bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 *Article*

2 Itk promotes the integration of TCR and CD28 3 costimulation, through its direct substrates, SLP-76 4 and Gads

5 **Enas Hallumi[1], Rose Shalah[1], Wan-Lin Lo[2], Jasmin Corso[3], Ilana Oz[1], Dvora Beach[1], Samuel 6 Wittman[1], Amy Isenberg[1], Meirav Sela[1], Henning Urlaub[3,4], Arthur Weiss[2,5] and Deborah 7 Yablonski[1*]**

8 [1] Department of Immunology, Ruth and Bruce Rappaport Faculty of Medicine, Technion—Israel Institute of 9 Technology, Haifa 3525433, Israel
10 [2] Division of Rheumatology, Rosalind Russell and Ephraim P. Engleman Arthritis Research Center, 11 Department of Medicine, University of California, San Francisco, San Francisco, CA, USA
12 [3] Bioanalytical Mass Spectrometry Group, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 13 37077 Göttingen, Germany
14 [4] Bioanalytics, Institute for Clinical Chemistry, University Medical Center Göttingen, Robert Koch Strasse 40, 15 37075 Göttingen, Germany
16 [5] Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA, USA
17 * Correspondence: debya@technion.ac.il

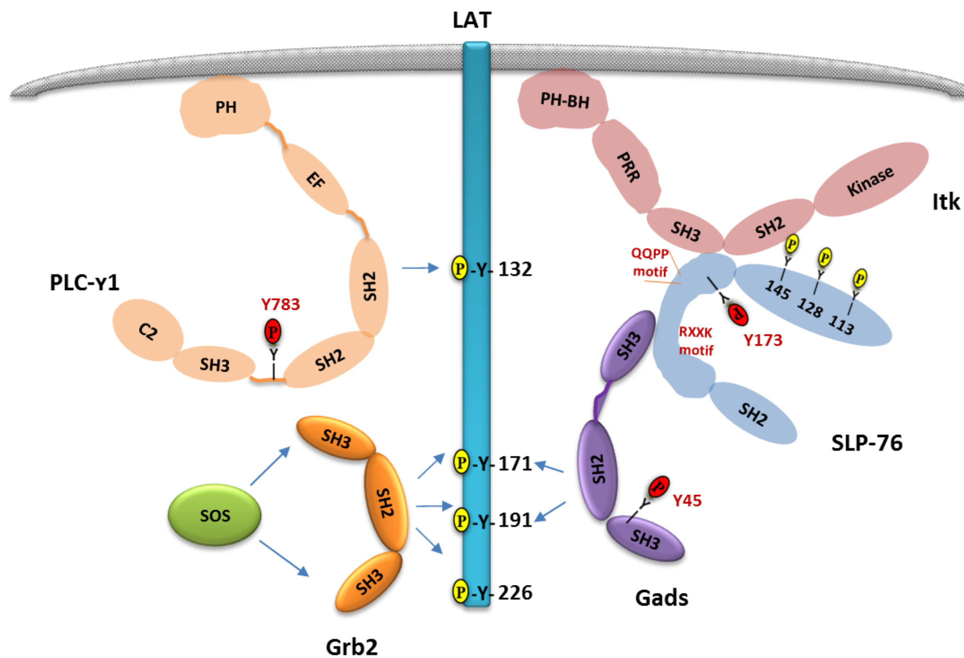
18 **Abstract:** The costimulatory receptor, CD28, synergizes with the T cell antigen receptor (TCR) to 19 promote IL-2 production, cell survival and proliferation. Despite their profound synergy, the 20 obligatory interdependence of the signaling pathways initiated by these two receptors is not well 21 understood. Upon TCR stimulation, Gads, a Grb2-family adaptor, bridges the interaction of two 22 additional adaptors, LAT and SLP-76, to form a TCR-induced effector signaling complex. SLP-76 23 binds the Tec-family tyrosine kinase, Itk, which phosphorylates SLP-76 at Y173 and PLC-γ1 at 24 Y783. Here we identified Gads Y45 as an additional TCR-inducible, Itk-mediated phosphorylation 25 site. Y45 is found within the N-terminal SH3 domain of Gads, an evolutionarily conserved domain 26 with no known binding partners or signaling function. Gads Y45 phosphorylation depended on the 27 interaction of Gads with SLP-76 and on the preferentially-paired binding of Gads to phospho-LAT. 28 Three Itk-related features, Gads Y45, SLP-76 Y173, and a proline-rich Itk SH3-binding motif on 29 SLP-76, were selectively required for activation of the CD28 RE/AP transcriptional element from 30 the IL-2 promoter, but were not required to activate NFAT. This study illuminates a new regulatory 31 module, in which Itk-targeted phosphorylation sites on two adaptor proteins, SLP-76 and Gads, 32 control the transcriptional response to TCR/CD28 costimulation, thus enforcing the obligatory 33 interdependence of the TCR and CD28 signaling pathways.

34 **Keywords:** TCR signaling pathway; costimulation; adaptor proteins; Gads; SLP-76; Itk
35

36 1. Introduction

37 The TCR signaling pathway [recently reviewed in 1, 2, 3] is initiated by a hierarchical tyrosine 38 kinase cascade, leading to the formation of a large effector signaling complex that is nucleated by 39 three interacting adaptor proteins, LAT, Gads and SLP-76 (Figure 1). Within this LAT-nucleated 40 complex, adaptor-associated enzymes are recruited to become activated and trigger downstream 41 responses. For example, phospholipase-Cγ1 (PLC-γ1) is a key signaling enzyme that is 42 phosphorylated and activated within the LAT-nucleated complex and produces second messengers, 43 IP_3 and DAG, which respectively trigger calcium flux and Ras-pathway activation. Further 44 downstream, these signaling events result in the activation of well-characterized transcription 45 factors, including NFAT and AP-1.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



46

47 **Figure 1. A web of interactions connects Itk to its substrates.** Upon TCR stimulation, ZAP-70
48 phosphorylates conserved tyrosine residues on LAT and SLP-76 (shown in yellow). Itk binds to
49 SLP-76 via a multivalent interaction, in which its SH2 may bind to SLP-76 p-Y145, and its SH3
50 domain may bind to the SLP-76 QQPP motif. Gads bridges the recruitment of SLP-76 to LAT, via its
51 C-SH3 domain, which binds with high affinity to a SLP-76 RxxK motif, and its central SH2 domain,
52 which binds in a cooperatively paired manner to LAT p-Y171 and p-Y191. Grb2 bridges the
53 recruitment of SOS to LAT p-Y171, p-Y191 and/or p-Y226. PLC-γ1 is recruited via its N-SH2 to LAT
54 p-Y132, and is thereby brought into the vicinity of SLP-76-bound Itk. Itk-targeted sites, including
55 Gads Y45, identified in this study, are shown in red.

56 In addition to the above events, full T cell responsiveness depends on costimulatory signals that
57 are triggered upon binding of B7-family ligands to the canonical costimulatory receptor, CD28. The
58 TCR and CD28 signaling pathways act synergistically to induce the activation of NFκB-family
59 members, which are required for the transcriptional activation of IL-2 and Bcl-xL, key response
60 markers that promote T cell proliferation and survival [4, 5].
61 The TCR and CD28 signaling pathways exhibit remarkable interdependence, which can be
62 most easily demonstrated by measuring the activity of RE/AP, a composite transcriptional element
63 that forms an essential part of the IL-2 promoter [6, 7]. Composed of adjacent AP-1 and NFκB sites
64 that bind to c-Jun and to c-Rel, the RE/AP element recapitulates the costimulation-dependence of
65 IL-2 transcription, as stimulation with either TCR or CD28 alone is insufficient to activate RE/AP, but
66 co-stimulation through both receptors produces profound RE/AP activation [7, 8].
67 The interdependence of TCR and CD28 responsiveness has important biological outcomes.
68 CD28 costimulatory ligands serve as "danger signals" to indicate the presence of microorganisms
69 that necessitate an adaptive immune response [9]. Yet, to avoid the induction of potentially
70 dangerous bystander activity, CD28-dependent T cell activation must necessarily be restricted to
71 those cells bearing an antigen-specific clonotypic TCR. This goal is achieved by profound
72 interdependence, such that CD28 activity is necessary but insufficient for the activation of naive T
73 cells. However, the mechanistic basis for TCR/CD28 interdependence is not well understood.
74 The basic outlines of the TCR signaling pathway are well-defined [reviewed in 1, 10]. Upon
75 TCR stimulation, a tyrosine kinase cascade is initiated by Lck, a Src-family tyrosine kinase that
76 phosphorylates characteristic ITAM motifs within the CD3 and ζ accessory chains of the TCR.
77 Doubly-phosphorylated ITAM motifs trigger the recruitment and activation of a Syk-family tyrosine
78 kinase, ZAP-70 [11]. Activated ZAP-70 phosphorylates LAT, a membrane-bound adaptor, on at least

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

79 four essential sites [12-15], and phosphorylates SLP-76, a cytoplasmic adaptor, at three N-terminal
80 phosphorylation sites [Figure 1 and 16, 17]. The ZAP-70-phosphorylated sites recruit additional
81 signaling molecules via SH2-mediated interactions. Phospho-LAT binds directly to PLC-γ1, Grb2
82 and Gads, whereas the three tyrosine phosphorylation sites on SLP-76 bind to Nck, Vav and Itk
83 [reviewed in 2, 18].
84 Itk, a Tec-family tyrosine kinase, is the third member of the TCR-induced tyrosine kinase
85 cascade, as it is activated downstream of both Lck and ZAP-70. Lck phosphorylates Itk at Y511 [19],
86 whereas ZAP-70 phosphorylates SLP-76 at three N-terminal tyrosines that are required for Itk
87 activation [16, 17, 20, 21]. Upon TCR stimulation, Itk associates with SLP-76, and this association is
88 required to maintain the active conformation of the kinase [20]. SLP-76-bound Itk phosphorylates
89 SLP-76 at Y173 [21]. In turn, SLP-76 p-Y173 promotes the subsequent Itk-mediated phosphorylation
90 of PLC-γ1 at Y783 [21, 22], a site that is required for PLC-γ1 activation [23].
91 Structural studies suggest that an inactive conformation of Itk is stabilized by intramolecular
92 interactions of its SH2 and SH3 domains, as well as by an inhibitory interaction between the
93 N-terminal PH domain and the kinase domain [24-26]. Upon TCR or CD28 stimulation, PIP_3 in the
94 plasma membrane is increased. Elevated PIP_3 binds to the Itk PH domain, relieving the inhibitory
95 influence of the PH domain on the kinase domain [26]; moreover, bivalent binding of the SH2 and
96 SH3 domains of Itk to SLP-76 is thought to stabilize the active confirmation of Itk [Figure 1, right and
97 27, 28, 29].
98 The SH2 domain of Itk is thought to bind to SLP-76 p-Y145 [25]; however, competitive binding
99 studies suggested that it may also bind to SLP-76 p-Y113 [28], which is equivalent to murine p-Y112.
100 A SLP-76 Y145F mutation closely phenocopied Itk-deficient mice, but was insufficient to eliminate
101 the TCR-induced binding of Itk to SLP-76 [30]. Unexpectedly, phosphorylation PLC-γ1 Y783 was
102 markedly reduced by either the SLP-76 Y145F mutation, or by the double SLP-76 Y112,128F
103 mutation [21]. Thus, although Itk is commonly thought to bind to SLP-76 Y145 [25, 31], evidence
104 suggests that at least two N-terminal tyrosines of SLP-76 are required, whether directly or indirectly,
105 for optimal activation of Itk.
106 The SH3 domain of Itk can bind to a conserved proline rich motif, QQPPVPPQRP,
107 corresponding to SLP-76 residues 184-195 [28, 29], which, for convenience, we shall refer to as the
108 QQPP motif. This motif was also reported to bind, albeit weakly, to the SH3 domains of Lck and
109 PLC-γ1 [32-35]. Precise removal of the QQPP motif in a transgenic mouse model reduced
110 TCR-stimulated PLC-γ1 phosphorylation and calcium flux, as would be expected if Itk activity were
111 disrupted [34, 36]. Moreover, a cell permeable peptide based on the QQPP motif inhibited the
112 TCR-inducible interaction of Itk with SLP-76, phosphorylation of Itk at Y511, recruitment of Itk to
113 the immune synapse, and consequently inhibited the production of Th2 cytokines [29], all consistent
114 with a defect in Itk-mediated signaling [36, 37]. Nevertheless, deletion of a 36 amino acid region
115 encompassing the QQPP motif only modestly reduced PLC-γ1 phosphorylation and calcium flux in
116 reconstituted J14 cells [32], suggesting that the role of the QQPP motif in regulating Itk activity may
117 be context-dependent.
118 Itk-mediated phosphorylation of PLC-γ1 is facilitated by Gads [38, 39], a Grb2-family adaptor
119 that bridges the TCR-inducible recruitment of SLP-76 to phospho-LAT [Figure 1A, center, reviewed
120 in 3]. Composed of a central SH2 domain, flanked by two SH3 domains and a unique linker region,
121 Gads binds constitutively to SLP-76, via a high affinity interaction of its C-terminal SH3 domain with
122 a conserved RxxK motif on SLP-76 [33, 40, 41]. The SH2 domain of Gads is capable of spontaneous
123 dimerization, and mediates the cooperatively-paired binding of Gads to LAT p-Y171 and p-Y191,
124 thereby recruiting SLP-76 to phospho-LAT [42]. Curiously, the bridging activity of Gads does not
125 require its N-terminal SH3 domain, an evolutionarily conserved domain that has no known ligand
126 or signaling function [3]. Gads bridging activity supports PLC-γ1 phosphorylation, by bringing
127 SLP-76-bound Itk (Figure 1, right) in proximity with its substrate, LAT-bound PLC-γ1 (Figure 1,
128 left).
129 Once formed, the LAT-nucleated signaling complex may be regulated by post-translational
130 events occurring within the complex. HPK1, a SLP-76-associated Ser/Thr kinase, can negatively

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

regulate TCR signaling by phosphorylating SLP-76 at S376 [43, 44], and by phosphorylating Gads at T262 [38, 45]. Conversely, SLP-76-associated Itk promotes TCR responsiveness by phosphorylating SLP-76 at Y173 [21]. Itk activity is highly dependent on docking interactions that target its catalytic activity to potential substrates [22, 46, 47]. Since Itk is inducibly docked onto SLP-76, which binds with high affinity to Gads, this paradigm suggested to us that additional Itk-mediated phosphorylation sites on SLP-76 or Gads might play an important role in regulating their signaling function.

To explore this hypothesis, we performed a phospho-mass spectrometry analysis of SLP-76 and Gads, which were isolated from TCR-stimulated cells. Here we identify Gads Y45 as a TCR-inducible substrate of Itk, which is phosphorylated within the SLP-76-Gads-LAT signaling complex. Y45 is found within the N-terminal SH3 domain of Gads, and may provide a first clue to the biological function of this conserved domain. Unexpectedly, we found that TCR/CD28-induced activation of the RE/AP transcriptional element depended on Gads Y45 and SLP-76 Y173, two Itk-targeted sites, and also depended on the QQPP motif, an Itk-binding site within SLP-76. Gads Y45 phosphorylation was strictly dependent on the TCR-induced cooperative binding of Gads to LAT, and thereby may enforce the dependence of CD28 responsiveness on TCR activation.

2. Materials and Methods

2.1. *Recombinant Gads proteins*

Recombinant, maltose-binding protein (MBP)-Gads fusion proteins were expressed and purified as previously described [42].

2.2. *Antibodies*

To prepare mouse anti-Gads, Gads deficient mice on the Balb/C background were immunized with recombinant MBP-Gads-ΔN-SH3 in Freund's adjuvant. Polyclonal, affinity-purified rabbit anti-phospho-Gads p-Y45 was prepared for us by GenScript, by immunizing rabbits with a phospho-peptide GSQEG{p-TYR}VPKNFIDIC, corresponding to amino acids 40–54 of human Gads, conjugated to KLH, followed by two steps of affinity chromatography, to remove antibodies that recognize the non-phosphorylated peptide and enrich for those that recognize the phosphorylated peptide. To decrease non-specific background without blocking the sequence-specific recognition of p-Y45, we supplemented the diluted, purified antiserum with 3 μg/ml phosphotyrosine-conjugated BSA (Sigma, P3967). Rabbit polyclonal anti-human SLP-76 [32] and rabbit anti phospho SLP-76 p-Tyr173 [21] were previously described. Polyclonal anti-Itk [BL12, 48] was provided by Michael G Tomlinson and Joseph Bolen and was used for immunoprecipitation. The monoclonal antibody C305 [49] was used to stimulate Jurkat-derived cell lines through the TCR. Purified anti-human CD28 clone CD28.2, anti-human CD3-APC, anti-human CD28-PE and anti-human CD69 PerCP/Cy5.5 (clone FN50) were from Biolegend. Rabbit anti-PLC-γ1 (sc-81) was from Santa Cruz Biotechnology. Rabbit anti-phospho PLC-γ p-Tyr783 (AT-7142) was from MBL International. Rabbit polyclonal anti-phospho LAT p-Tyr191 (#3584) was from Cell Signaling Technology. Anti p-Tyr (4G10) and anti-Itk (06-546, used for western blotting) were from Merck Millipore. Anti-phospho Itk p-Tyr511 (clone M4G3LN) was from ThermoFisher. Anti-phosho LAT p-Tyr171 (clone I58-1169) was from BD Biosciences. Anti-phosho Jnk pT183+pT221-PE (ab208843) was from Abcam. Anti-Human IL-2-PE (MQ1-17H12) was from eBioscience.

2.3. *Cell lines and retroviral reconstitution*

Cell lines used in this work are summarized in Table 1. Cells were grown in RPMI, supplemented with penicillin, streptomycin and glutamine (PSG) and 5% fetal calf serum (FCS) in a humidified incubator with 5% CO_2. Cells were retrovirally reconstituted with wild-type or mutant, N-terminally twin-strep tagged [50] or FLAG-tagged, human Gads or SLP-76, using the pMIGR retroviral vector, which bears an internal ribosome entry site (IRES)-GFP cassette to mark infected cells [51]. Approximately two weeks later cells were sorted by FACS for comparable GFP expression, and

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

179 comparable TCR and CD28 expression were verified by FACS. Where indicated, we used a
180 modified, IRES-less version of pMIGR [42] in which Gads was fused C-terminally to a
181 non-dimerizing form of GFP [GFP A206K, 52].
182

Parental Cell Line	Reconstituted with	Description
Gads-deficient cell line, dG32 [38]	Gads Y45F	Tyr 45 substituted with phenylalanine
	Gads ΔN (Δ2-53)	N-terminal SH3 deleted
	Gads F92D [42]	SH2 dimerization interface disrupted (disrupts binding to LAT)
	Gads F92A,R109A [42]	
	Gads P321L [53]	Disrupts binding of Gads C-SH3 to SLP-76 RXXK motif
SLP-76-deficient cell line, J14 [54]	SLP-76 Y173F [21]	Tyr 173 substituted with phenylalanine
	SLP-76 Y145F [17]	An Itk SH2-binding site disrupted
	SLP-76 Δ177-212 [32]	Potential Itk SH3-binding site disrupted by deletion encompassing the QQPP motif
Itk-deficient cell line, J.ITK [55]		
LAT-deficient cell line, J.LAT [56]		

183 **Table 1. A summary of the cell lines and point mutations employed in this study**.

184 2.4. *Cell stimulation and Purification of Gads or SLP-76 complexes*

185 Jurkat-derived T cell lines were washed and resuspended in stimulation medium (RPMI + 100 μg/ml
186 glutamine), preheated to 37°C for 10 min, and then stimulated for the indicated time at 37°C with
187 anti-TCR antibody (C305), with or without 1.5-2 μg/ml anti CD28 as indicated. Cells were lysed at
188 10^8 cells/ml in ice-cold lysis buffer (20 mM Hepes pH 7.3, 1% Triton X-100, 0.1%
189 *n*-dodecyl-β-D-maltoside (Calbiochem) 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 10
190 μg/ml aprotinin, 2 mM EGTA, 10 μg/ml leupeptin, 2 mM phenylmethanesulfonyl fluoride, 1 μg/ml
191 pepstatin, and 1 mM dithiothreitol). Naïve CD4 T cells were sorted by BD FACSAria II. Thymocytes
192 or purified naïve CD4 T cells from 4-6 week old C57BL/6 mice were stimulated with anti-CD3
193 (145-2C11) and anti-CD28 (clone 37.51) at the indicated concentration, followed by cross-linking
194 with anti-hamster IgG. Cells were lysed by directly adding 10% NP-40 lysis buffer to a final
195 concentration of 1% NP40 (containing the inhibitors: 2 mM NaVO4, 10 mM NaF, 5 mM EDTA, 2 mM
196 PMSF, 10 μg/ml Aprotinin, 1 μg/ml Pepstatin and 1 μg/ml Leupeptin). Cell lysates were placed on
197 ice and centrifuged at 16,000 × g for 10 min at 4°C to pellet cell debris, and Gads or SLP-76 complexes
198 were affinity purified by tumbling lysates end-over-end for 1-3 hours at 4°C with Strep-Tactin
199 Superflow high capacity beads (IBA), FLAG M2 magnetic beads (Sigma), or with anti-SLP-76,
200 prebound to protein A sepharose fast flow (GE Healthcare). After three rapid washes with cold lysis
201 buffer, the isolated complexes were analyzed by mass spectrometry (see below) or by Western
202 blotting. Western blots were developed with SuperSignal™ West Pico PLUS Chemiluminescent
203 Substrate and digital images of the membrane were produced by the ImageQuant LAS 4000 or the
204 Fusion Fx7 camera system, followed by quantification with Total-Lab Quant software.

205 2.5. *A SILAC-based kinetic analysis of TCR-induced SLP-76 and Gads phosphorylation sites*

206 TCR-inducible phosphorylation sites within the SLP-76-nucleated complex were quantitatively
207 identified by using a Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)-based
208 approach, exactly as we previously described [38]. In brief, J14 cells, stably reconstituted with
209 twin-strep-tagged SLP-76, were metabolically labeled with the heavy amino acids L-tyrosine $^{13}C_9{}^{15}N$
210 (+10), L-Lysine $^{13}C_6{}^{15}N_2$ (+8) and L-Arginine $^{13}C_6{}^{15}N_4$ (+10), or with the corresponding light amino
211 acids. Prior to lysis, SILAC-labeled cells were stimulated with anti-TCR (heavy label) or
212 mock-stimulated (light label). Heavy and light lysates derived from 120 million cells were mixed at a
213 1:1 ratio, followed by affinity purification of twin-strep-tagged SLP-76 and its associated proteins
214 with Strep-Tactin beads. Purified proteins were split into three samples that were analyzed in
215 parallel. Following SDS-PAGE, Coomassie-stained protein bands corresponding to Gads and SLP-76

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

216 were cut from the gel, followed by in gel digestion with trypsin, chymotrypsin or AspN.
217 Phospho-peptides were enriched with TiO_2 and were analyzed on an LTQ Orbitrap Velos (Thermo
218 Fischer) mass spectrometer coupled to a nanoflow liquid chromatography system (Agilent 1100
219 series, Agilent), as described [57]. Resulting raw files were processed with MaxQuant (v1.3.0.5)
220 against a UniProtKB/Swiss-Prot human database. Data presented are the median values from four
221 biological repeats.

222 2.6. *Kinase assays*

223 Polyclonal anti-Itk (BL12) was used to immunoprecipitate Itk from the lysates of 4 × 10^6
224 TCR-stimulated dG32 cells. IP beads were washed twice with lysis buffer and once with kinase
225 reaction buffer (25 mM HEPES pH 7.3, 7.5 mM $MgCl_2$, and 1 mM Na_3VO_4), resuspended in 30 µl of
226 kinase reaction buffer containing 1 µM of recombinant MBP- Gads protein, and preheated for 2 min
227 at 30 °C. Kinase activity was initiated by the addition of ATP to 1 µM, and was terminated after 30
228 min at 30 °C with end-over-end mixing, by the addition of EDTA to 12.5 mM.

229 2.7. *FACS-based functional assays*

230 To decrease experimental variation, cell lines were barcoded by differential labeling with fourfold
231 dilutions of CellTrace Far Red or CellTrace Violet (ThermoFisher), and mixed together prior to
232 stimulation as described [42]. For calcium assays, mixed, CellTrace Far Red-barcoded cells were
233 loaded with the fluorescent calcium indicator dye, Indo1-AM (eBioscience), and then washed twice
234 and resuspended in calcium buffer, consisting of 25 mM Hepes (pH 7.4), 1 mM $CaCl_2$, 125 mM NaCl,
235 5 mM KCl, 1 mM Na_2HPO_4, 0.5mM $MgCl_2$, 1 g/l glucose, 2 mM L-glutamine and 1 mg/ml
236 high-purity bovine serum albumin (Sigma A4378). Intracellular calcium was measured by FACS at
237 37°C, with C305 or C305+CD28.2 stimulant added at the 60s time point [38]. CellTrace
238 Violet-barcoded cells were stimulated as indicated in the figure legends, and stained with
239 anti-CD69, or were fixed, permeabilized and stained with anti-pJNK, or with anti-IL-2-PE
240 (eBioscience). Results were analyzed using FlowJo, while gating on a defined GFP window within
241 each barcoded population.

242 2.8. *Luciferase Assays*

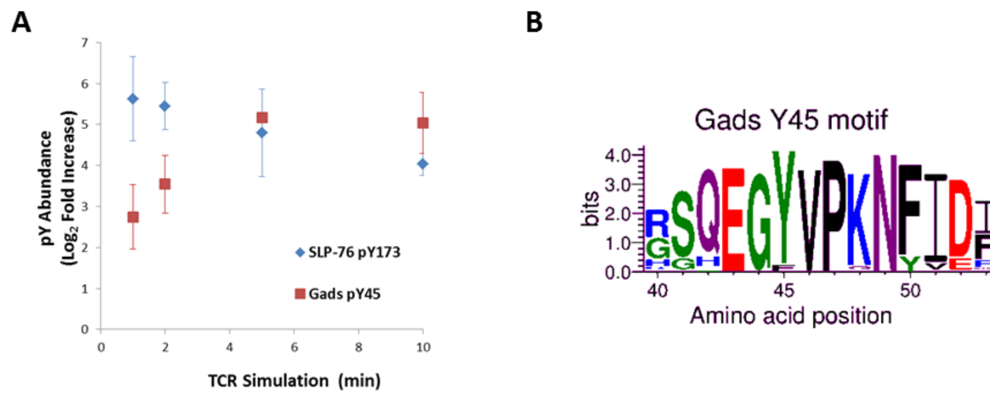
243 The firefly luciferase reporter plasmids pdelta-ODLO-3XNFAT and pdelta-ODLO-4XRE/AP [7]
244 were provided by Virginia Shapiro (Mayo Clinic). 5xκB-luciferase was from Stratagene. pRL-null,
245 which drives constitutive expression of renilla luciferase (Promega), was used for normalization.
246 $20X10^6$ cells were transfected by electroporation with 20 µg of firefly luciferase reporter plasmid and
247 3-5 µg of pRL-null, using the Gene Pulser (Bio-Rad Laboratories), at a setting of 234 V and 1000
248 microfarads. 16-20 hr after transfection $2X10^5$ cells per well were stimulated for 6 hr at 37^0C in a 96
249 well plate format with plate-bound anti-TCR (C305) and/or soluble anti-CD28 (CD28.2 1.5 µg/ml) or
250 were mock-stimulated, and activity was measured with the Dual Luciferase Kit (Promega). To
251 correct for variations in transfection efficiency, firefly luciferase activity for each well was
252 normalized the renilla luciferase activity measured in the same well.

253 **3. Results**

254 **3.1. *TCR-inducible phosphorylation of Gads at Y45***

255 We previously described a SILAC-based approach to identify TCR-inducible phosphorylation
256 sites within the SLP-76-nucleated complex [38]. Here, we used this approach to survey the
257 TCR-inducible phosphorylation sites on SLP-76 and Gads. Of the sites we identified, peptides
258 harboring SLP-76 p-Y173 and Gads p-Y45 exhibited the highest fold increase in intensity upon TCR
259 stimulation (Figure S1A and S1B). We previously characterized SLP-76 p-Y173, a TCR-inducible site
260 that is phosphorylated by Itk and facilitates the subsequent Itk-mediated phosphorylation of PLC-γ1
261 [21]; in contrast, the regulation and function of Gads p-Y45 are completely unknown.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



262
263 **Figure 2. A conserved Gads tyrosine phosphorylation site, identified by MS.** (**A**) **Kinetics of the**
264 **TCR-induced phosphorylation of Gads Y45 and SLP-76 Y173.** A SILAC approach was employed to
265 measure the TCR-induced fold change in SLP-76 and Gads phosphorylation sites. Shown is the
266 median Log_2-fold change for the two most highly-induced sites: Gads p-Y45 and SLP-76 p-Y173. Data
267 are from four independent biological replicates; error bars indicate the SD. (**B**) **Evolutionary**
268 **conservation of the sequence motif surrounding Gads Y45.** NCBI Protein Blast was used to identify
269 and select 66 vertebrate Gads orthologs from the mammalian, avian, cartilaginous and bony fish,
270 amphibian, and reptilian classes, including representatives of 55 different taxonomical orders [3].
271 Sequences were aligned with Clustal O [62], and WebLogo [63] was used to depict the conservation
272 of Gads residues 40-53.

273
274 We were intrigued by the high fold-induction of Gads p-Y45 upon TCR stimulation (Figure 2A)
275 and by the evolutionary conservation of the sequence motif surrounding this site (Figure 2B). Gads
276 Y45 is found within the N-terminal SH3 domain of Gads, which, like the C-terminal SH3 domain, is
277 highly conserved (Figure S2). Whereas the C-SH3 binds with high affinity to SLP-76; the N-SH3 has
278 no known function [3]. Phosphorylation of Gads Y45 was previously observed in high-throughput
279 phospho-MS studies [58-61]; yet its functional significance was not previously explored.
280 To fill the gaps in our fundamental knowledge regarding the potential roles of Gads Y45 in TCR
281 signaling, we took advantage of a Gads-deficient T cell line, dG32 [38], which we stably
282 reconstituted with N-terminally twin-strep-tagged Gads, either wild-type (WT) or bearing a
283 phenylalanine substitution at Y45 (Y45F). We also generated an affinity-purified, phospho-specific,
284 polyclonal antibody, to enable us to specifically detect the phosphorylation of Gads Y45.
285 For routine detection of Gads Y45 phosphorylation, cells were costimulated with anti-TCR and
286 anti-CD28, and strep-tactin-purified Gads complexes, which include Gads-associated SLP-76, LAT
287 and PLC-γ1, were probed by immuno-blotting. TCR/CD28-inducible phosphorylation of Gads was
288 clearly detectable using either the p-Y45 phospho-specific reagent or a global p-Tyr antibody (clone
289 4G10), and this band was substantially reduced by the Y45F mutation (Figure 3A, top three panels).
290 This result validates our phospho-specific reagent and suggests that Y45 is the major Gads tyrosine
291 phosphorylation site that can be detected by the anti-pTyr antibody, 4G10.
292 TCR-inducible phosphorylation of Gads Y45 was rapid and sustained, peaking at 2-5 minutes
293 and was still detectable 20 min after stimulation (Figure 3B, top). Phosphorylation of SLP-76 Y173
294 was similarly rapid and sustained, whereas the phosphorylation of Gads-associated PLCγ1
295 appeared to be more transient, perhaps reflecting the previously-reported dissociation of
296 phospho-PLCγ1 from LAT [64]. In some experiments, TCR-induced Gads Y45 phosphorylation was
297 moderately augmented by CD28 costimulation, but this difference was not statistically significant
298 (Figure 3B, bottom).
299 Inducible phosphorylation of Gads Y45 was also observed in primary mouse T cells across their
300 development stages, including thymocytes and naive T cells (Figure 3C). Together, these results
301 demonstrate TCR-inducible phosphorylation of Gads Y45 in both a human T cell line and in primary
302 murine T cells, suggesting a potentially important function, which we set out to investigate.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

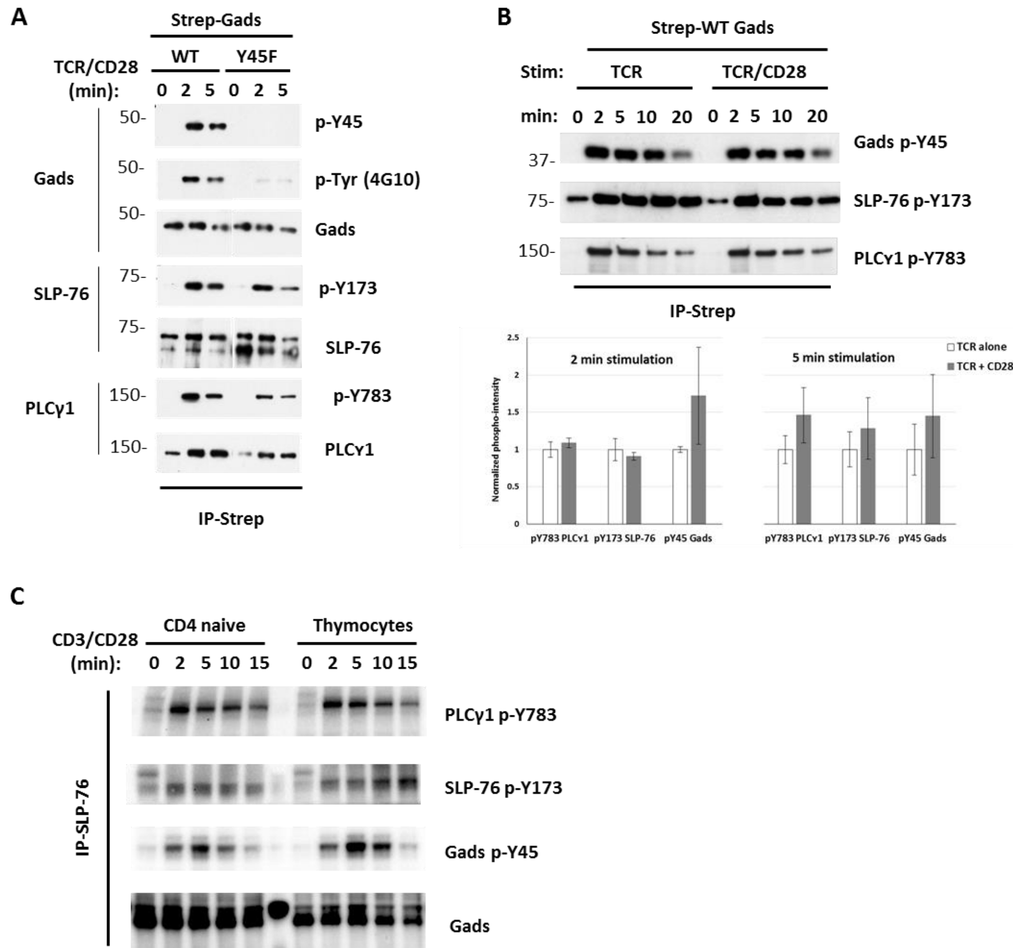


Figure 3. A phospho-specific reagent reveals TCR-induced Gads Y45 phosphorylation in mouse and human T cells. (**A**) **Validation of a p-Y45 phospho-specific reagent.** dG32 cells, stably reconstituted with twin strep-tagged Gads, either WT or Y45F, were stimulated with anti-TCR (C305, 1:1000 dilution) and anti-CD28 (CD28.2 1.5 μg/ml), and then lysed. Gads complexes were isolated with strep-tactin beads (IP-Strep) and probed with the indicated antibodies. Results are representative of at least 5 repeats. (**B**) **Gads Y45 phosphorylation is independent of CD28 co-stimulation.** dG32 cells reconstituted with WT Gads were stimulated with anti-TCR (C305, 1:1000 dilution), in the presence or absence of anti-CD28 (CD28.2 1.5 μg/ml), and lysed. Top: streptactin complexes were probed as in A. Bottom: cells were stimulated for 2 or 5 min in quadruplicate. Streptactin complexes were probed for phospho- and total Gads, whereas lysates were probed for phospho-and total SLP-76 and PLC-γ1, followed by quantification. Phospho-intensity was calculated as the ratio of phospho- to total protein in each band. Results are presented as the fractional phospho-intensity, relative to the average intensity observed in TCR stimulated WT cells from the same stimulation time. (**C**) **Inducible phosphorylation of Gads Y45 in primary mouse T cells.** Prior to lysis, thymocytes or naive CD4 peripheral T cells isolated from C57BL/6 mice were stimulated with 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28, followed by anti-Armenian Hamster IgG crosslinking to induce stimulation for the indicated time at 37°C. SLP-76 complexes were isolated by immunoprecipitation and were probed with the indicated antibodies.

3.2. *Gads Y45 phosphorylation is mediated by Itk*

TCR-proximal signaling is mediated by a cascade of three tyrosine kinases, Lck, ZAP-70 and Itk, acting in a strictly hierarchical order. Within this cascade, the activity of each kinase is limited by requirements for characteristic substrate motifs [65] and kinase docking sites [46, 47, 56]. To assess which kinase might be responsible for phosphorylating Gads at Y45, we compared the motif surrounding Gads Y45 (Figure 2B) to the known substrate preferences of Lck, ZAP-70, and Itk. Lck has a strong preference for a bulky hydrophobic residue at the Y-1 position, and does not tolerate

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

lysine at the Y+3 position [65]. Gads Y45 violates both of these rules, suggesting that it is not a substrate of Lck. ZAP-70-targeted sites are characterized by multiple negative residues surrounding the phosphorylated tyrosine; indeed, ZAP-70 is deterred from phosphorylating substrate motifs containing a positive charge anywhere within the surrounding motif [65]. The presence of glycine at the Y-1 position also slows substrate phosphorylation by ZAP-70 [55]. Gads Y45 is preceded by glycine at the Y-1 position, followed by a lysine at the Y+3 position, and has only one negatively charged residue in the surrounding motif, strongly suggesting that it is not a substrate of ZAP-70.
To the best of our knowledge, the substrate motifs favored by Itk have not been rigorously defined; however, they are clearly differentiated from ZAP-70 substrates. For example, ZAP-70 efficiently phosphorylates the three N-terminal tyrosines of SLP-76 but does not phosphorylate SLP-76 at Y173, whereas the reverse is true for Itk [21]. Since the conserved motif surrounding Gads Y45 bears some resemblance to known Itk-targeted sites [Fig S3A and 20, 21, 66-69], we decided to test the ability of Itk to phosphorylate Gads Y45, both in an *in vitro* kinase assay, and in an intact cellular environment.

For our *in vitro* experiments, Itk immune complexes were isolated from TCR-stimulated dG32 cells and were incubated in the presence or absence of ATP with recombinant MBP-Gads substrates, either full length (WT), lacking the N-terminal SH3 (ΔN, lacking residues 2-53), or with Phe substituted for Tyr45 (Y45F). All three substrates were phosphorylated by Itk in this assay system, as detected by immunoblotting with global anti-p-Tyr (Figure 4A, 2nd panel). Specific phosphorylation of Gads at Y45 was detected by the p-Y45 antibody, which, as expected, did not recognize the two substrates lacking this site (Figure 4A, top panel). Phosphorylation at all sites was abrogated upon addition of the Itk-specific inhibitor BMS-509744 [70], providing strong evidence that Gads tyrosine phosphorylation in this *in vitro* assay can be attributed directly to Itk (Figure S3B).

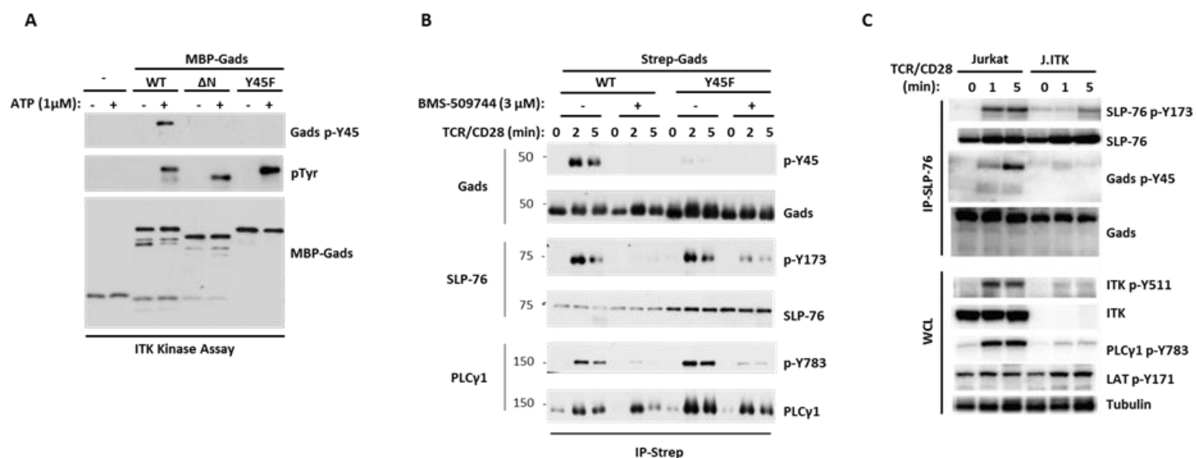


Figure 4. Itk mediates the TCR-inducible phosphorylation of Gads Y45 and SLP-76 Y173
(**A**) ***in vitro* phosphorylation of Gads Y45 by Itk.** Recombinant Gads, either full length (WT), lacking the N-terminal SH3 (ΔN) or with a substitution of phenylalanine for Tyr45 (Y45F), was phosphorylated *in vitro* by bead-bound Itk from TCR-stimulated cells, and the reaction supernatant was analyzed by blotting with anti-global p-Tyr (4G10), anti-p-Y45 or anti-MBP-Gads. (**B**) **An Itk inhibitor blocks TCR-induced Gads Y45 phosphorylation in intact cells.** dG32 cells, stably reconstituted with twin strep-tagged Gads, either WT or Y45F, were preincubated in stimulation medium for 30 min at 37°C, in the presence or absence of 3 μM BMS-509744, and were then stimulated with anti-TCR (C305, 1:1000 dilution) + CD28 costimulation (CD28.2 2 μg/ml). Streptactin complexes were probed with the indicated antibodies, as in Figure 3A. (**C**) **Gads Y45 phosphorylation is impaired in an ITK-deficient T cell line.** Jurkat or J.ITK cells were stimulated for the indicated time with anti-TCR + anti-CD28 and lysed. Anti-SLP-76 immune complexes and lysates were probed with the indicated phospho-specific antibodies, and then stripped, and reprobed for the total protein levels.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

368 Our *in vitro* results provided evidence that Itk can directly phosphorylate Gads Y45, as well as 369 an additional site or sites on Gads. In an effort to identify additional Itk-targeted sites, we performed 370 a mass spectromic analysis of phospho-Gads from our *in vitro* assay system. This analysis identified 371 Gads p-Y45 with high confidence, and also identified Gads phosphorylation at Y324 (data not 372 shown). However, Gads p-Y324 was not detected in our SILAC-based mass spectrometry study of 373 TCR-stimulated cells (Figure S1A), nor was it detected in other high throughput phospho-MS 374 studies of TCR-stimulated cells, which did identify Gads Y45 [59, 60]. In the absence of evidence that 375 Gads Y324 is phosphorylated in intact cells, we focused our attention on Gads Y45.

376 We employed pharmacologic and genetic approaches to test whether Itk mediates Gads Y45 377 phosphorylation in the context of intact T cells. In one approach, dG32 cells reconstituted with 378 twin-strep-tagged Gads were stimulated in the presence of BMS-509744, a selective Itk inhibitor [70], 379 and strep-tactin-purified Gads complexes were probed by immuno-blotting. BMS-509744 inhibited 380 the TCR/CD28-induced phosphorylation of SLP-76 Y173 and PLC-γ1 Y783, both known Itk 381 substrates, and likewise inhibited the phosphorylation of Gads Y45 (Figure 4B). As a control for 382 specificity, we note that BMS-509744 did not inhibit the TCR-induced association of Gads with 383 PLC-γ1, which is mediated by their mutual association with phospho-LAT (Figure 4B, bottom 384 panel). In a complementary approach, phosphorylation of Gads Y45, SLP-76 Y173 and PLC-γ1 Y783 385 were abrogated in an Itk-deficient derivative of the Jurkat T cell line, J.ITK; as a control for 386 specificity, the TCR-inducible phosphorylation of LAT Y171 was unaffected (Figure 4C).

387 Taken together, these results indicate that Gads Y45, like SLP-76 Y173 and PLC-γ1 Y783, is a 388 *bona-fide* Itk substrate that is phosphorylated in intact cells in response to TCR signaling.

389 *3.3.* ***SLP-76 targets active Itk to Gads Y45***

390 Specific docking interactions are generally required to target Itk catalytic activity to its 391 substrates [22, 46, 47]. Since active Itk associates with SLP-76 [20], and SLP-76 binds constitutively to 392 Gads [71-73], we reasoned that SLP-76 may bridge the docking of catalytically active Itk onto its 393 substrate, Gads Y45 (see Figure 1, right).

394 To test this idea, we reconstituted the Gads deficient cell line, dG32, with twin-strep-tagged 395 Gads, either wild-type or P321L, a mutant form of Gads that does not bind to SLP-76 [53]. As 396 expected, this mutation eliminated the constitutive association of SLP-76 with Gads (Figure 5A, 397 panels 2 and 3). The Gads P321L mutation did not abolish Itk activation, as measured by its ability to 398 phosphorylate SLP-76 at Y173; however, PLC-γ1 phosphorylation was substantially reduced (Figure 399 5A, bottom panels). These results are consistent with our previous observations in Gads-deficient T 400 cells, and further support the notion that Gads is not required for TCR-mediated activation of Itk, 401 but facilitates the Itk-mediated phosphorylation of PLC-γ1 by bringing SLP-76 associated Itk into the 402 vicinity of LAT-associated PLC-γ1 [38].

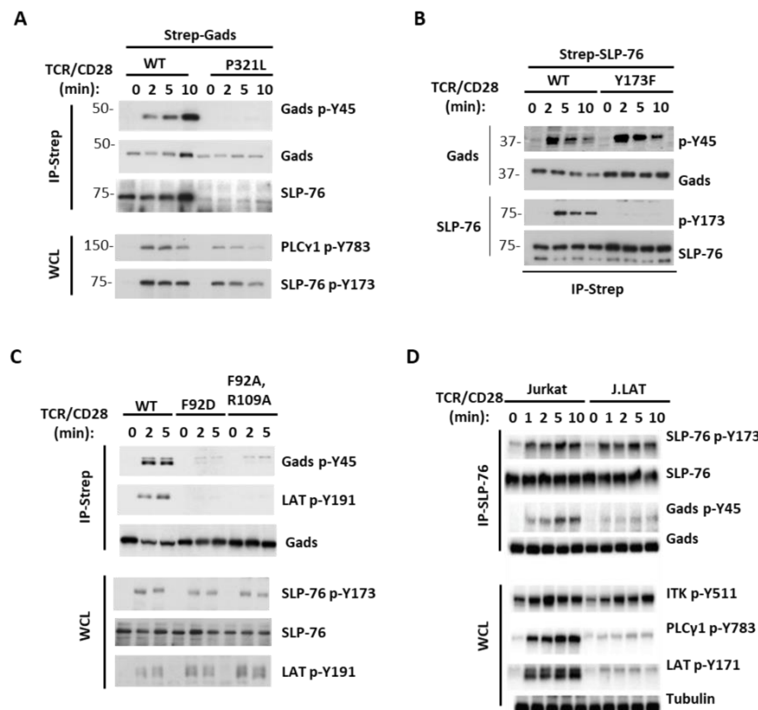
403 Whereas the Gads P321L mutation only partially reduced the phosphorylation of SLP-76 and 404 PLC-γ1, it eliminated detectable phosphorylation of Gads Y45 (Figure 5A, top panel). Thus, the 405 constitutive binding of SLP-76 to Gads is required in order to target SLP-76-bound Itk to Gads. It is 406 important to emphasize that this requirement does not imply any type of hierarchical, or ordered 407 phosphorylation of the two Itk-mediated sites; indeed, several of our results, presented above, 408 clearly demonstrated that Gads p-Y45 is not required for the phosphorylation of SLP-76 Y173 (Figure 409 3A, 4B and 5A). Conversely, SLP-76 Y173 was not required for the TCR-induced phosphorylation of 410 Gads Y45 (Figure 5B). These results suggest that within the SLP-76-Gads complex, SLP-76-bound Itk 411 can be independently targeted to two different substrates, SLP-76 Y173 and Gads Y45.

412 **3.4.** ***Gads Y45 phosphorylation occurs within the SLP-76-Gads-LAT complex.***

413 Next, we wondered whether the Gads-mediated recruitment of SLP-76 into the LAT-nucleated 414 complex is required for Itk-mediated phosphorylation events. We disrupted the bridging activity of 415 Gads by using two previously described mutant forms of Gads (Gads F92D, and Gads F92A,R109A), 416 in which targeted disruption of the Gads SH2 dimerization interface disrupts its 417 cooperatively-paired binding to LAT [Figure 5C and 42]. Whereas phosphorylation of SLP-76 Y173

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

418 was moderately reduced upon disruption of the Gads-LAT interaction; Gads Y45 phosphorylation
419 was undetectable (Figure 5C). These results suggest that the SH2-mediated binding of Gads to LAT
420 is required to direct Itk activity to Gads Y45.
421 To further validate this result, we disrupted LAT complex formation by CRISPR-mediated
422 deletion of LAT. As expected, deletion of LAT abrogated the TCR-inducible phosphorylation of
423 PLC-γ1; moreover, deletion of LAT greatly diminished the phosphorylation of Gads Y45, while only
424 mildly reducing the phosphorylation of Itk Y511 and SLP-76 Y173 (Figure 5D).
425 Taken together, these experiments provide evidence for the existence of mechanistically
426 independent pathways by which Itk activity is directed to its substrate sites on Gads and SLP-76.
427 Gads Y45 phosphorylation absolutely depends on the interaction of Gads with both SLP-76 and
428 LAT. In contrast, the TCR-inducible phosphorylation of SLP-76 Y173 is promoted by the binding of
429 Gads to LAT but can occur in the absence of these adaptors.
430

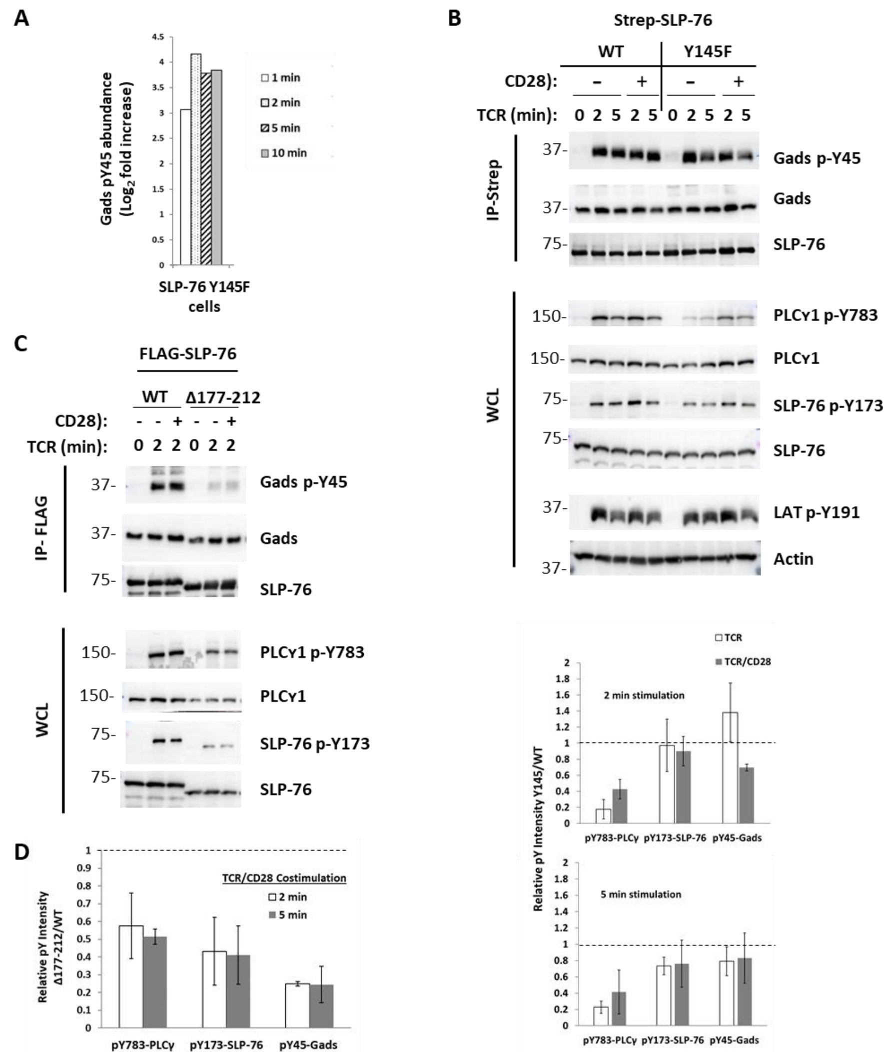


431

432 **Figure 5. Gads Y45 phosphorylation occurs within the SLP-76-Gads-LAT complex.** Jurkat-derived
433 cell lines bearing specific mutations, as described in Table 1, were used to determine the structural
434 requirements for Gads Y45 phosphorylation. Cells were stimulated for the indicated times with
435 anti-TCR (C305, 1:1000 dilution) + CD28 costimulation (CD28.2 2 μg/ml) and lysed. Whole cell lysates
436 (WCL) or affinity-purified complexes were probed with the indicated phospho-specific or total
437 protein antibodies. (**A**) **The Gads-SLP-76 interaction targets Itk activity to Gads.** phospho-sites in
438 dG32 cells, stably reconstituted with twin-strep-tagged Gads, either WT or bearing the
439 C-SH3-inactivating mutation, P321L. (**B**) **Gads Y45 phosphorylation is independent of SLP-76 Y173.**
440 Phospho-sites in J14 cells, stably reconstituted with twin strep-tagged SLP-76, either wild type or
441 bearing the Y173F mutation. (**C**) **Gads dimerization is required for Y45 phosphorylation.**
442 Phospho-sites were assessed in previously-described dG32 cell lines, stably reconstituted with WT or
443 dimerization-deficient forms of Gads-GFP. (**D**) **LAT is required to support Gads Y45**
444 **phosphorylation.** TCR/CD28-induced phosphorylation events were compared in Jurkat, or the
445 LAT-deficient cell line, J.LAT.

446

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



447

448 **Figure 6. Distinct docking interactions direct Itk activity to its substrates, Gads, SLP-76 and**
449 **PLC-γ1.** J14 cells, stably reconstituted with the indicated forms of N-terminally strep-tagged (**A-B**)
450 or FLAG-tagged (**C**) SLP-76, were stimulated as indicated and affinity-purified SLP-76 complexes or
451 whole cell lysates (WCL) were analyzed to determine site-specific phosphorylation. (**A**) **SLP-76 Y145,**
452 **an Itk SH2-binding site, is not required for Gads Y45 phosphorylation.** A SILAC approach was
453 employed, as in Figure 2A, to identify the TCR-induced change in Gads Y45 phosphorylation in J14
454 cells expressing twin strep-tagged SLP-76 Y145F. Results are the median of two independent
455 biological repeats. (**B**) **Differential effects of SLP-76 Y145 on Itk substrates.** TCR- and
456 TCR/CD28-induced phospho-sites in J14 cells, stably reconstituted with SLP-76 WT or Y145F. Top:
457 representative western blots. Bottom: Quantification of phospho-intensity. Gads pY45
458 phospho-intensity was normalized to total Gads protein. Results are expressed as the average
459 phospho-intensity observed in SLP-76 Y145F-expressing cells, relative to the intensity observed in
460 WT cells from the same stimulation time. n= 2 (TCR alone) or 3 (TCR/CD28) experiments, error bars
461 represent the standard deviation. (**C**) **Differential effect of SLP-76 QQPP motif on Itk substrates.**
462 TCR/CD28-induced phospho-sites in J14 cells, stably reconstituted with SLP-76 WT or Δ177-212. (**D**)
463 **Quantitative effect of QQPP motif on Itk substrates.** The cell lines shown in C were stimulated for 2
464 or 5 minutes with TCR/CD28. Anti-FLAG complexes were probed for phospho- and total Gads,
465 whereas lysates were probed for phospho- SLP-76 and phospho-PLC-γ1, and phospho-intensity was
466 quantified as in B. n= 2 (5 min) or 3 (2 min) experiments, error bars represent the standard deviation.

467

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

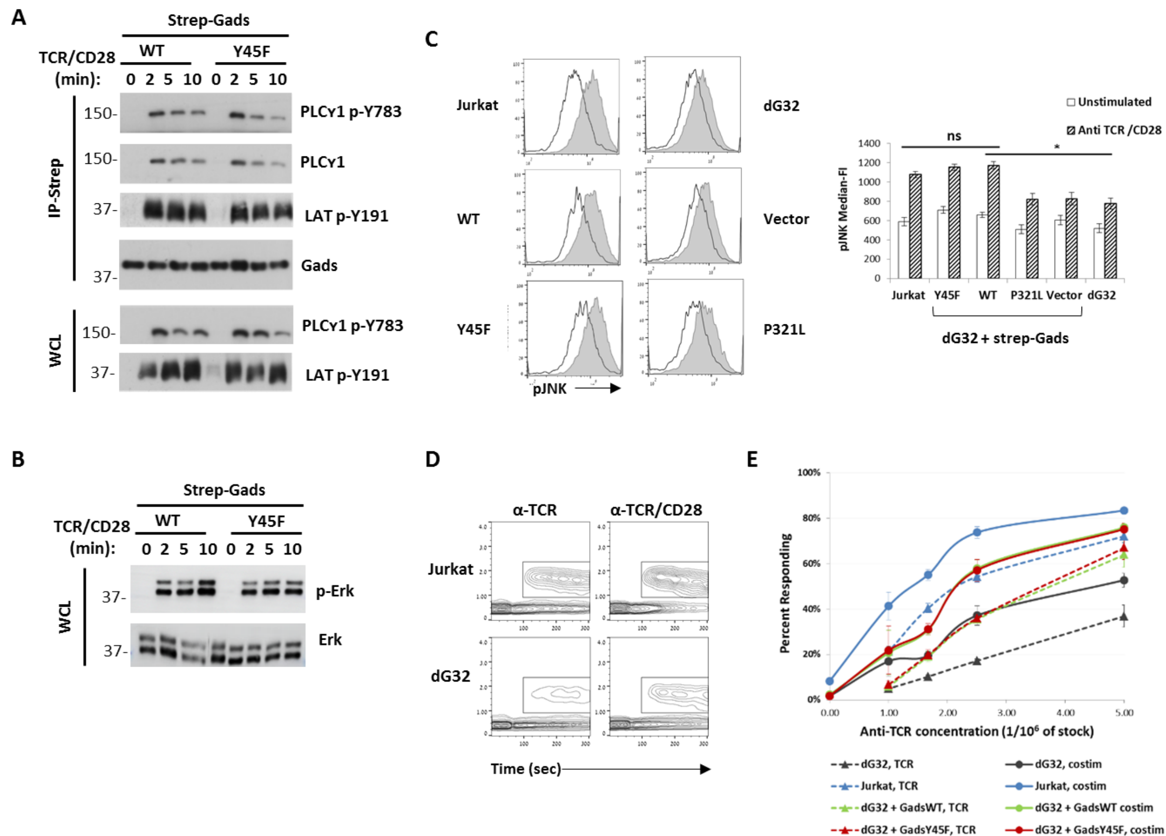
468 **3.5.** ***Distinct docking interactions direct Itk activity to its substrates, Gads, SLP-76 and PLC-γ1***

469 We next turned our attention to the molecular mechanisms by which SLP-76-bound Itk is
470 targeted to its different substrates. The SH2 and SH3 domains of Itk can mediate multiple, relatively
471 weak interactions with SLP-76 [28, 74], and perhaps also with SLP-76-associated Vav [75]. This
472 multiplicity of Itk-binding sites may allow for different conformations of SLP-76-bound Itk, each of
473 which may be competent to phosphorylate a different substrate. To test this notion, we examined the
474 role of two Itk-binding sites in mediating the phosphorylation of different Itk substrates.
475 Based on previous work [21, 30], we expected that the SH2-mediated binding of Itk to SLP-76
476 p-Y145 would be critical for phosphorylation of its substrates. To test this assumption, prior to the
477 development of our p-Y45 phospho-specific reagent, we used a SILAC approach to quantitatively
478 measure the TCR-induced fold-increase in Gads phosphorylation sites in TCR-stimulated J14 cells
479 expressing twin-strep-tagged SLP-76 Y145F. We were surprised to observe pronounced
480 TCR-induced phosphorylation of Gads Y45 (Figure 6A), which closely resembled our previous
481 observations in WT SLP-76-expressing cells (Figure 2A).
482 We later recapitulated this surprising result by directly comparing SLP-76 WT and
483 Y145F-expressing cells in immunoblotting experiments. Consistent with previous reports [21, 30],
484 disruption of the Itk-binding site at SLP-76 Y145 markedly and consistently reduced the
485 TCR-induced phosphorylation of PLC-γ1, although this effect was partially blunted by the addition
486 of CD28 co-stimulation (Figure 6B, WCL panels and quantitation in bottom panels). In contrast, the
487 SLP-76 Y145F mutation did not markedly reduce the TCR-induced phosphorylation of SLP-76 Y173
488 (Figure 6B WCL panels) or Gads Y45 (Figure 6B IP panels), either in the absence or in the presence of
489 costimulation (see quantitation in bottom panels). These results provide evidence that different
490 conformational states of SLP-76-bound Itk may be required to mediate its phosphorylation of
491 PLC-γ1, as compared to SLP-76 and Gads.
492 To further explore this hypothesis, we examined the role of the QQPP motif, found at SLP-76
493 residues 184-195, which can serve as a ligand for the SH3 domains of Itk or of PLC-γ1 [28, 29, 32]. J14
494 cells were stably reconstituted with FLAG-tagged SLP-76, either WT or bearing a 36 amino acid
495 deletion (Δ177-212) that encompasses the QQPP motif. Whereas the Δ177-212 deletion moderately
496 reduced PLC-γ1 p-Y783 and SLP-76 p-Y173, Gads p-Y45 was profoundly reduced (Figure 6C and
497 6D). Thus, the QQPP Itk SH3 domain-binding motif appears to be important for Itk activation in
498 general but is particularly required to direct Itk activity to Gads Y45.
499 Taken together, these experiments provide evidence that distinct conformations of
500 SLP-76-bound active Itk may be required to direct its activity to particular substrates. A Y145-ligated
501 conformation may primarily facilitate the phosphorylation of PLC-γ1 Y783 but not Gads Y45,
502 whereas a QQPP-ligated conformation may be required to direct Itk activity to Gads Y45.

503 **3.6.** ***Gads Y45 is not essential for TCR-proximal signaling to PLC-γ1***

504 Having established that SLP-76 p-Y173 and Gads p-Y45 are TCR-inducible Itk-mediated
505 phosphorylation sites within the LAT-nucleated complex, we next considered what the functional
506 significance of these sites might be. Since Itk, SLP-76 and Gads are all implicated in the
507 phosphorylation and activation of PLC-γ1 [20, 36, 38, 54, 76], we first explored the possibility that
508 Gads p-Y45 may play a role in regulating TCR signaling to PLC-γ1.
509 To this end, we compared TCR-proximal signaling events in Gads WT- and Y45F-reconstituted
510 dG32 cells. The Y45F mutation did not interfere with the TCR/CD28-induced association of Gads
511 with phospho-LAT or with its indirect, LAT-mediated association with PLC-γ1 (Figure 7A, IP
512 panels). The TCR-inducible phosphorylation of SLP-76 Y173 and LAT Y191 were likewise not
513 affected by the Gads Y45F mutation (Figure 3A and 7A). Notably, phosphorylation of PLC-γ1 Y783
514 was unaffected, both within strep-tactin-purified Gads complexes, and in whole cell lysates (Figure
515 7A), suggesting that PLC-γ1 recruitment to LAT, phosphorylation and its release from the LAT
516 complex all proceed independently of Gads Y45.
517

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



518

519 **Figure 7. Gads Y45 is not required for TCR-proximal signaling to PLC-γ1. (A) Recruitment of**
520 **Gads to LAT is independent of Gads p-Y45.** Lysates (WCL), or streptactin-purified Gads complexes
521 from stimulated cells were probed with the indicated antibodies. Results are representative of at least
522 3 repeats. (**B**) **Erk MAPK activation is independent of Gads pY45.** Cells were stimulated as in A and
523 lysates were probed with anti-phospho- and total Erk1/2. (**C-E**) FACS-based assays were used to
524 measure TCR responses in Jurkat, dG32 or dG32 cells that were stably reconstituted with the
525 indicated twin strep-tagged Gads alleles. Cells were differentially barcoded with CellTrace Violet (**C**)
526 or CellTrace Far Red (**D-E**), prior to stimulation. (**C**) **pJNK response is independent of Gads p-Y45.**
527 Cells were stimulated in triplicate for 15 min with anti-TCR (1:30,000) + anti-CD28 (2 μg/ml) (shaded
528 histogram), or mock stimulated (open histogram), prior to intracellular staining with anti-p-JNK-PE.
529 Results were analyzed while gating on matched GFP-expression gates, used as an indication of Gads
530 expression. Left: Representative results. Right: Average p-JNK median fluorescence intensity (n=3;
531 error bars indicate the SD). The unpaired two-tailed T test was used to identify statistically
532 significant differences, relative to TCR-stimulated WT-reconstituted dG32 cells (*, $p<0.05$). (**D-E**)
533 **Calcium response is independent of Gads p-Y45.** Intracellular calcium was measured by FACS,
534 with TCR or TCR/CD28 costimulation added at 60 sec. (**D**) Representative raw data observed upon
535 stimulation with anti-TCR (C305 1:400,000), in the presence or absence of anti-CD28 (1.5 μg/ml). Cells
536 within the rectangular gate are considered to be responding cells. (**E**) Cells were stimulated with the
537 indicated concentration of anti-TCR (C305), in the presence or absence of CD28 costimulation (1.5
538 μg/ml). Shown is the percent responding cells observed within a 100 second time window, beginning
539 1.5 min after the addition of stimulant (n=3, error bars indicate SD).

540 Further downstream, the Gads Y45F mutation did not reduce the TCR/CD28-induced
541 phosphorylation of MAPK family members, Erk (Figure 7B) or Jnk (Figure 7C), nor did it mitigate
542 the TCR-induced increase in CD69 expression (Figure S4A).
543 To more formally rule out a role for Gads Y45 in regulating PLC-γ1 activation, we examined its
544 effect on the TCR-induced calcium response, by assessing the frequency of cells that exhibited
545 increased intracellular calcium in response to low dose TCR stimulation. As we previously reported
546 [38], Gads-deficiency markedly reduced the frequency of responding cells (Figure 7D, left) over a

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

547 range of TCR stimulatory doses (Figure 7E). Stable reconstitution of Gads expression increased the
548 sensitivity of TCR responsiveness in this assay; however, the magnitude of the Gads-dependent
549 increase was not affected by the Y45F mutation (Figure 7E, dotted lines). CD28 costimulation further
550 increased the frequency of responding cells, both in the presence and in the absence of Gads (Figure
551 7D); moreover, the CD28-dependent increase in responsiveness was not affected by the Gads Y45F
552 mutation, as compared to WT-reconstituted cells (Figure 7E, solid lines).
553 Taken together, these results provide evidence that Gads p-Y45 is not required for TCR
554 signaling to PLC-γ1.

555 **3.7.** ***SLP-76 Y173 exerts a modest effect on TCR-proximal signaling to PLC-γ1***

556 Having excluded a role for Gads p-Y45 in mediating PLC-γ1 activation, we decided to take a
557 closer look at the role of SLP-76 p-Y173. Our previously published data suggested, but did not
558 definitively prove a role for SLP-76 Y173 in regulating PLC-γ1. Whereas the Y173F mutation
559 decreased the TCR-induced accumulation of phospho-PLC-γ1 in whole cell lysates; this mutation
560 only modestly reduced TCR-induced calcium flux [21]. To address this apparent contradiction, we
561 compared the accumulation of PLC-γ1 p-Y783 within two pools of PLC-γ1, the SLP-76-bound pool,
562 and the pool that is found in whole cell lysates. The latter pool includes PLC-γ1 that was
563 phosphorylated within the LAT-nucleated complex and subsequently released [64]. Whereas the
564 Y173F mutation markedly reduced the abundance of PLC-γ1 p-Y783 in whole cell lysates, the
565 SLP-76-bound pool of PLC-γ1 p-Y783 was not reduced (Figure S4B). This observation suggests that
566 SLP-76 Y173 is not required for PLC-γ1 phosphorylation *per se*, but may be required for the release of
567 phosphorylated PLC-γ1 from the LAT-nucleated complex into the cytosol.

568 **3.8.** ***The Itk-targeted sites on SLP-76 and Gads are selectively required for TCR/CD28 signaling to***
569 ***the RE/AP transcriptional element***

570 Having demonstrated that PLC-γ1 activation occurs independently of Gads Y45, and is
571 partially independent of SLP-76 Y173, we considered the possibility that the Itk-targeted sites on
572 SLP-76 and Gads may regulate a distinct aspect of the TCR/CD28 signaling pathway.
573 The LAT-nucleated complex controls different branches of the TCR signaling pathway, leading
574 to the activation of different transcriptional elements, which together drive the transcription of IL-2
575 [1]. PLC-γ1 produces two second messengers, IP_3 and DAG, which respectively bring about the
576 activation of NFAT and AP1 transcription factors that bind to a compound NFAT/AP1 site within
577 the IL-2 promoter. A second compound site, RE/AP, binds to AP1 and NFκB, and is activated in
578 response to TCR/CD28 costimulation [7].
579 Whereas SLP-76 is absolutely required for NFAT and AP-1 activation [54], the Y173F mutation
580 did not reproducibly affect the activity of an NFAT/AP1 luciferase reporter construct (Figure 8A,
581 left). Nevertheless, this mutation markedly reduced the TCR/CD28-induced production of IL-2
582 (Figure 8B), suggesting that it may affect signaling through a different branch of the TCR signaling
583 pathway. Consistent with this notion, the Y173F mutation eliminated TCR/CD28-induced activation
584 of an RE/AP-luciferase reporter construct (Figure 8A, middle) and markedly reduced the activation
585 of an NFκB-luciferase reporter construct (Figure 8A, right).
586 These results suggest that SLP-76 p-Y173 is selectively required for an NFκB-dependent branch
587 of the signaling pathway in which TCR and CD28 costimulation synergistically bring about the
588 activation of RE/AP. Moreover, these results imply that Itk may exert two mechanistically distinct
589 influences on IL-2 transcription. Via phosphorylation of PLC-γ1, Itk promotes activation of the
590 NFAT transcriptional element, whereas via phosphorylation of SLP-76 Y173, Itk promotes the
591 activation of RE/AP.
592 To further explore the dual role of Itk as a regulator of NFAT and RE/AP, we chose to compare
593 two mutations that disrupt Itk-mediated signaling in different ways. To this end, we reconstituted
594 J14 cells with FLAG-tagged SLP-76, either WT, Y173F, Δ177-212 (which lacks the QQPP motif), or
595 with an allele of SLP-76 bearing both mutations, Δ177-212+Y173F. This setup allowed us to compare
596 different effects of Itk: whereas the Y173F mutation disrupts Itk-mediated phosphorylation of

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

597 SLP-76, the Δ177-212 mutation primarily disrupts Itk-mediated phosphorylation of Gads Y45, and
598 the double mutation disrupts the phosphorylation of SLP-76, Gads and PLC-γ1 (Figure 8C and 8D)
599 Activation of NFAT was only modestly reduced by each of the single mutations, Y173F or
600 Δ177-212, but was dramatically reduced in cells bearing the double mutation, SLP-76
601 Y173F+Δ177-212 (Figure 8E, top). The inhibitory effect of the combined mutations may be
602 understood in terms of their effects on PLC-γ1 phosphorylation (Figure 8C). The Y173F mutation
603 decreased PLC-γ1 p-Y783 in whole cell lysates, but not within the SLP-76-bound pool. The Δ177-212
604 mutation had the opposite effect, dramatically decreasing PLC-γ1 p-Y783 within the SLP-76-bound
605 pool, but only moderately decreasing PLC-γ1 phosphorylation in whole cell lysates. The double
606 mutation synergistically impaired NFAT activity, most likely due to its ability to markedly reduce
607 the phosphorylation of PLC-γ1 both in both compartments.
608 Compared with their modest effect on NFAT activation (Figure 8E, top), each of the single
609 mutations markedly impaired the activation of RE/AP, which was even more profoundly impaired
610 by the double mutation (Figure 8E, bottom). These results suggest the presence of at least two
611 PLC-γ1-independent mechanisms by which Itk can influence downstream signaling events leading
612 to RE/AP. First, Itk is required for SLP-76 Y173 phosphorylation, which is required to support RE/AP
613 activation. Second, Itk may act via the QQPP motif to influence RE/AP activation by an additional
614 mechanism. While neither mechanism on its own is required for NFAT activation, removal of both
615 mechanisms abrogated NFAT activity as well as RE/AP activity. Since the Δ177-212 mutation
616 dramatically decreased Gads p-Y45 but only moderately affected SLP-76 p-Y173, we considered the
617 possibility that Gads p-Y45 may be required for RE/AP activation.
618 Gads plays a supporting role in the TCR signaling pathway, for example, it promotes, but is not
619 absolutely required for TCR-induced calcium flux and NFAT activation [38]; moreover, the thymic
620 developmental defect of Gads-deficient mice is much milder than the absolute thymic block
621 exhibited by SLP-76-deficient mice [3]. Consistent with this notion, TCR/CD28-induced RE/AP
622 activation was Gads-independent. Nevertheless, the Y45F mutation dramatically reduced activation
623 of the RE/AP reporter (Figure 8F). To verify this surprising result, we independently repeated the
624 reconstitution of dG32 cells, and again found that whereas RE/AP activation can occur equally well
625 in the absence or presence of Gads, reconstitution of the cells with Gads bearing a mutation at Y45,
626 either Y45F or Y45E, resulted in profound inhibition of RE/AP activity (Figure S4B).
627 This unexpected result suggested that Gads may perform an inhibitory signaling function,
628 which must be removed in order to activate RE/AP. According to this model, the inhibitory function
629 may be removed upon TCR-induced phosphorylation of Gads Y45. Consistent with this result,
630 removal of the N-SH3 did not impair RE/AP activation, and in some experiments activation was
631 modestly increased (Figure 8F and Figure S4B).
632 Taken together, these results suggest that Itk controls CD28 responsiveness via TCR-induced
633 phosphorylation of its targets on Gads and SLP-76. Moreover CD28 responsiveness appears to be
634 actively restrained by un-phosphorylated Gads. The fact that two independent pathways are
635 required for RE/AP activation - a SLP-76-dependent pathway leading to SLP-76 Y173
636 phosphorylation and a LAT-dependent pathway leading to Gads Y45 phosphorylation - may help to
637 insure the interdependence of CD28 responsiveness on TCR signaling.
638

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

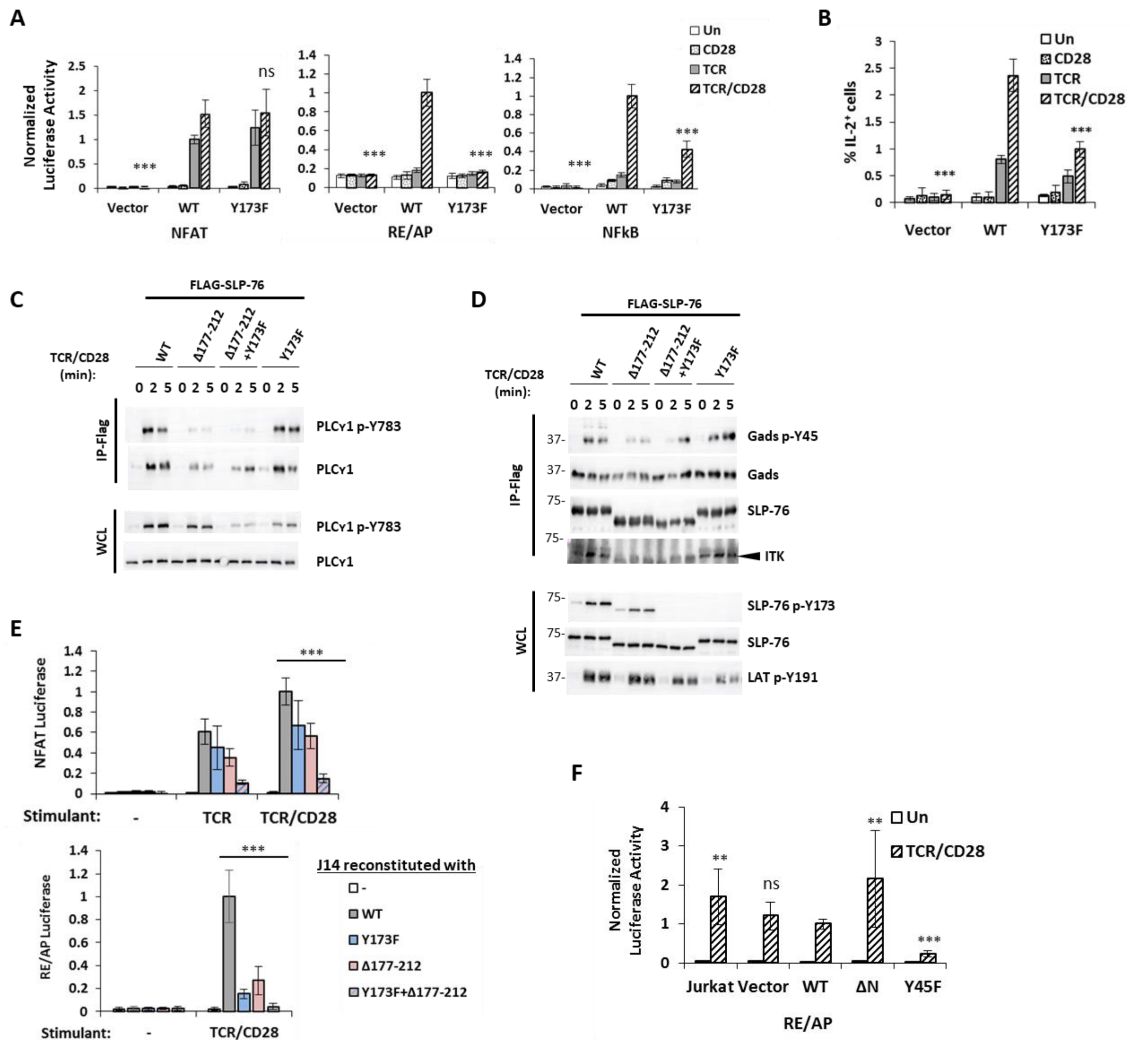


Figure 8. Itk-related features of SLP-76 and Gads are specifically required to activate the RE/AP transcriptional element. (A) Role of SLP-76 Y173 in transcriptional responses to TCR/CD28 stimulation. Luciferase reporter activity was measured in J14 cells that had been stably reconstituted with comparable expression of twin strep-tagged SLP-76 (WT or Y173F) or with a vector control. Normalized results are presented relative to that observed in TCR/CD28-stimulated WT cells from the same stimulation plate. Results are the average of 3 experiments, conducted in triplicate, error bars indicate the SD. **(B) Role of SLP-76 Y173 in TCR/CD28-induced IL-2 expression.** The cell lines shown in A were stimulated for 6 hours with plate-bound anti-TCR and soluble anti-CD28, in the presence of 5 μg/ml brefeldin A during the last 4 hours of stimulation, and intracellular staining with α-IL-2-PE was analyzed by FACS, to determine the percent IL-2+ cells. Results are the average of 2 experiments, conducted in duplicate, error bars indicate the SD. **(C-E) Two Itk-related features of SLP-76 are required for RE/AP activation.** J14 cells were reconstituted with the indicated forms of FLAG-tagged SLP-76 and were sorted for comparable expression level. **(C-D) Differential effect of the SLP-76 mutations on PLC-γ1 and Gads phosphorylation.** Cells were stimulated and western blots prepared from anti-FLAG-purified SLP-76 complexes (IP-FLAG) or whole cell lysates (WCL). **(E) NFAT and RE/AP luciferase activities** were measured as in A. Results are the average of 3 or 4 experiments, conducted in triplicate, error bars indicate the SD. **(F) Role of Gads Y45 in transcriptional response to TCR/CD28.** dG32 cells were stably reconstituted with twin-strep tagged Gads (WT, ΔN or Y45F) or with a vector control and were sorted for comparable expression level. RE/AP luciferase activity was measured as in A. Results are the average of four experiments, each conducted in triplicate, error bars indicate the SD. For all bar graphs in this figure, the unpaired student T test was used to compare TCR/CD28-stimulated cells to WT TCR/CD28-stimulated cells (**, p<0.005; ***, p<0.0005; ns, p>0.05).

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

663 **4. Discussion**

664 Prior studies established a clear role for Itk in the initiation of TCR-proximal signaling events,
665 leading to the phosphorylation and activation of PLC-γ1 [25]. As a Tec-family kinase that is
666 activated downstream of Lck and ZAP-70, Itk constitutes the third member of the TCR-induced
667 tyrosine kinase cascade. Active Itk can be found in complex with SLP-76, which is recruited to LAT
668 by Gads, and within this heterotrimeric adaptor complex, Itk promotes downstream responsiveness,
669 in part, by phosphorylating LAT-associated PLC-γ1 at Y783.
670 Here, we have uncovered a distinct Itk-dependent signaling module that is based on the
671 Itk-mediated phosphorylation of SLP-76 Y173 and Gads Y45. Our data document the profound
672 TCR-induced increase in the abundance of SLP-76 p-Y173 and Gads p-Y45, both in a human T cell
673 line and in primary mouse lymphocytes [Figure 2-3 and 21]. We provide several independent lines
674 of evidence to demonstrate that these phosphorylation events are mediated by Itk (Figure 4), and
675 that each site can be phosphorylated independently of the other (Figures 3A and 5B). Itk-mediated
676 phosphorylation of Gads occurs within the LAT-nucleated signaling complex (Figure 5) and appears
677 to depend on a particular conformation of Itk, in which the Itk SH3 domain binds to the QQPP motif
678 of SLP-76 (Figure 6). Whereas Gads p-Y45 and SLP-76 p-Y173 are largely expendable for signaling
679 through the PLC-γ1-calcium-NFAT axis; both are required to mediate the TCR/CD28-induced
680 activation of the RE/AP transcriptional element from the IL-2 promoter (Figure 7-8). This study
681 therefore establishes a PLC-γ1-independent mechanism by which SLP-76 and Gads regulate the
682 synergistic activity of the TCR and CD28 signaling pathways, leading to the activation of RE/AP.
683 The high fold phosphorylation and evolutionary conservation of the Gads p-Y45 motif, which is
684 found within the conserved N-terminal SH3 domain of Gads, all suggest a conserved function. Yet
685 the N-terminal SH3 domain of Gads, to date, has no known ligand or signaling function [3], and our
686 characterization of Gads p-Y45 therefore provides the first insight into the regulatory role played by
687 the N-SH3 of Gads. Moreover, our study may provide some insight into the widespread but poorly
688 understood phenomenon of SH3 domain tyrosine phosphorylation [77]. A recently published
689 bioinformatic survey revealed that of 273 human SH3 domains, 94 are phosphorylated on tyrosine,
690 and 20 of those are phosphorylated at the M2 position, which is the location of Gads Y45 [78]. Yet,
691 with the possible exception of Src p-Y133 [79] and Caskin 1 p-Y336 [80], the functional significance of
692 SH3 domain tyrosine phosphorylation at the M2 position is, to the best of our knowledge, unknown.
693 Upon the identification of Gads Y45 and SLP-76 Y173 as substrates of Itk, we sought to identify
694 the mechanisms by which the catalytic domain of Itk is directed to these particular substrates. This
695 question is complicated by the fact that Itk is regulated by multiple intra- and inter-molecular
696 protein-protein interactions [25]. In the resting state, intramolecular interactions of the SH2 and SH3
697 domains of Itk stabilize the inactive conformation of the enzyme; whereas upon TCR stimulation,
698 bivalent binding of SLP-76 to the SH domains of Itk is thought to stabilize the catalytically active
699 conformation [24, 28]. Consistent with this notion, elution of Itk from the SLP-76 complex abrogated
700 its activity, which was restored upon its reassociation with SLP-76 [20].
701 The association of active Itk with SLP-76 appears to be sufficient to target its activity to SLP-76
702 Y173. In support of this notion, we observed that the phosphorylation of SLP-76 at Y173 can occur in
703 the absence of either Gads or LAT, and was only moderately reduced by point mutations that
704 disrupt the Gads-mediated recruitment of SLP-76 to LAT [Figure 5 and 38].
705 In contrast, Itk-mediated phosphorylation of Gads was exquisitely dependent on the
706 association of Gads with both SLP-76 and LAT, suggesting that Gads Y45 phosphorylation must
707 occur within the heterotrimeric adaptor complex (Figure 5). These findings provide clues to the
708 specific docking interactions that target Itk catalytic activity to Gads. In particular, the high affinity,
709 constitutive interaction of Gads with SLP-76 [33, 40, 41] may serve to bring Gads Y45 into the vicinity
710 of SLP-76-bound Itk. Moreover, the cooperatively paired binding of the Gads SH2 to LAT [42] may
711 create a docking surface for the Itk kinase domain, which may facilitate the Itk-mediated
712 phosphorylation of the adjacent Gads Y45. This speculation is consistent with previous studies, in
713 which docking of the Itk kinase domain onto a non-classical SH2 domain surface within particular
714 substrate proteins was required for the Itk-mediated phosphorylation of an SH2-adjacent site [46,

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

715 47]. Alternatively, the dimeric binding of Gads to LAT may induce a conformational change that
716 may increase the surface exposure of Gads Y45. It is important to note that no structural information
717 is available for the N-terminal SH3 of Gads, and we expect that future structural studies will be
718 required to illuminate the mechanism by which Itk activity is targeted to Gads Y45.
719 To better understand the mechanisms that direct Itk to its individual substrates, we explored
720 how phosphorylation of each substrate may depend on two known Itk-binding motifs: SLP-76
721 p-Y145, which binds to the SH2 domain of Itk, and the SLP-76 QQPP motif, which binds to the Itk
722 SH3 domain (Figure 6). As previously reported [21, 30], ablation of SLP-76 Y145 markedly reduced
723 the Itk-mediated phosphorylation of PLC-γ1; however, its effects on the Itk-mediated
724 phosphorylation of SLP-76 and Gads were quite modest. Conversely, a deletion encompassing the
725 QQPP motif markedly reduced the TCR-induced association of Itk with SLP-76 and the Itk-mediated
726 phosphorylation of Gads Y45 but had a more modest effect on the phosphorylation of SLP-76 and
727 PLC-γ1. These results provide evidence that Itk may assume multiple conformations within the
728 SLP-76-Gads-LAT complex, each of which may be most suited to phosphorylation of a particular
729 substrate within this complex.
730 The concept that Itk can assume distinct, catalytically active, SLP-76-bound conformations may
731 help to resolve other previously puzzling observations. The SH2 of Itk is commonly thought to bind
732 to SLP-76 pY145, yet ablation of SLP-76 Y145 was not sufficient to disrupt the interaction of Itk with
733 SLP-76 [30]. The residual association of Itk with SLP-76 Y145F may be mediated by binding of the Itk
734 SH3 to the QQPP motif, by binding of the Itk SH2 to pY113 of SLP-76, or by indirect recruitment of
735 Itk, via its interaction with Vav [75], which binds directly to SLP-76 and may serve to stabilize the
736 SLP-76-Itk interaction.
737 The binding site of the Itk SH3 domain is likewise not completely clear. Whereas it may bind to
738 the QQPP motif [28, 29], this motif was also reported to bind to the SH3 domains of Lck and PLC-γ1
739 [32, 34]. One possible solution to this conundrum might involve the binding of the QQPP motif to
740 different SH3 domains at different stages in the signaling cascade, or via partially overlapping
741 binding sites, as was recently demonstrated for the binding of Itk and Lck to the related adaptor
742 TSAD [81]. A switch between different QQPP-binding partners may be facilitated by the
743 autophosphorylation of Itk at Y180 within its SH3 domain [66], as phosphorylation of this site alters
744 the affinity of the SH3 for different proline-rich ligands [82]. In this way, autophosphorylation of Itk
745 Y180 may alter its mode of binding to SLP-76.
746 As an integrative explanation for the above data, we propose that Itk may interact with SLP-76
747 via multiple distinct modes, each of which is most suitable for the phosphorylation of a particular
748 substrate.
749 Further downstream, Gads Y45 and SLP-76 Y173 appear to be dispensable for the canonical
750 SLP-76-Gads-LAT-mediated phosphorylation and activation of PLC-γ1, leading to calcium flux and
751 NFAT activation; rather both sites are required to mediate the TCR/CD28-induced activation of the
752 RE/AP transcriptional element (Figures 7-8). In a similar manner, a region encompassing the QQPP
753 motif on SLP-76, was required for phosphorylation of Gads Y45 and for the activation of RE/AP but
754 not NFAT (Figures 6C and 8C-E). Consistent with our findings, precise excision of the 10 amino acid
755 QQPP motif in SLP-76-reconstituted J14 cells moderately reduced NFAT nuclear translocation, but
756 dramatically reduced IL-2 production [34]. Our results therefore suggest that Itk regulates at least
757 two distinct signaling branches downstream of the TCR, one leading to PLCγ1-dependent calcium
758 flux, and the other acting via phosphorylation sites on SLP-76 and Gads to regulate the activity of the
759 RE/AP transcriptional element.
760 Whereas we previously reported that SLP-76 p-Y173 is required for optimal phosphorylation of
761 PLC-γ1 [21], a careful re-appraisal of this site revealed that SLP-76 p-Y173 is not required for PLC-γ1
762 phosphorylation *per se*, but rather contributes to the catalytic release of phospho-PLC from the
763 LAT-nucleated complex [as described by 64], thereby promoting the accumulation of
764 phospho-PLC-γ1 outside the confines of this complex (Supp Figure 4B). The ability of SLP-76 p-Y173
765 to promote the release of phospho-PLC-γ1 from LAT may relate to its ability to bind weakly to the
766 C-terminal SH2 of PLC-γ1 [22]. It is interesting to note a previous mutational study of PLC-γ1,

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

767 which presented evidence that the C-SH2 of PLC-γ1 is required for the activation of RE/AP,
768 independently of any influence on TCR-induced calcium flux [83]. Thus, we can speculate that the
769 pathway by which SLP-76 p-Y173 regulates RE/AP may be related to its ability to bind to the
770 C-terminal SH2 of PLC-γ1.

771 The mechanisms by which Itk-mediated phosphorylation of Gads and SLP-76 regulate the
772 activity of the RE/AP transcriptional element remain to be determined. One likely possibility is an
773 effect on NFκB signaling, as revealed by the partial inhibition of an NFκB reporter upon mutation of
774 SLP-76 Y173 (Figure 8A). Previous reports suggested that SLP-76 may regulate NFκB through HPK1,
775 a kinase that associates with the SH2 domain of SLP-76 [84] and is reported to phosphorylate
776 Carma1, a key element of the NFκB signaling pathway [85]. Another possibility is that SLP-76 and
777 Gads may directly influence transcriptional events via the ability of SLP-76 to translocate to the
778 nuclear pores, where it regulates the nuclear translocation NFκB [86]. It remains to be seen whether
779 this activity depends on Itk-targeted phosphorylation sites on the adaptors. Another possibility is a
780 direct effect on CD28 signaling. It has been known for some time that Gads can bind directly to a
781 membrane-proximal pYMNM motif found in the cytoplasmic tail of CD28 [87]; however, evidence
782 for the functional relevance of this interaction is mixed [88-90]. While it is possible that Gads p-Y45
783 may exert its effects directly within the CD28-nucleated signaling complex, we consider this
784 possibility unlikely, since Gads Y45 did not affect the CD28-induced augmentation of calcium flux
785 (Figure 7). In light of the large number of possibilities, a specific resolution of the mechanism by
786 which SLP-76 p-Y173 and Gads p-Y45 regulate RE/AP is outside the scope of this study.

787 The profound inhibition of RE/AP by the Gads Y45F mutation is especially intriguing, as Gads
788 itself was not required for RE/AP activation. This observation suggests that Gads performs an
789 inhibitory function, analogous to the closing of a gate, which limits T cell responsiveness by
790 inhibiting the activation of RE/AP. A similar phenomenon has been observed for the adaptor protein
791 ALX, which is dispensable for RE/AP activation, but profoundly inhibits RE/AP when
792 overexpressed [91, 92]. This example provides evidence for the existence of regulatory pathways that
793 are dedicated to the negative regulation of RE/AP, and we suggest that Gads is likely to constitute an
794 important component of this regulatory mechanism. The gating mechanism is likely to involve
795 particular ligands of the N-SH3 of Gads, or a phospho-dependent ligand of Y45, which remain to be
796 identified.

797 Based on this model, opening of the gate to allow RE/AP activation would depend on Gads SH2
798 domain dimerization, leading to the phosphorylation of Gads at Y45, which together may stabilize
799 an active conformation. In this respect, it is important to note the tight regulation of Gads Y45
800 phosphorylation, induction of which depends both on the TCR-induced binding of Gads to LAT,
801 and on the TCR-induced interaction of Itk with SLP-76. Since the gate remains closed and RE/AP is
802 inhibited in the absence of Gads Y45 phosphorylation, these requirements may prevent spurious
803 immune responses by limiting CD28 responsiveness to cells that have experienced signaling
804 through the TCR.

805 **Supplementary Materials:** Figure S1: Summary of SLP-76 and Gads phosphorylation sites identified in our
806 SILAC-based phospho-mass spectrometry analysis, Figure S2: Evolutionary conservation of the Gads N- and
807 C-terminal SH3 domains, Figure S3: Gads Y45 is an Itk-targeted site, Figure S4: Downstream signaling functions
808 of Gads p-Y45 and SLP-76 p-173.

809 **Author Contributions:** EH and DY developed the study objectives and experimental strategy with key inputs
810 from HU, AW and W-LL. Mass spectrometry samples were prepared by DB and were analyzed by JC under the
811 supervision of HU. Novel cell lines and immunological reagents used in this work were created by EH, RS,
812 W-LL, IO, SW, AI, MS and DB. Cellular responses to stimulation were measured and data were analyzed by
813 EH, RS, W-LL, IO, SW and DY. This manuscript was written by DY and EH, with key inputs from W-LL, AW
814 and HU. All authors have read and agreed to the published version of the manuscript.

815 **Funding:** This research was supported by grants to D.Y. from the Israel Science Foundation (1288/17) and the
816 Colleck Research Fund, and by a subsidy to D.Y. from the Russell Berrie Nanotechnology Institute. The research
817 was also supported by collaborative grants from the Volkswagenstfitung (VWZN2828) to DY and HU, and

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

818 from the United States - Israel Binational Science Foundation (2017195) to DY and AW, and by a grant to AW
819 from the NIH/NIAID (R37AI114575).

820 **Acknowledgments:** The Biomedical Core Facility (BCF) of the Rappaport Faculty of Medicine provided access
821 to FACS equipment, and BCF staff members Ofer Shenkar, Amir Grau, and Rotem Honen Kadosh provided
822 excellent technical support. Gads deficient mice on the Balb/C background were generously provided by C.
823 Jane McGlade (University of Toronto), We thank Dr. Rona Shofti from the Technion preclinical authority and
824 her staff for their professional assistance with the care and housing of our mice.

825 **Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the
826 study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to
827 publish the results.

828 **References**

829 1. Gaud, G., R. Lesourne, and P.E. Love. 2018. Regulatory mechanisms in T cell receptor signalling. *Nature*
830 *Reviews Immunology*.
831 2. Balagopalan, L., N.P. Coussens, E. Sherman, L.E. Samelson, and C.L. Sommers. 2010. The LAT Story: A
832 Tale of Cooperativity, Coordination, and Choreography. *Cold Spring Harbor Perspectives in Biology*. 2(8): p.
833 a00 5512.
834 3. Yablonski, D. 2019. Bridging the Gap: Modulatory Roles of the Grb2-Family Adaptor, Gads, in Cellular
835 and Allergic Immune Responses. *Frontiers in Immunology*. 10(1704): p. doi: 10.3389/fimmu.2019.01704.
836 4. Esensten, Jonathan H., Ynes A. Helou, G. Chopra, A. Weiss, and Jeffrey A. Bluestone. 2016. CD28
837 Costimulation: From Mechanism to Therapy. *Immunity*. 44(5): p. 973-988.
838 5. Tuosto, L. 2011. NF-κB family of transcription factors: Biochemical players of CD28 co-stimulation.
839 *Immunology Letters*. 135(1): p. 1-9.
840 6. Fraser, J.D., B.A. Irving, G.R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer
841 activity by the T cell accessory molecule CD28. *Science*. 251(4991): p. 313-6.
842 7. Shapiro, V.S., K.E. Truitt, J.B. Imboden, and A. Weiss. 1997. CD28 mediates transcriptional upregulation of
843 the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1
844 sites. *Mol Cell Biol*. 17(7): p. 4051-8.
845 8. Shapiro, V.S., M.N. Mollenauer, and A. Weiss. 1998. Nuclear factor of activated T cells and AP-1 are
846 insufficient for IL-2 promoter activation: requirement for CD28 up-regulation of RE/AP. *J Immunol*.
847 161(12): p. 6455-8.
848 9. Matzinger, P. 2002. The Danger Model: A Renewed Sense of Self. *Science*. 296(5566): p. 301-305.
849 10. Brownlie, R.J. and R. Zamoyska. 2013. T cell receptor signalling networks: branched, diversified and
850 bounded. *Nat Rev Immunol*. 13(4): p. 257-69.
851 11. Au-Yeung, B.B., N.H. Shah, L. Shen, and A. Weiss. 2018. ZAP-70 in Signaling, Biology, and Disease.
852 *Annual Review of Immunology*. 36(1): p. 127-156.
853 12. Lin, J. and A. Weiss. 2001. Identification of the minimal tyrosine residues required for linker for activation
854 of T cell function. *J. Biol. Chem*. 276(31): p. 29588-29595.
855 13. Zhang, W., R.P. Trible, M. Zhu, S.K. Liu, C.J. McGlade, and L.E. Samelson. 2000. Association of Grb2, Gads
856 and Phospholipase C-γ1 with phosphorylated LAT tyrosine residues. *J. Biol. Chem*. 275(30): p. 23355-23361.
857 14. Zhu, M., E. Janssen, and W. Zhang. 2003. Minimal requirement of tyrosine residues of linker for activation
858 of T cells in TCR signaling and thymocyte development. *J Immunol*. 170(1): p. 325-33.
859 15. Paz, P.E., S. Wang, H. Clarke, X. Lu, D. Stokoe, and A. Abo. 2001. Mapping the Zap-70 phosphorylation
860 sites on LAT (linker for activation of T cells) required for recruitment and activation of signalling proteins
861 in T cells. *Biochem J*. 356(Pt 2): p. 461-71.
862 16. Wardenburg, J.B., C. Fu, J.K. Jackman, H. Flotow, S.E. Wilkinson, D.H. Williams, R. Johnson, G. Kong,
863 A.C. Chan, and P.R. Findell. 1996. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is
864 required for T-cell receptor function. *J. Biol. Chem*. 271(33): p. 19641-19644.
865 17. Fang, N., D.G. Motto, S.E. Ross, and G.A. Koretzky. 1996. Tyrosines 113, 128, and 145 of SLP-76 are
866 required for optimal augmentation of NFAT promoter activity. *J. Immunol*. 157: p. 3769-3773.
867 18. Koretzky, G.A., F. Abtahian, and M.A. Silverman. 2006. SLP76 and SLP65: complex regulation of
868 signalling in lymphocytes and beyond. *Nat. Rev. Immunol*. 6: p. 67-78.
869 19. Heyeck, S.D., H.M. Wilcox, S.C. Bunnell, and L.J. Berg. 1997. Lck phosphorylates the activation loop
870 tyrosine of the Itk kinase domain and activates Itk kinase activity. *J Biol Chem*. 272(40): p. 25401-8.
871 20. Bogin, Y., C. Ainey, D. Beach, and D. Yablonski. 2007. SLP-76 mediates and maintains activation of the Tec
872 family kinase ITK via the T cell antigen receptor-induced association between SLP-76 and ITK. *Proc. Natl.*
873 *Acad. Sci. USA*. 104(16): p. 6638-43.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

874 21. Sela, M., Y. Bogin, D. Beach, T. Oellerich, J. Lehne, J.E. Smith-Garvin, M. Okumura, E. Starosvetsky, R.
875 Kosoff, E. Libman, G. Koretzky, T. Kambayashi, H. Urlaub, J. Wienands, J. Chernoff, and D. Yablonski.
876 2011. Sequential phosphorylation of SLP-76 at tyrosine 173 is required for activation of T and mast cells.
877 *EMBO J.* 30(15): p. 3160-72.
878 22. Devkota, S., R.E. Joseph, L. Min, D. Bruce Fulton, and A.H. Andreotti. 2015. Scaffold Protein SLP-76
879 Primes PLCγ1 for Activation by ITK-Mediated Phosphorylation. *Journal of Molecular Biology.* 427(17): p.
880 2734-47.
881 23. Poulin, B., F. Sekiya, and S.G. Rhee. 2005. Intramolecular interaction between phosphorylated tyrosine-783
882 and the C-terminal Src homology 2 domain activates phospholipase C-gamma1. *Proc. Natl. Acad. Sci. USA.*
883 102(12): p. 4276-81.
884 24. Andreotti, A.H., S.C. Bunnell, S. Feng, L.J. Berg, and S.L. Schreiber. 1997. Regulatory intramolecular
885 association in a tyrosine kinase of the Tec family. *Nature.* 385(6611): p. 93-7.
886 25. Andreotti, A.H., P.L. Schwartzberg, R.E. Joseph, and L.J. Berg. 2010. T-Cell Signaling Regulated by the Tec
887 Family Kinase, Itk. *Cold Spring Harbor Perspectives in Biology.* 2(7): p. a002287.
888 26. Devkota, S., R.E. Joseph, S.E. Boyken, D.B. Fulton, and A.H. Andreotti. 2017. An Autoinhibitory Role for
889 the Pleckstrin Homology Domain of Interleukin-2-Inducible Tyrosine Kinase and Its Interplay with
890 Canonical Phospholipid Recognition. *Biochemistry.* 56(23): p. 2938-2949.
891 27. Su, Y.-W., Y. Zhang, J. Schweikert, G.A. Koretzky, M. Reth, and J. Wienands. 1999. Interaction of SLP
892 adaptors with the SH2 domain of Tec family kinases. *Eur. J. Immunol.* 29: p. 3702-3711.
893 28. Bunnell, S.C., M. Diehn, M.B. Yaffe, P.R. Findell, L.C. Cantley, and L.J. Berg. 2000. Biochemical interactions
894 integrating Itk with the T Cell Receptor-initiated signaling cascade. *J. Biol. Chem.* 275(3): p. 2219-2230.
895 29. Grasis, J.A., D.M. Guimond, N.R. Cam, K. Herman, P. Magotti, J.D. Lambris, and C.D. Tsoukas. 2010. In
896 Vivo Significance of ITK-SLP-76 Interaction in Cytokine Production. *Mol. Cell. Biol.* 30(14): p. 3596-3609.
897 30. Jordan, M.S., J.E. Smith, J.C. Burns, J.E. Austin, K.E. Nichols, A.C. Aschenbrenner, and G.A. Koretzky.
898 2008. Complementation in trans of altered thymocyte development in mice expressing mutant forms of
899 the adaptor molecule SLP76. *Immunity.* 28(3): p. 359-69.
900 31. Jordan, M.S. and G.A. Koretzky. 2010. Coordination of receptor signaling in multiple hematopoietic cell
901 lineages by the adaptor protein SLP-76. *Cold Spring Harb Perspect Biol.* 2(4): p. a002501.
902 32. Gonen, R., D. Beach, C. Ainey, and D. Yablonski. 2005. T Cell Receptor-induced Activation of
903 Phospholipase C-γ1 Depends on a Sequence-independent Function of the P-I Region of SLP-76. *J. Biol.*
904 *Chem.* 280(9): p. 8364-70.
905 33. Houtman, J.C., Y. Higashimoto, N. Dimasi, S. Cho, H. Yamaguchi, B. Bowden, C. Regan, E.L. Malchiodi, R.
906 Mariuzza, P. Schuck, E. Appella, and L.E. Samelson. 2004. Binding specificity of multiprotein signaling
907 complexes is determined by both cooperative interactions and affinity preferences. *Biochemistry.* 43(14): p.
908 4170-8.
909 34. Kumar, L., S. Feske, A. Rao, and R.S. Geha. 2005. A 10-aa-long sequence in SLP-76 upstream of the Gads
910 binding site is essential for T cell development and function. *Proc Natl Acad Sci U S A.* 102(52): p. 19063-8.
911 35. Sanzenbacher, R., D. Kabelitz, and O. Janssen. 1999. SLP-76 Binding to $p56^{lck}$: A role for SLP-76 in
912 CD4-induced desensitization of the TCR/CD3 signaling complex. *J. Immunol.* 163: p. 3143-3152.
913 36. Liu, K.-Q., S.C. Bunnell, C.B. Gurniak, and L.J. Berg. 1998. T cell receptor-initiated calcium release is
914 uncoupled from capacitative calcium entry in Itk-deficient T cells. *J. Exp. Med.* 187(10): p. 1721-1727.
915 37. Fowell, D.J., K. Shinkai, X.C. Liao, A.M. Beebe, R.L. Coffman, D.R. Littman, and R.M. Locksley. 1999.
916 Impaired NFATc translocation and failure of Th2 development in Itk-deficient CD4+ T cells. *Immunity.*
917 11(4): p. 399-409.
918 38. Lugassy, J., J. Corso, D. Beach, T. Petrik, T. Oellerich, H. Urlaub, and D. Yablonski. 2015. Modulation of
919 TCR responsiveness by the Grb2-family adaptor, Gads. *Cell Signal.* 27(1): p. 125-134.
920 39. Bilal, M.Y., E.Y. Zhang, B. Dinkel, D. Hardy, T.M. Yankee, and J.C. Houtman. 2015. GADS is required for
921 TCR-mediated calcium influx and cytokine release, but not cellular adhesion, in human T cells. *Cell Signal.*
922 27(4): p. 841-50.
923 40. Berry, D.M., P. Nash, S.K. Liu, T. Pawson, and C.J. McGlade. 2002. A high-affinity Arg-X-X-Lys SH3
924 binding motif confers specificity for the interaction between Gads and SLP-76 in T cell signaling. *Curr Biol.*
925 12(15): p. 1336-41.
926 41. Seet, B.T., D.M. Berry, J.S. Maltzman, J. Shabason, M. Raina, G.A. Koretzky, C.J. McGlade, and T. Pawson.
927 2007. Efficient T-cell receptor signaling requires a high-affinity interaction between the Gads C-SH3
928 domain and the SLP-76 RxxK motif. *Embo J.* 26(3): p. 678-689.
929 42. Sukenik, S., M.P. Frushicheva, C. Waknin-Lellouche, E. Hallumi, T. Ifrach, R. Shalah, D. Beach, R. Avidan,
930 I. Oz, E. Libman, A. Aronheim, O. Lewinson, and D. Yablonski. 2017. Dimerization of the adaptor Gads

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

facilitates antigen receptor signaling by promoting the cooperative binding of Gads to the adaptor LAT. *Sci Signal.* 10(498): p. eaal1482.
43. Di Bartolo, V., B. Montagne, M. Salek, B. Jungwirth, F. Carrette, J. Fourtane, N. Sol-Foulon, F. Michel, O. Schwartz, W.D. Lehmann, and O. Acuto. 2007. A novel pathway down-modulating T cell activation involves HPK-1-dependent recruitment of 14-3-3 proteins on SLP-76. *J. Exp. Med.* 204(3): p. 681-91.
44. Shui, J.W., J.S. Boomer, J. Han, J. Xu, G.A. Dement, G. Zhou, and T.H. Tan. 2007. Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. *Nat. Immunol.* 8(1): p. 84-91.
45. Lasserre, R., C. Cuche, R. Blecher-Gonen, E. Libman, E. Biquand, A. Danckaert, D. Yablonski, A. Alcover, and V. Di Bartolo. 2011. Release of serine/threonine-phosphorylated adaptors from signaling microclusters downregulates T cell activation. *J. Cell. Biol.* 195(5): p. 839-53.
46. Joseph, R.E., L. Min, R. Xu, E.D. Musselman, and A.H. Andreotti. 2007. A remote substrate docking mechanism for the tec family tyrosine kinases. *Biochemistry.* 46(18): p. 5595-603.
47. Min, L., R.E. Joseph, D.B. Fulton, and A.H. Andreotti. 2009. Itk tyrosine kinase substrate docking is mediated by a nonclassical SH2 domain surface of PLCgamma1. *Proc. Natl. Acad. Sci. USA.* 106(50): p. 21143-8.
48. Tomlinson, M.G., T. Kurosaki, A.E. Berson, G.H. Fujii, J.A. Johnston, and J.B. Bolen. 1999. Reconstitution of Btk signaling by the atypical tec family tyrosine kinases Bmx and Txk. *J Biol Chem.* 274(19): p. 13577-85.
49. Weiss, A. and J.D. Stobo. 1984. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.* 160: p. 1284-1299.
50. Schmidt, T.G.M., L. Batz, L. Bonet, U. Carl, G. Holzapfel, K. Kiem, K. Matulewicz, D. Niermeier, I. Schuchardt, and K. Stanar. 2013. Development of the Twin-Strep-tag® and its application for purification of recombinant proteins from cell culture supernatants. *Protein Expression and Purification.* 92(1): p. 54-61.
51. Pear, W.S., J.P. Miller, L. Xu, J.C. Pui, B. Soffer, R.C. Quackenbush, A.M. Pendergast, R. Bronson, J.C. Aster, M.L. Scott, and D. Baltimore. 1998. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood.* 92(10): p. 3780-92.
52. Day, R.N. and M.W. Davidson. 2009. The fluorescent protein palette: tools for cellular imaging. *Chemical Society Reviews.* 38(10): p. 2887-2921.
53. Bourgin, C., R.P. Bourette, S. Arnaud, Y. Liu, L.R. Rohrschneider, and G. Mouchiroud. 2002. Induced Expression and Association of the Mona/Gads Adapter and Gab3 Scaffolding Protein during Monocyte/Macrophage Differentiation. *Molecular and Cellular Biology.* 22(11): p. 3744-3756.
54. Yablonski, D., M.R. Kuhne, T. Kadlecek, and A. Weiss. 1998. Uncoupling of nonreceptor tyrosine kinases from PLC-γ1 in an SLP-76-deficient T cell. *Science.* 281: p. 413-416.
55. Lo, W.-L., N.H. Shah, S.A. Rubin, W. Zhang, V. Horkova, I.R. Fallahee, O. Stepanek, L.I. Zon, J. Kuriyan, and A. Weiss. 2019. Slow phosphorylation of a tyrosine residue in LAT optimizes T cell ligand discrimination. *Nature Immunology.* 20(11): p. 1481-1493.
56. Lo, W.-L., N.H. Shah, N. Ahsan, V. Horkova, O. Stepanek, A.R. Salomon, J. Kuriyan, and A. Weiss. 2018. Lck promotes Zap70-dependent LAT phosphorylation by bridging Zap70 to LAT. *Nature Immunology.* 19(7): p. 733-741.
57. Oellerich, T., M. Gronborg, K. Neumann, H.H. Hsiao, H. Urlaub, and J. Wienands. 2009. SLP-65 phosphorylation dynamics reveals a functional basis for signal integration by receptor-proximal adaptor proteins. *Mol. Cell. Proteomics.* 8(7): p. 1738-50.
58. Hornbeck, P.V., B. Zhang, B. Murray, J.M. Kornhauser, V. Latham, and E. Skrzypek. 2014. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Research.* 43(D1): p. D512-D520.
59. Mayya, V., D.H. Lundgren, S.I. Hwang, K. Rezaul, L. Wu, J.K. Eng, V. Rodionov, and D.K. Han. 2009. Quantitative phosphoproteomic analysis of T cell receptor signaling reveals system-wide modulation of protein-protein interactions. *Sci Signal.* 2(84): p. ra46.
60. Kim, J.-E. and F.M. White. 2006. Quantitative Analysis of Phosphotyrosine Signaling Networks Triggered by CD3 and CD28 Costimulation in Jurkat Cells. *The Journal of Immunology.* 176(5): p. 2833-2843.
61. Ross, Sarah H., C. Rollings, Karen E. Anderson, Phillip T. Hawkins, Len R. Stephens, and Doreen A. Cantrell. 2016. Phosphoproteomic Analyses of Interleukin 2 Signaling Reveal Integrated JAK Kinase-Dependent and -Independent Networks in CD8⁺ T Cells. *Immunity.* 45(3): p. 685-700.
62. Sievers, F., A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Soding, J.D. Thompson, and D.G. Higgins. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol.* 7: p. 539.
63. Crooks, G.E., G. Hon, J.M. Chandonia, and S.E. Brenner. 2004. WebLogo: a sequence logo generator. *Genome Res.* 14(6): p. 1188-90.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

989 64. Cruz-Orcutt, N., A. Vacaflores, S.F. Connolly, S.C. Bunnell, and J.C.D. Houtman. 2014. Activated PLC-γ1
990 is catalytically induced at LAT but activated PLC-γ1 is localized at both LAT- and TCR-containing
991 complexes. *Cell Signal.* 26(4): p. 797-805.
992 65. Shah, N.H., Q. Wang, Q. Yan, D. Karandur, T.A. Kadlecek, I.R. Fallahee, W.P. Russ, R. Ranganathan, A.
993 Weiss, and J. Kuriyan. 2016. An electrostatic selection mechanism controls sequential kinase signaling
994 downstream of the T cell receptor. *eLife.* 5: p. e20105.
995 66. Wilcox, H.M. and L.J. Berg. 2003. Itk phosphorylation sites are required for functional activity in primary T
996 cells. *J Biol Chem.* 278(39): p. 37112-21.
997 67. van de Weyer, P.S., M. Muehlfeit, C. Klose, J.V. Bonventre, G. Walz, and E.W. Kuehn. 2006. A highly
998 conserved tyrosine of Tim-3 is phosphorylated upon stimulation by its ligand galectin-9. *Biochemical and*
999 *Biophysical Research Communications.* 351(2): p. 571-576.
1000 68. Hwang, E.S., S.J. Szabo, P.L. Schwartzberg, and L.H. Glimcher. 2005. T Helper Cell Fate Specified by
1001 Kinase-Mediated Interaction of T-bet with GATA-3. *Science.* 307(5708): p. 430-433.
1002 69. Hey, F., N. Czyzewicz, P. Jones, and F. Sablitzky. 2012. DEF6, a Novel Substrate for the Tec Kinase ITK,
1003 Contains a Glutamine-rich Aggregation-prone Region and Forms Cytoplasmic Granules that Co-localize
1004 with P-bodies. *Journal of Biological Chemistry.* 287(37): p. 31073-31084.
1005 70. Lin, T.A., K.W. McIntyre, J. Das, C. Liu, K.D. O'Day, B. Penhallow, C.Y. Hung, G.S. Whitney, D.J. Shuster,
1006 X. Yang, R. Townsend, J. Postelnek, S.H. Spergel, J. Lin, R.V. Moquin, J.A. Furch, A.V. Kamath, H. Zhang,
1007 P.H. Marathe, J.J. Perez-Villar, A. Doweyko, L. Killar, J.H. Dodd, J.C. Barrish, J. Wityak, and S.B. Kanner.
1008 2004. Selective Itk inhibitors block T-cell activation and murine lung inflammation. *Biochemistry.* 43(34): p.
1009 11056-62.
1010 71. Liu, S.K., N. Fang, G.A. Koretzky, and C.J. McGlade. 1999. The hematopoietic-specific adaptor protein
1011 Gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. *Curr. Biol.* 9: p. 67-75.
1012 72. Asada, H., N. Ishii, Y. Sasaki, K. Endo, H. Kasai, N. Tanaka, T. Takeshita, S. Tsuchiya, T. Konno, and K.
1013 Sugamura. 1999. Grf40, a novel Grb2 family member, is involved in T cell signaling through interactions
1014 with SLP-76 and LAT. *J. Exp. Med.* 189(9): p. 1383-1390.
1015 73. Law, C.-L., M.K. Ewings, P.M. Chaudhary, S.A. Solow, T.J. Yun, A.J. Marshall, L. Hood, and E.A. Clark.
1016 1999. GrpL, a Grb2-related adaptor protein, interacts with SLP-76 to regulate Nuclear Factor of Activated
1017 T Cell activation. *J. Exp. Med.* 189(8): p. 1243-1253.
1018 74. Brazin, K.N., D.B. Fulton, and A.H. Andreotti. 2000. A specific intermolecular association between the
1019 regulatory domains of a Tec family kinase. *J Mol Biol.* 302(3): p. 607-23.
1020 75. Dombroski, D., R.A. Houghtling, C.M. Labno, P. Precht, A. Takesono, N.J. Caplen, D.D. Billadeau, R.L.
1021 Wange, J.K. Burkhardt, and P.L. Schwartzberg. 2005. Kinase-independent functions for Itk in
1022 TCR-induced regulation of Vav and the actin cytoskeleton. *J. Immunol.* 174(3): p. 1385-92.
1023 76. Yoder, J., C. Pham, Y.-M. Iizuka, O. Kanagawa, S.K. Liu, J. McGlade, and A.M. Cheng. 2001. Requirement
1024 for the SLP-76 adaptor GADS in T cell development. *Science.* 291: p. 1987-1991.
1025 77. Tatárová, Z., J. Brábek, D. Rösel, and M. Novotný. 2012. SH3 Domain Tyrosine Phosphorylation – Sites,
1026 Role and Evolution. *PLoS ONE.* 7(5): p. e36310.
1027 78. Merő, B., L. Radnai, G. Gógl, O. Tőke, I. Leveles, K. Koprivanacz, B. Szeder, M. Dülk, G. Kudlik, V. Vas, A.
1028 Cserkaszky, S. Sipeki, L. Nyitray, B.G. Vértessy, and L. Buday. 2019. Structural insights into the tyrosine
1029 phosphorylation–mediated inhibition of SH3 domain–ligand interactions. *Journal of Biological Chemistry.*
1030 294(12): p. 4608-4620.
1031 79. Broome, M.A. and T. Hunter. 1996. Requirement for c-Src catalytic activity and the SH3 domain in
1032 platelet-derived growth factor BB and epidermal growth factor mitogenic signaling. *J Biol Chem.* 271(28): p.
1033 16798-806.
1034 80. Pesti, S., A. Balazs, R. Udupa, B. Szabo, A. Fekete, G. Bogel, and L. Buday. 2012. Complex formation of
1035 EphB1/Nck/Caskin1 leads to tyrosine phosphorylation and structural changes of the Caskin1 SH3 domain.
1036 *Cell Commun Signal.* 10(1): p. 36.
1037 81. Andersen, T.C.B., P.E. Kristiansen, Z. Huszenicza, M.U. Johansson, R.P. Gopalakrishnan, H. Kjelstrup, S.
1038 Boyken, V. Sundvold-Gjerstad, S. Granum, M. Sorli, P.H. Backe, D.B. Fulton, B.G. Karlsson, A.H.
1039 Andreotti, and A. Spurkland. 2019. The SH3 domains of the protein kinases ITK and LCK compete for
1040 adjacent sites on T cell-specific adapter protein. *J Biol Chem.* 294(42): p. 15480-15494.
1041 82. Joseph, R.E., D.B. Fulton, and A.H. Andreotti. 2007. Mechanism and functional significance of Itk
1042 autophosphorylation. *J Mol Biol.* 373(5): p. 1281-92.
1043 83. Irvin, B.J., B. Williams, L., A.E. Nilson, H.O. Maynor, and R.T. Abraham. 2000. Pleiotropic contributions of
1044 phospholipase C-γ1 (PLC-γ1) to T-cell antigen receptor-mediated signaling : reconstitution studies of a
1045 PLC-γ1-deficient Jurkat T-cell line. *Mol. Cell. Biol.* 20(24): p. 9149-9161.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.11.293316; this version posted September 11, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1046 84. Sauer, K., J. Liou, S.B. Singh, D. Yablonski, A. Weiss, and R.M. Perlmutter. 2001. Hematopoietic Progenitor
1047 Kinase 1 associates physically and functionally with the adaptor proteins B Cell Linker Protein and SLP-76
1048 in lymphocytes. *J. Biol. Chem.* 276: p. 45207-45216.
1049 85. Brenner, D., M. Brechmann, S. Rohling, M. Tapernoux, T. Mock, D. Winter, W.D. Lehmann, F. Kiefer, M.
1050 Thome, P.H. Krammer, and R. Arnold. 2009. Phosphorylation of CARMA1 by HPK1 is critical for
1051 NF-kappaB activation in T cells. *Proc Natl Acad Sci U S A.* 106(34): p. 14508-13.
1052 86. Liu, H., H. Schneider, A. Recino, C. Richardson, M.W. Goldberg, and C.E. Rudd. 2015. The Immune
1053 Adaptor SLP-76 Binds to SUMO-RANGAP1 at Nuclear Pore Complex Filaments to Regulate Nuclear
1054 Import of Transcription Factors in T Cells. *Mol Cell.* 59(5): p. 840-9.
1055 87. Ellis, J.H., C. Ashman, M.N. Burden, K.E. Kilpatrick, M.A. Morse, and P.A. Hamblin. 2000. GRID: a novel
1056 Grb-2-related adapter protein that interacts with the activated T cell costimulatory receptor CD28. *J.*
1057 *Immunol.* 164: p. 5805-5814.
1058 88. Watanabe, R., Y. Harada, K. Takeda, J. Takahashi, K. Ohnuki, S. Ogawa, D. Ohgai, N. Kaibara, O. Koiwai,
1059 K. Tanabe, H. Toma, K. Sugamura, and R. Abe. 2006. Grb2 and Gads exhibit different interactions with
1060 CD28 and play distinct roles in CD28-mediated costimulation. *J Immunol.* 177(2): p. 1085-91.
1061 89. Higo, K., M. Oda, H. Morii, J. Takahashi, Y. Harada, S. Ogawa, and R. Abe. 2014. Quantitative analysis by
1062 surface plasmon resonance of CD28 interaction with cytoplasmic adaptor molecules Grb2, Gads and p85
1063 PI3K. *Immunological Investigations.* 43(3): p. 278-291.
1064 90. Thaker, Y.R., H. Schneider, and C.E. Rudd. 2015. TCR and CD28 activate the transcription factor NF-κB in
1065 T-cells via distinct adaptor signaling complexes. *Immunology Letters.* 163(1): p. 113-119.
1066 91. Greene, T.A., P. Powell, C. Nzerem, M.J. Shapiro, and V.S. Shapiro. 2003. Cloning and characterization of
1067 ALX, an adaptor downstream of CD28. *J Biol Chem.* 278(46): p. 45128-34.
1068 92. Shapiro, M.J., P. Powell, A. Ndubuizu, C. Nzerem, and V.S. Shapiro. 2004. The ALX Src Homology 2
1069 Domain Is Both Necessary and Sufficient to Inhibit T Cell receptor/CD28-mediated Up-regulation of
1070 RE/AP. *Journal of Biological Chemistry.* 279(39): p. 40647-40652.