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Functions of the FAK family kinases in T cells: Beyond actin cytoskeletal rearrangement

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Abstract

T cells control the focus and extent of adaptive immunity in infectious and pathological diseases. The activation of T cells occurs when the T cell antigen receptor (TCR) and costimulatory and/or adhesion receptors are engaged by their ligands. This process drives signaling that promotes cytoskeletal rearrangement and transcription factor activation, both of which regulate the quality and magnitude of the T cell response. However, it is not fully understood how different receptor-induced signals combine to alter T cell activation. The related non-receptor tyrosine kinases focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2) are phosphorylated downstream of the TCR and several costimulatory and adhesion receptors. FAK family proteins integrate receptor-mediated signals that influence actin cytoskeletal rearrangement and effector T cell responses. In this review, we summarize the receptor-specific roles that FAK and Pyk2 control to influence T cell development and activation.

Keywords

T cells; FAK; Pyk2; signal transduction; Csk; cytoskeletal rearrangement

Introduction

T cells are critical regulators of the adaptive immune response to foreign antigens. After an infection occurs, pathogen-derived components promote antigen-presenting cell (APC) activation, which leads to the subsequent processing and presentation of pathogen-derived peptides in the context of major histocompatibility complexes (MHC) [1, 2]. In secondary lymphoid tissues or at sites of infection, APCs interact with T cells that express antigen-specific T cell antigen receptors (TCRs). To facilitate this process, adhesion and costimulatory receptors, including CD28, CD44, leukocyte factor of adhesion (LFA)-1 and very late antigen (VLA)-4, are also activated on T cells [1–5]. The integration of these receptor-mediated signals leads to the secretion of cytokines, alterations in the expression of intracellular and surface proteins and enhancement of T cell proliferation and survival [3, 6].

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These events are critical to promote adaptive immune response-mediated pathogen clearance and to induce T cell memory development. Unfortunately, dysregulation of TCR, adhesion receptor, and/or costimulatory signals also induces aberrant T cell activation that contributes to asthma, allergy, transplanted organ rejection, cardiovascular disease, autoimmune disorders, and cancer progression [1, 2, 4].

Because co-stimulatory and adhesion receptors cooperate with the TCR to induce effective T cell responses, investigators have aimed to identify what proteins mediate TCR and costimulatory/adhesion receptor signaling crosstalk. Focal adhesion kinase (FAK; gene name Protein Tyrosine Kinase 2 (PTK2)) and proline-rich tyrosine kinase 2 (Pyk2; gene name Protein Tyrosine Kinase 2 β (PTK2 β)) are non-receptor tyrosine kinases that are phosphorylated downstream of the TCR and various costimulatory or adhesion receptors, but are also activated by cytokine and chemokine receptors [7–21]. Pyk2 is also called cellular adhesion kinase (CAK) β [22], related adhesion focal tyrosine kinase (RAFTK) [23], calcium-dependent tyrosine kinase (CADTK) [24], and FAK2 [25]. FAK is expressed at various levels in all tissues, while Pyk2 expression is restricted to neurons, epithelial cells, and hematopoietic cells [19, 24, 26]. Hematopoietic cells also express a splice variant of Pyk2 called Pyk2-H [13, 27, 28], which will be discussed in more detail below. Here, we review our current understanding of the roles that FAK and Pyk2 serve in receptor-mediated functions that control T cell development and mature T cell activation.

Structure of FAK and Pyk2

FAK and Pyk2 are 46% identical and 65% similar at the amino acid level. These kinases have an N-terminal four point ezrin/radixin/moesin (FERM) domain, a central tyrosine kinase domain, and a C-terminal focal adhesion targeting (FAT) domain [29]. The FERM domain facilitates the interaction of FAK and/or Pyk2 to growth-factor receptors, phospholipids, and additional proteins that are reviewed elsewhere [30, 31, 29]. The FAT domain binds to the intracellular domains of integrins to directly or indirectly recruit FAK or Pyk2 to focal adhesions [31, 29, 19]. Structural studies have revealed that FAK is auto-inhibited by an intra-molecular interaction between the FERM and kinase domains (Figure 1). This inhibition is released when the FERM domain binds to other proteins and FAK Y397 is phosphorylated [30, 31]. FAK and Pyk2 also have several proline-rich sequences near their FERM and FAT domains that bind SH3 domain-containing proteins, including the adaptor protein, p130Cas and the guanine nucleotide exchange factor (GEF), Vav1 [32, 33]. Pyk2-H, the more abundantly expressed isoform of Pyk2 in immune cells, lacks 42 amino acids and expresses a truncated proline-rich region near its C-terminus [13, 28, 27]. Pyk2-H thus associates with distinct signaling proteins compared to full-length Pyk2 [34, 13].

In addition to modular domains, FAK and Pyk2 have key tyrosine residues that regulate their functions. FAK Y397 and Pyk2 Y402 are found in the linker regions between the FERM and kinase domains. These residues are auto-phosphorylation sites, but other mechanisms also induce their phosphorylation as discussed below. The SFKs, including Src, Lck, and Fyn, bind FAK and Pyk2 on these residues and phosphorylate FAK Y576/577 and Pyk2 Y579/Y580, which are found in the kinase domains of these proteins. These phosphorylation events induce maximum catalytic function. FAK Y925 and Pyk2 Y880 are

found in the FAT domain, and, upon phosphorylation, these sites bind to Grb2 family adaptor proteins. Interestingly, Grb2 has only been demonstrated to interact with Pyk2 in transformed T cells and not primary T cells [19]. In later sections of this review, we highlight additional proteins that bind to these modular domains and phosphotyrosine residues and discuss their relevance to T cell function. Many additional reviews describe in more detail studies examining FAK and Pyk2's structure and interacting partners [19, 30, 31, 35, 29].

Phosphorylation mechanisms and kinetics

Fyn and/or Lck associate with FAK and Pyk2 and control FAK and Pyk2 phosphorylation downstream of the TCR [7–10, 23, 34, 36–38]. In Fyn^{-/-} T cells, anti-CD3 antibody induced phosphorylation of FAK and Pyk2 is reduced [36, 37]. Similarly, after treatment with the SFK inhibitor PP2, the TCR-inducible phosphorylation of Pyk2 Y402 and Y580 is dramatically decreased in the human T cell lines and activated primary human T cells [8–10]. Inhibition of Ca²⁺ influx, PI3K function, actin cytoskeletal reorganization or ZAP-70 expression does not alter TCR-mediated Pyk2 phosphorylation on Y402 or Y580 [10]. Other studies appear to contradict these findings, demonstrating that Ca²⁺ flux, actin cytoskeletal reorganization, PI3K activation, and ZAP-70 kinase function control Pyk2 phosphorylation downstream of the TCR [23, 39, 40]. However, off-target effects of the pharmacological inhibitors used in these studies or contaminating stimulation from integrin receptors likely contributed to the different results.

Recent work has further elucidated how Lck/Fyn activity regulates FAK phosphorylation. In activated human CD4⁺ T cells, FAK is constitutively phosphorylated on Y397, Y576/577, and Y925 [38]. This basal phosphorylation is dramatically reduced upon PP2 treatment, while only Y925 treatment is affected by treatment with PF-562,271, a FAK/Pyk2 dual kinase inhibitor [38]. Moreover, treatment with either PP2 or PF-562,271 reduces the levels of TCR-inducible FAK phosphorylation at all these sites, with the most dramatic effect observed using PP2. Lck and/or Fyn's constitutive kinase activities [41] may maintain a pre-activated pool of FAK that can subsequently phosphorylate FAK Y397 after TCR stimulation. This event would allow Lck/Fyn to bind to this site and phosphorylate FAK Y576/77 and Y925. It is unknown if FAK is constitutively phosphorylated in naïve T cells.

The TCR-inducible tyrosine phosphorylation of FAK and Pyk2 occurs within 1–5 min post-stimulation and is maintained 60–120 min after activation [9, 10, 18]. Interestingly, Pyk2 has two unique bursts of Y402 and Y580 phosphorylation, and these early (within 1–2 min) and late (within 30–60 min) peaks are independently controlled by Lck and/or Fyn activity [9]. The phosphorylation of these sites appears to induce Pyk2's catalytic function, as the Pyk2 substrate paxillin is also phosphorylated with similar magnitude and kinetics as Pyk2 [9]. Anti-CD3 antibody-induced FAK phosphorylation also appears to occur in two phases [18], although the phosphorylation kinetics of individual tyrosine residues have not been examined.

The activation of costimulatory and adhesion receptors also influences FAK and Pyk2 phosphorylation. CD28 is an important costimulatory molecule that induces T cell activation

[1, 2]. Although it is unclear if CD28 ligation alone induces Pyk2 phosphorylation, CD28 activation augments FAK and Pyk2 phosphorylation in response to suboptimal TCR stimulation [32, 40, 42]. Adhesion receptors, including VLA-4, LFA-1, and CD44, facilitate T cell-APC or target cell interactions in secondary lymphoid tissues or at sites of inflammation and also serve as costimulatory receptors for T cells [1, 2, 5, 17, 19, 31, 35, 42–44]. The simultaneous activation of the TCR and LFA-1 or VLA-4 enhances the TCR-induced phosphorylation of FAK and Pyk2 [11, 12, 18, 42]. Activation of the adhesion and costimulatory molecule CD44 also stimulates the phosphorylation of Pyk2 and FAK. It is unknown whether CD44 and TCR signals cooperate to induce Pyk2 or FAK phosphorylation.

Natural ligands or agonistic antibodies against subunits of LFA-1 and VLA-4 induce FAK and Pyk2 activation independently of TCR stimulation [11, 12, 18, 21, 45–48]. Compared to the TCR, anti-LFA-1 antibody-induced phosphorylation of FAK and Pyk2 is delayed, occurring between 10 to 15 minutes after receptor stimulation [48]. Moreover, anti-LFA-1 antibody-induced Pyk2 phosphorylation peaks more rapidly and is more transient than FAK phosphorylation [48]. By contrast, FAK is phosphorylated within 1 minute of fibronectin stimulation; this phosphorylation peaks at 15 min after stimulation and is maintained out to 120 min after fibronectin stimulation [18]. FAK and Pyk2 phosphorylation is also observed after recombinant vascular cellular adhesion molecule (VCAM)-1 and agonistic monoclonal antibody stimulation [45–47].

It has been proposed that integrin activation drives the localized accumulation of FAK and/or Pyk2, leading to the *trans*-autophosphorylation of FAK Y397 or Pyk2 Y402. Subsequently, SFKs will bind to the autophosphorylation site and induce maximum enzymatic function of FAK and Pyk2 [19, 35]. It appears that the cytoskeletal network increases the localized concentration of FAK and Pyk2 after LFA-1 stimulation, as FAK and Pyk2 phosphorylation requires actin and microtubule cytoskeletal stability [48]. Extracellular Ca^{2+} flux also serves to activate LFA-1 function, leading to Pyk2 phosphorylation [49]. Pyk2 phosphorylation downstream of LFA-1 is inhibited by the small GTPase and its effector Rho kinase (ROCK), as these proteins limit localized actin cytoskeletal responses that increase LFA-1 avidity [50]. The cytoskeletal network also appears to regulate FAK and Pyk2 phosphorylation downstream of VLA-4 in T cells. In this regard, VLA-4 induced FAK and Pyk2 phosphorylation are linked to paxillin binding to the α_4 subunit of VLA-4 [47], suggesting paxillin directly recruits FAK and Pyk2 to this receptor to promote their activation. Finally, CD44-induced Pyk2 phosphorylation requires SFK and PLC- γ 1 activity [20]. Together, these studies demonstrate that the activation of multiple receptors can induce the phosphorylation and activation of Pyk2 and Fak, but that different environmental stimuli promote FAK and Pyk2 phosphorylation via distinct mechanisms.

Subcellular localizations of FAK and Pyk2 are regulated by receptor stimulation

Upon TCR-mediated binding to peptide-MHC ligand, the morphology of T cells changes dramatically. First, the T cell membrane forms lamellipodia over the surface of the APC or

target cell. Afterward, integrin-induced adhesion causes the T cell to arrest and adopt a polarized configuration (reviewed in [51, 52]). At the T cell-APC interface, an organized structure called the immunological synapse (IS) is formed. The TCR, adhesion, and costimulatory receptors are localized into defined regions within the IS, and intracellular signaling proteins and cytoskeletal networks facilitate the crosstalk between receptors at this site [51, 52]. We recently demonstrated that FAK coimmunoprecipitates with the TCR-CD4 complex in activated human CD4⁺ T cells, and that FAK colocalizes with LFA-1 in T cells [53]. The TCR-FAK interaction may be mediated by FAK binding directly to Lck/Fyn and/or CD4 [7, 54, 53]. TCR stimulation alone drives Pyk2's re-localization to the IS, while Pyk2 is recruited to this region with faster kinetics and/or more efficiently upon simultaneous TCR and CD28 or LFA-1 stimulation [55, 56]. Upon activation, Pyk2 translocates to the IS, and the recruitment to this sites requires Lck/Fyn, but not Pyk2, catalytic function [57, 55]. Further, translocation of Pyk2 to the IS is also impaired in T cells lacking functional ITAMs on the receptor, Lck, the Tec kinases Itk and Rlk, and to a lesser extent, ZAP-70 [56, 55]. The fact that approximately 50% of Pyk2 still reorients to the IS after anti-CD3 antibody stimulation in ZAP-70-deficient Jurkat T cells suggests that other ITAM-associated proteins associate with Pyk2. At the IS, the scaffolding and/or enzymatic functions of FAK and Pyk2 is likely important to couple multiple receptor-mediated signals to drive efficient T cell responses. We discuss some of these functions later in this review.

The microtubule organizing complex (MTOC) reorients toward the IS upon antigen recognition. In resting CD8⁺ T cells and activated human T cells [57, 49], Pyk2 colocalizes with the MTOC. Upon receiving TCR stimulation, Pyk2 and the MTOC localize near the T cell-APC interface [58, 57, 55]. Paxillin, which constitutively binds to Pyk2's FAT domain [59, 19], may allow Pyk2 to associate with the MTOC [57]. FAK could potentially also localize to this site, as LFA-1 stimulation drives MTOC, FAK, and Pyk2 reorientation in human T cells [48]. Treatment with the protein kinase C (PKC) inhibitor bisindolylmaleimide (BIM)-II modestly suppressed Pyk2 translocation induced by anti-CD3 antibody stimulation [55]; therefore, the activation of PKC θ , PKC η , and/or PKC ϵ may regulate Pyk2 reorientation similarly to their known roles in controlling MTOC polarization [58]. Because the MTOC-associated fraction of Pyk2 is distinguishable from the IS-associated pool of Pyk2 [57], the presence of these two fractions may explain why Pyk2 has two separate bursts of tyrosine phosphorylation after TCR stimulation [9]. Future studies will investigate the mechanisms that drive Pyk2 into two subcellular localizations. Relative to IS-associated Pyk2, MTOC-bound Pyk2 is hypophosphorylated on serine/threonine residues. As protein kinase A promotes and calcineurin inhibits Pyk2 serine/threonine phosphorylation in neurons [60], these two proteins may regulate the functional localization of Pyk2 in activated T cells.

FAK and Pyk2 in T cell development

T cell development occurs in the thymus, where immature T cells, or thymocytes, respond to various environmental stimuli that promote their differentiation and survival. Developing thymocytes progress sequentially from the CD4⁻CD8⁻ double negative stages (DN) to the CD4⁺CD8⁺ double positive (DP) stage to the CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive (SP) stage before exiting the thymus as mature T cells [61]. In the DN and DP stages, pre-TCR

and TCR signaling, respectively, regulate survival, proliferation, and differentiation [61, 62]. TCR stimulation in thymocytes promotes FAK and Pyk2 phosphorylation [36, 63, 37], suggesting that both proteins may serve important roles in thymocyte development. To investigate this possibility, FAK and Pyk2-deficient mice have been generated. While FAK^{-/-} mice are embryonic lethal [64], whole animal Pyk2^{-/-} mice are viable and have no defects in T cell development [65], suggesting that Pyk2 is not a major regulator of TCR signaling in the thymus. FAK's role in thymocyte development is unclear. T cell development is normal in FAK^{+/-} mice and CD4⁺ T cell lineage-specific FAK knockout mice [38, 63]. However, breeding FAK^{+/-} mice with Fyn^{-/-} mice reduces both the phosphorylation and *in vitro* kinase activity of FAK more efficiently than suppression of Fyn or FAK alone [63]. The FAK^{+/-}Fyn^{-/-} mice have 100-fold fewer thymocytes, but higher percentages of CD4 or CD8 SP and DN thymocytes than the parental strains [63]. Thus, incomplete inhibition of FAK function may explain why no defects are seen when FAK expression is suppressed by only 50–60%. Alternatively, Fyn deficiency may also reduce Pyk2's function in the FAK^{+/-}Fyn^{-/-} thymocytes [8–10, 37], which would suggest that these kinases have compensatory roles in T cell development.

Functions of FAK and Pyk2 in mature T cells

Overview of TCR signal transduction and function in mature T cells

TCR stimulation by cognate peptide-MHC ligand drives intracellular signaling, which is initiated by Lck and/or Fyn [1, 2, 41]. These Src family kinases phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) found in the intracellular chains of the TCR. Subsequently, ZAP-70 is recruited to the TCR and activated [1, 2]. ZAP-70 phosphorylates the critical adaptor proteins, LAT and SLP-76 [1, 2, 4]. Several adaptor proteins and enzymes are then recruited to LAT and SLP-76. Phospholipase C- γ 1, which induces Ca²⁺ flux and diacylglycerol (DAG) production, is one such protein. In addition, the Grb2 family of adaptors and phosphatidylinositol-3-kinase (PI3K) proteins, which activate the MAPKs and Akt signaling pathways, respectively, also localize to the LAT/SLP-76 complex. These pathways influence cytokine production, proliferation, and survival. Many actin cytoskeletal-associated proteins also interact with the LAT/SLP-76 complex to induce T cell spreading, adhesion, and migration [1, 2, 4].

FAK and Pyk2 differentially influence functions downstream of the TCR

No consensus has formed with regard to Pyk2's function in effector T cell responses. Overexpression studies in Jurkat cells suggested that TCR and CD28-induced c-Jun N-terminal kinase (JNK) and p38 MAPK activation and IL-2 production are partially dependent upon Pyk2 [32]. Using T cells from Pyk2 knockout mice, Weiss and colleagues demonstrated that both Pyk2-deficient CD4⁺ and CD8⁺ T cells have reduced proliferation *in vitro* following high dose anti-CD3 antibody stimulation, and that IL-2 and interferon (IFN)- γ production by Pyk2^{-/-} CD8⁺ T cells are impaired under the same conditions [66]. In the CD8⁺ T cells, these defects are more pronounced after stimulation with low dose anti-CD3 antibody in combination with the LFA-1 ligand, intracellular adhesion molecule (ICAM)-1 [66]. Lymphocytic-choriomeningitis virus (LCMV) antigen-specific proliferative responses are also impaired *in vitro* [66]. While these studies suggest that Pyk2 serves a vital role in

generating effector T cell responses, only antigen-specific proliferation and short-lived effector CD8⁺ T cell generation are impaired in the absence of Pyk2 following an *in vivo* LCMV challenge, while IL-2 and IFN- γ production are not altered [66]. Although CD25 expression is comparable between the Pyk2 sufficient and deficient CD8⁺ T cells [66], these proliferation defects may be in part linked to defective IL-2 signaling, as overexpression of kinase-dead Pyk2 suppresses IL-2 induced proliferation [15]. It is unknown why Pyk2 deficiency has a more profound effect on CD8⁺ T cell effector function *in vitro* than *in vivo*, but it is possible that increased FAK expression *in vivo* and/or FAK compensating for the loss of Pyk2 account for these differences. Indeed, FAK expression increases upon T cell activation [53, 67], and Pyk2 partially compensates for FAK in other systems [68, 69].

The role that Pyk2 serves in CD4⁺ T cell responses is even less clear. As noted above, anti-CD3 antibody-induced proliferation of naïve CD4⁺ T cells is partially dependent upon Pyk2 [66]. However, other effector functions like cytokine production were not examined. Interestingly, when Pyk2's expression or catalytic function is suppressed in activated CD4⁺ T cells, anti-CD3 and anti-CD28 antibody-induced IL-2 production is not inhibited [70]. Thus, Pyk2 may serve different functions in naïve versus effector/memory CD4⁺ T cells. In support of this role, recent work has demonstrated that Pyk2 is hyper-activated in PTP-PEST^{-/-} CD4⁺ T cells, and that these cells have impaired secondary but not primary responses to antigen stimulation [70]. PTP-PEST^{-/-} CD4⁺ T cells are also more susceptible to becoming anergic [70], a state that occurs after T cells receive TCR signals in the absence of adequate costimulation [71], and Pyk2 inhibition reverses the anergic phenotype of the PTP-PEST^{-/-} CD4⁺ T cells [70]. These data indicate that Pyk2 may regulate distinct functions in T cells depending upon which receptors are activated, the activation status of the cells, or the specific T cell lineage examined.

Like Pyk2, FAK's role in T cell biology is controversial. When activated human CD4⁺ T cells were treated with PF-562,271, the TCR-inducible phosphorylation of ZAP-70, LAT, and Erk1/Erk2 is reduced. Moreover, anti-CD3 and anti-CD28 antibody-induced proliferation was impaired when either human or murine CD4⁺ T cells were treated with this inhibitor [38]. Although these data suggest that FAK positively regulates TCR signaling and proliferative responses, no proliferation defects are observed when FAK is conditionally deleted in murine CD4⁺ T cells [38]. The differences in these experiments could be explained by the simultaneous inhibition of other kinases, including Pyk2, in the inhibitor experiments or by the incomplete deletion of FAK in the murine CD4⁺ T cells. To clarify the functional role that FAK serves in TCR activation, we selectively suppressed FAK's expression in both Jurkat T cells and activated human CD4⁺ T cells [53]. Although we observe slight decreases in ZAP-70 and Erk1/Erk2 phosphorylation in FAK-deficient T cells, the TCR-inducible phosphorylation of LAT, SLP-76, PLC- γ 1, and Akt is elevated in these cells compared to controls. The FAK-deficient T cells also have enhanced TCR antigen sensitivity, as they produce more IL-2 and IFN- γ and have increased CD69 surface expression after stimulation with suboptimal doses of anti-CD3 antibody [53]. Thus, our data demonstrate that FAK negatively regulates TCR function in activated human CD4⁺ T cells. Ongoing work in our laboratory is determining how FAK integrates TCR and co-stimulatory signals to influence T cell activation and differentiation.

Csk is a cytosolic tyrosine kinase that associates with membrane-bound adaptor proteins via its SH2 domain, which facilitates its ability to suppress SFK function [72, 73]. This inhibition is important to suppress both tonic and TCR-inducible signaling [74, 75]. FAK binds to Csk in other cell lineages [72, 76], and FAK and Csk-deficient T cells have similar signaling and functional defects after TCR stimulation. We found that, after TCR stimulation, less Csk associates with the plasma membrane, the TCR, and Lck in FAK-deficient T cells [53]. FAK-deficient T cells also have decreased Lck Y505 phosphorylation, the site that is directly phosphorylated by Csk to suppress Lck function, while the magnitude and kinetics of phosphorylation of the Lck substrates, Lck Y394 and TCR ζ -ITAM Y142, are higher and faster, respectively [53]. These data collectively suggest that FAK regulates Csk's functional localization to negatively regulate Lck activation after TCR stimulation. Although the precise mechanisms remain elusive, FAK Y397 is contained within the consensus binding motif for the SH2 domain of Csk [77], and Pyk2 Y402 binds the Csk-related protein, Csk homologous kinase (Chk) [78].

Control of actin cytoskeletal responses downstream of the TCR

The actin cytoskeleton serves multifaceted roles in regulating T cell responses by controlling intracellular signaling, driving cellular adhesion, and promoting cellular migration [52, 51]. Previous studies in non-T cells suggest redundant roles for FAK and Pyk2 as actin cytoskeletal remodeling-inducing proteins (reviewed in [19, 35]). Indeed, we found that two phases of Pyk2 phosphorylation and actin polymerization are observed after TCR stimulation [9], and that these two bursts of actin polymerization appear to promote two phases of adhesion to anti-CD3 antibody-coated plastic plates [8]. We demonstrated that Pyk2's catalytic function is partially required for the first wave of TCR-driven adhesion, while its scaffolding function regulated the second phase [8]. In further support of Pyk2's role as an actin cytoskeleton regulatory protein, anti-CD3 antibody-induced spreading is reduced in Pyk2-deficient Jurkat T cells after 7.5 minutes of stimulation (Figure 2); this defect is strikingly similar to Jurkat T cells that are treated with actin depolymerizing agents [79]. Although these data suggest that Pyk2 regulates TCR-induced adhesion via two distinct mechanisms, whether the two separate pools of Pyk2 described by St-Pierre *et al.* [57] differentially regulate these functions remains uninvestigated. To control actin cytoskeletal responses, Pyk2 may promote the activation of actin cytoskeletal remodeling proteins that associate with the LAT and/or SLP-76. For instance, Pyk2 may regulate small Rho GTPase activation to control actin cytoskeletal-induced spreading and adhesion. In support of this view, Pyk2 interacts with many modulators of Rho family GTPase function, including Vav1 [32, 33], and is linked to Rac, Rho, and Cdc42 function [33, 65, 70, 80]. As discussed below, Pyk2's scaffolding function may stabilize TCR-induced adhesion by facilitating crosstalk between the TCR and the actin cytoskeleton network to induce contractile forces.

Coating anti-CD3 antibody onto rigid surfaces drives T cell spreading early after activation, but later leads to the generation of stable cell-stimulatory interface contacts that are facilitated by TCR-induced contractile forces at the periphery of the cell [8, 79, 81–83]. The TCR is a mechanosensor for substrate rigidity, and this process regulates efficient intracellular signaling, IL-2 production, and proliferation [81, 82]. Anti-CD3 and/or anti-

CD28-induced traction forces are generated in the cell periphery [81, 83], where F-actin is co-localized with TCR microclusters [5, 51]. Moreover, phosphorylated SFKs and Pyk2 are also localized to the periphery of the cell, where the largest forces are generated upon anti-CD3 and anti-CD28 antibody stimulation, whereas phosphorylated ZAP-70 is distributed throughout the cell [81, 82]. SFK inhibition using PP2 leads to a correlative decrease in both Pyk2 phosphorylation and traction forces induced by anti-CD3 and anti-CD28 antibodies [81], suggesting that Pyk2 is a critical mechanosensor downstream of the TCR. This potential function of Pyk2 may explain, in part, why Pyk2-deficient T cells do not stably adhere or proliferate after TCR stimulation [8, 66].

In contrast to Pyk2, FAK does not appear to control actin cytoskeletal responses downstream of the TCR. We demonstrated that anti-CD3 antibody-induced spreading, adhesion, and TCR downregulation are normal in FAK-deficient Jurkat T cells [8, 53]. However, FAK could regulate TCR and chemokine receptor-induced “inside-out” signaling that promotes integrin clustering and affinity maturation, which increases adhesion receptor function. Although these data suggest that Pyk2 is the primary regulator of TCR-dependent actin cytoskeletal responses, FAK and Pyk2 are also phosphorylated downstream of many chemokine receptors and facilitate adhesion and migration in B cells, neutrophils, and macrophages [19, 65, 84]. Therefore, their roles in chemokine-induced T cell responses need to be investigated.

FAK and Pyk2 regulate adhesion receptor function

Although FAK and Pyk2 are activated downstream of the adhesion receptors, VLA-4 and CD44 [6, 14, 18, 20, 45, 46, 21], few studies have examined functions that FAK or Pyk2 serve downstream of these receptors. Overexpressing dominant-negative truncation mutants of FAK or Pyk2 into Jurkat T cells inhibits VLA-4-induced *trans*-regulation of LFA-1, resulting in reduced migration on ICAM-1 coated surfaces [47]. FAK is also implicated in regulating VLA-4 induced costimulation, as expressing a truncation mutant of Crk-associated substrate lymphocyte-type (Cas-L) that cannot be phosphorylated by FAK inhibits TCR and VLA-4-induced IL-2 production in Jurkat T cells, whereas cells that overexpress full-length Cas-L retain VLA-4 costimulatory function [85]. Further, FAK scaffolding function regulates matrix metalloproteases downstream of VLA-4 [86]. Anti-CD44 antibody treatment promotes actin cytoskeletal rearrangement which induces T cell spreading and elongation, and the catalytic function of Pyk2 controls these processes [20]. Whether Pyk2 controls CD44-induced costimulation or adhesion has not been investigated, and FAK's role downstream of CD44 is unexplored.

Catalytic inhibition of FAK alone or in combination with Pyk2 inhibition reduces T cell-APC conjugate formation [38], suggesting that these kinases may serve functionally redundant roles in controlling T cell adhesion events regulated by integrins. T cell-APC interactions are stabilized after TCR stimulation promotes LFA-1 integrin activation. Weiss and colleagues demonstrated that adhesion to ICAM-1 in the presence or absence of TCR stimulation is dramatically reduced in the Pyk2^{-/-} CD8⁺ T cells. These data, combined with those linking Pyk2 to TCR-induced actin remodeling ([8]; Figure 2), suggest that Pyk2 regulates both TCR-induced “inside-out” and LFA-1-mediated “outside-in” signals to

promote adhesion to ICAM-1. A reduction in FAK phosphorylation is also correlated with impaired TCR-induced LFA-1 clustering and adhesion to ICAM-1 in Jurkat T cells [87], and a modest reduction in adhesion to ICAM-1 was reported in FAK-deficient CD4⁺ T cells [38]. Thus, FAK and Pyk2 may serve redundant functions downstream of LFA-1 to induce T cell adhesion to APC. FAK and Pyk2 may also regulate CD2-mediated or chemokine-induced adhesion, as these receptors induce FAK and Pyk2 activation [19].

T cells also form self-aggregates that promote cytokine exchange and augment T cell activation. Like T cell-APC interactions, T cell-T cell aggregate formation is also regulated by LFA-1/ICAM-1 interactions [88]. Interestingly, FAK and Pyk2 seem to play opposing roles in LFA-1-induced T cell homo-aggregate formation. Hyperphosphorylation of Pyk2 in PTP-PEST^{-/-} CD4⁺ T cells is correlated with reduced T cell-T cell aggregate formation [70], and this finding likely explains why these cells have reduced secondary T cell responses [70]. By contrast, Jurkat T cells that overexpress the low molecular weight-protein tyrosine phosphatase (LMW-PTP) have reduced FAK phosphorylation and T cell homoaggregate formation [87], likely as a result of impaired LFA-1 clustering. Future work will utilize FAK and Pyk2-deficient systems to better determine the roles these proteins serve in LFA-1-mediated homoaggregate formation.

Conclusions and future perspectives

Recent studies have challenged the view that FAK and Pyk2 serve functionally redundant roles to regulate receptor-mediated actin cytoskeletal responses in T cells. Instead, FAK and Pyk2 have receptor-specific functions, which are likely regulated by a receptor's unique spatiotemporal and mechanistic regulation of FAK and Pyk2 activation and by distinct protein-protein interactions that occur downstream of these receptors. Pyk2 controls actin cytoskeletal responses, including spreading and adhesion, downstream of the TCR and CD44 and both positively and negatively regulates LFA-1-induced functions. By contrast, FAK is not required for actin cytoskeletal rearrangements downstream of the TCR but is instead a novel inhibitor of TCR function. Further, FAK positively regulates LFA-1-induced responses in T cells (Figure 3). Thus, these related kinases serve important and distinct functions downstream of the TCR, but have some level of functional redundancy downstream of LFA-1. Future work will characterize if these and other unique functions extend to CD44 and VLA-4 and will examine the signaling events that FAK and Pyk2 induce to control these redundant and unique functions.

What role does each pool of Pyk2 serve in T cell responses? MTOC polarization regulates directional lytic granule and cytokine secretion and is also implicated in asymmetric cell division [58], which is involved in effector versus memory T cell generation [89]. Given that Pyk2 regulates MTOC polarization in NK cells [90, 91], it is reasonable to hypothesize that Pyk2 controls this process in T cells. This prediction may help explain why effector T cell generation and anti-CD3 antibody induced spreading are impaired in Pyk2-deficient CD8⁺ T cells and Jurkat T cells, respectively (Figure 2; [66, 79]). The IS-associated fraction of Pyk2 may associate with TCR signaling molecules, including Lck/Fyn, ZAP-70, Grb2, and/or PI3K [7, 32, 34, 37, 92, 93]. Therefore, Pyk2 may promote proliferation and cytokine responses in CD4⁺ and CD8⁺ T cells by controlling proximal TCR signaling events leading

to LAT/SLP-76 complex formation, MAPK activation, or PI3K function. Future work will also ascertain how Pyk2 promotes T cell anergy. In this regard, Pyk2's scaffolding or enzymatic may regulate the function of the Cbl family of E3 ubiquitin ligases [94], which promote T cell anergy [95].

Although FAK negatively regulates TCR function in human CD4⁺ T cells [53], the precise molecular mechanisms that govern its control of Csk are unknown. Moreover, it is unclear how costimulatory signals impact this function of FAK. Does FAK deficiency allow T cells to robustly respond to low dose or affinity antigens in the absence of costimulation? If so, could this feature be manipulated therapeutically to increase T cell responses to weak antigens? Conversely, does loss of FAK function contribute to T cell hyperactivation in autoimmunity or facilitate T cell leukemia and lymphoma development? The roles FAK and Pyk2 serve in CD4⁺ T cell differentiation and memory T cell formation is also an interesting topic for future study, given that FAK and Pyk2 are clinical targets of interest [31]. Ultimately, further investigation into the roles FAK and Pyk2 serve in normal and dysfunctional T cell responses is needed before clinicians can safely harness FAK and Pyk2 inhibitors to treat human diseases without deleterious T cell-mediated effects.

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FAK **Pyk2-H** **Figure 1.**

Domain structure of FAK and Pyk2-H, the predominant Pyk2 isoform expressed in T cells. Black circles indicate phosphorylation sites, and PPPP represent proline-rich regions. PP is the truncated proline-rich region expressed in Pyk2-H. Proteins that interact with FAK and Pyk2/Pyk2-H are shown in italics.

Figure 2.

Anti-CD3 antibody-induced actin polymerization and spreading are impaired in Pyk2-deficient Jurkat T cells. Jurkat T cells were transfected with the control, luciferase (Luc)-specific microRNA (miRNA) or the Pyk2-specific miRNA. These cells were then stimulated on glass chamber slides using 10 μ g/ml of anti-CD3 antibody for 7.5 minutes. The fixed cells were then stained for F-actin using FITC-phalloidin. One representative image is shown, and the cell area of 25 cells was calculated using Fiji software. The graph shows the mean $\pm$ s.d. from 25 cells.

Figure 3.

Summary of FAK and Pyk2 functions downstream of the TCR, CD28, and LFA-1. In resting T cells, FAK and Pyk2 are localized near the microtubule organizing complex (MTOC; not shown). Upon TCR activation, FAK and Pyk2 are phosphorylated by Lck/Fyn, while additional receptor-induced signals from CD28 and LFA-1 synergize to enhance FAK and Pyk2 phosphorylation. The subcellular localization of FAK and Pyk2 changes after these receptors are activated. FAK is recruited to the TCR complex, where it associates with Csk to inhibit TCR-dependent signaling and function. LFA-1 stimulation also drives FAK-

MTOC relocation, and FAK positively regulates LFA-1-induced T cell adhesion to APC and other T cells. After antigen recognition, a fraction of Pyk2 remains associated with the MTOC, while a different pool of Pyk2 is recruited to the T cell-APC interface. The latter pool of Pyk2 may associate with the LAT/SLP-76 complex to couple TCR and CD28 signaling that drives MAPK and/or PI3K activation. Additionally, this pool of Pyk2 may regulate actin cytoskeletal polymerization to activate LFA-1 and promote T cell spreading. In this manner, Pyk2 positively regulates LFA-1 induced adhesion to APC and LFA-1 mediated costimulation that induces T cell proliferation and cytokine production. We propose that the MTOC-associated pool of Pyk2 regulates signaling that drives MTOC reorganization to control directional cytokine and lytic granule secretion and asymmetric cell division that promotes effector T cell formation.