθ; Vav1; and anti-goat HRP (Santa Cruz Biotechnology); anti-CD3, CD3-biotin, TCR, and CD11a-FITC (BD Pharmingen); anti-LFA-1 mAb I21/7 (Endogen); rabbit anti-Vav1 for IF (gift from D. Billadeau, Mayo Clinic, Rochester, MN); sheep anti-ADAP (gift from G. Koretzsky, University of Pennsylvania, Philadelphia, PA); anti-phospho ZAP-70 (Cell Signaling Technologies); anti-rabbit HRP (Chemicon International); anti-mouse HRP (Roche); anti-sheep HRP (Bio-Rad); Alexa Fluor 594 phalloidin and Alexa Fluor 594 anti-goat (Molecular Probes); and anti-rabbit rhodamine (Jackson ImmunoResearch Laboratories).

Flow cytometry

Splenic or lymph node T cells were stained with anti-CD11a-FITC and analyzed by flow cytometry.

Adhesion assay

Adhesion assays were performed as previously described by assessing the adhesion of purified lymph node T cells to 96-well Nunc MaxiSorp plates precoated with 0.3 μg/well mouse rICAM-2/Fc (R&D Systems) (3). T cells were isolated from mouse lymph nodes by MACS column with anti-B220 microbeads (Miltenyi Biotec), labeled with calcein-AM (Molecular Probes; 15 min, 37°C), washed, and incubated for 20 min on ice in the presence or the absence of anti-CD3 biotin (15 μg/4 $\times$ 10^6 cells). Cells were washed twice and added to the ICAM-coated wells (100,000 cells/well) containing 50 μl of the appropriate 2$\times$ stimulatory agent (medium, 200 ng/ml PMA or 16 mg/ml streptavidin), then incubated for 1 h on ice to allow cells to settle. Plates were read on a fluorescent plate reader (Bio-Tek; Flx800TBE) using 485-nm excitation and 516-nm emission filters, then warmed to 37°C in a floating bath for 10 min. Wells were gently washed three or four times with cold wash buffer, the plate was read again, and the percent adhesion per well was calculated (final read/preread $\times$ 100). Data are presented as the average of triplicate wells for each sample.

Immunoprecipitation and immunoblotting

A six-well plate was coated overnight with 5 μg/ml anti-CD3 or anti-LFA-1 in PBS. Mouse splenic T cells were isolated by T cell subset columns (R&D Systems) and resuspended in serum-free RPMI 1640 (SFRPMI) at 10^8/ml, and 100 μl of cells were added per well as the plate floated in a 37°C water bath. At the indicated times, 100 μl 1% SDS in PBS (plus protease inhibitor minitab (Roche) and sodium orthovanadate) was added to each well, followed by the addition of 1 ml of 1% Triton in PBS (plus inhibitors). Lysates were sheared through a 25-gauge needle with a 1-cc syringe five or six times and spun at 13,000 rpm for 15 min at 4°C.

For TCR stimulation of Pyk2, 15 $\times$ 10^6 cells were resuspended in 2 ml of SFRPMI containing 22.5 μg of anti-CD3 biotin and incubated on ice for 20 min. Samples were washed once in cold SFRPMI and resuspended in 1.5 ml of SFRPMI, and 0.5 ml (5 $\times$ 10^6 cells) was aliquoted in Eppendorf tubes containing 5 μg of streptavidin. Samples were stimulated at 37°C and then spun for 2 min at 4°C at 10,000 rpm, lysed in 0.5 ml of Nonidet P-40 lysis buffer (1% Nonidet P-40, 10 mM Tris-Cl (pH 7.5), and 150 mM NaCl plus inhibitors) for 10–15 min on ice, and then spun as described above.

Gamma BindPlus Sepharose beads (20 μl; Amersham Biosciences) were prebound to 1–2 μg of Ab/sample for 30 min at 4°C and washed with lysis buffer; cleared lysates were added, and proteins were immunoprecipitated overnight at 4°C while rotating. Beads were washed twice with lysis buffer, and samples were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted. All experiments were performed at least three times.

T cell:bead assay and immunofluorescence

Latex beads (Interfacial Dynamics) were coated with 3 or 1 μg/ml anti-TCR (H57) in PBS for 1.5 h at 37°C. Beads were then spun and resuspended in PBS, and 3 μg/ml anti-$H2K^b$ or anti-CD28 or ICAM-2/Fc was added for an additional 1.5 h. Beads were washed in PBS/3% BSA, then resuspended in complete RPMI 1640 or SFRPMI and stored at 4°C. For these assays, we did not observe differences with the inclusion of serum. Relative Ab and ICAM concentrations on beads were confirmed by flow cytometry. The concentrations of anti-TCR Ab used were determined by titration of Ab levels on the beads to achieve maximal and suboptimal protein polarization. Mouse splenic T cells were resuspended at 2.5 $\times$ 10^7/ml in SFRPMI, mixed with coated beads in a 1:1 ratio, spun at 100 $\times$ *g* for 1 min to form a loose pellet, flicked to mix, and incubated in a 37°C water bath for 5–30 min based on observed optimal times for recruitment (5 min for pZAP-70; 30 min for all other stains). Paraformaldehyde was added to a final concentration of 4% (0.25% for pZAP-70) at room temperature for 10 min, 0.5 ml of SFRPMI was added, and conjugates were stored at 4°C. Cells were permeabilized with 0.1% Triton in PBS (or 500 μg/ml digitonin in PBS for pZAP-70) and stained with primary Ab, followed by washing, and with secondary Ab for 1 h each at room temperature. Conjugates were washed twice and vortexed gently, and 1.5 μl was placed on a printed slide (Carlson Scientific) and sealed with a coverslip. Conjugates were viewed with a $\times$100 oil objective on a Zeiss Axiophot fluorescence microscope, and images were captured with IP Lab software. Cells were scored as having polarized actin or signaling molecules if they bound a single bead and showed increased staining (concentrated fluorescence) at the contact site between bead and cell. A minimum of 60 single-cell plus single-bead conjugates per sample were scored per experiment. Data are presented as the percentage of cells with polarized actin or signaling molecules per conjugates scored $\pm$ SEM for a minimum of three experiments (except as noted in the figure legends). Data were analyzed by one-way ANOVA. and post-hoc analysis was completed using Bonferroni's multiple comparison test to determine statistical significance, which was accepted at a value of $p < 0.05$ (GraphPad PRISM software).

Results

Tec kinases regulate TCR-mediated increases in LFA-1 adhesion

To address the role of Tec kinases in cytoskeleton-regulated processes, we examined adhesion via the β_2 integrin LFA-1 in primary T lymphocytes from mice that were wild type (WT) or deficient in Itk and Rlk. Stimulation of WT T cells with the phorbol ester PMA or an anti-CD3 Ab, which activates the TCR, led to enhanced adhesion to the LFA-1 ligand ICAM-2 (Fig. 1*A*). However, stimulation of $Rlk^{-/-}Itk^{-/-}$ T cells with anti-CD3 failed to promote adhesion despite intact PMA-stimulated adhesion in the mutant cells. This adhesion defect was not due to reduced integrin receptor expression, because ex vivo freshly isolated $Rlk^{-/-}Itk^{-/-}$ T cells expressed slightly higher levels of surface LFA-1 than WT cells (Fig. 1*C*), possibly accounting for the enhanced basal adhesion of these T cells.

T cells deficient in Rlk and Itk show graded defects in proliferation and IL-2 production in response to TCR stimulation, associated with decreased activation of PLC-γ (17). Defects are minimal in $Rlk^{-/-}$ cells, moderately severe in $Itk^{-/-}$ cells, and most severe in $Rlk^{-/-}Itk^{-/-}$ cells. Although Itk-deficient cells show less severe defects in Ca^{2+} mobilization than $Rlk^{-/-}Itk^{-/-}$ cells, we found no difference between $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ T cells in the static adhesion assay (Fig. 1, *A* and *B*). Similar results were obtained for actin polarization in response to TCR stimulation (14)

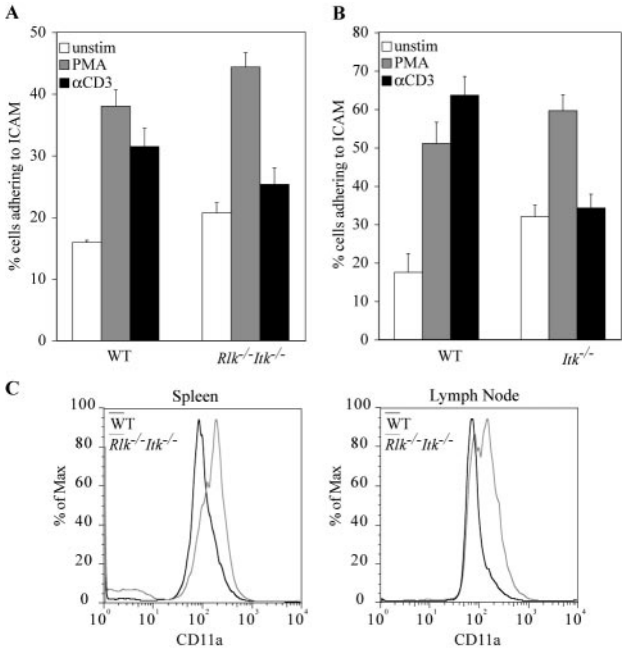

FIGURE 1. Itk is required for TCR-mediated up-regulation of LFA-1 integrin adhesion. WT and $Rlk^{-/-}Itk^{-/-}$ (*A*) or $Itk^{-/-}$ (*B*) T cells were left unstimulated or were stimulated with PMA or anti-CD3 biotin plus streptavidin for 10 min in a 96-well plate coated with the LFA-1 ligand ICAM-2. Samples were prepared in triplicate, and the percent adhesion was determined. Results are representative of at least three experiments. *C*, WT and $Rlk^{-/-}Itk^{-/-}$ splenic or lymph node T cells were stained with anti-CD11a-FITC and analyzed by flow cytometry.

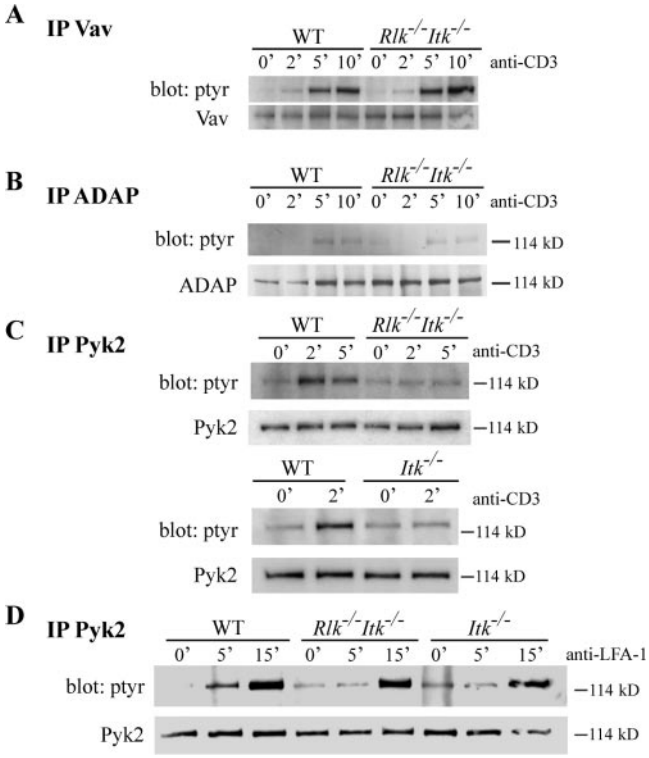

FIGURE 2. TCR-stimulated tyrosine phosphorylation of Vav1 and ADAP, but not Pyk2, is intact in Tec kinase-deficient cells. *A* and *B*, Splenic T cells from WT or $Rlk^{-/-}Itk^{-/-}$ mice were stimulated with plate-bound anti-CD3, Vav1 (*A*) or ADAP (*B*) immunoprecipitated, and immunoblotted to detect phosphotyrosine as well as the immunoprecipitated protein. *C*, Splenic T cells from WT, $Itk^{-/-}$, or $Rlk^{-/-}Itk^{-/-}$ mice were stimulated with soluble anti-CD3 biotin and streptavidin. Lysates were immunoprecipitated with anti-Pyk2 and analyzed as described above. *D*, Splenic T cells were stimulated with plate-bound anti-LFA-1, and Pyk2 analyzed as described in *C*. Results are representative of at least three experiments.

(see below). These results may reflect a lower level of sensitivity in the actin and adhesion assays compared with assays for Ca^{2+} mobilization. Alternatively, Itk deficiency may have a more profound effect on the ability of T cells to polarize actin and adhere upon TCR stimulation. Thus, Itk appears to be the major Tec kinase required for both actin reorganization and integrin regulation downstream of the TCR.

TCR-stimulated tyrosine phosphorylation in Tec-kinase deficient T cells

To explore the biochemical basis of these defects in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ cells, we examined two critical components required for TCR-mediated integrin adhesion, the guanine nucleotide exchange factor Vav1, and the adaptor molecule ADAP (5–7). Both ADAP and Vav1 become tyrosine phosphorylated upon TCR stimulation. Despite the differences in adhesion, similar levels of Vav1 and ADAP tyrosine phosphorylation were observed in WT and $Rlk^{-/-}Itk^{-/-}$ T cells (Fig. 2, *A* and *B*). Similar to results in Jurkat cells treated with short-interfering RNA directed against Itk (20), relatively normal phosphorylation of Vav1 was also detected in $Itk^{-/-}$ T cells (data not shown). Although we cannot rule out that Tec kinases could phosphorylate a specific regulatory tyrosine residue on either protein that is undetectable with the use of anti-phosphotyrosine or available site-specific phosphotyrosine Abs, our data argue that loss of the Tec kinases does not affect adhesion by influencing gross patterns of Vav1 or ADAP tyrosine phosphorylation.

In contrast, although tyrosine phosphorylation of many proximal signaling molecules appears intact in $Rlk^{-/-}Itk^{-/-}$ T cells (17), we observed that Tec kinases were required for tyrosine phosphorylation of the focal adhesion kinase family tyrosine kinase Pyk2. Pyk2 is tyrosine phosphorylated upon TCR engagement in a Fyn- and Vav1-dependent manner (Fig. 2*C*) (5, 21). TCR-induced phosphorylation of this protein was strongly reduced in T cells from $Rlk^{-/-}Itk^{-/-}$ or $Itk^{-/-}$ mice (Fig. 2*C*). Pyk2 is also phosphorylated downstream of integrin receptors in T cells (22). Although Tec kinase-deficient T cells showed a delay in LFA-1-stimulated Pyk2 tyrosine phosphorylation, at later time points, maximal levels of phosphorylation were still achieved (Fig. 2*D*). These results suggest that Tec kinases also influence the efficiency of signaling downstream of integrins.

Tec kinases are required for localization of molecules to the site of TCR stimulation

Although the role of Pyk2 in T lymphocytes is not well understood (23), a defect in TCR-stimulated Pyk2 tyrosine phosphorylation was also found in T cells deficient in Vav1 (5). Intriguingly, we have recently shown that Vav1 is not localized properly in $Itk^{-/-}$ T cells upon activation with Ag-loaded APCs (14). $Vav1^{-/-}$ T cells, like Itk-deficient T cells, also show defective actin polarization and integrin-mediated adhesion (5, 24, 25). In $Vav1^{-/-}$ cells, these defects are associated with defective recruitment and clustering of LFA-1 at the site of TCR signaling, which is thought to play a major role in increasing the avidity of integrins and stabilizing the adhesion to APCs. Nonetheless, Vav1-deficient cells show normal recruitment of TCR and tyrosine-phosphorylated proteins to peptide-pulsed APCs when they successfully form conjugates (26).

To explore the similarities between Tec kinase- and Vav1-deficient mice, we examined localization of molecules in T cells stimulated with anti-TCR-coated latex beads, a model system that permitted evaluation of the requirements for recruitment of signaling molecules. Using this method, we consistently observed fewer conjugates with $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ cells compared with WT cells (data not shown). However, when conjugates formed, we observed that localization of phosphorylated ZAP-70 to the contact site, a proximal signaling event, was only modestly affected in $Rlk^{-/-}Itk^{-/-}$ T cells (Fig. 3*A*). In contrast, recruitment of Vav1 was significantly impaired in both $Rlk^{-/-}Itk^{-/-}$ and $Itk^{-/-}$ T cells, whereas $Rlk^{-/-}$ cells appeared similar to WT (Fig. 3*B*). Consistent with previous findings and the abnormal recruitment of Vav1, we also observed defective recruitment of polymerized actin to the site of TCR stimulation in both $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ T cells (Fig. 3*C*). Thus, using the bead activation system, we confirmed that $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ T cells show defective actin recruitment and Vav1 polarization that results specifically from impaired responses to TCR alone, in the absence of effects of costimulatory molecules.

Because $Vav1^{-/-}$ T cells show defective clustering of the integrin LFA-1 at the site of TCR signaling, we asked whether Tec kinase-deficient T cells also showed defects in polarization of LFA-1 as well as talin, a cytoskeletal protein that is required for up-regulation of integrin adhesion. Talin binds to integrin cytoplasmic tails and helps mediate a conformational change that promotes integrin ligand binding (27, 28). Defective recruitment of both talin and LFA-1 to the T cell-bead contact site was observed in $Rlk^{-/-}Itk^{-/-}$ and $Itk^{-/-}$ T cells (Fig. 3, *D–F*). Thus, $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ T cells fail to accumulate key adhesion molecules at the site of TCR stimulation, suggesting that this may be a major cause of the adhesion defect in these cells.

Defective recruitment of multiple proteins found in the immune synapse

LFA-1 and talin are used as markers that define the peripheral region of the immune synapse, the peripheral supramolecular activation complex. The defects in recruitment of LFA-1 and talin in Itk-deficient cells suggest that other markers of the immune synapse may be abnormally localized. One of the key molecules recruited to the site of TCR stimulation is the novel PKC, PKCθ, which localizes to the central region of the mature immune synapse, the central supramolecular activation complex, via a pathway mediated by Vav1 and the Rho family GTPase, Rac (29). Indeed, consistent with their defect in Vav1 localization, T cells from either $Rlk^{-/-}Itk^{-/-}$ or $Itk^{-/-}$ mice showed defective polarization of this PKC isoform to anti-TCR-coated beads (Fig. 4, *A* and *B*).

Pyk2 has also been found to accumulate at the T cell-bead contact site in primary T cells stimulated with anti-CD3-coated beads (30). Again, we observed defective Pyk2 polarization in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ T cells (Fig. 4*C*), suggesting that recruitment of multiple signaling proteins to the area of the immune synapse is affected in these mutant cells. Moreover, the defects in Pyk2 localization and phosphorylation suggest that these two processes may be linked and may both require Itk.

LFA-1 costimulation potentiates recruitment of molecules to the immune synapse

Optimal activation of T cells requires costimulation via molecules such as CD28. Indeed, the recruitment of molecules such as PKCθ to the site of TCR stimulation is potentiated by engagement of the CD28 costimulatory receptor (31). Given the ability of LFA-1 to costimulate TCR signaling events (32), we examined whether LFA-1 engagement also influenced the recruitment of molecules to the site of TCR stimulation. Cells were stimulated with beads coated with anti-TCR plus anti-H2K^b or ICAM-2, and recruitment of molecules was evaluated. With ICAM alone on the beads, T cell-bead conjugates formed, but minimal levels of protein polarization were detected in WT T cells (data not shown). However, costimulation with ICAM-2 in conjunction with anti-TCR stimulation increased the number of conjugates showing polarized Vav1 in WT T cells compared with those stimulated with anti-TCR alone (Fig. 5*A*). Consistent with Vav1's role in the recruitment of PKCθ and the activation of Pyk2, costimulation with ICAM also

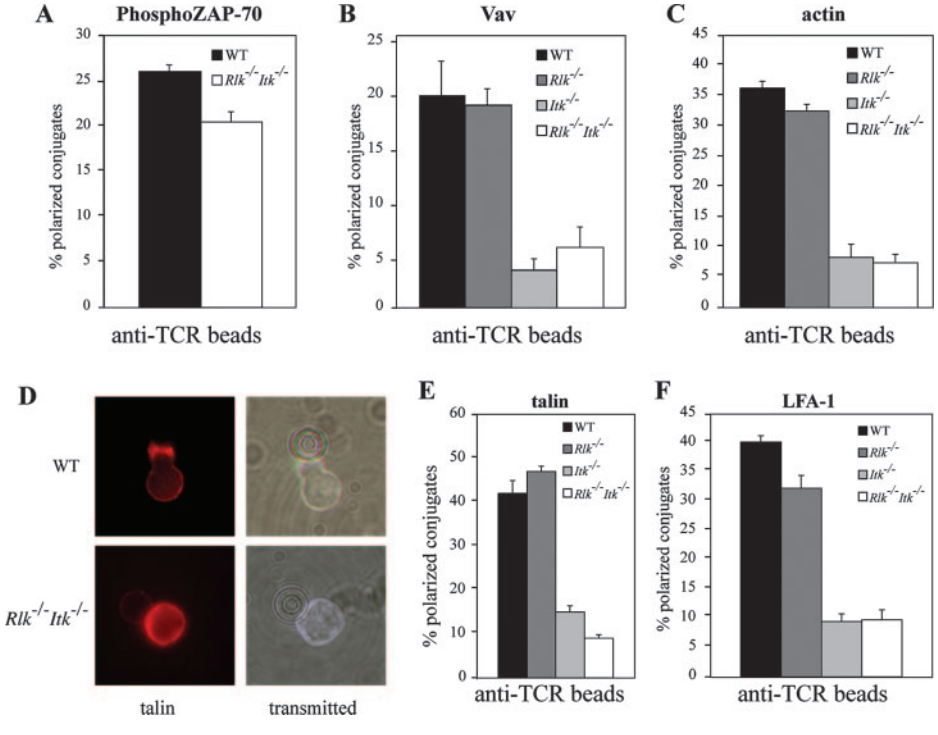

FIGURE 3. Tec kinase-deficient T cells are defective in Vav1, actin, talin, and LFA-1 polarization to the site of TCR stimulation. WT, $Rlk^{-/-}$, $Itk^{-/-}$, and/or $Rlk^{-/-}Itk^{-/-}$ splenic T cells were stimulated with anti-TCR-coated beads, and conjugates were stained for phosphoZAP-70 (*A*), Vav1 (*B*), actin (*C*), talin (*D* and *E*), or LFA-1 (*F*). Graphs of data represent results from at least three (two for pZAP-70) independent experiments, scoring a minimum of 60 conjugates/sample/experiment. The percentage of polarized conjugates represents the percentage of conjugates with a given protein localized to the T cell-bead contact site.

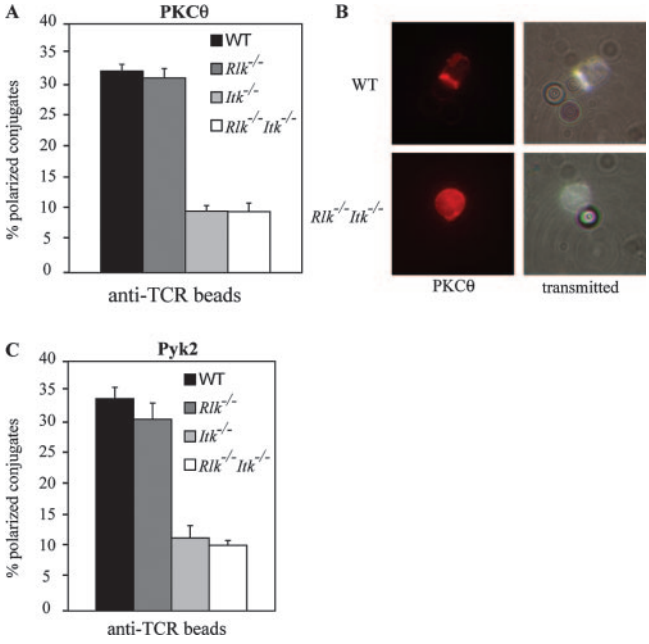

FIGURE 4. Tec kinase-deficient T cells are defective in PKCθ and Pyk2 polarization to the site of TCR stimulation. Splenic T cells were stimulated with anti-TCR-coated beads, and conjugates were stained for PKCθ (*A* and *B*) or Pyk2 (*C*). Graphs represent results from three independent experiments.

led to increased recruitment of PKCθ and Pyk2 in WT T cells (Fig. 5*A*). Thus, combined signals through the TCR and integrins can enhance the recruitment of certain signaling molecules. These data are consistent with the known activation of Vav1 and Pyk2 in response to integrin engagement (22, 33). However, a role for integrins in the regulation of PKCθ has not been previously appreciated.

Interestingly, costimulation with ICAM did not increase actin recruitment in WT T cells (Fig. 5*B*), suggesting that integrin engagement did not increase cell polarization per se. Moreover, ICAM did not significantly increase the recruitment of talin, arguing that TCR signaling alone may be sufficient to achieve maximal recruitment of this molecule, perhaps as part of the mechanism by which TCR signaling increases integrin adhesion. Although ICAM increased TCR-mediated LFA-1 localization in WT T cells, this increase was highly variable and did not reach statistical significance compared with stimulation with anti-TCR/$H2K^b$ beads under these conditions of high anti-TCR stimulation. Importantly, none of the protein polarization defects found in Tec kinase-deficient T cells was rescued upon costimulation through the LFA-1 integrin under these conditions (Fig. 5). These results suggest that the lack of Itk or Rlk/Itk disrupts signaling pathways downstream of the TCR to an extent that prevents LFA-1 from enhancing protein localization.

However, because effects of costimulation are often more apparent under conditions of suboptimal TCR stimulation, we repeated the studies described above with lower concentrations of anti-TCR on the beads. Under these conditions, we obtained higher levels of ICAM on the beads than we could obtain with beads coated with higher levels of anti-TCR Abs (data not shown). Using these suboptimal anti-TCR stimulation conditions, WT cells showed decreased recruitment of all signaling molecules (Fig. 6). Nonetheless, the ICAM-mediated recruitment of PKCθ, Vav1, and Pyk2 to the cell-bead contact site was actually more pronounced in WT T cells (Fig. 6*A*). In addition, the recruitment of LFA-1 to the site of TCR stimulation was clearly enhanced by ICAM costimulation in WT cells (Fig. 6*B*). Interestingly, under these suboptimal TCR stimulation conditions, protein recruitment in *Itk*$^{-/-}$ T cells was improved by costimulation by LFA-1 for all proteins examined (Fig. 6), emphasizing the importance of LFA-1 costimulation under conditions of decreased TCR signaling. In contrast, ICAM did not significantly increase the recruitment of any of the proteins examined in *Rlk*$^{-/-}$*Itk*$^{-/-}$ T cells, perhaps reflecting the very poor activation potential of these cells (17). Together, these data demonstrate an important role for LFA-1 costimulation in potentiating the recruitment of signaling proteins to the site of TCR stimulation.

Discussion

We present in this study data demonstrating a critical role for the Tec kinases in TCR-mediated regulation of integrin adhesion and the recruitment of key signaling proteins involved in adhesion, including LFA-1 and talin. Our data suggest that the defective cell polarization and organization of signaling molecules into the region of the immune synapse in Itk-deficient cells may lead to decreased adhesion in these cells. Our results also support the idea that a major role of recruitment of integrins and signaling molecules to the area of the immune synapse may be to help stabilize the adhesive interactions between a T cell and an APC, thus facilitating the prolonged signaling through the TCR that is required for a productive immune response. The normal phosphorylation of many signaling molecules in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ T lymphocytes is consistent with the view that initiation of TCR signaling does not require synapse formation (34). In particular, the relatively normal Vav1 phosphorylation despite its abnormal localization implies that phosphorylation of Vav1 can occur before or independent of its stable polarization to the site of TCR stimulation.

Mutations affecting the Tec kinases, Itk and Rlk, impair T cell development and functional responses, defects that have been attributed to decreased PLC-γ phosphorylation and Ca^{2+} mobilization (15). However, the defects in actin polarization and adhesion in *Itk*$^{-/-}$ and *Rlk*$^{-/-}$*Itk*$^{-/-}$ cells may also contribute to their TCR signaling defects by limiting the duration of TCR signaling. Indeed, many of the phenotypes of Tec kinase-deficient cells, including defective thymic selection, decreased proliferation, impaired IL-2 production, and defective Th2 cytokine production by mature T cells, might result in part from decreased signal duration and resemble those seen in *Vav1*$^{-/-}$ T cells (15, 24, 35).

It is therefore of interest that many of the biochemical defects we and others have observed in Itk-deficient cells are similar to those seen in *Vav1*$^{-/-}$ thymocytes. These include the previously described defects in PLC-γ activation, Ca^{2+} mobilization, and ERK activation despite normal early tyrosine phosphorylation (17, 18, 24, 25, 36, 37). Our new findings that Itk-deficient cells also exhibit defective TCR-stimulated cell adhesion, phosphorylation of Pyk2, and recruitment of LFA-1 and PKCθ highlight additional similarities between Itk- and Vav1-deficient cells (5, 29). A recent report has characterized TCR signaling defects in mice deficient in downstream of Crk (DOCK)2, another Rac guanine nucleotide exchange factor involved in TCR signaling (38). Although *DOCK2*$^{-/-}$ cells, like *Vav1*$^{-/-}$ cells, show defects in TCR-mediated activation of Rac, DOCK2-deficient cells exhibit normal LFA-1 and PKCθ recruitment and Pyk2 phosphorylation, but fail to recruit the TCR and lipid rafts. In contrast, Vav1-deficient T cells show normal polarized localization of TCR and phosphotyrosine (26). Although we have evaluated different proximal signaling molecules, the relatively minor defects in recruitment of phosphorylated ZAP-70 support a phenotype in Itk-deficient cells similar to that in Vav1-deficient cells, with normal early tyrosine

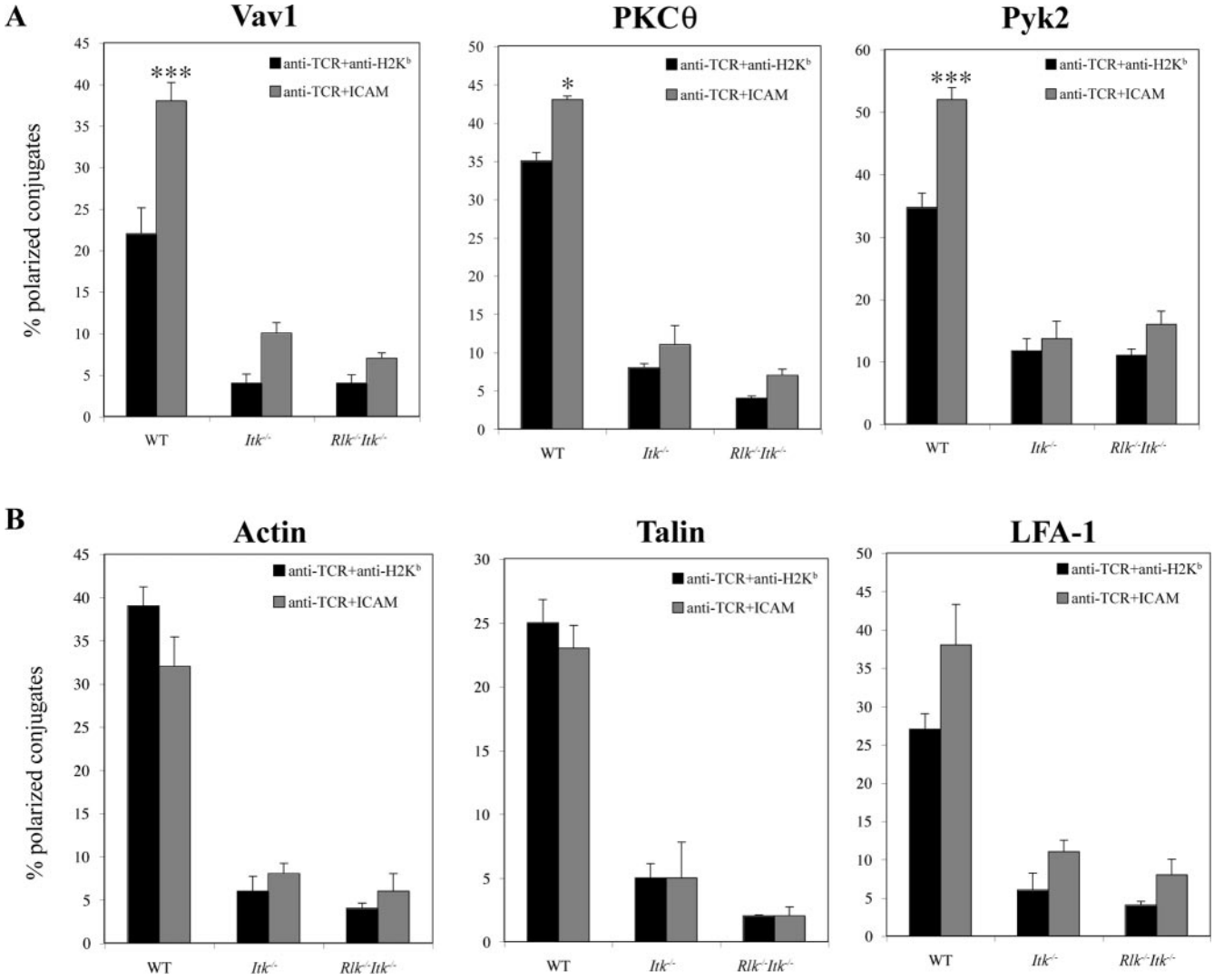

FIGURE 5. LFA-1 costimulation enhances protein polarization in WT, but not Tec kinase-deficient, T cells. Splenic T cells from WT, $Itk^{-/-}$, or $Rlk^{-/-}Itk^{-/-}$ mice were stimulated with beads coated with anti-TCR plus anti-H2K^b or ICAM-2. Conjugates were stained for Vav1, PKCθ, or Pyk2 (*A*) or actin, talin, or LFA-1 (*B*). Graphs represent results from at least three independent experiments (two for talin). *, $p < 0.05$; ***, $p < 0.001$ (for anti-TCR plus anti-H2K^b vs anti-TCR plus ICAM-2).

phosphorylation and recruitment of proximal signaling molecules. Therefore, Itk-deficient cells appear to closely resemble cells lacking Vav1, rather than DOCK2, particularly with respect to defective LFA-1 and PKCθ recruitment, Pyk2 phosphorylation, and cell adhesion. It is noteworthy that parallel findings of defects in stable adhesion have been reported in neutrophils from Vav1/Vav3 double-knockout mice, whereas initial adhesion events are intact (39). Thus, although the Tec kinases have been generally recognized for their roles in regulating PLC-γ and Ca^{2+}, the defect in stable Vav1 recruitment may be a major factor contributing to the phenotype of $Itk^{-/-}$ T cells. Given the recent report implicating Vav1 defects in a subset of patients with common variable immunodeficiency (40), Itk may be another important molecule to examine in this disorder.

LFA-1 has been proposed to facilitate TCR signaling by enhancing T cell-APC adhesion, although recent data argue that LFA-1 also generates intracellular signals that affect T cell function (32, 41). Our results suggest that LFA-1 engagement also contributes to TCR signaling by increasing recruitment of molecules that can enhance TCR signaling pathways, revealing a new function for LFA-1 in TCR signaling. The early immune synapse may therefore function as an equivalent to the focal adhesion, serving as a site of convergence of multiple signaling molecules at the adhesive interface, thereby potentiating signals from multiple receptors.

The inability of LFA-1 to rescue recruitment of signaling molecules in Tec kinase-deficient cells under high TCR stimulation conditions (Fig. 5) supports the idea that the defective TCR signaling in these cells prevents effective signaling through LFA-1. Interestingly, under conditions of suboptimal TCR stimulation, ICAM could improve protein recruitment in $Itk^{-/-}$ T cells (Fig. 6). Although this observation was surprising, this effect could be due to the slightly higher levels of ICAM on these beads compared with beads coated with optimal anti-TCR concentrations (data not shown). Alternatively, under conditions of suboptimal TCR signaling, the effects of ICAM may be more pronounced, perhaps due to the effects of outside-in signaling that may be obscured under conditions of higher TCR signaling. Our observations raise the possibility that there may be a balance between the activation of signaling pathways by the TCR and integrins, so that although these pathways can potentiate each other, they may also compete by using the same signaling molecules. Under lower TCR stimulation conditions, more signaling molecules may be available to

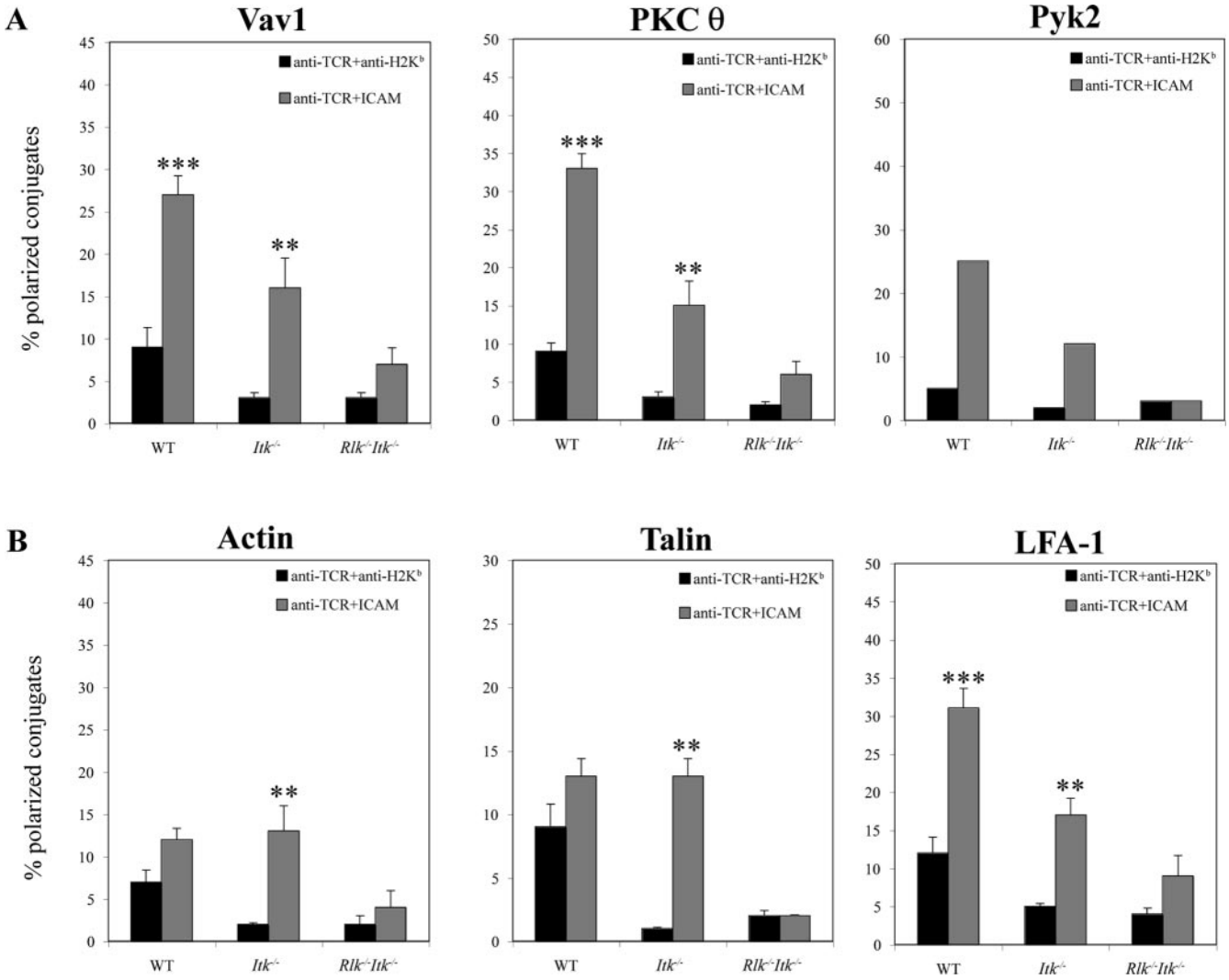

FIGURE 6. LFA-1 costimulation under suboptimal TCR stimulation improves protein recruitment in WT and Itk-deficient T cells. Splenic T cells from WT, $Itk^{-/-}$, or $Rlk^{-/-}Itk^{-/-}$ mice were stimulated with beads coated with suboptimal anti-TCR plus anti-H2K^b or ICAM-2. Conjugates were stained for Vav1, PKCθ, or Pyk2 (*A*) or actin, talin, or LFA-1 (*B*). Note that the *y*-axes are scaled to the same maximal values shown in Fig. 5 for comparison. Graphs represent results from four independent experiments (two for talin or one for Pyk2). **, $p < 0.01$; ***, $p < 0.001$ (for anti-TCR plus anti-H2K^b vs anti-TCR plus ICAM-2).

integrin-mediated activation, including signaling pathways that may be partially engaged, but not be effectively activated, by TCR signaling in the Itk-deficient background.

Nonetheless, costimulation with ICAM was not able to significantly improve recruitment of signaling molecules in $Rlk^{-/-}Itk^{-/-}$ T cells under any of the conditions tested. Thus, although we saw no difference between $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ T cells for cell polarization and adhesion in response to TCR stimulation, ICAM revealed that $Itk^{-/-}$ cells were more responsive under certain conditions. These observations may be secondary to the more severe TCR stimulation defects in $Rlk^{-/-}Itk^{-/-}$ T cells (17), such that the TCR is inadequate to supply enough signal to respond to costimulation. Alternatively, although we have not found evidence for a contribution of Rlk to LFA-1-mediated signaling, it is possible that such a role is only revealed in the absence of Itk. Of note, similar to our observations with LFA-1, we have found that costimulation initiated by CD28 can also overcome the protein localization defects in Itk-deficient, but not RlkItk-deficient, T cells (L. D. Finkelstein and P. L. Schwartzberg, unpublished observations). Thus, under certain conditions, protein recruitment to the site of TCR stimulation exhibits the graded defects previously observed with these knockout animals, highlighting the ability of LFA-1 costimulation to potentiate the recruitment of signaling proteins in response to signals from the TCR.

Recent evidence suggests that talin is required for a zone of clustered LFA-1 that forms in migrating T cells (42). Other studies have revealed that talin binding to integrin cytoplasmic domains may be a final common step in multiple signaling pathways leading to up-regulation of integrin adhesion (28). The defects we observed for talin and LFA-1 polarization, therefore, may not only affect TCR-mediated up-regulation of integrin adhesion, but may also have consequences for other signaling pathways that activate integrin adhesion. Indeed, Tec kinases are also important for chemokine-mediated cell polarization, adhesion, and migration (43, 44). Thus, cell polarization defects in Tec kinase-deficient T cells may impair multiple biological processes, suggesting that these defects may have important contributions to many phenotypes associated with mutations affecting these kinases.

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Disclosures

The authors have no financial conflict of interest.

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