Focal Adhesion Kinase Negatively Regulates Lck Function Downstream of the T Cell Antigen Receptor

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Focal Adhesion Kinase Negatively Regulates Lck Function Downstream of the T Cell Antigen Receptor

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Focal adhesion kinase (FAK) is a critical regulator of signal transduction in multiple cell types. Although this protein is activated upon TCR engagement, the cellular function that FAK plays in mature human T cells is unknown. By suppressing the function of FAK, we revealed that FAK inhibits TCR-mediated signaling by recruiting C-terminal Src kinase to the membrane and/or receptor complex following TCR activation. Thus, in the absence of FAK, the inhibitory phosphorylation of Lck and/or Fyn is impaired. Together, these data highlight a novel role for FAK as a negative regulator TCR function in human T cells. These results also suggest that changes in FAK expression could modulate sensitivity to TCR stimulation and contribute to the progression of T cell malignancies and autoimmune diseases. The Journal of Immunology, 2013, 191: 000–000.

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uman T cells control the extent of the adaptive immune response following infection and in many pathological conditions (1–6). T cells are activated upon stimulation of the TCR by peptide-bound MHC complexes in combination with one or more costimulatory receptors (2). Constitutively active Lck is recruited to the TCR complex after Ag stimulation, where it phosphorylates ITAMs found in multiple TCR subunits (2, 7, 8). This event is critical for ZAP70 activation (2). The adaptor proteins, linker for activation of T cells (LAT) and SLP-76, are then phosphorylated by ZAP70. Together, LAT and SLP-76 recruit and control the activation of multiple effector proteins such as phospholipase C (PLC)-γ1 and PI3K, thereby triggering downstream signaling events like calcium influx and Akt activation (2, 9, 10). TCR activation culminates in transcriptional and morphological changes that regulate cytokine production, receptor expression, and the migratory properties of T cells (2).

The phosphorylation of Lck Y394 and Y505 controls Lck enzymatic activity to prevent inappropriate T cell responses. Lck Y505 phosphorylation stabilizes the protein in a closed, inactive conformation, which limits TCR function (11–15). This tyrosine residue is phosphorylated by C-terminal Src kinase (Csk) and dephosphorylated by CD45 (11). The activity of Lck is also enhanced by the trans autophosphorylation of Y394, a residue found in the activation loop of the kinase domain (8, 11). Importantly, increases in Lck Y394 and decreases in Y505 phosphorylation are correlated with enhanced Lck activity (11). Thus, Lck activity is dictated by the balance of Lck Y394 and Y505 phosphorylation, and perturbations in the phosphorylation ratio of these two residues can increase or decrease TCR-induced signaling and T cell activation.

To phosphorylate Lck Y505, cytoplasmic Csk is recruited to the T cell membrane, a process that is vital for its function (12, 16–18). The current model is that in unstimulated T cells Csk binds to phospho-Y317 on phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), also known as Csk-binding protein (18, 19–23). This interaction localizes Csk to the plasma membrane and enhances its catalytic function, which allows Csk to phosphorylate Lck Y505 (24). Upon TCR activation, PAG/Csk-binding protein is dephosphorylated, after which Csk is transiently displaced from detergent-insoluble membrane lipid rafts (18, 22). This transient displacement allows CD45 to dephosphorylate Lck Y505, resulting in the enhanced enzymatic function of Lck (11, 25). Within 5 min after TCR activation, Csk reassociates with lipid rafts, presumably because PAG Y317 is rephosphorylated (18, 19). However, contrary to this model, Lck Y505 phosphorylation remains unchanged or increases after TCR stimulation (7, 8, 12, 26). Furthermore, the observation that PAG-deficient T cells do not have enhanced T cell activation suggests that alternative mechanisms exist to regulate Csk’s recruitment to the membrane after TCR activation (20, 21, 27). Therefore, the mechanisms that regulate Csk’s recruitment to the membrane after TCR stimulation are not clear.

Actin cytoskeletal responses are essential for cytokine release and cellular spreading downstream of the TCR (28, 29). Focal adhesion kinase (FAK) is phosphorylated by Lck and/or Fyn upon TCR induction (30, 31). Previously, FAK was found to control cellular processes linked to actin polymerization. In line with this role, inhibiting FAK’s expression or function in T cells, B cells, macrophages, and neutrophils impaired actin-dependent processes such as adhesion or spreading (31–35). Thus, the observation that FAK regulates actin-dependent responses is likely to have important implications in TCR function. However, because FAK is expressed at low levels in human T cells compared with B cells (36), it may serve an alternative function downstream of the TCR.

In this study, we show that the transient knockdown of FAK results in enhanced or extended TCR-induced signal transduction, cytokine production, and CD69 expression in Jurkat E6.1 cells and
CD4 human activated peripheral blood T cells (hAPBTs). Using total internal reflection fluorescence (TIRF) microscopy and immunoprecipitations, we found that Csk recruitment to the membrane and TCR complex following TCR induction requires FAK expression. After TCR activation, FAK-deficient cells also displayed decreased Lck Y505 phosphorylation and increased Lck Y394 and TCRζ-chain phosphorylation. Together, these findings demonstrate that FAK negatively regulates TCR signaling by controlling Lck activation.

Materials and Methods

Human samples

All human subject studies were in compliance with the Declaration of Helsinki. Healthy donors between the ages of 18 and 55 y old were recruited by the DeGowin Blood Center at the University of Iowa’s Hospitals and Clinics. These donors provided written consent for their blood products to be used in research projects conducted at the University of Iowa in compliance with the University of Iowa’s Institutional Review Board. Whole blood was passed through leukocyte reduction system cones (37), and these completely deidentified cones were then provided to investigators at the University of Iowa. Because the blood products from LRS cones were normally discarded, we did not require further Institutional Review Board approval to use these blood samples.

Reagents and Abs

RPMI 1640, t-glutamine, penicillin-streptomycin, and PBS were purchased from Life Technologies. The FBS was obtained from Atlanta Biologicals and Denville. Human rIL-2 used for ELISA was purchased from R&D Systems, whereas the human rIL-2 used in growth media was obtained from AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: human rIL-2 from M. Gately (Hoffman-La Roche) (38). The anti-human γc TCR, PE/Cy5 anti-CD69, Dylight 488 goat anti-mouse IgG1, DyLight 488 donkey anti-rabbit IgG, anti-CD3 (OKT3), and anti-CD4 (RPA-T4) Abs were obtained from BioLegend. The anti-Akt pS473, Alexafluor 568 anti-rabbit, and Alexafluor 568 anti-mouse IgG1 Abs and the magnetic Dynabeads were from Invitrogen. The goat anti-mouse IgG Ab was acquired from Rockland International. The Fak inhibitor, PF-573228, was purchased from Tocris Bioscience. Criterion Prestain 4–15% polyacrylamide gels were purchased from Bio-Rad. Oligonucleotides were generated at Integrated DNA Technologies. All chemicals used were research grade and purchased from a variety of sources.

Plasmids

The Luc microRNA (miRNA) cassette was a gift of A. McCaffrey (TriLink BioTechnologies). Sequences for Fak-specific miRNAs were selected (Supplemental Table I) and used to replace the Luc targeting sequence, as previously described (39). Two copies of the Luc and Fak 3183 miRNAs were PCR amplified and ligated into the pVetLeGFP vector developed by the University of Iowa’s Gene Vector Transfer Core. The Luc miR- and Fak miR-GFP reporter lentiviruses were generated by the Gene Vector Transfer Core at the University of Iowa.

Transfection and stimulation of Jurkat E6.1 cells

Jurkat E6.1 T cells were grown to a density of 3–4 × 10^5 cells/ml in complete RPMI (RPMI 1640 supplemented with 10% FBS, 2 mM t-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin). Cells were transfected with Luc or Fak miRNA or the various FAK knockdown/reexpression vectors (5 µg per 5 × 10^6 cells) using the Amaxa Cell Line Nucleofector Kit V (Lonza) and program X-001 and then incubated for 72 h. Prior to stimulation, cells were grown to a density of 2–5 × 10^6 cells/ml. Cells were stimulated with 10 µg/ml soluble anti-CD3 for various times, as previously described (9, 40–43).

Isolation and growth of CD4 hAPBT

PBMCs were obtained from LRS cones (37), as previously described (44). CD4 T cells were purified from nonadherent PBMCs by negative selection...
using the human CD4 T cell isolation kit (Stem Cell Technologies). Cells were routinely >95% pure. Human CD4 T cells then were activated with anti-CD3 and anti-CD28 beads in the presence of 100 U/ml IL-2.

**FAK knockdown and stimulation of CD4 hAPBT**

Purified CD4 T cells were activated for 3 d with the Ab-coated beads. The cells were then resuspended at 2 × 10^5 cells/ml in complete RPMI plus 100 U/ml rIL-2 and 8 μg/ml polybrene. Luc or Fak miRNA-containing lentivirus were then incubated with the cells (multiplicity of infection 2) for 24 h. The virus was removed, and the cells were incubated for 48–72 h. These cells were then stimulated by cross-linking with 2 μg/ml anti-CD3 and 2 μg/ml anti-CD4, as previously described (40, 41, 43). For imaging experiments, CD4 T cells were activated for 5–7 d with the Dynabeads as above, and transfections were carried out as described for the Jurkat cells.

**Immunoblotting**

Proteins in cellular lysates were separated by PAGE using 4–15% Criterion gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat dry milk, 1% BSA, or in SEA BLOCK Blocking Buffer (Thermo Scientific) diluted 1:1 in PBS. Immunoblotting was performed, as described (9, 41, 43). Ab binding was detected using ECL and a Fiji imager or using the Licor Odyssey Infrared Imager. Pan Abs do not always recognize the phosphorylated forms of TCR signaling proteins, including those for LAT and SLP-76 (40). Therefore, stripped membranes were reprobed with a pan-actin Ab to ensure equivalent protein loading.

**Analysis of immunoblotting**

The relative intensity of bands was determined using the gel-plotting macro of ImageJ or the analysis software in the Odyssey v3.0 program. Normalization of the phospho-protein intensity to the actin intensity was conducted, as we have described previously (40–43). Upon normalization, the percentage of maximal Luc phosphorylation was calculated as follows: % maximum Luc phosphorylation = (normalized intensity of time point ÷ normalized intensity of maximum Luc time point) × 100%. The average percentage from each least three independent experiments ± SEM for every time point was then plotted using Microsoft Excel.

**Correlation analyses**

Human CD4⁺ T cells were isolated by negative selection and activated using anti-CD3– and anti-CD28–coated beads in the presence of rIL-2. On the indicated days, equal numbers of these cells were lysed using hot 2× sample buffer and were boiled and sonicated, as described above. The expression of FAK, Csk, phospho-Lck Y505, and/or Pyk2 was detected by immunoblotting and normalized to actin expression in each lane. These normalized values obtained from each independent experiment were used to produce the scatter plots shown in Fig. 10E and 10G. To generate the graphs shown in Fig. 10F and 10H, equal numbers of freshly isolated CD4⁺ T cells (unstimulated CD4 T cell), unstimulated Jurkat E6.1 cells, unstimulated HuT78 T cells, and CD4⁺ T cells that were activated for 6 d using the anti-CD3– and anti-CD28–coated beads plus rIL-2 (activated CD4 T cell) were lysed with hot 2× sample buffer. Each of these samples was loaded in duplicate onto a polyacrylamide gel, and immunoblotting was used to detect differences in FAK, Csk, and phospho-Lck Y505 expression.

**FIGURE 2.** FAK controls the magnitude of early TCR-mediated signaling in Jurkat T cells. Control and FAK-deficient Jurkat cells were stimulated with soluble anti-TCR for various times. (A) Total changes in TCR-induced phosphorylation were analyzed by immunoblotting with antiphosphotyrosine (Y). (B) The expression of FAK, PLC-γ1 SLP-76, Lck, Csk, LAT, or actin was analyzed by immunoblotting. (C) The site-specific phosphorylation of LAT Y191, SLP-76 Y128, and PLC-γ1 Y783 in TCR-stimulated control or FAK-deficient Jurkat T cells was analyzed by immunoblotting. (D) The percentage of phosphorylation for LAT Y191, PLC-γ1 Y783, and SLP-76 Y128 compared with the luciferase miRNA-transfected cells was calculated, as described in Materials and Methods. The mean of three to four experiments ± SEM is shown. *p ≤ 0.05, **p ≤ 0.01.
in each sample. The densitometric intensity for FAK, Csk, and phospho-Lck Y505 in each lane was then normalized to actin expression. To generate the plots shown in Fig. 10F and 10H, the average expression of the duplicate samples was first calculated. Next, the average of these duplicate values from each of the four independent samples was determined and graphed. The Pearson correlation coefficient and linear regression analysis were used to evaluate the relationship between FAK or Csk expression and Lck Y505 phosphorylation.

Immunoprecipitations

Control or FAK-deficient cells were stimulated with 2 μg/ml soluble anti-CD3 for various times. These cells were lysed with immunoprecipitation lysis buffer, and Csk was then immunoprecipitated using 2 μg anti-Csk Ab and protein A/G beads, as described (9, 40). To precipitate the TCR complex from CD4 hAPBTs, the cells were treated with soluble anti-CD3 and anti-CD4 with or without anti-mouse IgG, and the samples were lysed as above. The protein A/G beads were then added to the supernatants and rotated overnight. Bound proteins were eluted using 25 μl hot 2× buffer, and samples were boiled at 95˚C for 4 min.

Cytokine production

Control and FAK-deficient cells were washed and resuspended at 6 × 10⁵ cells/ml in complete RPMI and stimulated with various doses of plate-bound OKT3 for 24 h. The production of IL-2 or IFN-γ was determined by standard tetramethylbenzidine sandwich ELISA. The absorbance at 490 nm was measured using the Epoch plate reader (Biotek). Data were then normalized to account for the variations between human donors and/or independent experiments. The relative amount of cytokine that was produced in each sample was determined using this formula: percentage of maximum cytokine value in Fak miRNA-treated sample = (concentration of sample / maximum concentration in Fak miRNA-treated sample) × 100%. The mean ± SEM from three to four independent experiments was then calculated.

CD69 expression

Control and FAK-deficient Jurkat cells were suspended at 1 × 10⁷ cells/ml in RPMI 1640 and stimulated with various doses of anti-CD3 for 4 h at 37˚C. Cells were washed with FACS buffer and stained on ice with PE/Cy5 anti-human CD69 for 30 min. Samples were collected on an Accuri flow cytometer. The MFI of each sample was determined using the Accuri Cflow Plus software. The data were normalized such that CD69 expression at 0 min was equal to 1, and the average normalized value from three experiments ± SEM was plotted using Microsoft Excel.

Cellular imaging

Control or Fak-deficient T cells were stimulated on glass chamber slides coated with anti-CD3 (45). Cells were fixed with 3.2% paraformaldehyde and permeabilized with 0.25% Triton X-100. After blocking, primary Abs against ZAP70 pY319 or total Csk were then diluted in SEA BLOCK buffer prepared in PBS and incubated overnight at 4˚C. Secondary Abs were also incubated overnight at 4˚C. The images were obtained using the Leica AM TIRF MC imaging system found in the University of Iowa’s Central Microscopy Core. Images from 6–12 randomly selected fields per treatment were obtained using the 100X oil objective and a TIRF alignment of 150 nm at room temperature. The GFP-TIRF or mCherry-TIRF laser intensity and exposure times were set using the control cells stimulated for 3 min for the Jurkat cells or 7.5 min for CD4 hAPBTs, and these settings were kept constant for the remaining samples. Alternatively, the cells were stained using FITC-conjugated phalloidin overnight at 4˚C and imaged using the 100X objective and the GFP-epifluorescence filter. All images were acquired using the Leica AF software and processed using the Fiji software package.

Imaging analysis

All image analyses were performed using the Coloc 2 plug-in for the Fiji software package. To determine percentage of colocalization, 25 randomly selected cells from each of the four independent Jurkat or three independent

FIGURE 3. The kinetics or extent of TCR signaling is altered in CD4 hAPBTs that lack FAK. (A) CD4 hAPBTs were transduced with a lentivirus containing miRNAs against luciferase (Luc) or FAK. The cells were then stimulated with soluble anti-CD3 and anti-CD4 cross-linking for various times. The site-specific phosphorylation of LAT Y191, SLP-76 Y128, and Akt S473 was analyzed by immunoblotting. The expression of FAK, Pyk2, and actin was also examined. (B) Percentage of phosphorylation for LAT Y191, SLP-76 Y128, and Akt S473 relative to the luciferase miRNA-transduced cells is depicted. Data are shown as mean of three experiments ± SEM. *p ≤ 0.05, **p ≤ 0.01.
CD4 haPBT experiments were analyzed. The Manders correlation coefficients were then determined and plotted using GraphPad Prism 5. Any cell that had a Costes p value <0.9 was excluded from further analysis to reduce the likelihood of false-positive colocalization. The mean channel intensity from these cells was also recorded to determine the amount of Csk and ZAP70 phosphotyrosine 319 staining. Stimulated cells that were stained with the secondary Abs alone served as the negative control for these experiments.

**Statistical analysis**

Statistical analysis was performed in Microsoft Excel using a two-tailed t test assuming equal variance.

**Results**

*miRNAs repress FAK expression in human T cells*

To elucidate whether FAK is required for TCR function in mature human T cells, we transiently suppressed FAK expression using miRNAs. Seven RNA interference sequences were selected and used to replace the endogenous targeting sequences of human miR30 (39). We then tested the silencing efficacy of these sequences by transiently transfecting the Jurkat E6.1 human T cell line. By 72 h after transfection, only the sequence corresponding to nt 3183–3202 inhibited FAK protein expression by >90% compared with the control sequence against firefly luciferase (Luc miRNA) (Fig. 1A). As shown in Fig. 1B, FAK knockdown was detectable at 48 h and maximal suppression occurred at 72–96 h after transfection.

Pyk2 is also expressed in human T cells and is activated by TCR stimulation (43, 46). Pyk2 shares 45% amino acid identity to FAK and is overexpressed in FAK−/− cells, where it compensates for multiple cellular functions (47–49). We found that transient knock-down of FAK in Jurkat cells did not affect Pyk2 expression, nor were Lck, Csk, SLP-76, LAT, Grb2, and PLC-γ1 protein levels altered (Fig. 1C, 1D). Thus, the Fak 3183 miRNA selectively and potently inhibits FAK expression in Jurkat cells.

**FAK negatively regulates TCR-induced signaling in Jurkat cells and CD4 haPBTs**

Because FAK appears to integrate receptor-mediated signals that control actin reorganization in immune cells (32–35), we examined actin polymerization upon TCR induction. Jurkat cells expressing the Luc or FAK-specific miRNAs were stimulated on glass chamber slides treated with anti-TCR and stained to detect actin filaments that form upon T cell spreading (45). As shown in Supplemental Fig. 1A and 1B, the intensity of phalloidin staining and the degree of cellular spreading as measured by changes in cell area were comparable in control and FAK-deficient Jurkat cells. Therefore, it does not appear that TCR-induced actin remodeling that drives cellular spreading requires FAK.

The actin cytoskeleton also regulates TCR expression on resting and activated T cells (29). As such, we next examined TCR levels by flow cytometry. As shown in Supplemental Fig. 1C, the steady-state expression of the TCR was comparable in both cell lines. Additionally, no differences in the loss of TCR surface expression were observed following receptor activation in the control or FAK-deficient cells (Supplemental Fig. 1D). Thus, basal TCR expression and ligand-induced TCR downregulation are normal in the absence of FAK.

The actin cytoskeleton network also regulates signaling downstream of the TCR (28, 29). Therefore, we next investigated whether

![FIGURE 4](https://www.jimmunol.org/)

**FIGURE 4.** TCR-induced effector functions are enhanced in FAK-deficient T cells. 
(A) CD69 expression in control or FAK-depleted Jurkat cells stimulated for 4 h with the indicated doses of soluble OKT3. The graph shows the mean fold change over the unstimulated samples ± SEM from three independent experiments. (B) Control or FAK-deficient Jurkat cells were stimulated for 24 h with various doses of plate-bound anti-TCR, and IL-2 production was measured by ELISA. The mean normalized value ± SEM from three independent experiments is shown. (C) CD4 haPBTs were transduced with luciferase (Luc) or Fak-specific miRNAs. Cells were then stimulated with various concentrations of plate-bound anti-TCR for 24 h. The production of IL-2 and IFN-γ was measured by ELISA. The mean normalized value ± SEM of at least three independent replicates is shown.
TCR-induced signaling was altered in the absence of FAK. To that end, control and FAK-deficient Jurkat cells were stimulated for various times with a soluble, agonistic anti-TCR Ab. We then examined broad changes in early TCR-induced signaling using an antiphosphotyrosine immunoblot. Surprisingly, TCR-inducible signaling appeared to be enhanced in the FAK-deficient Jurkat cells as measured by an antiphosphotyrosine immunoblot (Fig. 2A). This global increase in tyrosine phosphorylation was not due to altered protein expression of Lck, LAT, SLP-76, PLC-γ1, or Csk after TCR induction (Fig. 2B). To confirm these findings, we employed a quantitative method to examine changes in TCR-induced signaling using phospho-specific Abs and immunoblotting (42). Following activation with the stimulatory anti-TCR Ab, the site-specific phosphorylation of LAT Y191, PLC-γ1 Y783, and SLP-76 Y128 was examined. As shown in Fig. 2C and 2D, the phosphorylation kinetics of LAT, SLP-76, and PLC-γ1 were comparable between Luc and Fak miRNA-treated Jurkat cells, with maximum phosphorylation occurring at 1–2 min after stimulation and returning to baseline by 15 min. The magnitude of the phosphorylation of these proteins was strikingly different, however. When FAK expression was silenced in Jurkat cells, the site-specific phosphorylation of LAT, PLC-γ1, and SLP-76 was enhanced by 2- to 3-fold at 1–5 min after TCR stimulation (Fig. 2C, 2D). These data demonstrate that FAK suppresses the level of early TCR-mediated signaling in Jurkat cells.

In Jurkat cells, many proximal signaling molecules including PLC-γ1 and Pyk2 have exaggerated TCR-induced activation compared with hAPBTs (40). Because of these differences, we also examined whether FAK deficiency altered TCR-induced signaling in primary human T cells. Transducing CD4 hAPBTs with GFP-expressing lentiviruses containing the Luc- or FAK-specific miRNAs led to >75% silencing of FAK expression while having no effect on Pyk2 protein levels (Fig. 3A), demonstrating that this suppression vector is also highly specific in primary human CD4 T cells. FAK-deficient CD4 hAPBTs initially displayed lower LAT Y191 phosphorylation after TCR activation. However, the phosphorylation of LAT appeared to last longer in FAK-deficient cells, although this change was not significant (Fig. 3B). The TCR-inducible phosphorylation of SLP-76 was also significantly extended in the

**FIGURE 5.** Recruitment of Csk to membrane and TCR complex is impaired in FAK-deficient Jurkat T cells. (A) Cells were stimulated on anti-TCR–coated glass chamber slides for various times and stained with Abs to detect ZAP70 phosphotyrosine 319 and total Csk. Cells were also stained with secondary Abs alone (US) as a negative control. The images are representative of four independent experiments. The white scale bar is equal to 5 μm. (B) For every experiment, 25 cells from each time point were analyzed using the Fiji analysis software. The intensity of Csk and ZAP70 phosphotyrosine 319 staining from 100 cells analyzed from four independent experiments was plotted. The horizontal line depicts the mean value (top graphs). The Manders correlation coefficients were used to calculate Csk and phospho-ZAP70 Y319 colocalization. The Manders coefficients from 100 total Jurkat cells analyzed from four independent experiments were plotted. The horizontal line represents the mean value (bottom graphs). The p values reflect the statistical differences between the control and FAK-deficient cells at the indicated times. n.s. means p > 0.05.
absence of FAK (Fig. 3B). We next determined whether Akt phosphorylation was altered, because this kinase regulates many T cell functions, including cytokine production (50). Interestingly, the phosphorylation of Akt S473 was significantly enhanced in FAK-deficient cells compared with the control cells (Fig. 3B). Together, these data reveal that TCR-induced signaling is enhanced and/or prolonged in the absence of FAK, suggesting that FAK feedback inhibits TCR-mediated signaling in human T cells.

**FAK controls the threshold of TCR activation in human T cells**

We next addressed whether the changes in TCR-induced signaling described above altered downstream functions in human T cells. To that end, we measured differences in CD69 expression and cytokine production. As shown in Fig. 4A, Jurkat cells treated with the Luc miRNA showed a modest upregulation in the expression of CD69 when stimulated with 100 ng/ml soluble anti-TCR. CD69 expression in these cells had increased CD69 by ~2-fold after stimulation with 250 ng/ml anti-TCR. By comparison, CD69 upregulation in FAK-deficient cells was similar to the control cells following stimulation with 2- to 4-fold lower doses (25–50 ng/ml) of anti-TCR. The control and Fak miRNA-treated cells showed similar CD69 responses at 250 ng/ml anti-TCR (Fig. 4A). We also found FAK-deficient Jurkat cells produced 2- to 3-fold more IL-2 production after stimulation with 0.5–1 μg/ml anti-TCR (Fig. 4B).

**FIGURE 6.** Csk membrane and TCR localization are altered in FAK-deficient CD4 hAPBTs. (A) CD4 hAPBTs were transfected with the Luc or FAK-specific miRNAs and incubated for 72 h. The cells were stimulated for 7.5 min with anti-TCR coated onto glass chamber slides. These cells were then stained with anti–phospho-ZAP70 Y319 and anti-Csk, followed by the appropriate secondary Abs. Representative cells from three separate human donors are shown. The white scale bar is equal to 5 μm. (B) The intensity of Csk and ZAP70 pY319 staining and colocalization between these proteins was analyzed as in Fig. 9. The values from 75 cells taken from three independent experiments are shown, and the mean values are represented by the horizontal lines. The p values reflect the statistical differences between the control and FAK-deficient cells at the indicated times. n.s. means p > 0.05.
FAK-deficient CD4 hAPBTs secreted ∼5-fold more IL-2 and ∼2-fold more IFN-γ following stimulation with 0.25–0.5 μg/ml anti-TCR. No significant differences were observed at the 1 μg/ml dose (Fig. 4C). Thus, TCR induction occurs at lower doses of stimulation in the absence of FAK, suggesting that this protein fine-tunes the responsiveness of T cells to TCR activation.

The localization of Csk is altered in FAK-deficient cells

In other cell types, FAK associates with Csk (51–53). TCR-induced signaling is enhanced in Csk-deficient human T cells, and these cells are also more responsive to TCR induction (12, 13). Because this phenotype is strikingly similar to what we observed in FAK-deficient human T cells, we investigated whether FAK controlled Csk function in human T cells. Csk is active when it is recruited to the plasma membrane (16–18). To assess whether Csk was differentially recruited to the T cell membrane after TCR stimulation, we used TIRF microscopy. This type of microscopy will only excite proteins labeled with fluorochromes when they are within 100–200 nm from the cell-chamber slide interface, and is therefore a useful way to track the recruitment of cytoplasmic proteins to the membrane (54). As seen in Fig. 5, Csk was detectable in the membranes of TCR-stimulated control Jurkat cells at 3 min after receptor activation, and its membrane expression increased over time. These results are consistent with previous membrane fractionation studies (17, 18). Csk was also found in the membrane of the control CD4 hAPBTs after TCR stimulation (Fig. 6). Strikingly, there was a significant decrease in the intensity of Csk staining after TCR stimulation in FAK-deficient Jurkat cells and CD4 hAPBTs (Figs. 5, 6). We also analyzed phospho-ZAP70 Y319 staining as an internal staining control, and we observed no detectable differences in the amount of phospho-ZAP70 Y319 that was present in the membrane fractions (Figs. 5, 6). The latter observation is most likely due to the fact that ZAP70 turnover at the TCR is very rapid at these times (55). We did not select for FAK-deficient cells in these assays; therefore, those cells that had high Csk intensity were most likely still FAK sufficient. These results show that Csk membrane recruitment is defective in FAK-deficient T cells.

Csk interacts with the TCR complex (56, 57). Therefore, we next determined whether the Csk and TCR association was mediated by FAK. Using phospho-ZAP70 Y319 as a surrogate marker for the TCR (55), we found that a small fraction (15–20%) of membrane-associated Csk colocalized with phosphorylated ZAP70 at 3–7.5 min after TCR stimulation. Similarly, 25–30% of phosphorylated ZAP70 colocalized with Csk in Jurkat cells (Fig. 5B, bottom graphs). When FAK expression was suppressed, the amount of phosphorylated ZAP70 that colocalized with Csk was significantly decreased at 5–7.5 min after stimulation (Fig. 5). Similar results were also obtained using CD4 hAPBTs, although there was substantially more colocalization between Csk and phosphorylated ZAP70 in these cells (Fig. 6). Jurkat cells are of thymocyte origin and do not express CD4 (40), which may explain these differences. These data indicate that Csk recruitment to the TCR complex is also impaired in the absence of FAK.

The results described above suggest that FAK associates with Csk at the TCR. To test this possibility, we stimulated CD4 hAPBTs for various times and pulled down the TCR/CD4 complex using the stimulatory anti-CD3 and anti-CD4 Abs in the absence or presence of an anti-mouse cross-linking Ab. We found that FAK and Csk coimmunoprecipitated with the TCR/CD4 complex (Fig. 7A). Consistent with published data (11, 51, 58, 59), Lck was also associated with the TCR/CD4 complex (Fig. 7A). Interestingly, FAK and Lck were found to coimmunoprecipitate with the TCR/CD4 complex in the absence of cross-linking Ab, suggesting that these proteins constitutively associate with CD3 and/or CD4 in CD4 hAPBTs. At the 0-min time point, the addition of the cross-linking Ab caused Csk to associate with the TCR/CD4 complex. This increase in Csk’s association with the TCR/CD4 complex could be the result of postlysis stimulation. These data may also indicate that, upon cross-linking CD3 with CD4, the association between Csk and CD3 is stabilized (57), allowing us to detect the interaction by immunoprecipitation. We also attempted to verify these interactions using TIRF microscopy; however, FAK-specific Abs detected many nonspecific bands and could therefore not be used for imaging studies (Supplemental Fig. 2 and N. Chapman and J. Houtman, unpublished observations). These data show that FAK is recruited to the TCR/CD4 complex, where it associates with Lck and its inhibitor Csk. Thus, FAK could regulate the function of these kinases.

FAK, Csk, and Lck formed a stable complex in human T cells, and FAK-deficient T cells displayed hyperactive TCR function similar to Csk-deficient T cells. Therefore, FAK could serve as a scaffolding protein to recruit Csk to its substrate, Lck. To test this possibility, we stimulated control and FAK-deficient Jurkat cells with soluble anti-TCR for various times and performed Csk immunoprecipitations. We found that a cellular fraction of Lck was bound with Csk in TCR-stimulated cells. Strikingly, the TCR-inducible association between Lck and Csk was greatly reduced in the absence of FAK (Fig. 7B). Thus, FAK facilitates complex formation between Csk and Lck after TCR induction.

The TCR-inducible phosphorylation and function of Lck are altered in FAK-deficient T cells

The recruitment of Csk to the membrane, the TCR, and/or Lck was defective in FAK-deficient T cells (Figs. 5, 6). In Csk-deficient T cells, the phosphorylation of Lck Y394 and Y505 is enhanced and reduced, respectively, and these alterations result in enhanced Lck activity (12, 13). To examine whether the defects in Csk localization were correlated with differences in Lck phosphorylation, we stimulated control or FAK-deficient Jurkat cells and CD4 hAPBTs and examined the phosphorylation of Lck Y394 and Y505. We used an anti-Src pY416 Ab to detect changes in Lck Y394 phosphorylation, which recognizes all Src kinases when

![FIGURE 7.](http://www.jimmunol.org/)  
(A) Activated CD4 T cells were stimulated by anti-CD3 and anti-CD4 cross-linking for various times. The TCR/CD4 complex was then immunoprecipitated, and the expression of FAK, Csk, and Lck was analyzed by immunoblotting. Data are representative of three independent experiments. (B) Control or Fak-deficient Jurkat T cells were stimulated for various times with anti-CD3. Csk was immunoprecipitated, and its association with Lck was assessed by immunoblotting. Data are representative of three experiments.
they are phosphorylated on their activating sites. Consistent with previous reports (7, 8, 12), both Lck Y394 and Y505 were phosphorylated in unstimulated Jurkat cells and CD4 hAPBTs, and TCR stimulation enhanced the phosphorylation of these sites in the control cells. Strikingly, the levels of Lck Y505 induced by TCR activation were significantly reduced in the FAK-deficient Jurkat cells and CD4 hAPBTs. The phosphorylation of Lck Y394, the autophosphorylation site, was also increased by ~2-fold in the FAK-deficient cells (Fig. 8). This result suggests that Lck’s enzymatic activity is enhanced in FAK-deficient cells. Thus, the defects in Csk recruitment observed in FAK-deficient cells are correlated with decreased Lck Y505 and increased Y394 phosphorylation.

In Csk-deficient Jurkat cells, TCRζ-chain phosphorylation occurs with faster kinetics and is modestly elevated at early times after TCR stimulation (13). Therefore, we also examined whether TCRζ Y142 phosphorylation was altered in FAK-deficient Jurkat cells. In the Jurkat cells expressing the Luc-specific miRNA, the site-specific phosphorylation of TCRζ Y142 was detected in unstimulated cells and increased by ~40% after 15 min of stimulation (Fig. 9A, 9B). By comparison, the phosphorylation of TCRζ Y142 was enhanced by ~1.5-fold in the FAK-deficient T cells at 0–5 min after TCR stimulation (Fig. 9A, 9B). Thus, FAK-deficient T cells have higher levels of TCRζ phosphorylation in resting conditions and early after TCR stimulation. TCRζ Y142 phosphorylation was comparable in the control and FAK-deficient T cells >5 min after receptor activation. Because ITAM phosphorylation appears to be the limiting factor that mediates ZAP70 recruitment to the TCR (60), these data may also explain why no significant differences in phospho-ZAP70’s membrane recruitment were observed by TIRF microscopy (Figs. 5, 6). Collectively, these results suggest that Lck’s function is enhanced in FAK-deficient T cells and that FAK-deficient T cells are more poised for TCR activation, resulting in increased TCR-dependent signaling and function.

**FAK expression correlates with Lck Y505 phosphorylation in human T cells**

FAK expression has been reported to increase upon T cell activation (61). We found that FAK protein levels were upregulated by 3- to 10-fold in primary CD4 T cells that were stimulated for 1–4 d with anti-CD3 and anti-CD28, whereas Pyk2 levels remained unchanged (Fig. 10A, 10B). The human T cell lines Jurkat E6.1 and HuT78 also expressed slightly more FAK protein relative to primary CD4 T cells (Fig. 10C, 10D), indicating that FAK-dependent functions may be differentially regulated in these cell lines compared with primary human CD4 T cells.

Like FAK expression, Lck Y505 phosphorylation was also increased in activated CD4 hAPBTs (Fig. 10A, 10B). Basal Lck Y505 phosphorylation was also augmented in HuT78 T cells and comparable in the control and FAK-deficient T cells >5 min after receptor activation. Because ITAM phosphorylation appears to be the limiting factor that mediates ZAP70 recruitment to the TCR (60), these data may also explain why no significant differences in phospho-ZAP70’s membrane recruitment were observed by TIRF microscopy (Figs. 5, 6). Collectively, these results suggest that Lck’s function is enhanced in FAK-deficient T cells and that FAK-deficient T cells are more poised for TCR activation, resulting in increased TCR-dependent signaling and function.

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FAK INHIBITS TCR FUNCTION

In this study, we evaluated the role that FAK plays in TCR-induced effector function in human T cells. Surprisingly, we found that TCR-mediated signaling is enhanced or extended and that TCR-induced effector functions are augmented in Jurkat cells and CD4 hAPBTs that lack FAK. It appears that FAK controls the function of the Lck by recruiting Csk to the membrane and/or receptor complex in TCR-stimulated cells (Supplemental Fig. 3). These studies reveal that FAK regulates Csk activity and Lck/Fyn function downstream of the TCR and, to our knowledge, demonstrate for the first time that FAK is a negative regulator of TCR function.

While this manuscript was under review, Wiemer et al. (31) published a report examining how FAK controlled CD4 T cell activation. To do so, FAK’s function was impaired using PF562271, an inhibitor that suppresses the catalytic function of both FAK and its related kinase, Pyk2. In contrast to the results we obtained using FAK-deficient human CD4 T cells, treatment with PF562271 appeared to suppress proximal TCR-mediated signaling and proliferation. Wiemer et al. (31) used doses of PF562271 that were substantially higher than those required to suppress FAK’s enzymatic activity in vitro and in cell-based assays (62). This is a major limitation of their study, as higher doses of PF562271 have been reported to suppress the in vitro catalytic functions of Pyk2, cyclin-dependent kinases, Fyn, and Lck (62). To confirm the results of their inhibitor experiments, this group also conditionally deleted FAK in murine CD4 T cells, which resulted in a ~65% knockdown of FAK protein expression. In contrast to the results obtained with PF562271, anti-CD3- and anti-CD28–induced proliferation was normal to elevated in these FAK-deficient murine CD4 T cells (31). Therefore, it is likely that the differences seen in this published study and ours are due to off-target effects of PF562271.

The activation of Lck and Fyn is tightly regulated to prevent inappropriate T cell activation. This regulation is achieved by altering Lck’s localization or phosphorylation (11, 63). Lck Y505 plays a seminal role in inhibiting Lck activity: the in vitro kinase activity of Lck is reduced when Lck Y505 is phosphorylated, and T cells that express a Lck Y505F mutant are hyperactivated (64–66). Defective TCR-inducible Lck Y505 phosphorylation has been observed in hyperresponsive CD4 T cells isolated from patients with acute coronary syndromes (67). Thus, deregulated Lck Y505 phosphorylation can promote T cell hyperactivation. In this study, we showed that the TCR-inducible phosphorylation of Lck Y505 was significantly decreased in the absence of FAK. This defect was also accompanied by an increase in Lck Y394 autophosphorylation (8) and enhanced TCR-induced function. Our data support the idea that Lck Y505 phosphorylation is vitally important to inhibit TCR-induced responses and demonstrate that FAK is a critical regulator of Lck Y505 phosphorylation downstream of the TCR. This inhibitory function may be achieved by suppressing Lck’s catalytic function and/or limiting its accessibility to its protein substrates, including the TCR-γchain (14, 15).

Several reports have demonstrated that Csk regulates TCR function in both the basal and activated states. Indeed, knocking down Csk or inhibiting its function increases TCR-dependent signaling and effector responses (12, 13, 18), demonstrating that this kinase is indispensable for proper mature T cell function. Csk has been reported to bind to the plasma membrane, where it exerts its inhibitory effects on TCR function (12, 16–18). We have confirmed by high-resolution TIRF imaging that Csk is brought to the membrane following TCR activation. Our data suggest that FAK regulates the membrane localization and function of Csk in activated T cells. We found that the translocation of Csk to the membrane following TCR activation was dependent upon FAK. This reduction in Csk protein found in the membrane was correlated with increased Lck Y394 and TCR-γ-chain phosphorylation, decreased Lck Y505 phosphorylation, and increased sensitivity to TCR induction. However, we did not see an increase in the magnitude of ZAP70 Y319 or Erk1/Erk2...
phosphorylation (data not shown). These observations are strikingly similar to those made using Csk-deficient Jurkat cells or human CD4 T cells (13). Thus, FAK and Csk appear to cooperatively inhibit TCR function following its activation.

We also demonstrated that FAK associated with Csk at the activated TCR. Using TIRF microscopy, we also found that a small fraction of the membrane-associated Csk colocalized with phosphorylated ZAP70. Thus, Csk may be actively sequestered from the activated TCR complex, as was recently reported in murine CD8 T cells (56). The Csk and phosphorylated ZAP70 interaction was significantly reduced in FAK-deficient Jurkat cells and CD4 T cells. Therefore, it appears that FAK regulates Csk recruitment to or retention at the TCR, where Csk could inhibit the pool of active Lck that is found at the stimulated receptor (14, 58, 59). Further work is needed to address how FAK regulates Csk’s function. We hypothesize that FAK Y397 serves to localize Csk to the plasma membrane after TCR induction. Interestingly, this site has the consensus-binding motif for the Src homology 2 (SH2) domain of Csk (68). The SH2 domain of Csk has been reported to bind to FAK (51, 53); therefore, Csk may directly interact with FAK Y397. Furthermore, FAK may be recruited to the plasma membrane and/or TCR by directly binding Lck/Fyn or the TCR coreceptor CD4 (69, 70). These hypotheses will be addressed in future experiments.

The current model states that Csk’s function is regulated by the plasma-membrane anchored protein, PAG. According to this model, Csk is associated with PAG via its SH2 domain, which binds to phospho-Y317 on PAG (22, 23). This interaction allows Csk to phosphorylate Lck Y505 to suppress tonic TCR signals to prevent aberrant T cell development or activation (12, 13, 24, 71). After TCR stimulation, the current paradigm suggests that PAG is dephosphorylated, which subsequently releases Csk from the plasma membrane (18, 19, 22). After Csk moves into the cytoplasm, this model then states that CD45 must dephosphorylate Lck Y505 to induce T cell activation (11, 25, 72). Csk is then recruited back into the plasma membrane once activated Fyn phosphorylates PAG Y317. Indeed, PAG overexpression inhibits TCR-dependent signaling and function (19, 22), supporting the idea that PAG is an important regulator of Csk function in T cells.

Although this model persists as the mode by which Csk feedback inhibits TCR signaling, several more recent publications have challenged this paradigm. First, we and others have demonstrated that Lck Y505 phosphorylation does not decrease after TCR activation. Instead, Lck Y505 phosphorylation remains unchanged or modestly increases upon TCR stimulation (7, 8, 12, 26). Second, despite the fact that Lck Y505 is hyperphosphorylated in CD45-deficient T cells, Lck’s catalytic function is actually increased in the absence of CD45, most likely because CD45 also dephosphorylates Lck Y394 (64, 72–74). Finally, Lck is catalytically active when it is phosphorylated on both Y394 and Y505 (8). Thus, Lck Y505 dephosphorylation does not appear to be required to induce Lck’s catalytic function; however, it may serve to localize Lck to the TCR, where it can induce T cell activation (14).
Several reports have also called into question the relevance of the PAG-Csk axis in regulating TCR signaling events. Csk’s SH2 domain associates with PAG, and Csk’s enzymatic function is enhanced when bound to PAG (19, 22); however, the loss of PAG does not appear to limit Csk function. Consistent with this idea, genetic deletion of PAG in murine T cells did not dramatically alter Csk’s localization to lipid rafts or inhibit T cell development or activation (20, 21). Similarly, knocking down PAG in human T cells modestly increased early TCR-mediated signaling events, but actually suppressed downstream effector functions (27, 75). These results are in sharp contrast to Csk-deficient T cells that have developmental defects and enhanced TCR-induced activation (12, 13, 71). Additionally, after it is transiently displaced, Csk appears to be recruited back into the plasma membrane before PAG is phosphorylated (18, 19). These data strongly suggest that PAG is not the primary regulator of Csk function in activated T cells. It is possible that other membrane adaptors like LIME or Dok1 may recruit Csk to the membrane in the absence of PAG (12, 76). Alternatively, PAG may regulate Csk’s function in unstimulated T cells to suppress tonic TCR signaling, whereas other mechanisms may control Csk’s function following TCR activation. Moreover, PAG may only regulate Csk’s function at specific cellular locations distinct from sites of TCR activation. Our data strongly suggest that FAK is a protein that recruits Csk to the TCR complex after Ag stimulation in mature CD4 T cells and that this process suppresses TCR-induced function.

Immune cell activation via Ag receptors is intricately linked to protective immunity against pathogens and in the development of allergic disease and autoimmunity (77–80). Like the TCR, signal transduction downstream of the BCR and FcR is initiated by members of the Src family kinases and suppressed by Csk (11, 81, 82). Our data support a model wherein changes in FAK expression regulate the activity of Lck by recruiting its inhibitor Csk to the TCR complex. Interestingly, we found that FAK associated with both Lck and the TCR/CD4 complex, an interaction that was previously reported for the related proteins Lyn and IgM-BCR (83). Thus, FAK could similarly inhibit BCR function by recruiting Csk to the TCR complex to block Lyn activation. We also showed that changes in FAK expression regulated the Ag sensitivity of the TCR. Similar to our observations, the magnitude of FcR-mediated signaling was higher in cells that expressed low levels of FAK (84). Thus, the expression of FAK may also fine-tune FcR-mediated signaling. Together, these data suggest that FAK may be a universal negative regulator of Ag receptor signaling, a finding that would have broad implications in the treatment of a variety of human disorders.

Collectively, our data demonstrate that FAK is a negative regulator of TCR function in human T cells. The fact that FAK expression appears to fine-tune TCR activation suggests that FAK signaling could be regulated to manipulate T cell responses in different disease states. FAK function could be dampened to enhance T cell responsiveness to low-affinity tumor or pathogen-derived Ags. Moreover, conditions that arise due to hyperreactive T cell responses may benefit from enhancing the function of FAK to downmodulate TCR activation. The balance between Th1- and Th2-driven immune responses could possibly be skewed by altering FAK signaling, because strength of TCR signal controls the polarization of these cells (85). Ultimately, FAK signaling could serve as an important node to amplify or dampen normal and aberrant T cell function in numerous human diseases.

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Disclosures
The authors have no financial interests of conflict.

References


