T cells transduce T-cell receptor signal strength by generating different phosphatidylinositol

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T-cell receptor (TCR) signaling strength is a dominant factor regulating T-cell differentiation, thymic development, and cytokine signaling. The molecular mechanisms by which TCR signal strength is transduced to downstream signaling networks remains ill-defined. Using computational modeling, biochemical assays, and imaging flow cytometry, we found here that TCR signal strength differentially generates phosphatidylinositol species. Weak TCR signals generated elevated phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and reduced phosphatidylinositol (3,4,5)-trisphosphate (PIP3) levels, whereas strong TCR signals reduced PI(4,5)P2 and elevated PIP3 levels. A proteomics screen revealed that focal adhesion kinase bound to PI(4,5)P2, biochemical assays disclosed that focal adhesion kinase is preferentially activated by weak TCR signals and is required for optimal Treg induction, and further biochemical experiments revealed how TCR signaling strength regulates AKT activation. Low PIP3 levels generated by weak TCR signals were sufficient to activate phosphoinositide-dependent kinase-1 to phosphorylate AKT on Thr-308 but insufficient to activate mTOR complex 2 (mTORC2), whereas elevated PIP3 levels generated by a strong TCR signal were required to activate mTORC2 to phosphorylate Ser-473 on AKT. Our results provide support for a model that links TCR signaling to mTORC2 activation via phosphoinositide 3-kinase signaling. Together, the findings in this work establish that T cells measure TCR signal strength by generating different levels of phosphatidylinositol species that engage alternate signaling networks to control cell fate decisions.

TCR2 signal strength is a dominant factor that regulates T cell–mediated immunity including thymic selection (1), T-cell differentiation (2–4), and sensitivity to cytokine signaling (3). Downstream from proximal signaling, the strength of the TCR signal regulates multiple transcription factors, including FOXO1 (5), IRF4 (6), and the induction of cytokine receptor genes (3). Further, TCR signal strength shapes CD4+ T-cell differentiation programs, where weak TCR signals promote regulatory T cell (Treg) induction and strong TCR signals induce T helper cells (Th) (7, 8). Although the role of TCR signal strength in shaping T-cell immune responses is appreciated (9), the biochemical mechanisms used to grade TCR signal strength are less established.

One signaling network responsive to TCR signal strength is the AKT/mTOR pathway (10–12). T-cell signaling activates PI3K to phosphorylate PI(4,5)P2 to yield PIP3 (13). Indeed, PIP3 is important for TCR signaling (14, 15). PIP3 recruits kinases including PDK1 (16, 17) and AKT (18) to the plasma membrane and activates downstream signaling networks. There are two major phosphorylation sites on AKT that have been studied in T cells: threonine 308 and serine 473 (17, 19). AKT-T308 is phosphorylated by PDK1 (17) downstream of PI3K and TCR signaling (20). AKT-S473 is phosphorylated by mTORC2 (19); however, the precise mechanism by which mTORC2 is activated in a T cell is not well-established. In CD4+ T cells, weak TCR signals are sufficient to stimulate PDK1 phosphorylation of AKT-T308 to promote Treg induction, whereas strong TCR signals are required to activate both PDK1 and mTORC2 to phosphorylate Thr-308 and Ser-473 on AKT to drive Th induction (11, 21). The AKT proteoforms that result from a weak or a strong TCR signal appear to have alternate substrate specificities and engage different signaling networks to drive Treg (weak signal) versus Th (strong signal) induction (11). These data suggest that the PI3K/AKT signaling axis functions in grading TCR signal strength.

In addition to kinases, lipid phosphatases function in establishing the set point for TCR signaling thresholds. Previous work demonstrated that TCR signal strength regulates PTEN (5), which is a lipid phosphatase that dephosphorylates PIP3 at the 3’ position to generate PI(4,5)P2. Strong TCR signals suppress PTEN activity via ubiquitin- and caspase-mediated degradation pathways, whereas weak TCR signals maintain PTEN (5). In addition to dephosphorylating the 3’ position of PIP3, PTEN can dephosphorylate PI(3,4)P2 at the 3’ position (22). Thus, differential regulation of PTEN via TCR signal strength
**Results**

**T cells encode TCR signal strength by generating different phosphatidylinositols**

We built a computational model to better conceptualize how PTEN suppression via TCR signal strength regulates PI3K signaling. The following assumptions were included in the model (Fig. 1A) (2, 5, 11, 21): 1) ligation of peptide-MHC (p-MHC) to a TCR and stimulation through CD28 activates multiple kinases that phosphorylate phosphatidylinositols, including PI3K, which converts PI(4,5)P2 to PIP3; 2) increased TCR signal strength promotes PTEN degradation as previously demonstrated (5); and 3) PDK1 is activated by lower levels of PIP3 (23) ($K_d$ of 1.6 ns) than mTORC2 (24, 25) ($K_d$ of 141 ns) via SIN1 (a target of rapamycin complex 2 subunit MAPKAP1) component (26).

TCR signal strength was modeled by altering the amount of TCR-pMHC in the simulation. The resulting simulations captured that strong TCR signals decrease PTEN protein levels (5) (Fig. 1B). PDK1 activation via autophosphorylation and resulting phosphorylation of Thr-308 on AKT required lower TCR signaling levels than mTORC2 activation (Fig. 1C) and phosphorylation of Ser-473 on AKT (Fig. 1D). An unexpected feature of the model occurred at the level of phosphatidylinositol metabolism. Maintenance of PTEN during a weak TCR signal promoted the accumulation of PI(4,5)P2 and reduced PIP3 (Fig. 1E and F). Conversely, PTEN reduction resulting from a strong TCR signal diminished PI(4,5)P2 accumulation and allowed for elevated PIP3 generation. Taken together, these simulations predicted that the regulation of PTEN abundance by TCR signal strength results in a redistribution of phosphatidylinositol species generated.

Based on the predictions from the computational model that TCR signal strength regulated the amounts of PI(4,5)P2 and PIP3 (Fig. 1, E and F), phosphatidylinositol abundance generated by T cells activated across a range of TCR signal strengths was measured biochemically (27, 28). Weak TCR stimulation yielded robust generation of PI(4,5)P2 with peak intensity at 10 min (Fig. 1G) and diminished PIP3 generation (Fig. 1H). Conversely, a strong TCR signal generated reduced levels of PI(4,5)P2 (Fig. 1G) and robust PIP3 (Fig. 1H) relative to a weak TCR signal. Finally, a strong TCR signal increased PI(3,4)P2 relative to a weak TCR stimulus, which was significant at the 10-min time point (Fig. 1I).

To determine whether PTEN inhibition impacted the distribution of phosphatidylinositols generated from a weak or strong TCR signal, murine CD4$^+$ T cells were activated in the presence of a PTEN inhibitor (Fig. 1, G–I). In T cells activated with a weak TCR stimulus, PTEN inhibition markedly decreased PI(4,5)P2 (Fig. 1G) and significantly increased PIP3 to levels observed in T cells activated with a strong TCR signal (Fig. 1H). PTEN inhibition slightly increased PI(3,4)P2 in T cells stimulated with a weak TCR signal (Fig. 1I); however, the difference was not statistically significant. In T cells activated with a strong TCR stimulus, PI(4,5)P2 levels were reduced (Fig. 1G), whereas PIP3 and PI(3,4)P2 levels were increased by PTEN inhibition (Fig. 1, H and I).

To confirm the results from the PTEN inhibitor studies, PTEN was knocked down in CD4$^+$ T cells using siRNA (Fig. 1J), and levels of PI(4,5)P2 (Fig. 1K), PIP3 (Fig. 1L), and PI(3,4)P2 (Fig. 1M) were measured in T cells stimulated with either a weak or strong TCR signal. Knockdown of PTEN reduced PI(4,5)P2 levels and increased both PIP3 and PI(3,4)P2 levels in T cells activated with a weak TCR stimulus. PTEN knockdown did not impact PIP generation in T cells versus scrambled control in T cells stimulated with a strong TCR stimulus. This was expected because strong TCR signals result in the degradation of PTEN protein to promote PIP3 synthesis. Taken together, these data demonstrated that PTEN was essential for PI(4,5)P2 accumulation during a weak TCR stimulus.

**Weak TCR signals generate more PI(4,5)P2 than strong TCR signals**

The heightened generation of PI(4,5)P2 from a weak TCR stimulus was unexpected. Therefore, we performed a detailed dose-response time course study to better characterize the kinetics of PI(4,5)P2 generation in both murine CD4$^+$ and CD8$^+$ T cells. A flow cytometric assay was utilized to measure PI(4,5)P2 abundance using an antibody that specifically binds PI(4,5)P2. The CD4$^+$ T-cell population was defined as being plate-bound anti-CD3 antibody and constant amounts of soluble anti-CD28 antibody (1 μg/ml). Following fixation, the cells were stained with antibodies that bound CD4, CD8, TCR, and PI(4,5)P2. The CD4$^+$ T-cell population was defined as being double positive for CD4 and TCR. Likewise, the CD8 population was positive for both CD8 and TCR.

Stimulation of CD4$^+$ T cells resulted in the synthesis of PI(4,5)P2 across multiple anti-CD3 doses (Fig. 2A). In comparing PI(4,5)P2 generation as a function of anti-CD3 antibody dose, lower doses resulted in increased generation of PI(4,5)P2 across multiple time points (Fig. 2A) as highlighted for the 2.5-min time point (Fig. 2B). Time course data for PI(4,5)P2 for the 0.25 and 1 μg/ml anti-CD3 antibody doses were plotted (Fig. 2C). These data further highlighted that weak TCR stimuli increased generation of PI(4,5)P2 and are consistent with the mass ELISA results (Fig. 1G). The generation of PI(4,5)P2-positive cells was plotted as a function of both anti-CD3 antibody dose and activation time (Fig. 2D), which further illustrated that PI(4,5)P2 generation peaks at early activation time points and is more sustained in CD4$^+$ T cells stimulated with weaker TCR signals. Similar to CD4 T cells, weaker TCR signals also generated elevated PI(4,5)P2 levels in CD8$^+$ T cells (Fig. S1).

We tracked the generation of PI(4,5)P2 (Fig. 2E), PIP3 (Fig. 2F), and PI(3,4)P2 (Fig. 2G) with imaging flow cytometry to further confirm that different TCR stimuli generated different levels of PIPs. As measured by biochemical assays (Fig. 1G) and by flow cytometry (Fig. 2C), the imaging flow cytometry mea-
Figure 1. T cells generate a different landscape of PIPs in response to TCR signal strength. A, a simplified model of T-cell activation focusing on AKT activation was constructed. Simulations in Matlab were performed where TCR signal strength was modulated by altering the amount of TCR-pMHC in the simulation. B–F, the abundance of PTEN (B), P-PDK1 (C), mTORC2 (C), phosphorylated AKT (D), PI(4,5)P2 (E), and PIP3 (F) were plotted as a function of TCR signal strength. G–I, mass ELISA assays were used to measure the amount of PI(4,5)P2 (G), PIP3 (H), and PI(3,4)P2 (I) generated in murine CD4+ T cells isolated by negative selection stimulated using a low (0.25 μg/ml) or high (1.0 μg/ml) dose of plate-bound anti-CD3 antibody and a constant amount of soluble anti-CD28 antibody (1 μg/ml) in the presence or absence of 10 μM PTEN inhibitor (SF1670). J, CD4+ T cells were nucleofected with either scrambled control (SC) or siRNA targeting PTEN (siRNA) for 48 h, Western blotting was utilized to monitor PTEN levels, and actin was utilized as a loading control. K–M, mass ELISA assays were used to measure the amount of PI(4,5)P2 (K), PIP3 (L), and PI(3,4)P2 (M) generated in murine CD4+ T cells treated with the scrambled control or siRNA targeting PTEN that were activated for 10 min with either a low (0.25 μg/ml) or high (1.0 μg/ml) dose of plate-bound anti-CD3 antibody and a constant amount of soluble anti-CD28 antibody (1 μg/ml). Each experiment was repeated three times, and error bars are ± standard deviation. A two-way ANOVA statistical test was performed. ****, <0.0001; ***, <0.001; **, <0.01; *, <0.05. Symbols over data points are comparisons between the low- and high-dose groups, and symbols in the legend are between the untreated and SF1670-treated groups.
Figure 2. Weaker TCR signal strengths generate more P(4,5)P2. Total murine splenocytes were activated with varying doses of plate-bound anti-CD3 antibody indicated in each panel and a constant amount of soluble anti-CD28 antibody (1 μg/ml) for various time points. A, flow cytometry was utilized to measure P(4,5)P2 in CD4+ T cells stained with an antibody that specifically binds to P(4,5)P2. CD4+ T cells were defined as cells positive for both TCR and CD4 receptors. B, the percentage of CD4+ T cells positive for P(4,5)P2 at 10 min of activation were plotted versus anti-CD3 antibody dose. C, the kinetics of P(4,5)P2 positive CD4+ T-cell formation were plotted for the 0.25 and 1.0 μg/ml anti-CD3 antibody doses. D, the percentage of P(4,5)P2 positive CD4+ T cells were plotted as a function of both anti-CD3 antibody dose and time. E–G, imaging flow cytometry was utilized to track generation of P(4,5)P2 (E), PIP3 (F), and P(3,4)P2 (G) from a low (0.25 μg/ml) or high (1.0 μg/ml) dose of plate-bound anti-CD3 antibody and a constant amount of soluble anti-CD28 antibody (1 μg/ml) in murine CD4+ T cells isolated by negative selection. A one-way ANOVA was utilized to experiments comparing the impact of α-CD3 antibody dose on P(4,5)P2 (B). A two-way ANOVA statistical test was performed to analyze the kinetic profiles (C and E–G). For all statistical tests, p values were summarized as follows: ****, <0.0001; ***, <0.001; **, <0.01; *, <0.05. Each experiment was repeated three times, and error bars are ± standard deviation.
Figure 3. TCR signal strength regulates PIP colocalization to the TCR and stability of TCR capping. The localization of the TCR and either PI(4,5)P2 (A), PIP3 (B), or PI(3,4)P2 (C) was measured in murine CD4+ T cells that were purified by negative selection stimulated using low (0.25 μg/mL), medium (0.5 μg/mL), and high (1.0 μg/mL) doses of plate-bound anti-CD3 antibody and a constant amount of soluble anti-CD28 antibody (1 μg/mL) using imaging flow cytometry. The yellow scale bars correspond to 5 μm. D, the colocalization score between the TCR and PI(4,5)P2, PIP3, and PI(4,5)P2 was calculated in the IDEAS software package from at least 1000 individual cells. E, different levels of TCR capping were observed in the imaging flow cytometry data. The yellow scale bars correspond to 5 μm. F, the Delta centroid function in the IDEAS software package was utilized to calculate the level of TCR capping from the imaging flow cytometry data as a function of activation time in CD4+ T cells that received a weak or strong TCR signal. A one-way ANOVA statistical test was performed to analyze the data in D. A two-way ANOVA statistical test was performed to analyze the kinetic profiles (F). For all statistical tests, p values were summarized as follows: ****, <0.0001; **, <0.01; *, <0.05. Each experiment was repeated three times, and error bars are ± standard deviation.

% measurement demonstrated that a weak TCR stimulus generated elevated PI(4,5)P2 levels, whereas a strong TCR stimulus generated elevated PIP3 levels (Fig. 1H). Together, these results demonstrated that CD4+ T cells generated different levels of PIPs in response to TCR signal strength.

**TCR signal strength regulates the extent and duration of TCR capping**

Previous reports demonstrated that generation of phosphatidylinositols occurred in close proximity to TCR clusters, and PIP3 sequestration to TCR clusters controlled the architecture of the immunological synapse to promote signaling (30). Other work illustrated that PI(4,5)P2 inhibits TCR signaling by interacting with the intracellular signaling domains of CD3, preventing LCK binding, which would dampen downstream signaling (31). Thus, the differential generation of phosphatidylinositols induced by TCR signal strength might impact proximal signaling by regulating the local membrane environment around the TCR.

Imaging flow cytometry was utilized to track the spatial distribution of the TCR relative to PI(4,5)P2, PIP3, and PI(3,4)P2 in T cells activated with a weak or strong TCR signal (32). As expected, PIP generation occurred in proximity to TCR cap structures (Fig. 3, A–C). The colocalization between the TCR, PI(4,5)P2, PIP3, and PI(3,4)P2 was analyzed in the IDEAS software package. T cells activated with a weak TCR signal had an increase in colocalization of PI(4,5)P2 with the TCR relative to T cells activated with a strong TCR signal (Fig. 3D). Conversely, a strong TCR stimulus increased the colocalization between PIP3 and the TCR relative to a weak TCR stimulus (Fig. 3D). The colocalization score between PI(3,4)P2 and TCR was lower for T cells stimulated with either a strong or a weak TCR signal (Fig. 3D). These results demonstrated that TCR caps generated in response to a weak TCR stimulus are enriched with PI(4,5)P2, whereas TCR caps generated in response to strong TCR signals are enriched with PIP3.

The imaging flow cytometry data were analyzed to measure TCR capping during activation (Fig. 3E). The IDEAS software package was utilized to calculate TCR capping using the Delta centroid function between the nuclear stain and the TCR. In resting CD4+ T cells, few TCR caps were observed (Fig. 3F). Stimulation with either a weak or strong TCR signal induced TCR capping (Fig. 3F). At 30 min, a strong TCR stimulus induced more TCR caps than a weak TCR stimulus (Fig. 3F). At later time points, a strong TCR signal increased TCR capping, whereas TCR clustering was diminished in T cells stimulated with a weak TCR signal. These results demonstrated that TCR signal strength regulated TCR capping.

**Proteomic profiling identifies proteins in T cells that bind different phosphatidylinositols**

Our data demonstrated that TCR signal strength regulated the PI(4,5)P2/PIP3 ratio. Many proteins bind to specific phosphatidylinositols. Therefore, differential PIP generation in T cells could regulate different proteins and downstream signaling pathways. We adapted a proteomic profiling approach to identify proteins in a resting CD4+ T cell that could bind PI(4,5)P2, PIP3, or PI(3,4)P2 (33) (Fig. 4A). A 3-fold enrichment cutoff was utilized to classify a protein as uniquely pulled down by a specific phosphatidylinositol bead (Fig. 4B). A pathway analysis was performed on the protein lists for each PIP pulldown sample group. As expected, pathways known to be regulated by phosphatidylinositols including PI3K/AKT signaling, MTOR, actin-based signaling and RHO signaling were involved in protein ubiquitination and metabolic pathways including glycolysis and the TCA cycle were enriched. Together, these data demonstrated that generation of different phosphatidylinositols could potentially regulate fundamental biological processes in a CD4+ T cells.

The relative amount of specific proteins in the PIP pulldown experiments were plotted for proteins in specific functional
classes. Multiple kinases associated with proximal TCR signaling are regulated by phosphatidylinositol signaling (Fig. 4D). ITK, TEC, PDK1, and AKT all bound PIP3-coated beads. PDK1 and AKT also bound PI(3,4,P2, which was previously reported (34). Interestingly, our screen identified that focal adhesion kinase (FAK) specifically bound to PI(4,5,P2 (Fig. 4D). Previous reports demonstrated that PI(4,5,P2 activated FAK enzymatic activity (35). Therefore, FAK activation triggered by PI(4,5,P2 might be important for transducing a weak TCR signal where elevated PI(4,5,P2 levels are generated.

Proteins involved with chromatin remodeling (Fig. 4E), splicing (Fig. 4F), and transcription (Fig. 4G) also bound...
phosphatidylinositols. Smarce1, Smacra1, and Smarcad2 are components of the Swi/Snf chromatin remodeling complex and associated with PI(4,5)P2 beads. This is consistent with previous reports that the Swi/Snf complex associated with PI(4,5)P2 (36). Smarca1 was also pulled down by the PIP3 beads. Also, components of the INO80 chromatin remodeling complex, Ruvbl1, Ruvbl2, and Yy1, were pulled down by the PI(4,5)P2 beads. Protein involved with splicing and mRNA processing including SFB4, HNRNPK, and HNRNPL also associated with the PI(4,5)P2 beads (Fig. 4F), consistent with previous reports that demonstrated the involvement of PI(4,5)P2 in regulating transcription (39, 40).

Weak TCR signals activate FAK via elevated PI(4,5)P2 in CD4+ T cells, which is essential for optimal FOXP3 induction

Previous signaling studies in T cells focused on the role of PIP3 in activating downstream signaling pathways including AKT. In T cells, the role function of PI(4,5)P2 has focused on controlling the actin cytoskeleton and dynamics of the TCR. An interesting observation from our proteomic screen is that FAK bound PI(4,5)P2 (Fig. 4D). Additionally, FAK has established roles in T-cell signaling (41, 42). Previous reports demonstrated that PI(4,5)P2 binding activated FAK activity (35, 43, 44). Therefore, it is possible that PI(4,5)P2 generated by a weak TCR signal could activate FAK to serve as transducer to downstream signaling pathways.
FAK autophosphorylation on Tyr-397 serves as a marker of FAK activation (45). Western blotting was used to monitor the phosphorylation of Tyr-397 on FAK in CD4+ T cells that received a weak or strong TCR signal (Fig. 5A). Weak TCR signaling generated more Tyr-397 phosphorylation than a strong TCR signal (Fig. 5, A and B), corresponding to elevated PI(4,5)P2 generated from a weak TCR signal (Fig. 1G). Because PTEN was critical in maintaining PI(4,5)P2 levels (with a low-dose TCR stimulus (0.25 g/ml plate-bound anti-CD3 antibody and 1 µg/ml soluble anti-CD28 antibody) for 72 h in the presence of multiple doses of a FAK inhibitor. T cells were stained with antibodies that recognized CD4, FOXP3, and CD25 and analyzed by flow cytometry. The generation of CD4+FOXP3+CD25+ T cells was reduced with the FAK inhibitor in a dose-dependent manner (Fig. 5, C and D). These data demonstrated that FAK was necessary for the optimal induction of FOXP3+CD25+ T cells.

To confirm the results from the FAK inhibitor studies, FAK was knocked down in CD4+ T cells using siRNA (Fig. 5E) and activated with either a low or high dose of anti-CD3 antibody and constant amount of anti-CD28 antibody. Activation with a low-dose stimulus drove the generation of FOXP3+CD25+ T cells in cells treated with the scrambled control, whereas cells treated with siRNA targeting FAK had reduced generation of FOXP3+CD25+ T cells (Fig. 5, F and G). Conversely, FAK knockdown promoted the generation of FOXP3−CD25+ T cells in similar proportion to T cells activated with a high dose anti-CD3 stimulus (Fig. 5, F and H). Taken together, these data demonstrated that FAK is essential for optimal FOXP3 induction from a weak TCR signal.

**Differential PDK1 and mTORC2 PIP3 thresholds regulate AKT activation in CD4+ T cells**

Our model predicts that only low PIP3 levels are necessary to activate PDK1 to phosphorylate Thr-308 on AKT, whereas mTORC2 activation required high PIP3 levels to phosphorylate Ser-473 on AKT (Figs. 1, A, C, and D, and 6A). The differential activation thresholds between PDK1 and mTORC2 are likely controlled by the difference in binding affinity for PIP3 (Kd of 1.5 versus 141 nM, respectively) (23, 26).

The relationship between TCR signal strength and PIP3 generation was first established by measuring PIP3 abundance as a function of anti-CD3 antibody dose and constant anti-CD28 antibody concentration (1 µg/ml) using a mass ELISA kit at 10 min poststimulation from murine CD4+ T cells. PIP3 generation yielded a sigmoidal response as a function of anti-CD3 antibody dose and plateaued at 1 µg/ml of anti-CD3 antibody (Fig. 6B). One approach to alter the PIP3/PI(4,5)P2 ratio was to inhibit PTEN, which should increase PIP3 levels. In CD4+ T cells pretreated with PTEN inhibitor, the amount of anti-CD3 antibody required to achieve maximal PIP3 was reduced to 0.25 µg/ml (Fig. 6B). Previous work demonstrated that TCR signal strength modulated PTEN protein abundance, where weak signals maintained PTEN and strong signals reduced PTEN (5). We monitored PTEN levels as a function of TCR signal strength and confirmed that PTEN levels are inversely correlated to TCR signal strength (Fig. 6, C and D), further demonstrating that CD4+ T cells modulate the balance of PI(4,5)P2/PIP3 by regulating PTEN.

T-cell activation assays were performed to determine activation thresholds for PDK1 and mTORC2. Autophosphorylation of Ser-241 is an activation marker for PDK1 (46). Phosphorylation Thr-1135 on the RICTOR subunit of mTORC2 is a repressive modification, so disappearance of pThr-1135 RICTOR is a marker of mTORC2 activity (47). PDK1 achieved maximal activation at the lowest anti-CD3 antibody dose tested, 0.15 µg/ml (Fig. 6, C and E). Maximal phosphorylation of Thr-308 on AKT, which is phosphorylated by PDK1, was also achieved at 0.15 µg/ml of anti-CD3 antibody (Fig. 6F). However, maximal mTORC2 activation required 1 µg/ml of anti-CD3 antibody as measured by dephosphorylation of mTORC2 (Fig. 6, C and E) and phosphorylation of AKT Ser-473 (Fig. 6, C and F). To characterize the relationship between PIP3 levels and activation thresholds for PDK1 and mTORC2, the levels of p-PDK1, mTORC2 p-T308-AKT, and p-S473-AKT were plotted versus PIP3 levels (Fig. 6, C and H). These data confirmed that mTORC2 has a higher PIP3 activation threshold than PDK1 to become activated in a CD4+ T cell, which in turn regulates the proteoform of AKT generated.

We reasoned that if mTORC2 required more PIP3 for maximal activation, then PTEN inhibition would reduce the dose of anti-CD3 stimulatory antibody needed for mTORC2 activation (Fig. 6B). In experiments were T cells were pretreated with the PTEN inhibitor, the dose of anti-CD3 antibody required to achieve maximal RICTOR dephosphorylation was reduced to 0.25 µg/ml (Fig. 6, C and E). Additionally, PTEN inhibition reduced the anti-CD3 dose required for mTORC2 to phosphorylate Ser-473 on AKT (Fig. 6, C and F). Addition of a PTEN inhibitor did not alter the threshold for phosphorylation of Thr-308 (Fig. 6, C and F), which is consistent with PDK1 having a lower PIP3 activation threshold. Together, these results supported the proposed model where low levels of PIP3 generated by a weak TCR signal were sufficient to activate PDK1 to phosphorylate Thr-308 on AKT, whereas stronger TCR signals were required to generate higher PIP3 levels to activate mTORC2 to phosphorylate AKT-S473.

**Discussion**

Herein, we identified that the balance between PI(4,5)P2 and PIP3 is a key determinant in grading the strength of a TCR signal (Fig. 7). In our measurements, generation of PIP3 and PI(4,5)P2 has sharp TCR signal strength thresholds that resemble a digital signaling circuit. A weak TCR signal is encoded by high PI(4,5)P2 and low PIP3, whereas a strong TCR signal is encoded by low PI(4,5)P2 and high PIP3. In cytotoxic lympho-
cytes, PIP5 kinases are expelled from the membrane around TCRs in the immunological synapse, which prevents PI(4,5)P2 replenishment. These data demonstrate that the coordination of PIPs around the TCR is a mechanism of controlling T-cell activation and effector functions. Our model predicts that the activation of proteins sensitive to PIP3 levels would also have a sharp activation threshold as a function of TCR signal strength. Indeed, we observe sharp activation thresholds for proteins that are activated by PIP3, including AKT and mTORC2. Additionally, PTEN protein levels demonstrated a sharp diminution as a function of TCR signal strength, which further demonstrates the commitment of a T cell to actively modulate the PI(4,5)P2 to PIP3 ratio to interpret TCR signal strength.

Although most T-cell activation mechanisms ascribe PI(4,5)P2 as an intermediary metabolite in the synthesis of PIP3, our work suggests that PI(4,5)P2 has an active function in encoding a weak TCR signal. T cells activated with weak TCR stimulation employ multiple mechanisms to sustain PI(4,5)P2 levels including maintenance of PTEN activity at the transcriptional and posttranscriptional levels. We find that maintenance of PTEN enzymatic activity is crucial for generating PI(4,5)P2 during a weak TCR signal and is involved with the activation of downstream kinases including FAK. PTEN inhibition diminishes Treg function and FAK inhibition also reduced Treg induction. In other studies that utilized microscopy, weak TCR stimulation resulted in a ring-like structure around TCR microclusters that contained the FAK-interacting protein paxillin. Possibly, the elevated PI(4,5)P2 production around the TCR that we observed could facilitate the organization of this structure. FAK inhibition could have therapeutic potential in the context of...
TCR strength is encoded with phosphatidylinositols

Figure 7. Model describing how the balance of PI(4,5)P2/PIP3 is used to measure TCR signal strength. Stimulation of a T cell with a weak TCR signal results in maintenance of PTEN, elevated PI(4,5)P2, and lower PIP3 levels. In this mode, there is sufficient PIP3 generation to activate PDK1 to phosphorylate AKT on Thr-308. The elevated PI(4,5)P2 levels generated from a weak signal activate FAK. Stimulation with a strong TCR signal reduces PTEN levels, which allows for higher PIP3 levels and reduced PI(4,5)P2. Higher levels of PIP3 activate both PDK1 and mTORC2, which results in phosphorylation of AKT on both Thr-308 and Ser-473. Diminished PI(4,5)P2 results in weak FAK activation. The AKT proteoforms generated by a weak versus high TCR signal have different substrate specificities and activate divergent downstream signaling pathways to program different T-cell fate decisions.

In conclusion, the work presented here provides a molecular mechanism illustrating how CD4⁺ T cells measure TCR signal strength. In this model, T cells generate different levels of multiple phosphatidylinositols, which in turn engage different signaling pathways to drive alternate cell fate decisions. Generally, phosphatidylinositol-binding proteins display a wide range of binding affinities and specificities. Therefore, cells contain signaling networks that are responsive to qualitative and quantitative fluctuations in phosphatidylinositol metabolism, which could be manipulated by receptor signaling to drive alternate signaling programs and integrate multiple receptor signaling inputs. We propose that generation of different phosphatidylinositols is a driver of T-cell fate decisions. Because of the number of possible phosphatidylinositols that can be generated, T cells could utilize phosphatidylinositol metabolism to encode the type of TCR and cytokine stimuli received and possibly integrate receptor signaling inputs to generate coherent effector outputs.

Experimental procedures

Computational modeling of AKT activation in a CD4⁺ T cell

A model of AKT activation in a T cell was constructed in the SimBiology application of Matlab R2017B. The model was simulated using the ode15s (stiff/NDF) solver. TCR strength was modeled by changing the initial amount of the TCR–pMHC complex in the simulation.

Murine CD4⁺ T-cell isolation and activation assays

Spleens from C57BL/6 mice were a generous gift from the laboratory of Dr. Louise D’Cruz at the University of Pittsburgh.
The mice were housed at the University of Pittsburgh in a pathogen-free facility and handled under Institutional Animal Care and Use Committee-approved guidelines. CD4+ T cells were isolated from C57BL/6 spleens using a CD4+ -negative selection kit (Miltenyi Biotech), and CD25+ T cells were removed using CD25 microbeads. Following isolation, T cells were incubated for 1 h at 37°C. T cell activation assays were performed with various doses plate-bound anti-CD3 mAb (clone 17A2 BioLegend) noted specified throughout the manuscript in the presence of 1 μg/ml soluble anti-CD28 mAb (Clone 37.51 BioLegend). For experiments using inhibitors, isolated CD4+ T cells were incubated with 10 μM PTEN inhibitor (SF1670) for 1 h prior to activation.

**Mass ELISA assay to measure phosphatidylinositol abundance during T-cell activation**

Following activation, 10 million CD4+ T cell pellets were washed with 1 ml of ice cold 0.5 mM TCA. Neutral lipids were extracted by adding 750 μl of MeOH:CHCl3:12 N HCl (80:40:1), vortexing for 30 min, and centrifuging for 10 min at 3000 rpm. The supernatant was transferred to a new 2-ml tube to which 250 μl of CHCl3 and 450 μl of 0.1 N HCl were added. The sample was then centrifuged to separate the aqueous and organic phases. The organic phase was collected and dried under a stream of nitrogen gas. The sample was reconstituted in PBS. Mass ELISA kits from Echelon Biosciences to measure PI(4,5)P2, PIP3, and PI(3,4)P2 following the manufacturer’s protocol. The mass ELISA results were measured at 450 nm on a Molecular Devices SpectraMax i3 plate reader. For each sample, three biological samples were measured, and two technical replicates were performed per sample. The standard curve was fit assuming a sigmoidal dose response with variable slope, and replicates were performed per sample. The standard curve was used to determine the relative abundance of proteins in each sample was extrapolated in the GraphPad Prism 8 software package.

**siRNA knockdown**

A murine PTEN siRNA kit (Origene) was used to knock down expression. A murine FAK kit (Origene) was used to knock down FAK expression. The siRNAs were introduced into isolated CD4+ T cells using a standard protocol (Lonzo Nucleofector kit for mouse T cells). Western blotting analysis for either PTEN, FAK, or PTEN was performed after 48 h of incubation.

**Staining for PI(4,5)P2, PIP3, and PI(3,4)P2**

Single cell suspensions of splenocytes were prewarmed in DMEM at 2 × 106 cells/ml for 1 h. The cells were activated with various doses of plate-bound anti-CD3 mAb noted in the manuscript in the presence of soluble anti-CD28 mAb (1 μg/ml). To stop stimulation, one volume of 2× Cytofix/wash buffer (3% PFA + BD perm/wash buffer (catalog no. 554723)) was added directly to cell culture and incubated at room temperature for 15 min and on ice for 30–60 min. The cells were washed twice with perm/wash buffer and resuspended in perm/wash buffer with FC blocking antibody (2.4G2). Antibodies against TCR-APC (BD Biosciences clone H57-597), CD4-PerCP-5.5 (BD Biosciences clone RM4-5), and CD8-BUV395 (BD Biosciences clone 53-6.7). The cells were either stained with PI(4,5)P2-PE (Echelon Biosciences Z-B045), PIP3-PE (Echelon Biosciences Z-B3345B), or PI(3,4)P2-PE (Echelon Biosciences Z-B034) and were incubated with the samples for 60 min. The cells were washed with perm/wash buffer and resuspended in PBS containing 3% fetal serum, 2 mM EDTA, 0.02% azide.

**Analysis of cells by flow cytometry**

The samples were analyzed on LSR II flow cytometer, and the data were analyzed with the Flowjo 10 software package. For each sample, five thousand CD4+ or CD8+ T cells were acquired.

**Analysis of cells by imaging flow cytometry**

Samples were analyzed on an Image Stream MarkII imaging flow cytometer. 1000 cells/sample were collected. The IDEAS software package was utilized to compensate, process, and analyze all of the imaging flow cytometry data.

**Mass spectrometric analysis of PI(4,5)P2-, PIP3-, and PI(3,4)P2-binding proteins**

20 million CD4+ T cells were lysed in a buffer containing 1% Nonidet P-40, 50 mM Tris (pH 8.0), and 150 mM NaCl containing Complete C phosphatase inhibitor mixture. Lysates were incubated with beads coated with either PI(4,5)P2, PIP3, and PI(3,4)P2 from Echelon Biosciences at 4°C for 12 h. Filter-aided sample preparation was utilized to generate tryptic peptide fragments. The samples were analyzed by reverse phase LC in tandem with MS using a Waters nanoAcquity LC system using a New Objective PicoChip nanospray column in line with a ThermoFisher LTQ Velos Orbitrap Pro mass spectrometer. Raw spectra were processed using the PEAKS 8 software package. The PEAKS 8 software package was used to identify proteins in the IP using the UNIPROT mouse protein database and a 1% false discovery rate. The quantitative module of PEAKS 8 was utilized to determine the relative abundance of proteins in each IP.

**Bioinformatics**

The proteins that bound to specific phosphatidylinositol-coated beads were analyzed with the Ingenuity software package (Qiagen). A standard core analysis with default settings was utilized to analyze the data set and identify pathways that were overrepresented in each phosphatidylinositol IP. The right-tailed Fisher test was utilized to calculate p values, and a cutoff for significance was set to <0.05.

**Western blotting**

PAGE was performed using Bio-Rad precast Protein TGX gels. Proteins were transferred to polyvinylidene difluoride membranes using a Bio-Rad Trans-Blot Turbo transfer system using the mixed molecular weight transfer setting. All antibodies used for Western blotting were purchased from Cell Signaling Technology, which included Pdk1 (D37A7), pPDK11 (C49H2), RICTOR (D15H9), pRICTOR (D30A3), AKT (C67E7), p308AKT (D25E6), p473AKT (D9E), ACTIN (13E5), H3 (D1H2), FAK (D2R2E), and p397FAK (D20B1). All antibodies utilized were rabbit. An anti-rabbit IgG-HRP antibody (Cell
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Signaling Technology 7074) was used with the SuperSignal West Pico Plus chemiluminescent substrate for detection on a protein simple FluorChem M system.

Statistics

For all experiments, the statistics were calculated with Prism GraphPad 8 software. Two-way ANOVA tests were calculated using Bonferroni after analysis correction.

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