Small molecule inhibition of Csk alters affinity recognition by T cells

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Abstract

The C-terminal Src kinase (Csk), the primary negative regulator of Src-family kinases (SFK), plays a crucial role in controlling basal and inducible receptor signaling. To investigate how Csk activity regulates T cell antigen receptor (TCR) signaling, we utilized a mouse expressing mutated Csk (Csk^{AS}) whose catalytic activity is specifically and rapidly inhibited by a small molecule. Inhibition of Csk^{AS} during TCR stimulation led to stronger and more prolonged TCR signaling and to increased proliferation. Inhibition of Csk^{AS} enhanced activation by weak but strictly cognate agonists. Titration of Csk inhibition revealed that a very small increase in SFK activity was sufficient to potentiate T cell responses to weak agonists. Csk plays an important role, not only in basal signaling, but also in setting the TCR signaling threshold and affinity recognition.

Introduction

SFKs are ubiquitous regulators of basal and inducible receptor signaling. The seven family members are expressed in various combinations in different cell types, have unique substrate specificity and are differentially regulated by phosphatases and localization[1]. However, they share a common negative regulator Csk. How Csk maintains basal and inducible receptor signaling is still unclear.

In T cells, the strength and duration of TCR signaling in response to antigen stimulation dictates the magnitude and quality of primary and secondary immune responses[2,3]. The TCR activation threshold, agonist affinity discrimination and signal termination must therefore be tightly regulated. This is achieved through the concerted action of multiple positive and negative regulators acting basally and during inducible signaling. The SFK Lck regulates TCR signaling by phosphorylating tyrosines in the cytoplasmic segments of the TCR ζ and CD3 chains, basally and during antigen recognition, as well as by phosphorylating downstream kinases such as ZAP-70 and ITK [2,4,5]. The availability and recruitment of active Lck has been proposed to be the rate-limiting step in discriminating agonist affinity[6]. Weak agonists with shorter half-lives of pMHC (peptide-bound major histocompatibility complex)-TCR interaction have less time to recruit active CD4 or CD8 co-receptor bound Lck. Active Lck (phosphorylated on Y394) is critical for downstream signaling and can be detected in the basal state, but does not change appreciably after TCR stimulation[7].

Although the molecular regulation of Lck is understood, its localization and changes in activity during TCR signaling are still unclear[3]. Lck is tightly regulated by phosphorylation on two conserved tyrosines. Trans-autophosphorylation of its kinase

domain activation loop tyrosine, Y394, increases its catalytic activity, whereas phosphorylation of its C-terminal tail inhibitory tyrosine, Y505, promotes its closed, inactive conformation[3,8]. In T cells, the receptor-like protein tyrosine phosphatase CD45 regulates Lck activity positively and negatively by dephosphorylating its inhibitory tyrosine and activation loop tyrosine[9,10]. However, the major negative regulator of Lck is Csk, which phosphorylates its inhibitory tail tyrosine [11,12].

Csk is a cytoplasmic protein, yet its predominant role is to phosphorylate the inhibitory tyrosine of SFKs that are localized to membranes[11]. Hence, multiple adaptors have been implicated in regulating its activity by influencing its localization. Transmembrane PAG and Lime, as well as the cytosolic PH-domain containing Dok1/2, may play roles in recruiting Csk to the cell membrane in a phosphorylation-dependent manner. The association of Csk with PAG in biochemically defined lipid rafts has been observed to diminish in response to TCR stimulation, leading to its proposed involvement in regulating TCR signaling[13,14]. Since Csk returns to these fractions upon signal termination, it has also been implicated in the down-regulation of signaling[13,14]. However individual genetic deficiencies in PAG, LIME or Dok1/2 have had little effect on TCR signaling and on T cell function[15-18], indicating that our understanding of Csk regulation during TCR signal initiation, propagation and termination remains incomplete.

The precise role of Csk in TCR signaling has also been difficult to study by genetic inactivation[19-21]. Germline deletion of Csk leads to embryonic lethality[19,20]. Conditional deletion of Csk in the T lineage leads to TCR-MHC-independent, but Lck-dependent, passage through both thymic beta selection and positive selection checkpoints, both of which require perception of pre-TCR or TCR signaling,

79 respectively[21]. However, since Csk deficiency leads to aberrant T cell development and
80 compensatory changes in the basal state, studying the role of Csk in ligand-induced
81 signaling in unmanipulated primary T cells has been hindered.
82 We recently generated BAC transgenic mice that express a mutant of Csk (Csk^{AS})
83 in the absence of endogenous Csk [22]. Csk^{AS} kinase activity can be specifically and rapidly
84 inhibited by an analog of the general kinase inhibitor PP1, 3-iodo-benzyl-PP1 (3-IB-
85 PP1). Studies in thymocytes in this model system support the notion that the combined
86 actions of CD45 and Csk establish the basal activity of SFK[22]. However, it is not clear
87 whether Csk plays a role in setting the TCR signaling threshold or in signal termination.
88 In this study, we utilized $CD4^+$ and $CD8^+$ T cells from Csk^{AS} mice to investigate how Csk
89 modulates the threshold for TCR signal activation, affinity recognition and TCR signal
90 termination. Our data suggest that by controlling the amount of available active Lck, Csk
91 regulates the TCR signaling threshold and affinity recognition. Notably, even at a low
92 level of Csk inhibition, T cell activation by weak agonists is greatly enhanced.
93

94 **Results**

95 To determine whether Csk activity regulates the TCR activation threshold, we
96 stimulated Csk^{AS} $CD4^+$ T cells or wild type $CD4^+$ T cells (as a specificity control) by
97 titrating anti-CD3ε antibody in the absence or presence of a high dose of the Csk^{AS}
98 inhibitor, 3-IB-PP1, and examined proximal TCR signaling tyrosine phosphorylation
99 events. As previously shown, inhibition of Csk^{AS} alone induced strong activation of Lck,
100 as well as Fyn, which was indicated by the hyperphosphorylation of their activation loop
101 tyrosines and the reduced phosphorylation of their inhibitory tyrosines [22]. In contrast,

102 ligation of the TCR alone did not detectably alter Lck phosphorylation, consistent with an
103 earlier report [7]. The striking Lck activation after Csk^{AS} inhibition was associated with
104 only relatively weak downstream phosphorylation of ZAP-70, LAT and PLC-γ1 (Figure
105 1A). However, this downstream signaling was very strongly enhanced when the Csk^{AS}
106 inhibitor treatment was combined with anti-CD3ε crosslinking, as evidenced by the
107 marked increase in ZAP-70, LAT and PLC-γ1 phosphorylation (Figure 1A). To
108 determine whether this early enhanced proximal signaling was transmitted further
109 downstream, we examined ERK1/2 phosphorylation (p-ERK). The magnitude of p-ERK
110 was increased by Csk^{AS} inhibition in a 3-IB-PP1 dose-dependent manner, with the
111 greatest enhancement seen when using a low dose of anti-CD3ε (Figure 1B). At a high
112 dose of anti-CD3ε the increased p-ERK signal intensity, as indicated by the slight peak
113 shift for 3-IB-PP1 treated cells, might reflect a more rapid or more complete peak
114 response at the time point assayed. Notably, Csk^{AS} inhibition alone induced only a small
115 amount of protein phosphorylation downstream of ZAP-70 (i.e., LAT and ERK1/2),
116 likely due to spurious activation of a few cells (Figure 1A and 1B). Instead, Csk^{AS}
117 inhibition synergized with TCR stimulation leading to downstream signaling. Our data
118 strongly suggest that Csk activity plays a crucial role in establishing the TCR activation
119 threshold. Specifically, a reduction in Csk activity lowered the threshold for signal
120 activation and increased TCR sensitivity.

121 It has been proposed that Csk plays an important role in TCR signal
122 termination[13,14]. To test this model we examined a time-course of Csk^{AS} $CD4^+$ T cells
123 stimulation with anti-CD3ε antibody. Csk^{AS} inhibition prolonged phosphorylation of
124 ZAP-70, LAT and PLC-γ1 (Figure 1-figure supplement 1a). Moreover, there was still a

clear digital response to anti-CD3 stimulation that was augmented by Csk^{AS} inhibition at the earliest time point (Figure 1-figure supplement 1b). There was evidence of 3-IB-PP1 dose-dependent delay in downregulation of p-ERK, with the down-regulation seeming more heterogeneous and, perhaps, asynchronous compared to the digital up-regulation of the response (Figure 1-figure supplement 1b). However, Csk inhibition did not prevent eventual signal attenuation over time, indicating that Csk has only a partial role in signal termination. Other negative regulatory mechanisms must contribute to the termination of TCR signaling, albeit more slowly in the absence of Csk[23,24].

To examine more physiologically relevant regulatory mechanisms induced by peptide-MHC stimulation, we generated Csk^{AS} mice expressing the ovalbumin peptide/MHCII-reactive OTII TCR transgene[25,26]. A tetramer was used as the stimulus to allow for detailed biochemical analyses that would not have been possible with the confounding contribution of proteins from antigen presenting cells (APCs). Stimulation of Csk^{AS};OTII $CD4^{+}$ T cells with an agonistic OVA pMHC tetramer during Csk^{AS} inhibition resulted in markedly enhanced phosphorylation of ZAP-70, LAT and PLC-γ1 (Figure 2a) and led to increased and prolonged ERK1/2 phosphorylation (Figure 2b). Thus, our findings with the physiologic OVA pMHC TCR ligand parallel those using anti-CD3ε, confirming that Csk plays an important role in setting the TCR activation threshold and **has a partial role** in signal termination.

To assess whether the reduced TCR activation threshold for proximal signaling events following Csk inhibition had a functional impact on downstream T cell activation, we monitored cell proliferation of Csk^{AS};**OT**II T cells stimulated with plate-bound anti-

147 CD3ε (Figure 2-figure supplement 1a) or OVA pMHC tetramer (Figure 2-figure
148 supplement 1b) in the presence of anti-CD28 costimulation. **Each stimulus was titrated**
149 **to doses that yield minimal to maximum responses since antibody and tetramer have**
150 **different molecular weights and physiological potency.** At low doses, both anti-CD3ε
151 and OVA pMHC tetramer induced greater cellular proliferation when Csk^{AS} was
152 inhibited, consistent with the increased magnitude of proximal TCR signaling. Therefore,
153 by controlling Csk activity, the threshold for TCR signaling and resultant responses can
154 be modulated.

155 Next, we investigated how much perturbation of Csk and Lck is necessary to
156 induce physiological changes in the T cell response. Here we used Csk^{AS}-expressing
157 $CD8^+$ T cells or transgenic $CD8^+$ T cells with the OTI TCR specific for an OVA
158 peptide/MHC class I complex. Titration of 3-IB-PP1 in Csk^{AS} $CD8^+$ T cells revealed a
159 maximum 3-4 fold enhancement of Lck pY394, which correlated with increased Lck
160 activity (Figure 3a)[3]. Thus at resting state 25-33% of Lck was phosphorylated at Y394,
161 which agrees with **some** previous estimates[7] **and is higher than others[27]. It should be**
162 **noted that this is only an inference from the plateau with high drug doses. This**
163 **study is not designed to estimate the percentage of active Lck at the basal state, since**
164 **some Lck may not be available for activation by Csk inhibition.** Low doses of 3-IB-
165 PP1 (1 μM or less) induced at most a 50% upregulation of pY394, while doses of 5-10
166 μM induced a 3-4 fold upregulation. Surprisingly, phosphorylation of the inhibitory tail
167 Y505, the direct target of Csk, did not decrease as appreciably. This observation suggests
168 that Lck is actively phosphorylated by Csk, but **a substantial proportion of** pY505 is
169 either inaccessible for dephosphorylation by CD45 or is dephosphorylated over a longer

timescale. **This, however, does not explain how the small reduction in pY505, particularly so in CD8$^+$ T cells, results in a large increase of pY394.**

We explored whether the effects of Csk inhibition has differential effects on CD4$^+$ vs CD8$^+$ T cells. However, we found that CD4$^+$ and CD8$^+$ T cells have identical responses to Csk inhibition when normalized to their basal level of Lck phosphorylation (Figure 3- figure supplement 1). Interestingly, CD8$^+$ T cells express ~15% more Lck[28] and have a higher (~20%) basal amount of inhibitory tail pY505 phosphorylation. In both CD4 and CD8 T cells, pY394 increased much more markedly than pY505 in response to low doses of the Csk inhibitor. The molecular mechanism for this response is unknown. One possibility to consider is that even a small pool of active Lck that is dephosphorylated at Y505 and becomes phosphorylated at Y394 enables a trans-autocatalytic mechanism which can become amplified within the larger pool of Lck molecules that are not phosphorylated on pY394. Interestingly, it has also been observed that stimulation with anti-TCR and anti-CD4 antibody leads to substantial increase in pY394 without much loss of pY505[27].

Short-term Csk^{AS};OTI CD8$^+$ T cell stimulation with bead-bound OVA-MHC tetramer was enhanced even by low levels of Csk inhibition (< 1μM) (Figure 3b), as demonstrated by the degree of PLC-γ1 and ERK1/2 phosphorylation. At this inhibitor dose, Lck pY394 did not increase by more than 50%, suggesting that even subtle changes in Lck activity can potentiate proximal signaling. Higher doses of the inhibitor potentiated Lck signaling even further, suggesting Csk and Lck activity can be titrated

192 over a wide range.

193 Lck not only initiates TCR-dependent signaling but is also proposed to be the
194 crucial gate-keeper in kinetic proof-reading models that attempt to explain the exquisite
195 affinity discrimination of T cells of agonists with only slightly different half-lives [6,29].
196 We hypothesized that Lck hyperactivation may differentially regulate strong and weak
197 agonists. To address this question, we utilized a panel of well-characterized altered OVA
198 peptides[30]. When Csk^{AS};OTI $CD8^+$ T cells were stimulated by APCs preloaded with
199 peptides of different agonist potency during strong Csk^{AS} inhibition (5μM), we observed
200 marked augmentation of CD69 expression in responses to weak (T4, Q4H7, G4) agonists,
201 contrasting with only slight enhancement of the responses to strong (OVA, Q4R7)
202 agonists (Figure 4a). Although the OVA response was not fully saturated at this early
203 time point (4 hours), it was only slightly enhanced by strong Csk inhibition. Upon
204 stimulation with OVA, but not altered peptides, the TCR is quickly downregulated due to
205 its phosphorylation and retention in intracellular vesicles[31]. Csk inhibition alone induced
206 some ligand-independent TCR downregulation, but additional downregulation was
207 observed with all peptides, even the weakest G4, which argues for altered signaling of
208 low-affinity peptides at a very upstream step (Figure 4 – figure supplement 1).
209 Importantly, the null agonist VSV-loaded APCs did not induce similar activation or TCR
210 downregulation, demonstrating that Csk inhibition works strictly in synergy with a
211 cognate TCR ligand. Moreover, although the spleen-derived APCs should still present
212 endogenous self-peptides, only loading of cognate peptides led to sustained T cell
213 activation after Csk inhibition.

214 We speculated that the striking enhancement of weak agonist recognition is due to

215 the strong inhibition of Csk and activation of Lck (3-fold at 5μM 3-IB-PP1). To test the
216 extent of Csk inhibition necessary to boost weak agonist signaling, we titrated 3-IB-PP1
217 and assayed responses to weak agonists Q4H7 and G4 (Figure 4b). Surprisingly we
218 observed dose-dependent enhancement of signaling at 100-1000 nM 3-IB-PP1, where
219 increased Lck pY394 was weakly detectable and at most 50% above the basal level.
220 Beyond this inhibitor dose, we observed a saturating effect, despite the lower overall
221 plateau with lower peptide dose. Thus, relatively weak Lck hyperactivation was sufficient
222 to enhance the recognition of weak agonists. The lower plateau suggests that there are
223 Csk-independent mechanisms for sensing agonist dose. Remarkably, the EC50
224 concentration of Csk inhibition for CD69 upregulation was still dependent on agonist
225 quality and dose (Figure 4c). At a lower agonist dose, the weaker peptide G4 required
226 double the amount of 3-IB-PP1 than Q4H7. Similarly, the EC50 depended on peptide
227 dose. These observations argue that Csk inhibition fine-tunes the response to agonist
228 dose and affinity sensing in peripheral T cells.

229

230 **Discussion**

231 Our results raise the question of how Csk activity controls SFK activity and
232 specifically the TCR activation threshold. Since a substantial amount of active Lck is
233 present in the basal state and does not change with TCR ligation, it has been hypothesized
234 that the pre-existing pool of active Lck is responsible for the highly sensitive and rapid
235 initiation of TCR signaling in response to ligand engagement[7]. Hence, a likely
236 explanation for our observations is that by controlling the amount of active Lck present,

Csk regulates the responsiveness of the T cell to receptor stimulus.

Our data supports the model that the size of the active pool of Lck is especially critical for affinity discrimination of different agonistic peptides. Strong agonists have a long half-life of pMHC-TCR and have sufficient time to recruit active Lck and initiate downstream events, while weak agonists with short half-lives are most sensitive to the availability of active Lck before the pMHC-TCR complex falls apart. The different amounts of Lck loaded on the CD4 and CD8 co-receptors can explain the different half-life range of positively and negatively selecting ligands for CD4 and CD8 cells[6]; however, this is a genetically established parameter. Our data supports the model that recruitment of active Lck is critical for affinity discrimination, but offers an alternative mechanism for its regulation. By controlling the fraction of active Lck, Csk can also modulate the activation threshold and affinity discrimination of T cells. Notably, since Csk localization and activity is dynamically controlled by phosphorylation-dependent recruitment to transmembrane and cytoplasmic adaptors, this could enable flexibility in affinity recognition in different settings. For example, Csk has been found to be differentially localized in naïve and antigen-experienced T cells[32]. Our results add to the emerging understanding of how signaling by agonists of dissimilar affinity are differentially regulated. Recently, the adaptor molecule Themis was characterized as critical for the suppression of weak agonists in thymocytes, while PTPN22 serves a similar role in in effector cells[25,26]. Here, we show that Csk, via its regulation of Lck, regulates naïve T cell priming to agonists of different potency.

The ability to acutely titrate the activity of Csk, and consequently Lck, allowed us to assess how graded increases of Lck activity affects TCR signaling without concerns

about basal level adaptation and long-term compensation that occurs when using genetic manipulation of Csk amounts or function. Surprisingly, even very small changes in Lck activity, caused by Csk^{AS} inhibition at 200 nM 3-IB-PP1, **led to relatively large pY394 changes in the setting of small changes in pY505. Since Y394 phosphorylation is the result of trans-autophosphorylation, it seems likely that a small reduction in pY505 induced by low levels of Csk inhibition, could lead to autoactivation of a large pool of Lck. Such a larger pool of active Lck could then** show strong synergy with the TCR stimulus and are sufficient to markedly enhance the response to very weak agonists. The synergy we observed between TCR stimulus and Csk inhibition, strikingly had its greatest impact on the activation of downstream signaling molecules. This is most likely due to positive feedback/signal amplification along the pathway. Eventually, the integration of stronger signaling over hours led to profound differences in the fraction of cells upregulating CD69 or proliferating. On the other hand, this minimal change in Lck activity highlights how tightly it must be regulated in both the basal and inducible states, by kinases and phosphatases and their respective adaptors. It is possible that, although small changes in Lck activity greatly sensitize the TCR to weak agonists, such small changes do not as effectively engage negative feedback mechanisms.

Loss of Csk from the cell membrane, or biochemically defined lipid raft fractions has been proposed to lead to TCR signal initiation via activation of Lck[13,14]. This model is contradicted by the lack of appreciable change in Lck phosphorylation upon TCR stimulation and by unaltered signaling in cells deficient in the lipid-raft-resident Csk adapter PAG[16,17]. Our data demonstrate that acute activation of Lck, via weak or strong Csk inhibition, does not lead to full TCR signaling. Strong Csk inhibition in mature T

283 cells leads to small amounts of phosphorylation of downstream targets like ZAP-70 and
284 PLC-γ1, but not ERK activation or long-term proliferation in the absence of TCR
285 engagement. Thus Lck activation alone is insufficient to trigger complete TCR signaling.
286 However, when combined with cognate TCR ligands, very small amounts of Csk
287 inhibition and resultant Lck activation, synergize to enhance early biochemical events
288 and downstream activation. Therefore, it is possible that during physiological TCR
289 stimulation, localization of only a small number of Csk molecules is altered and leads to
290 the activation of a small fraction of Lck molecules that would be difficult to detect with
291 current biochemical or imaging methods[7,33] but might be sufficient to influence
292 downstream signaling.

293 Csk has also been proposed to regulate TCR signal downregulation by returning
294 to the plasma membrane after signal initiation. It has been challenging to study this role
295 using genetic ablation approaches since Csk deficiency affects TCR basal state signaling.
296 Using acute inhibition of Csk concurrently with TCR stimulation, we were able to follow
297 TCR signal downregulation in the absence of Csk activity and observed that it is delayed
298 but not abolished. Our data suggest that other molecules are involved in signal
299 termination more prominently than Csk.

300 New insights in how Csk is regulated will enable the development of strategies to
301 manipulate Csk activity and hence the sensitivity of T cells to various activation stimuli.
302 Such manipulation of Csk activity by small molecule inhibitors could be useful
303 therapeutically, for example in pathologic settings where suppression (i.e., autoimmunity)
304 or augmentation (i.e., vaccines or cancer) of T cell responses to antigen is desirable.
305

Materials and Methods

Mice

Mice used for these studies were 6-12 weeks of age. All mice were housed in a specific pathogen-free facility at UCSF according to the University Animal Care Committee and National Institutes of Health (NIH) guidelines.

Inhibitors

3-IB-PP1 has been described (24).

Antibodies and Reagents

CD4 PerCP-Cy5.5 (550954), TCR Vα2 PE (553289) and Lck-pY505 (BD Biosciences 612390) are from BD Biosciences; p44/42 MAPK pThr202/Tyr204 (4377), Src 416 (2101), ZAP-70–pY319 (2701) are from Cell Signaling; LAT-pY132 (44-224), PLC-γ1-pY783(44-696G) and CFSE (C34554) are from Life Technologies; actin (Sigma Aldrich A2066); GAPDH (Abcam ab8245); Goat anti-armenian hamster IgG(H+L) (127-005-160) and donkey anti-rabbit IgG Ab conjugated to APC (711-136-152) are from Jackson Immunoresearch; Horseradish peroxidase (HRP)–conjugated goat antibody α-rabbit IgG (H+L) (4050-05), α-mouse IgG (H+L) (1031-50), Alexa647- conjugated α-mouse IgG (H+L) (1010-31) are from Southern Biotech; I-A(b) tetramers loaded with human class II-associated invariant chain peptide 103-117 or chicken OVA peptide 328-337 are from NIH tetramer core facility.

Flow Cytometry and data analyses

Stained cells were analyzed on a BD Fortessa (BD Biosciences). Data analysis was performed using FlowJo software (Treestar Incorporated) and GraphPad Prism (GraphPad Software, Inc.) and ImageLab (BioRad Inc.).

Cell stimulations and intracellular phosphoflow

Before stimulations, cells were serum-starved at 37 °C for at least 20 min. Stimulations were performed in serum-free RPMI at 37 °C. CD3ε crosslinking was induced by addition of anti-CD3ε followed by goat anti-armenian hamster IgG(H+L) to a final concentration of 50 μg/ml. For bead-based stimulations, 4.5 μm styrene beads (Polyscience) were coated overnight at 4°C or for 1 hr at room temperature with p-MHC tetramers or αCD3 (2c11) in PBS under continuous rotation at 4°C. Biotinylated p-MHC for CD8 stimulation were precoated with 5 μg/ml streptavidin. Beads were then saturated with 1% BSA in PBS under continuous rotation for 2 h at room temperature, and washed with serum-free RPMI before use. Ice-cold rested cells and beads were span together and signaling was initiated by transfer to 37°C. Intracellular phospho-ERK was performed as previously described[34].

Immunoblotting

Immunoblotting was performed as previously described[22].

Cell enrichments

Enriched populations of pan T cells were obtained by negative selection according to manufacturer's protocol (STEMCELL Technologies, 19751, 19851). Enriched populations of $CD4^+$ or $CD8^+$ T cells were obtained by negative selection according to manufacturer's protocol (STEMCELL Technologies 19852) or with an in-house procedure as follows: The following biotinylated antibodies were combined and dialyzed in 1x PBS with Slide-a-lyzer 10,000 Molecular Weight cutoff dialysis cassette (Thermo catalog# 66810), then filter sterilized: biotin anti-CD8a (clone 53-6.7) or biotin anti-CD4 (clone GK1.5), biotin anti-CD11b (clone M1/70), biotin anti-CD11c (clone N418), biotin

anti-CD19 (clone 1D3) from were Tonbo biosciences; biotin anti-CD24 (clone M1/69), biotin anti-CD45R (B220) (clone RA3-6B2), biotin anti-CD49b (clone DX5), biotin anti-TER119 (clone TER-119) were from Biolegend. ACK-lysed splenocytes and lymphocytes were incubated at room temperature with the antibody cocktail for 10 minutes in PBS with 1% FBS, 2mM EDTA and 5% normal rat serum at 10^8 cells/mL. Cells were then washed once with PBS with 1% FBS, 2mM EDTA, resuspended at 0.85 mL per 10^8 cells, and mixed with 0.15 mL anti-biotin MACSiBead per 10^8 cells (Miltenyi Biotec). After 5 minutes at room temperature, cell-bead mix was placed in separation magnet for 5 minutes and the unbound cells were collected.

Cell proliferation

Purified $CD4^+$ or $CD8^+$ T cells were resuspended in PBS, labeled for 4 min at room temperature with 2μM CFSE, and quenched with 100% FBS. Labeled $CD4^+$ T cells were stimulated by plate-coated OVA-MHC or α-CD3 (2c11) for 72 hrs. At indicated timepoint, DAPI were analyzed for CFSE dilution.

CD69 upregulation

Purified $CD8^+$ OTI T cells were stimulated for indicated time by Mitomycin C-treated $Calpha^{-/-}$ or BoyJ splenocytes, preloaded for 1 hr with indicated peptide and washed of excess peptide. At indicated time, cells were fixed and stained for CD69, TCRValpha2 and CD45.1, and analyzed by flow cytometry.

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Author Contributions

B.N.M., Y.X.T. and A.W. designed the research and wrote the manuscript, B.N.M., Y.X.T., and A.C. performed the research and analyzed the data. K.M.S. and F.R. designed the Csk^{AS} allele and provided 3-IB-PP1. E.P. provided OTI pMHC. All authors commented on the manuscript.

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FIGURE 1. Inhibiting Csk increases the magnitude of ligand-induced TCR signaling and reduces the threshold for TCR activation. (**A**) Purified Csk^{AS} (AS) or wildtype (WT) $CD4^+$ T cells stimulated for 2 min with 1 μg/mL, 5 μg/mL or 10 μg/mL anti-CD3ε antibody in the presence of DMSO or 10 μM 3-IB-PP1 were analyzed by immunoblotting for the phosphorylation of the activation loop tyrosine of Lck and Fyn (Src pY416 antibody) and the inhibitory tyrosine of Lck (Lck pY505), phosphorylated ZAP-70 (ZAP70 pY319), LAT (LAT pY132) and PLC-γ1 (PLCγ1 pY783), as well as total actin (loading control). Data are representative of at least three independent experiments. (**B**) Purified total Csk^{AS} (AS) or wildtype (WT) T cells stimulated with the indicated dose of anti-CD3ε antibody for 2 min in the presence of DMSO or 5 μM, 1 μM or 0.4 μM 3-IB-PP1 were analyzed for phosphorylated ERK (p-ERK) by phosphoflow. Histograms were gated on $CD4^+$ cells. Data are representative of at least three independent experiments for AS cells and two independent experiments for WT cells.

Figure 1 – figure supplement 1. Inhibiting Csk during TCR stimulation prolongs TCR signals. (**A**) Purified Csk^{AS} $CD4^+$ T cells stimulated for 2, 5 or 10 min with anti-CD3ε antibody in the presence of DMSO or 10 μM 3-IB-PP1 were analyzed by immunoblotting for the phosphorylated ZAP-70, LAT and PLC-γ1 as well as total GAPDH (loading control). Data are representative of at least three independent experiments. (**B**) Purified total Csk^{AS} T cells stimulated with anti-CD3ε antibody for 2, 5 or 10 min in the presence of DMSO or 5 μM, 1 μM or 0.4 μM 3-IB-PP1 were analyzed for phosphorylated ERK (p-ERK) by phosphoflow. Histograms were gated on $CD4^+$ cells. Data are representative of at least three independent experiments.

FIGURE 2. Inhibiting Csk reduces the threshold for TCR activation and prolongs signaling induced by p-MHC engagement. (**A**) Purified Csk^{AS};OTII $CD4^+$ T cells stimulated for 3 min with 5 μg/mL bead-bound control p-MHC tetramer or 5 μg/mL, 2.5 μg/mL or 1.25 μg/mL bead-bound OVA p-MHC tetramer in the presence of DMSO or 10 μM 3-IB-PP1 were analyzed by immunoblotting for the phosphorylated ZAP-70, LAT and PLC-γ1, as well as total actin (loading control). Data are representative of three independent experiments.

(**B**) Purified Csk^{AS};OTII $CD4^+$ T cells stimulated with 5 μg/mL bead-bound control-pMHC tetramer or 2.5μg/mL bead-bound OVA p-MHC tetramer for 2, 5 or 10 min in the presence of DMSO or 5 μM 3-IB-PP1 were analyzed for phosphorylated ERK (p-ERK) by phosphoflow. Histograms were gated on $CD4^+$ $V\alpha2^+$ cells. Data are representative of three independent experiments.

Figure 2 – figure supplement 1. Csk inhibition shifts the threshold for cellular proliferation in response to anti-CD3 or p-MHC tetramer stimulation. (**A**) Purified $CD4^+$ T cells from Csk^{AS} mice were loaded with CFSE and stimulated with 3 μg/mL, 1 μg/mL or 0.3 μg/mL plate-bound anti-CD3ε and 1 μg/mL anti-CD28 for 72 h in the presence or absence of 5μM 3-IB-PP1. Data are representative of three independent experiments. (**B**) Purified $CD4^+$ T cells from Csk^{AS};OT-II mice were loaded with CFSE and stimulated with 0.3 μg/mL, 0.1 μg/mL or 0.04 μg/mL plate-bound OVA p-MHC tetramer and 1 μg/mL anti-CD28 for 72 h in the presence or absence of 5 μM 3-IB-PP1. (**A, B**) Cells were then analyzed by flow cytometry for CFSE intensity. Histograms were gated on $CD4^+$ cells. Data are representative of three independent experiments.

FIGURE 3. Weak Csk inhibition and Lck activation potentiate agonist signaling.

(**A**) Csk^{AS} $CD8^+$ T cells were rested and stimulated with indicated doses of 3-IB-PP1 for 3 min, lysed, and immunoblotted for activating (pY394) and inhibitory (pY505) site Lck phosphorylation. Below is quantification (+/-sem) of three independent experiments. immunoblot.

(**B**) Csk^{AS};OTI naive $CD8^+$ T cells were rested, stimulated with bead-bound BSA or 10 µg/ml pMHC-OVA for 3 min, lysed and assayed by immunoblot. Data are representative of at least two independent experiments.

Figure 3 – figure supplement 1. Comparison of Csk inhibition and Lck activation in $CD4^+$ versus $CD8^+$ T cells.

(A) Csk^{AS} $CD4^+$ and Csk^{AS} $CD8^+$ T cells were rested and stimulated with indicated doses of 3-IB-PP1 or DMSO for 2 min and then lysed. Lysates were immunoblotted for activating (pY394) and inhibitory (pY505) site Lck phosphorylation, total Lck, and GAPDH (loading control). Data are representative of three independent experiments. (B and C) Quantification of Lck pY505 and Lck pY394 of three independent experiments (as in A), with levels normalized to Lck levels per cell type. Error bars are standard deviation.

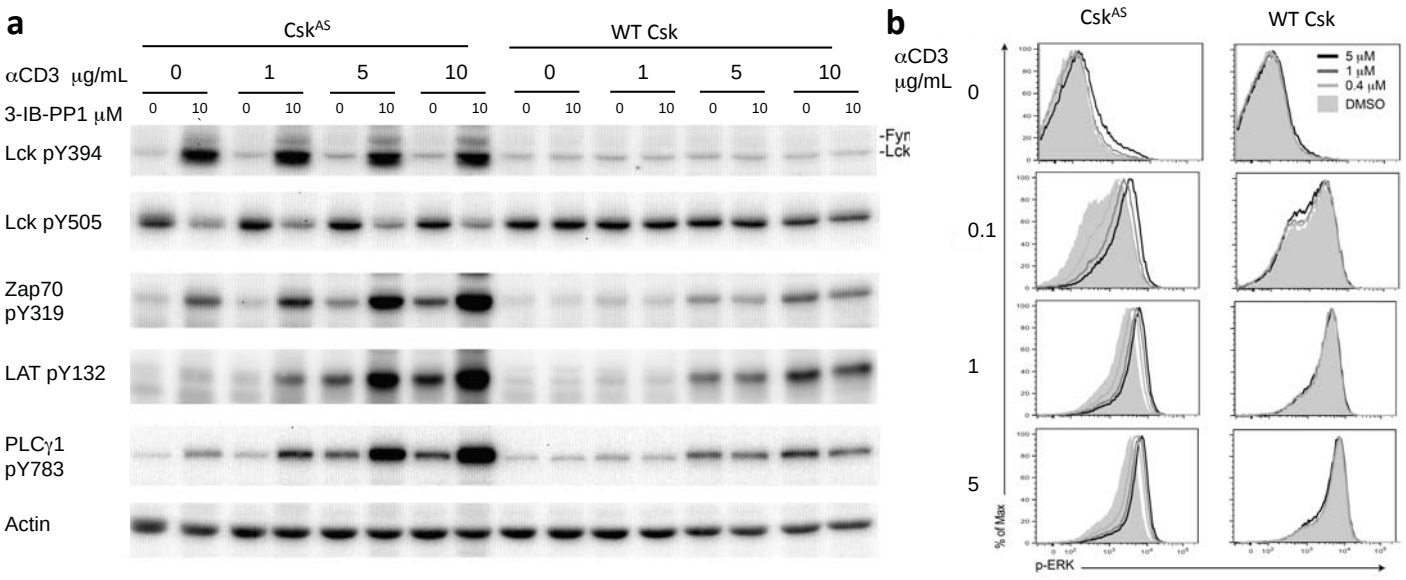
FIGURE 4. Csk inhibition potentiates response to weak agonists. (**A**) Csk^{AS};OTI naïve $CD8^+$ T cells were stimulated with MitoC-treated WT splenocytes, preloaded with indicated peptides, in presence of DMSO or 5 μM 3-IB-PP1 for 4 hrs and CD69 upregulation was measured by flow cytometry.

(**B**) Csk^{AS};OTI naïve $CD8^+$ T cells were stimulated as in **A** with varying doses of 3-IB-PP1 with dose-response fit.

(**C**) EC50 of 3-IB-PP1 (nM) for % CD69 positive cells for data in **B**. Data are representative of two independent experiments.

Figure 4 – figure supplement 1. TCR Valpha2 downregulation in cells in Figure 4A. Only OVA triggers peptide-dependent TCR downregulation. 5 μM 3-IB-PP1 induces ligand-independent downregulation of TCR, as seen in VSV peptide. In the presence of Csk^{AS} inhibition, all the other altered peptides induce further, dose-dependent loss of TCR.

Figure 1.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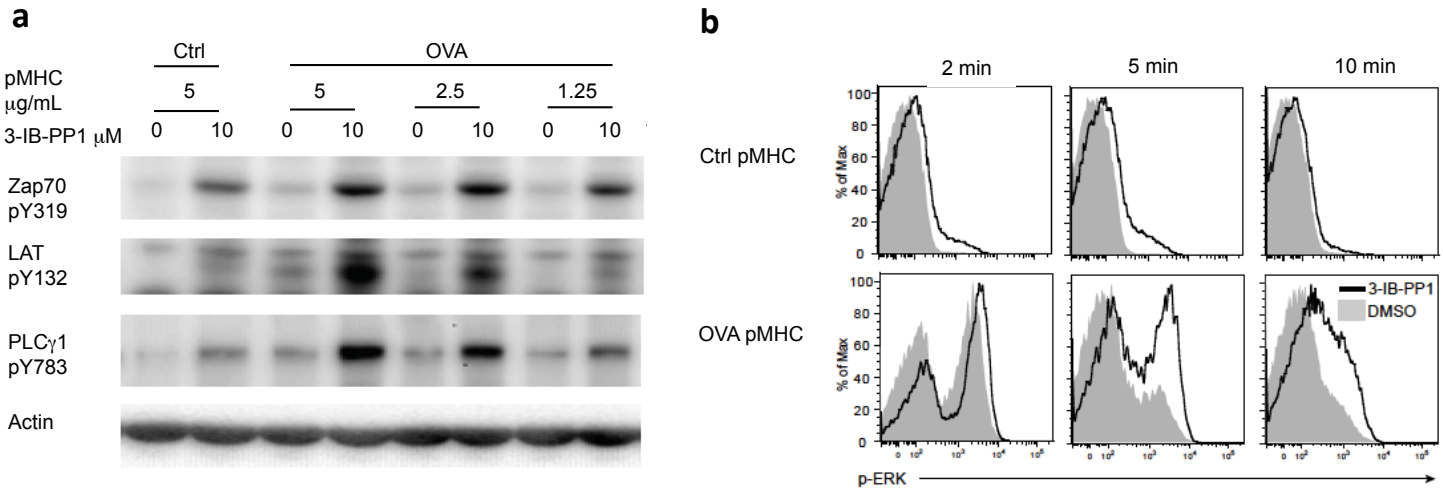
Csk^AS
WT Csk
αCD3 μg/mL
0
1
5
10
3-IB-PP1 μM
Lck pY394
Lck pY505
Zap70 pY319
LAT pY132
PLCγ1 pY783
Actin
-Fyn
-Lck
b
Csk^AS
WT Csk
αCD3 μg/mL
0
0.1
1
5
5 μM
1 μM
0.4 μM
DMSO
% of Max
p-ERK

Figure 2.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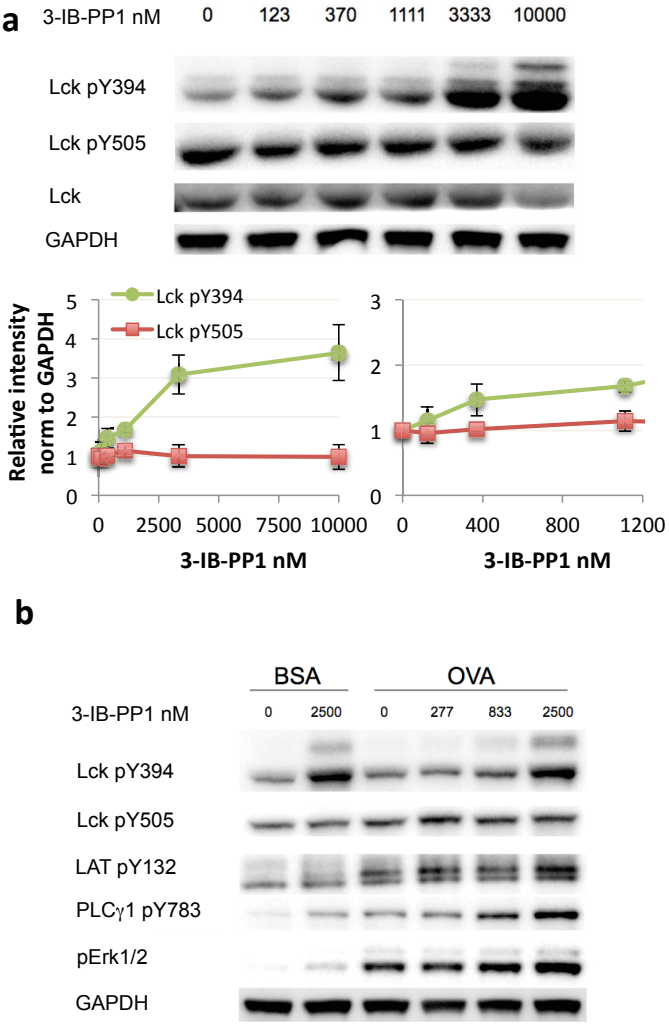
Ctrl
OVA
pMHC
μg/mL
5
5
2.5
1.25
3-IB-PP1 μM
0
10
0
10
0
10
0
10
Zap70
pY319
LAT
pY132
PLCγ1
pY783
Actin
b
2 min
5 min
10 min
Ctrl pMHC
OVA pMHC
% of Max
100
80
60
40
20
0
3-IB-PP1
DMSO
p-ERK

Figure 3.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0
123
370
1111
3333
10000
Lck pY394
Lck pY505
Lck
GAPDH
Relative intensity norm to GAPDH
Lck pY394
Lck pY505
0
1
2
3
4
5
0
2500
5000
7500
10000
3-IB-PP1 nM
0
400
800
1200
3-IB-PP1 nM
b
BSA
OVA
3-IB-PP1 nM
0
2500
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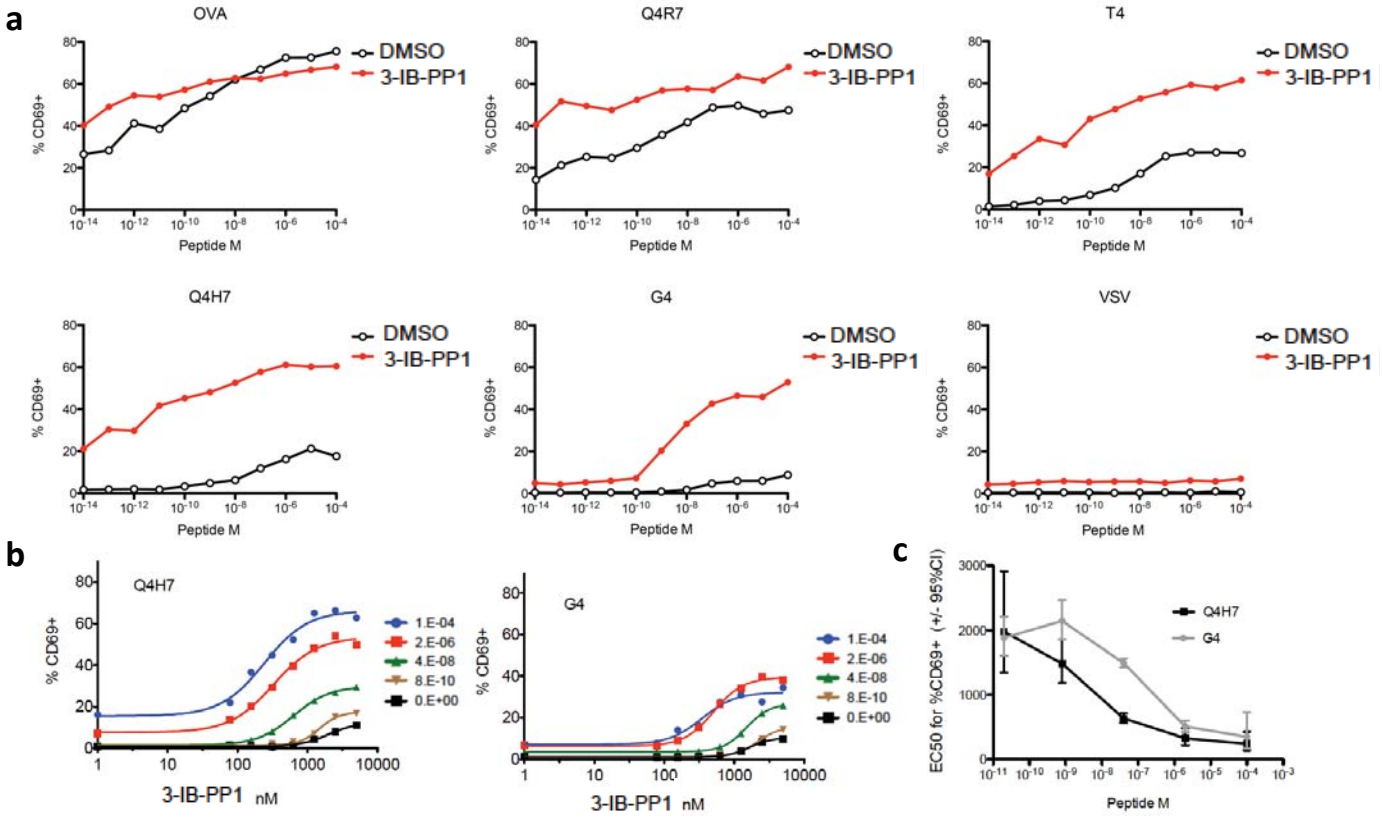
2500
Lck pY394
Lck pY505
LAT pY132
PLCγ1 pY783
pErk1/2
GAPDH

Figure 4.


a
OVA
Q4R7
T4
Q4H7
G4
VSV
% CD69+
Peptide M
DMSO
3-IB-PP1
b
Q4H7
G4
1.E-04
2.E-06
4.E-08
8.E-10
0.E+00
3-IB-PP1 nM
c
EC50 for %CD69+ (+/- 95%CI)
Q4H7
G4
Peptide M