Title
The role of Csk in the dynamic negative regulation of T cell receptor signaling

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The role of Csk in the dynamic negative regulation of T cell receptor signaling

by

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DISSERTATION

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Contributions to thesis work

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mutant of Csk^{AS}, the identification of Dok-1 as a hyperphosphorylated protein following Csk^{AS} inhibition and its increased association with Csk (Fig 2.12). Chao Zhang and Kevan Shokat provided reagents and critical review of the manuscript. The UCSF Sandler-Moore MS core facility, especially Eric Johansen, Halina Ewa Witkowska, and Steven Hall, carried out the mass spectrometric analyses. (Schoenborn, J.R., Tan, Y.X., Zhang, C., Shokat, K.M., and Weiss, A. (2011). Feedback circuits monitor and adjust basal Lck-dependent events in T cell receptor signaling. Science Signaling *4,* ra59.)

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The role of Csk in the dynamic negative regulation of T cell receptor signaling

by

Ying Xim Tan

Abstract

The cytoplasmic tyrosine kinase, C-terminal Src kinase (Csk), negatively regulates T cell receptor (TCR) signaling by inhibiting Src family kinase (SFK) activity. Its function in T cells is poorly defined since genetic ablation in the T lineage causes abnormal thymic development. To circumvent this, we have generated a novel allele of Csk, Csk^{AS}, that can be inhibited rapidly and specifically by an analog of the common kinase inhibitor, PP1. We further generated bacterial artificial chromosome (BAC) transgenic mice expressing the Csk^{AS} allele in the absence of endogenous Csk expression. Initial characterization of Csk^{AS} by ectopic expression in Jurkat T cells revealed that in the basal state, Csk actively restrains Lck activity. Inhibition of Csk^{AS} induced ligand-independent initiation of TCR signaling and downstream activation events, indicating the presence of a feedback circuit sensitive to the basal signaling state. Dok-1 was identified as a candidate protein involved in this feedback rewiring. In contrast, we observed a block in TCR signaling at the level of PLCγ1 hydrolysis of phosphatidylinositol-(4,5)-bisphsophate (PIP_2) following inhibition of Csk^{AS} in the BAC transgenic murine thymocytes. This implies that in primary thymocytes, activation of SFKs alone is insufficient for full TCR signaling . We further demonstrate that actin remodeling, most likely mediated by CD28 costimulation, is necessary for downstream propogation of proximal TCR signals by PLCγ1 to the calcium and MAPK pathways.

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Chapter 1

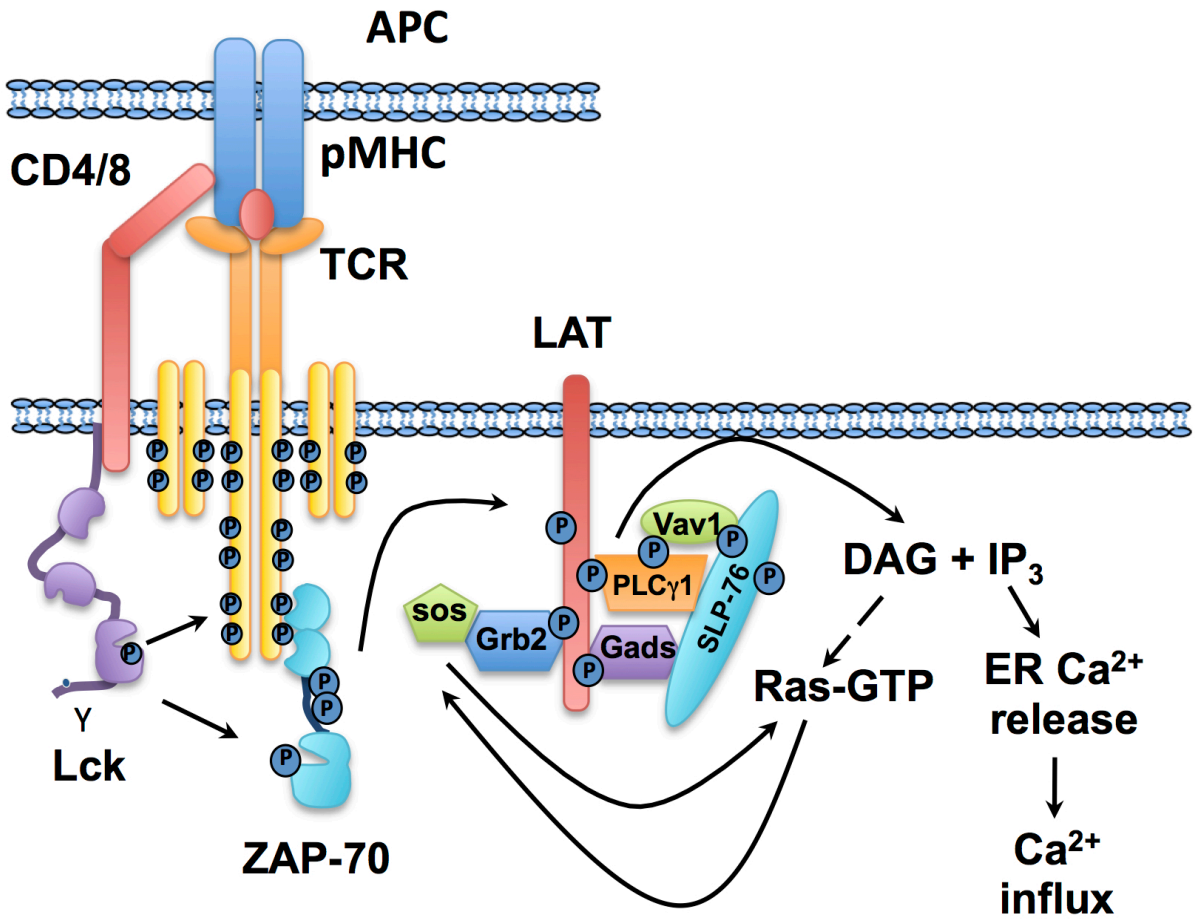
Csk and basal and inducible TCR signaling

Overview of TCR signaling

Signals emanating from the TCR drive thymocyte selection and maturation, peripheral T cell homeostasis and activation, as well as specification of effector and memory cell fates. The initiation of TCR signaling in response to self or foreign antigens of diverse affinities during different stages of T cell development must be tightly regulated. This ensures the selection of a protective T cell repertoire and the mounting of efficacious immune responses against foreign pathogens while preventing aberrant immune activation towards self. Apart from ligand-induced signaling, it has been demonstrated that a steady-state level of ligand-independent signaling is maintained in T cells. This so-called basal or tonic signaling shares many of the same signal transduction machinery as ligand-induced signaling and has important biological function in the maintenance of peripheral homeostasis and may help fine-tune TCR activation thresholds.

The TCR consists of distinct antigen recognition components (TCRαβ chains) and signal transduction components (heterodimers of CD3γε and δε and homodimer of ζ chains). Upon peptide/MHC (pMHC) recognition, the TCR activates a complex signal transduction cascade beginning with tyrosine phosphorylation events that culminate in the activation of key transcription factors. However, the TCR itself does not possess any intrinsic tyrosine kinase activity. Instead, the CD3 and ζ chains bear critical pairs of tyrosine residues that lie within immunoreceptor tyrosine based activation motifs (ITAMs), defined by the sequence (D/E)xxYxxI/L$x_{(6-8)}$YxxI/L. Each TCR has ten ITAM motifs, one from each CD3 chain and three from each ζ chain. During ligand recognition, the TCR interacts with the CD4/CD8 coreceptor-associated Src family tyrosine kinase (SFK) Lck which phosphorylates these ITAM tyrosines (Figure 1.1). The doubly phosphorylated ITAMs favor the recruitment of the Syk

Figure 1.1. TCR signaling. TCR engagement by pMHC on an APC results in phosphorylation of TCR ITAMs and ZAP-70 by Lck. Active ZAP-70 phosphorylates LAT and SLP-76, thus inducing the recruitment of multiple effector and adapter proteins that together form the proximal signaling complex. PLCγ1 hydrolyzes plasma membrane PIP_2 into IP_3 and DAG. IP_3 activates IP_3 receptors, stimulating Ca^{2+} release from the ER into the cytoplasm. Depletion of ER calcium stores leads to STIM oligomerization and activation of CRAC channels that allows for influx of extracellular Ca^{2+}. DAG recruits RasGRP to the plasma membrane where it is phosphorylated and activated by PKCθ. RasGRP then induces Ras to exchange GDP for GTP and become activated. Ras-GTP allosterically activates SOS, resulting in a positive feedback loop that ensures robust Ras-GTP production, and hence activation of the MAPK pathway.


APC
CD4/8
pMHC
TCR
LAT
Vav1
PLCγ1
SLP-76
sos
Grb2
Gads
DAG + IP3
Ras-GTP
ER Ca2+ release
Ca2+ influx
Y
Lck
ZAP-70

family kinase ZAP-70 by allowing it to dock via its tandem Src homology 2 (SH2) domains. Binding of the tandem SH2 domains of ZAP-70 to phosphorylated ITAMs relieves its autoinhibited coformation and allows it to be phosphorylated by Lck, which results in full activation of ZAP-70 (Wang et al., 2010; Weiss, 1993). Lck forms a complex with ZAP-70 via binding its SH2 domain to phospho-Y319 (Straus et al., 1996; Thome et al., 1995). This latter event positions Lck in close proximity to ZAP-70 to amplify signaling and also may contribute to the stabilization of the interaction of the CD4/CD8 coreceptors with pMHC complexes (Artyomov et al., 2010; Xu and Littman, 1993). Active ZAP-70 phosphorylates two critical adaptor molecules, LAT and SLP-76, leading to the recruitment and phosphorylation of multiple effector proteins, including SOS, PLCγ1 and Vav-1, that assemble into the TCR 'signalosome' (Koretzky et al., 2006).

PLCγ1 hydrolyzes phosphatidylinositol-(4,5)-bisphosphate (PIP_2) into the second messengers, diacyglycerol (DAG) and inositol triphosphate (IP_3). DAG activates the Ras guanine nucleotide exchange factor (GEF) RasGRP, resulting in the production of Ras-GTP that allosterically activates the Ras-GEF SOS, thus driving a positive feedback loop that results in robust Ras activation and mitogen-activated protein kinases (MAPK) signaling (Roose et al., 2007). On the other hand, IP_3 binds to the IP_3 receptor on the endoplasmic reticulum (ER) membrane, initiating release of ER calcium stores, stromal interaction molecule (STIM) aggregation and apposition to the plasma membrane and the subsequent opening of the calcium release activated calcium (CRAC) channels that allows for the influx of extracellular calcium. Increased intracellular calcium drives NFAT dephosphorylation and translocation into the nucleus, thereby promoting amongst other things, IL-2 transcription (Oh-hora and Rao, 2008). Vav-1 is a GEF for the Rho-GTPases Rac and Cdc42, which are important mediators of actin

polymerization (Tybulewicz, 2005). The activation of the MAPK cascade, calcium signaling pathways and dynamic actin remodeling events downstream of the TCR are critical for the induction of a variety of biological outcomes including cellular activation, proliferation, differentiation, or death, depending on cellular context. The physiologic importance of these pathways is clearly demonstrated by the defects in T cell development and function in mice deficient for SOS, PLCγ1 and Vav1 (Fu et al., 2010; Kortum et al., 2012; Turner et al., 1997).

Initiation of TCR signaling

Despite extensive studies of TCR signal transduction, how ligation of the TCRαβ chains is coupled to downstream signaling events remains uncertain. Various models that are not mutually exclusive have been proposed. They can be broadly grouped into three categories: 1) aggregation models, 2) conformational change models and 3) redistribution models (van der Merwe and Dushek, 2011).

The earliest models invoked to explain the initiation of TCR signaling following ligand binding were the aggregation models. Davis and coworkers as well as others observed that multimerized but not monomeric pMHC could activate T cells (Boniface et al., 1998; Cochran et al., 2000). This led to the idea that clustering of TCR-CD3 complexes was required for signaling to occur. However, given that very low densities of agonist pMHC on antigen presenting cells (APCs) can activate T cells and that even a single pMHC can initiate Ca^{2+} responses, this model was called into question (Irvine et al., 2002; Sykulev et al., 1996). The pseudodimer model was then proposed to reconcile the conflicting data. It postulates that a single agonist pMHC-engaged TCR can be bridged by one CD4 molecule to a self pMHC-binding TCR, thus overcoming the issue of low agonist pMHC density (Krogsgaard et al., 2005). An alternative model to TCR

oligmerization is the heterodimerization model which posits that MHC-recruitment of CD4/CD8 coreceptor-associated Lck positions it close to the CD3 ITAMs and thus couples ligand-recognition to signal transduction (Artyomov et al., 2010; Weiss, 1993; Xu and Littman, 1993). However, studies of T cells lacking co-receptors have shown that coreceptors impose MHC restriction but can be dispensible for ligand-mediated TCR signaling (Locksley et al., 1993; Van Laethem et al., 2007).

Conformational change models propose that TCR engagement results in conformational changes in the CD3 and ζ chains. These models gained popularity in part due to the fact that they can explain TCR triggering with a very low frequency of agonist pMHC. Alarcon and coworkers found that TCR ligation leads to a conformational change in CD3ε, which exposes a proline rich sequence (PRS) that can associate with the adaptor protein non-catalytic region of tyrosine kinase (NCK) (Gil et al. 2002)**.** However, deletion of this PRS in mice did not affect development and activation of mature T cells in response to TCR stimulation (Mingueneau et al., 2008; Szymczak et al., 2005). Using fluorescence resonance energy transfer (FRET) and nuclear magnetic resonance (NMR), Wucherpfennig and coworkers demonstrated that basic residues in the cytoplasmic tail of CD3ε mediates its close association with the negatively charged plasma membrane such that the ITAM tyrosines are buried inside the lipid bilayer in the resting T cell. This association was diminished following TCR ligation, thereby exposing the ITAM for phosphorylation by Lck (Xu et al., 2008). However, mutation of these basic residues in the CD3ε cytoplasmic tail impairs thymic development and abrogates T cell signaling (Deford-Watts et al., 2009). Other conformational change models propose that mechanical forces such as pulling, pushing or shearing produced when TCRαβ binds membrane-bound pMHC can be transmitted to the CD3 chains (Li et al., 2010; Ma and Finkel, 2010; Sun et al., 2001). Yet precisely how the

force transmission to the CD3 cytoplasmic tails is achieved and how the transduced forces then trigger signaling is not known.

The two principal redistribution models used to explain the initiation of TCR signaling are the lipid raft model and the kinetic segregation model. The lipid raft model proposes that TCR engagement by pMHC favors its partitioning into lipid rafts, which are enriched in active Lck and deficient in CD45 (Montixi et al., 1998; Xavier et al., 1998; Zech et al., 2009). This is thought to bring the TCR ITAMs into close proximity of Lck and encourage their phosphorylation. However, the concept of the lipid raft remains highly controversial and how TCR ligation drives its redistribution into lipid rafts is unresolved (Kenworthy, 2008). Over the past decade, there has been growing experimental support for the kinetic segregation model of TCR activation, first proposed by Van der Merwe and Davis in 1996 (Davis and van der Merwe, 1996; Davis and van der Merwe, 2006). This model proposes that physical proximity of MHC and TCR on the surface of an APC and a T cell respectively drives size-based exclusion of bulky inhibitory transmembrane molecules such as CD45, in turn permitting TCR signaling by favoring ITAM phosphorylation. Evidence in support of this model include the observations that CD45 is excluded from the T cell-APC contact zone and that deletion of the large ectodomains of CD45 inhibits TCR signaling (Irles et al., 2003; James and Vale, 2012). However, the time scale for CD45 relocalization and the earliest detectable biochemical events upon TCR stimulation seem incommensurate as the latter occur far more quickly than the former. This suggests that CD45 relocalization may help to maintain signaling at the immune synapse but may not account for the initiation of TCR triggering. Nonetheless, one of the key assumptions of the kinetic segrgation model is that the basal state of T cells actually represents an active and dynamic equilibrium rather than a static ‘off’ state.

Basal TCR signaling

Although signaling events triggered by the TCR have been extensively studied, much less is understood about the basal state of T cells. Active basal or tonic signaling through antigen receptors has been postulated for many years. Klausner and colleagues were the first to show that treatment of unstimulated T cells with pervanadate, a general PTPase inhibitor, was sufficient to trigger ligand-independent phosphorylation of cellular proteins (Garcia-Morales et al., 1990; Secrist et al., 1993). These data imply that an active equilibrium of kinase and phosphatase activity must exist even in the absence of ligand-mediated receptor stimulation. The finding that unstimulated, resting thymocytes and peripheral T cells exhibit high basal ζ chain ITAM phosphorylation and constitutive association with unphosphorylated ZAP-70 provided further evidence for active signaling in the basal state. This phenomenon requires expression of components of the TCR signaling pathway, including Lck and non-selecting MHC (van Oers et al., 1993; van Oers et al., 1994; van Oers et al., 1996). Given the presence of basal ζ phosphorylation in unstimulated T cells, it is possible that it is actually ZAP-70 phosphorylation and activation, rather than merely ζ phosphorylation, that serves as the key initiating event in TCR signaling initiation in vivo.

Consistent with these early observations, it has been shown that antigen receptors can indeed signal in a ligand-independent manner in vivo, and this signaling has biological significance. For instance, the pre-TCR mediates signal in an antigen-independent manner during thymic development and promotes TCRβ-selection (Irving et al., 1998). Expression of the BCR is required for B cell survival and inducible deletion of the BCR can be rescued with low-level constitutive PI3K activity (Lam et al. 1997; Srinivasan et al. 2009). This provides evidence for a

required role for tonic BCR signaling in B cell survival. However, by contrast to the BCR, inducible deletion of the TCR is associated with relatively long-term T cell survival, raising the question of which biological function is subserved by putative tonic TCR signaling (Polic et al., 2001). One potential function for basal antigen receptor signaling identified in both T and B cells is repression of RAG gene expression (Roose et al., 2003; Srinivasan et al., 2009). It has also been suggested that tonic TCR signaling is important to maintain TCRα expression (Markegard et al., 2011).

In addition to a direct function in lymphocyte survival and differentiation, it has also been suggested that tonic ζ phosphorylation and basal signaling in general might serve to sensitize or tune receptors to improve ligand recognition and discrimination (Germain and Stefanova, 1999; Stefanova et al., 2002). A related hypothesis is that basal signaling enhances the speed of cellular responses to stimuli (Artomov et al., 2010). It has been difficult to study basal signaling directly. One approach towards unmasking basal equilibrium is to perturb either positive or negative regulators of this balance genetically. Although mice harboring constitutively active SFKs (Csk-deficient mice and LckY505F mutant mice) indeed exhibit constitutive TCR signaling that is ligand-independent, the resting basal state in such mutant T cells is quite perturbed, obscuring normal tonic signaling (Baker et al., 2000; Imamoto and Soriano, 1993; Nada et al., 1993; Pingel et al., 1999). Optimal strategies for studying the basal state require the use of more subtle, temporally refined perturbations such as chemical inhibitors.

Reciprocal regulation of Lck activity by Csk and CD45

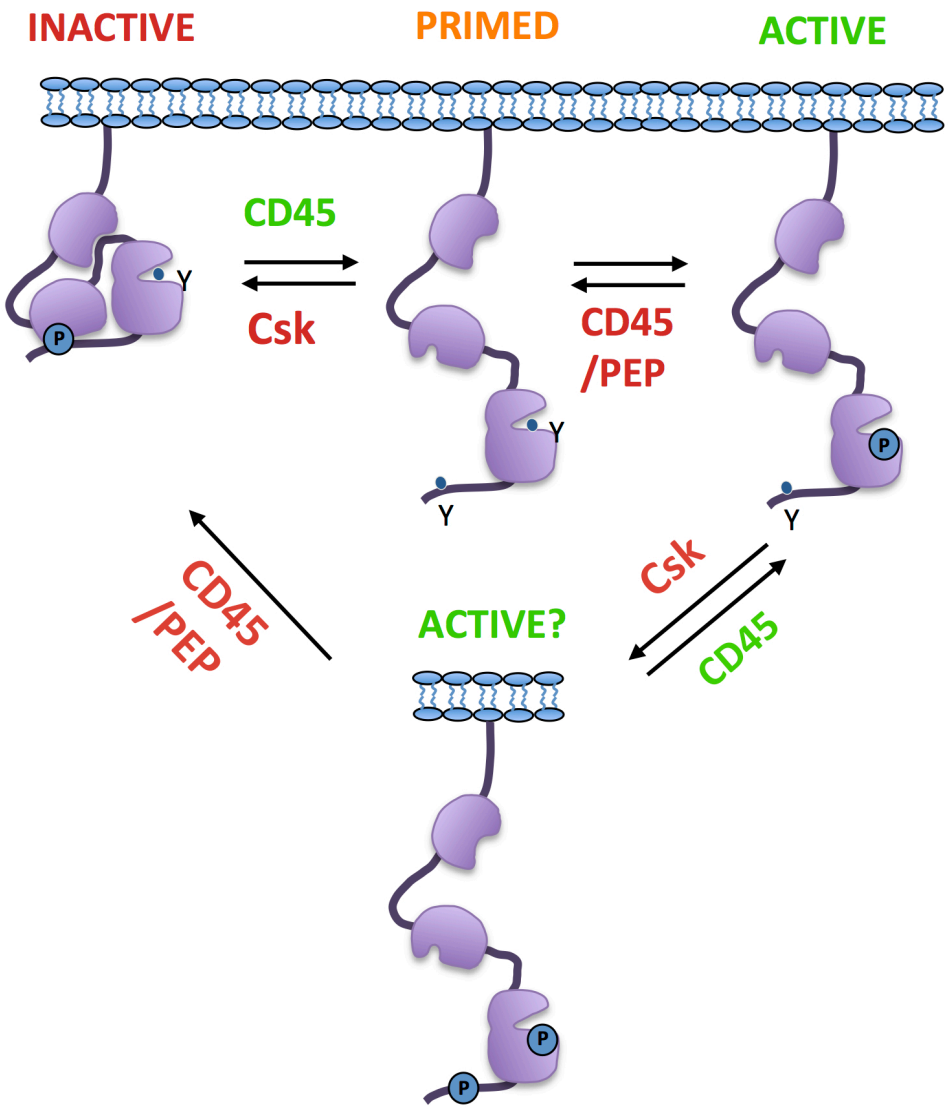
Given its critical proximal role in initiating TCR signaling, it is not surprising that Lck is itself tightly regulated (Hermiston et al., 2009). Like the other SFKs, Lck has an N-terminal

acylated region that is myristoylated and palmitoylated to allow it to anchor to the lipid bilayer. This acylated region is followed by a unique domain, an SH3 domain, an SH2 domain, a kinase domain and a C-terminal tail. The unique domain is involved in mediating the association with the CD4 and CD8 coreceptors, while the SH2 and SH3 domains mediate various intermolecular and intramolecular protein-protein interactions. Lck has two highly conserved tyrosine residues, one in the activation loop of the kinase domain, and the other in the C-terminal tail. Phosphorylation of the activation loop tyrosine (Y394) is required for full Lck kinase activity, while phosphorylation of the C-terminal inhibitory site (Y505) favors adoption of a closed auto-inhibited conformation which stabilizes the inactive conformation of the catalytic domain. This inhibitory tyrosine is in turn reciprocally regulated by the enzymatic activities of the receptor-like tyrosine phosphatase CD45 and the cytoplasmic tyrosine kinase Csk (Figure 1.2). Together this phosphatase/kinase pair impose tight, constitutive control over Lck activity and shapes both inducible and basal signaling tone.

CD45 phosphatase activity is essential to counteract the function of Csk in regulating Lck C-terminal tyrosine phosphorylation. Indeed, CD45-deficient T cells and cell lines exhibit constitutive hyper-phosphorylation of Y505 (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999; Sieh et al., 1993; Stone et al., 1997). As a result, CD45 is absolutely required for TCR stimulation and consequently for T cell development. An Lck Y505F transgene is sufficient to rescue TCR signaling and T cell development in CD45-deficient mice, establishing this tyrosine as a physiologically relevant substrate and Lck as a critical mediator of CD45 function in vivo (Pingel et al., 1999; Seavitt et al., 1999; Stone et al., 1997).

However, it has been suggested that in addition to its critical positive regulatory role, CD45 also plays a negative regulatory role, and indeed this forms the basis for its putative

Figure 1.2. SFK activity is reciprocally regulated by Csk and CD45. Phosphorylation of a c-terminal tail negative regulatory tyrosine of SFKs by Csk facilitates its interaction with its SH2 domain, resulting in a closed, catalytically inactive conformation. Dephosphorylation of this site by CD45 favors an open conformation. Phosphorylation of the catalytic site tyrosine is required for full kinase activity. CD45 and PTPN-22/PEP can negatively regulate SFK activity by dephosphorylating the catalytic site tyrosine.


INACTIVE
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ACTIVE?
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function in the kinetic segregation model of TCR triggering. Dephosphorylation of the activation loop tyrosine of Lck (Y394) may be the biochemical basis for the negative regulatory role of CD45 (Ashwell and D'Oro, 1999; McNeill et al., 2007; Zikherman et al., 2010). Dual hyperphosphorylation of Lck at both inhibitory and activating tyrosines has been reported in many CD45 deficient cell lines and mice (Ashwell and D'Oro, 1999). Several groups have independently generated CD45 single isoform transgenic mice with subphysiologic CD45 expression levels (Kozieradzki et al., 1997; McNeill et al., 2007; Ogilvy et al., 2003). Our laboratory has also taken advantage of an ENU-generated variant of CD45, *lightning*, with low surface expression but preserved splicing to generate normal isoform expression patterns. By further combining this allele with wild type and knockout CD45 alleles, we have generated an allelic series of mice in which expression of normally spliced CD45 is varied across a broad range on all hematopoietic cells (Zikherman et al., 2010). The dual positive and negative regulatory roles of CD45 are unmasked when CD45 expression levels are altered.

Signaling through the pre-TCR complex at the thymic TCRβ-selection checkpoint occurs in a ligand-independent, SFK-dependent manner, and is partially blocked in CD45-/- mice (Byth et al., 1996; Irving et al., 1998). Increasing CD45 expression across the CD45 *lightning* allelic series partially rescues this beta-selection defect, but surprisingly, very high CD45 doses were required for complete rescue (Zikherman et al., 2010), as they were for rescue of other basal TCR signaling markers, such as expression of CD5 and TCRβ on pre-selection double positive thymocytes, as well as basal TCR ζ-chain phosphorylation. Increasing CD45 levels also correlated with decreasing phosphorylation at Lck Y505 in the basal state, suggesting the presence of constitutive counter-regulation of this tyrosine site by Csk. These data suggest that CD45 plays a predominantly positive regulatory role during ligand-independent signaling and

that CD45 phosphatase activity must be continuously counter-regulated in the basal state by Csk. Indeed, in the CD45 allelic series mice in which the dose of Csk was reduced by half, there is partial rescue of these basal signaling phenotypes.

In contrast to basal signaling, very different sensitivity to CD45 dose in the context of ligand-dependent TCR signaling is observed. As previously reported (Koretzky et al., 1990; Koretzky et al., 1991; Stone et al., 1997), CD45-/- T cells are refractory to stimulation through the TCR. Even very low levels of CD45 expression can rescue in vitro TCR signaling in response to anti-CD3 ligation. In fact, cells with very low CD45 expression were super-sensitive to such TCR-ligation. Increasing CD45 expression down-regulated TCR signaling and flattened the dose response curve. In addition, low levels of CD45 correlated with higher levels of basal phosphorylation at the activation loop tyrosine of Lck, suggesting that CD45 directly or indirectly is capable of dephosphorylating this site. However, it should be mentioned that there are other phosphatases, such as PTPN-22/PEP, that have also been implicated in the dephosphorylation of Y394 (Cloutier and Veillette, 1999). Overall, this work clearly unmasks a negative role for CD45 in response to in vitro TCR cross-linking.

Together, the existing data suggest that the positive regulatory role of CD45 is directed at the inhibitory SFK tyrosine phosphorylation, while the negative regulatory role of CD45 is directed at the activation loop tyrosine of Lck. Moreover, the work from our laboratory demonstrates the importance of Csk in the countering CD45 activity, particularly in the basal state. The dynamic regulation of Csk might account for differential requirements for CD45 during basal and inducible signaling. One possible model is that under basal conditions, Csk is recruited to the plasma membrane by the phosphorylated adaptor PAG (and/or other as yet unidentified adaptors). High doses of CD45 are required to dephosphorylate the inhibitory

tyrosine of Lck because local concentrations of Csk are high and therefore the positive regulatory role of CD45 prevails. Upon antigen receptor ligation, Csk is rapidly removed from the proximity of its substrate by an unclear mechanism, such as, perhaps, rapid dephosphorylation of an adaptor molecule like PAG. Indeed, one must consider alternative adaptors, since the PAG deficient cells have little phenotypic change (Dobenecker et al., 2005). Moreover recent work suggest that Csk may relocalize to other regions of the cell (Borger et al., 2013). CD45 now acts unopposed upon the inhibitory tyrosine of Lck, such that the dominant factor influencing Lck activity under these circumstances is the phosphorylation status of the activation loop tyrosine. Now the negative regulatory role of CD45 prevails. Indeed, reducing the dose of Csk by half has no effect on in vitro anti-CD3 stimulation of allelic series thymocytes. These differences may in turn reflect dependence upon different pools of Lck in each of these processes. However, it remains unclear which pools of Lck are most relevant for ligand-dependent and ligand-independent TCR signaling, and how access of Csk to these pools is regulated dynamically.

Work from the Acuto lab has investigated the phosphorylation state of different subcellular pools of Lck in T cells and provides evidence for a basal equilibrium of four subsets of Lck with distinct phosphorylation states and activity levels in resting T cells (Figure 1.2) (Nika et al., 2010). One notable feature of this study is the identification of active forms of Lck in unstimulated T cells that does not appear to increase significantly upon TCR stimulation. This raises the possibility that such active Lck pools are relocalized/partitioned relative to the TCR before and after activation of the T cell and that additional activation of Lck may not be important for TCR triggering (Davis and van der Merwe, 2011).

However, recent work from the Schraven lab with a FRET-based Lck conformational reporter and FLIM technology suggests that TCR activation by anti-CD3 ligation or superantigen

(SAg) correlated with conformational changes in a small pool of Lck with presumed increased Lck kinase activity (Stirnweiss et al., 2013). This has very different implications for the mechanism of TCR triggering and the role of Csk and CD45 in this process. It will be critical to study individual functionally relevant pools of Lck to resolve the conflicting data.

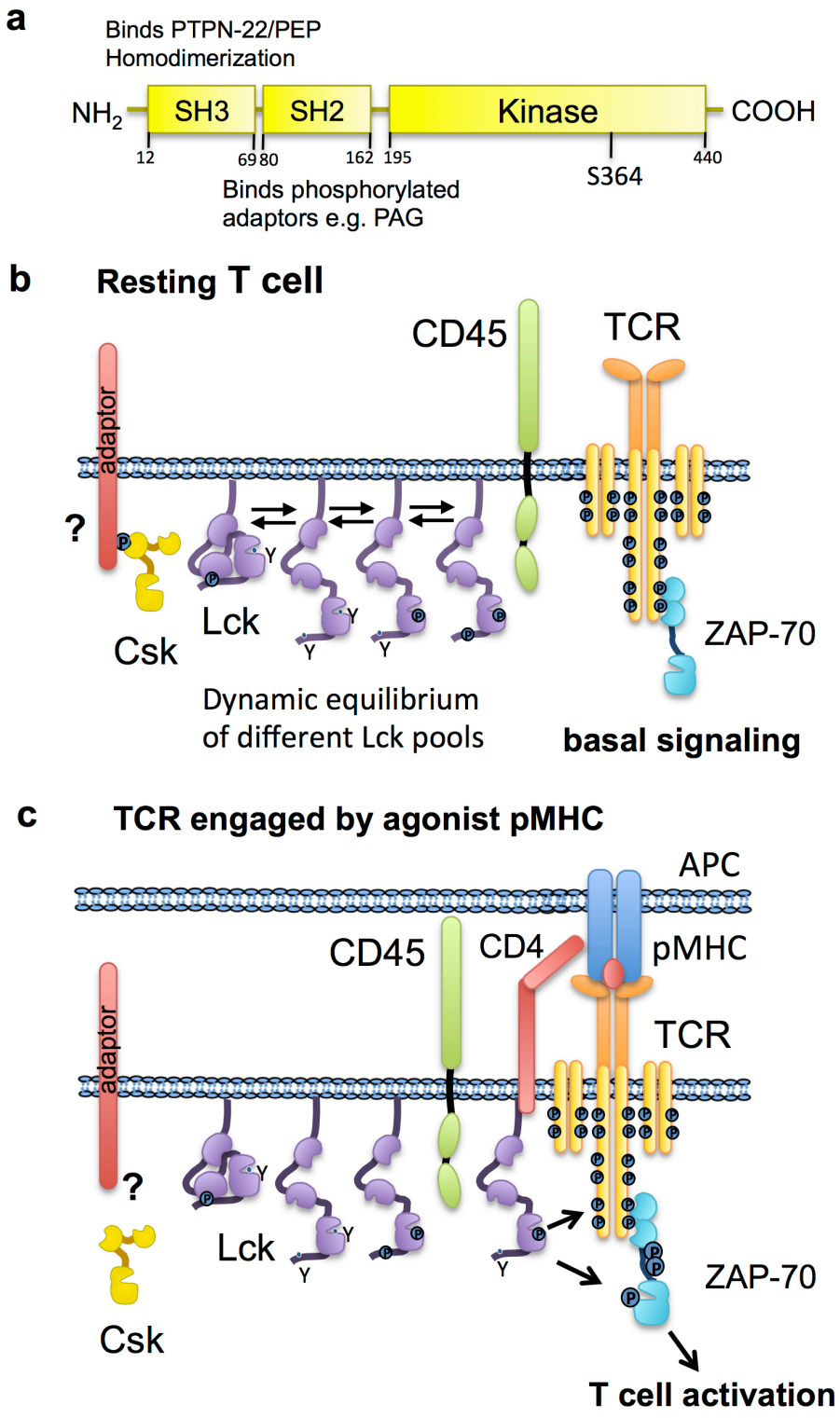
Csk structure, function and regulation

Csk is a ubiquitous cytosolic protein and serves as an essential negative regulator of SFK activity by phosphorylating their c-terminal inhibitory tyrosine (Bergman et al., 1992; Chow et al., 1993; Okada et al., 1991). The physiologic relevance of Csk as an inhibitor of SFK activity and cellular activation has been revealed in multiple cell types using various animal models. Constitutive deletion of Csk causes embryonic lethality due to defective neural development that is associated with increased SFK activity (Imamoto and Soriano, 1993; Nada et al., 1993). Conditional deletion of Csk in granulocytes results in lung and skin inflammation that is correlated to increased spontaneous and ligand-induced degranulation, as well as hyperadhesion and defective migration towards chemokines ex vivo (Thomas et al., 2004). Mice with Csk-deficient squamous epithelium basal cells display impaired cell-cell adhesion in the basal cell layer of the skin as well as epidermal hyperplasia and inflammation (Yagi et al., 2007). Importantly, deletion of Csk in immature thymocytes results in TCR and MHC-independent development of T cells with abnormal expression levels of TCR and coreceptors (Schmedt et al., 1998; Schmedt and Tarakhovsky, 2001). This aberrant thymic development is dependent on the presence of Lck and Fyn and suggests that loss of Csk results in the generation of TCR-like signals in the absence of TCR engagement.

In light of the essential role that Csk plays in the negative regulation of SFK activity, it is not surprising that its activity strongly influences TCR signaling. Overexpression of Csk in a mouse T cell line results in diminished tyrosine phosphorylation and IL-2 production following TCR stimulation (Chow et al., 1993). Conversely, knocking down Csk in Jurkat T cells increases basal ζ chain phosphorylation and results in larger increases in ζ chain phosphorylation and intracellular calcium upon TCR ligation (Vang et al., 2004). However, since the genetic ablation of Csk prevents normal T cell development, the study of Csk function in primary, unmanipulated T cells has not been possible.

Csk has a similar domain architecture as the SFKs, with an SH3 domain, and an SH2 domain followed by a kinase domain and a c-terminal tail (Fig. 1.3a). However, unlike the SFKs, it lacks the unique N-terminal acylation domain that undergoes myristoylation and palmitoylation and enables SFKs to anchor to the membrane. It also lacks the two regulatory tyrosines in the kinase activation loop and the c-terminal tail, suggesting that Csk has a mode of regulation different than that of the SFKs (Chong et al., 2005; Cole et al., 2003). Indeed, the arrangement of the domains of Csk in its tertiary structure is distinct from the SFKs in that the binding pockets of the SH3 and SH2 domains face outwards, suggesting the ability to interact with other proteins. Moreover, the kinase domain of Csk is in an active conformation despite the lack of activation loop phosphorylation and in stark contrast to the SFKs, Csk is constitutively active in vitro (Ogawa et al., 2002; Sun and Budde, 1999). The SH2 and SH3 domains of Csk play a key role in maintaining its activity, since the deletion of these domains reduces Csk kinase activity (Sondhi and Cole, 1999). The Csk crystal structure further supports this finding, since the SH2-kinase and SH2-SH3 linkers are tightly associated with the N-terminal lobe of the kinase domain and the SH2 and kinase domains are in direct contact only in the active Csk

Figure 1.3. Csk regulation of basal and inducible TCR signaling (a) Csk domain architecture. The SH3 and SH2 domains of Csk interact with the kinase domain and promote an active conformation. The SH3 domain binds the tyrosine phosphatase PTPN-22/PEP and may be involved in homodimerization. The SH2 domain interacts with phosphorylated adpators such as PAG and is important for Csk localization to the plasma membrane. Serine 364 is phosphoryalated by PKA and increases Csk activity 2 fold. (b) In resting T cells, Csk is recruited to the plasma membrane by membrane-resident adaptors that have yet to be fully identified. Together with CD45, they control the phosphorylation status of the negative regulatory tyrosine of Lck, resulting in the generation of an equilibrium of distinct species of Lck with different levels of activity. This equilibrium level of Lck activity sets the T cell basal signaling tone. (c) When the T cell encounters an APC bearing its cognate pMHC, the TCR is engaged, inducing the phosphorylation of the TCR ITAMs and ZAP-70 by active Lck, eventually leading to T cell activation. Precisely how this occurs remains poorly defined, and may involve an increase in the amount of active Lck due to a change in the relative activities of Csk and CD45 at the membrane, or a relocalization of preexisting active Lck to the proximity of the TCR. A change in the balance of Csk and CD45 activity may be achieved by relocalization of Csk off the membrane or away from Lck. However, when and how Csk is relocalized is unclear.


a
Binds PTPN-22/PEP
Homodimerization
NH2
SH3
SH2
Kinase
COOH
12
69 80
162 195
S364
440
Binds phosphorylated
adaptors e.g. PAG
b
Resting T cell
CD45
TCR
adaptor
?
Lck
Csk
ZAP-70
Dynamic equilibrium
of different Lck pools
basal signaling
c
TCR engaged by agonist pMHC
APC
CD45
CD4
pMHC
TCR
adaptor
?
Lck
Csk
ZAP-70
T cell activation

molecules in the crystal. It appears that these interactions help stabilize the active conformation of the kinase domain and further suggests that interaction of the SH2 domain with other proteins may regulate its overall activity. In fact, it has been demonstrated that binding of Csk SH2 domain to its phosphorylated adaptor PAG increases its catalytic activity (Takeuchi et al., 2000).

The SH2 and SH3 domains of Csk are known to interact with numerous different proteins. These interactions are functionally important because deleting the SH3 domain or mutating the SH2 domain of Csk to reduce its affinity for phospho-tyrosine diminishes its ability to inhibit TCR activation. In addition, mutation of the SH2 domain of untargeted Csk but not membrane-targeted Csk reduces its ability to inhibit TCR activation, implicating the SH2 domain in membrane recruitment (Cloutier et al., 1995). The binding partners that have been identified for the Csk SH2 domain are generally adaptor molecules. They include transmembrane adaptor molecules such as PAG, LIME and SIT, as well as cytosolic proteins such as paxillin and are thought to play a role in the recruitment of Csk to sites of SFK localization (Brdicka et al., 2000; Brdickova et al., 2003; Pfrepper et al., 2001; Sabe et al., 1994). On the other hand, the SH3 domain is involved in binding to phosphatases including PTPN-22/PEP. It is believed that PTPN-22/PEP acts synergistically with Csk in the negative regulation of SFKs, since it can dephosphorylate SFK activation loop tyrosine, thus further dampening SFK activity (Cloutier and Veillette, 1999). More recently, the SH3 domain has been suggested to mediate homodimerization of Csk, which may prevent the recruitment of phosphatases (Levinson et al., 2009). However, the functional significance of this dimerization has not been validated in-vivo.

Since the SFKs are membrane-anchored but Csk is a cytosolic protein, an important way by which Csk activity on SFKs is regulated is its reversible recruitment to the plasma membrane. The importance of this dynamic membrane localization is underscored by the finding that forcing

Csk to localize to the plasma membrane using membrane-targeting motifs completely inhibits TCR activation in response to TCR crosslinking (Chow et al., 1993). Csk is recruited to the plasma membrane via its SH2 domain by phosphorylated transmembrane adaptors such as PAG and LIME (Fig. 1.3b and c) (Brdicka et al., 2000; Brdickova et al., 2003; Davidson et al., 2003; Torgersen et al., 2001). In resting T cells, PAG is constitutively phosphorylated and associated with Csk in lipid rafts. However, following TCR stimulation, PAG is rapidly dephosphorylated by as yet unclear mechanisms and loses its ability to anchor Csk to the lipid rafts. It is proposed that the decrease in local concentration of Csk at the membrane allows CD45 phosphatase activity to dominate at the inhibitory tyrosine of SFKs, culminating in SFK activation and TCR signal transduction. However, in contrast to mice deficient in Csk, mice deficient in PAG or LIME or both develop normally, implying the existence of alternative adaptors or recruitment mechanisms (Dobenecker et al., 2005; Gregoire et al., 2007; Xu et al., 2005). Moreover studies examining Csk localization before and after TCR ligation has been limited to crude biochemical fractionation to determine the amount present in lipid rafts, which remain a controversial membrane domain. Therefore, the regulation of the spatio-temporal localization of Csk in response to TCR engagement remains poorly understood. Higher resolution methods such as total internal reflection (TIRF) microscopy as well as characterization of pools of Csk outside of lipid rafts are needed to truly understand the regulation of Csk localization.

One other mechanism described for regulating Csk activity is the phosphorylation of a serine residue in its kinase domain by protein kinase A (PKA). Activation of G-protein coupled receptors by ligands such as prostaglandins activates adenylate cyclase and the production of cyclic AMP (cAMP), leading to the activation of PKA, which then phosphorylates Csk and enhances its catalytic activity two to four-fold. Indeed, cAMP treatment of Jurkat T cells inhibits

ζ chain phosphorylation and IL-2 production following TCR stimulation (Vang et al., 2001). It remains unclear how this serine phosphorylation activates Csk.

Analog-sensitive kinases

The use of small molecule inhibitors to perturb kinase activity has been a powerful loss-of-function strategy for investigating the role of specific kinases in various signaling networks. The biggest advantages that small molecule inhibitors have over other loss-of-function strategies are that they work rapidly and the level of inhibition is tunable and may be reversible. The ability to rapidly inhibit protein function eliminates the confounding factor of cellular or developmental compensation in response to protein loss that often occur in other systems, including cell lines deficient in the protein, RNAi transient knockdown of protein expression and knockout animal models. Moreover the refined temporal control combined with reversibility of the inhibition allows one to carefully dissect the temporal role of the protein in the different stages of its given pathways. The ability to titrate the level of inhibition also enables one to discover differential sensitivities of the various pathways regulated by the protein to the loss of its function. Another important advantage that small molecule inhibitors have over other loss-of-function strategies is that they allow one to probe for additional functions of the protein that are independent of its enzymatic activity. For example, essential scaffolding functions may be revealed, as is the case for ZAP-70 in regulatory T cells (Au-Yeung et al., 2010).

Unfortunately, developing highly selective small molecule inhibitors has been difficult, due to the highly conserved kinase domain of protein tyrosine kinases (PTKs). Off-target effects are a major concern for kinase inhibitors. In basic research, to avoid misinterpretation of experimental results due to off-target effects, it is preferable to use more than one type of inhibitor against the molecule of interest or in conjunction with other loss-of-function strategies,

particularly if the specificity of the inhibitor has not been well characterized. Clinically, a prominent example of an inhibitor with an off target is Gleevec, which was developed against the Abl kinase, but has subsequently been found to also target the kinase c- KIT and is now also used to treat gastrointestinal stromal tumor (GIST) (Capdeville et al., 2002).

An approach that has now been widely adopted in basic research to overcome the challenges of developing a highly specific inhibitor to one's kinase of interest is the analog-sensitive kinase alleles (ASKAs) chemical genetic approach pioneered by Kevan Shokat and coworkers (Bishop et al., 2000). With this protein-engineering technology, any tyrosine or serine/threonine kinase can potentially be modified to confer sensitivity to an ATP-competitive inhibitor that cannot target wildtype kinases but still retain its kinase activity. This strategy takes advantage of the fact that in the ATP-binding pockets of all known eukaryotic kinases, there is a conserved, large amino acid residue, called the gatekeeper residue, that controls accessibility of the pocket to small molecules (Liu et al., 1998b). For example, by mutating this residue to the smaller residue glycine in v-Src, the pocket could be enlarged and allow N-6 substituted ATP analogs to bind (Liu et al., 1998a). Similarly, for the ASKA strategy, changing the bulky gatekeeper residue to a smaller one enables larger analog-inhibitors to bind. The inhibitors used for the ASKAs are bulkier analogs of the common kinase inhibitor 4-amino-1-tertbutyl-3-(p-methylphenyl) pyrazolo[3,4-d]pyrimidine (PP1). These larger molecules cannot enter the ATP-binding pockets of wildtype kinases, due to steric clash between their bulky gatekeeper residues and the enlarged group of the analog inhibitor (Bishop et al., 1998).

The first ASKA studied in vivo is the analog-sensitive cyclin dependent kinase Cdc28 (CDK1) in Saccharomyces cerevisiae (Bishop et al., 2000). In contrast to the G1 cell cycle arrest exhibited by Cdc28 temperature sensitive mutants, inhibiting analog-sensitive Cdc28 induced a

pre-mitotic cell-cycle arrest, highlighting the utility of ASKAs. Since this first in-vivo proof-of-concept, many different groups have adopted the ASKA technology and generated a multitude of analog-sensitive kinases. In lymphocytes, analog-sensitive alleles of Lck, Syk and ZAP-70 have been generated (Denzel et al., 2003; Levin et al., 2008; Oh et al., 2007). Through these studies, important limitations of the ASKA strategy have been revealed. Introduction of the gatekeeper mutation may greatly diminish kinase activity, such as for Syk. The resultant AS kinase may also not be sensitive to the existing panel of PP1 analogs. Moreover, not all the PP1 analogs are potent or biostable enough to allow for important in-vivo studies in an animal model. Nevertheless, compensatory second-site suppressor mutations can be introduced to restore catalytic function (Oh et al., 2007). Recently, it was demonstrated that structure-guided redesign of existing inhibitors can be utilized to enhance their potency towards AS kinases (Zhang et al., 2013). On the other hand, the power of the ASKA strategy has also been revealed. For instance, generation of mice bearing the analog-sensitive ZAP-70 has allowed the study of ZAP-70 function in mature T cells which do not develop in the constitutive knockout mouse. Moreover, the ability to mix analog-sensitive regulatory T cells with wildtype naïve T cells and specifically inhibit ZAP-70 activity only in AS cells has uncovered the kinase-independent scaffolding function of ZAP-70 in regulatory T cells (Au-Yeung et al., 2010).

The proven power of the ASKA-inhibitor system as well as our previous success with the analog-sensitive ZAP-70 kinase, combined with the the lack of understanding of Csk function and regulation in T cells and the fact that Csk-deficient T cells do not develop normally, prompted us to developed the analog-sensitive Csk (Csk^{AS}) allele and generate BAC transgenic mice expressing Csk^{AS}. We believe that this novel mouse model will allow us to shed light on

the role of Csk in regulating both basal signaling tone in T cells and the initiation and termination of TCR signaling.

Topics to be Covered

The following two chapters present data from the analysis of the Csk^{AS} allele. Chapter 2 describes the development of the Csk^{AS} allele and its characterization in the Jurkat T cell line, revealing a role for Csk in shaping the basal state of T cells. Chapter 3 covers the development and the utilization of BAC transgenic Csk^{AS} mice to examine the role of Csk in the initiation of TCR signaling. Finally, chapter 4 discusses the implications and potential future directions of this research.

Chapter 2

Feedback circuits monitor and adjust basal Lck-dependent events in T cell antigen receptor signaling

Summary

The Src family kinase Lck is crucial for the initiation of TCR signaling. The activity of Lck is tightly controlled to prevent erroneous immune activation, yet it enables rapid cellular responses over a range of sensitivities to antigens. Here, in experiments with an analog-sensitive variant of the tyrosine kinase Csk, we report that Lck in T cells is dynamically controlled by an equilibrium between Csk and the tyrosine phosphatase CD45. By rapidly inhibiting Csk, we showed that changes in this equilibrium were sufficient to activate canonical TCR signaling pathways independently of ligand binding to the TCR. The activated signaling pathways showed sustained and enhanced phosphorylation compared to that in TCR-stimulated cells, revealing a feedback circuit that was sensitive to the basal signaling machinery. We identified the inhibitory adaptor molecule Dok-1 (downstream of kinase 1) as a candidate that may respond to alterations in basal signaling activity. Our results also suggest a role for Csk in the termination or dampening of TCR signals.

Introduction

Cell surface receptors, such as the T cell receptor (TCR), are studied in the context of ligand activation and are controlled by a threshold of activation that is dependent on ligand affinity and avidity. TCR signaling is critical for the development, survival, and activation of mature lymphocytes, and TCR signal strength greatly influences the repertoire of TCRs on the T cells that populate the immune system (Love and Chan, 2003; Starr et al., 2003b). Sufficient activation of TCR signaling is necessary for the differentiation of naïve T cells into effector and memory T cells during an immune response (Farber, 2009). Comparatively little work has focused on the basal state of the TCR before ligand binds. Here, we uncover an unexpected amount of basal TCR signaling in the absence of ligand, which suggests that the cytoplasmic signaling network downstream of the TCR is poised to rapidly respond, yet is restrained by a single inhibitory kinase.

The TCR complex contains no endogenous kinase function, but uses the Src family kinase (SFK) Lck to phosphorylate paired tyrosine residues in cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) in each of the CD3 and ζ chains of the TCR complex (Palacios and Weiss, 2004). The tyrosine kinase C-terminal Src kinase (Csk) is a critical inhibitor of SFK activity and phosphorylates the conserved C-terminal inhibitory Tyr505 in Lck. Phosphorylation of Lck Tyr505 results in stabilization of an inactive conformation that inhibits the catalytic function of Lck and prevents its access to substrates (Palacios and Weiss, 2004). In T cells, Csk-mediated phosphorylation of Tyr505 is functionally opposed by the receptor-like tyrosine phosphatase CD45, which dephosphorylates Tyr505, enabling Lck to phosphorylate ITAMs (Hermiston et al., 2003). In contrast to Tyr505, phosphorylation of the conserved Tyr394 in the activation loop of the catalytic domain of Lck is associated with increased kinase activity,

although some work suggests that TCR stimulation may not markedly alter the total extent of Tyr394 phosphorylation (Nika et al., 2010).

Within the immune system, Csk is crucial for controlling lymphocyte development and preventing aberrant activation of immune cells. Csk is regulated primarily by its subcellular localization and by interactions with other proteins through its Src homology 2 (SH2) and SH3 domains. In unstimulated T cells, Csk is enriched in lipid raft fractions of the plasma membrane, the result of putative SH2-mediated interactions with lipid raft–enriched adaptor proteins, including phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) (Brdicka et al., 2000; Kawabuchi et al., 2000) and presumably other proteins. After TCR stimulation, PAG is rapidly dephosphorylated by an unknown mechanism, resulting in release of Csk into the cytoplasm. Dissociation of active Csk from the plasma membrane favors the action of CD45, thus promoting the activity of Lck and other SFKs (Davidson et al., 2007). Because PAG-deficient T cells have no obvious phenotype, other as yet unknown membrane recruitment mechanisms for Csk are likely to exist.

The regulation of Lck is critical for orchestrating the threshold sensitivity and strength of TCR signaling; however, it remains unclear whether the activation state of Lck is "fixed" in resting T cells or is the result of a dynamic equilibrium between ongoing Csk and CD45 activities. In a fixed state, Lck activation would require specific changes in the localization or catalytic activities of its regulatory proteins, whereas a dynamic equilibrium between Csk and CD45 might continuously alter the phosphorylation status and activity of Lck. Hence, a small imbalance in the activities of either CD45 or Csk would be sufficient to alter Lck activity. Rapid perturbation of Csk function has been hampered because of the prolonged time needed to express exogenous alleles of mutant signaling proteins. No selective small-molecule inhibitor of Csk is

available, because Csk inhibitors invariably also inhibit SFKs. Furthermore, Csk–/– mice suffer early embryonic lethality, and loss of Csk during early T cell development results in aberrant T cell development (Imamoto and Soriano, 1993; Schmedt and Tarakhovsky, 2001).

To characterize the role of Csk in regulating basal and ligand-induced signaling downstream of the TCR, we established a small-molecule controlled Csk allele, Csk^{AS}, that is capable of inhibiting TCR activation, particularly when CskAS is localized to the plasma membrane. Rapid and specific inhibition of membrane-targeted Csk^{AS} resulted in potent and sustained signal transduction and cell activation. This activation was independent of TCR ligation but used canonical TCR signaling components. We also suggest a previously uncharacterized role for Csk in the termination or dampening of the antigen receptor response. These findings reveal a control mechanism that is sensitive to the extent of basal signaling by the TCR pathway, which may feed back and alter the basal signal transduction machinery. Finally, we identify the inhibitory adaptor protein Dok-1 (downstream of kinase 1), which is involved in the membrane recruitment of Csk and inhibition of antigen receptor signaling in T cells (Yasuda et al., 2007), as a candidate component of the altered basal signaling circuitry.

Results

Generation of the Csk^{AS} T266G allele enables rapid and specific chemical inhibition of Csk

To achieve selective inhibition of Csk, we mutated the "gatekeeper" residue of the catalytic site, thereby enlarging the adenosine triphosphate (ATP)–binding pocket of Csk. The resulting analog-sensitive allele is referred to as Csk^{AS} (Fig. 2.1), and the protein that it encodes can accommodate bulky analogs of the nonselective kinase inhibitor PP1 (Bishop et al., 2000). In addition, we fused the N terminus of Lck to Csk^{AS} to enrich its localization to lipid rafts, and we refer to this construct as membrane Csk^{AS}. Because no Csk-null T cell lines exist, and because ablation of Csk in early T cell development results in aberrant maturation of T cells with altered expression of TCR and co-receptors, we expressed Csk^{AS} alleles in Jurkat cells, a human T cell line, which has low amounts of Csk present at the membrane in resting cells and for which signaling pathways have been well characterized.

Transfection of Jurkat cells with plasmids encoding either membrane-localized wild-type Csk (membrane Csk^{WT}) or membrane Csk^{AS} efficiently blocked TCR-mediated activation of the cells, as assessed by the change in abundance of the early activation marker CD69 on the cell surface (Fig. 2.2), but was insufficient to block activation in response to phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C (PKC) downstream of the TCR. We screened a library of PP1 analogs for their selective ability to block the inhibitory effects of membrane Csk^{AS} on T cell activation (Fig. 2.2). Whereas the nonselective kinase inhibitor PP1 blocked activation of all cell types, bulky PP1 analogs were unable to block TCR-mediated activation of cells transfected with empty plasmid. We identified 3-IB-PP1 [3-(3-iodobenzyl)-1-

Figure 2.1. Generation and characterization of the Csk^{AS} system. (a) Mutation of the conserved gatekeeper residue of Csk (T266G) enables larger analogs of PP1 to access the catalytic site. The 11 N-terminal amino acid residues of Lck were fused to Csk^{AS} to enforce its localization to lipid rafts in the plasma membrane. Abbreviations for the amino acid residues are as follows: C, Cys; D, Asp; E, Glu; G, Gly; M, Met; N, Asn; S, Ser; and V, Val. (b) Structures of PP1 and 3-IB-PP1. (c) Jurkat cells were transiently cotransfected with plasmid encoding GFP and plasmid encoding either cytoplasmic Csk^{AS} or membrane Csk^{AS}. After 16 hours, cells were starved of serum, stimulated with antibody against TCR for 5 min in the presence of DMSO or the indicated doses of 3-IB-PP1, and then analyzed for the presence of pERK. Data show the percentages of live GFP– and GFP+ cells that contain pERK. Data are representative of three independent experiments.

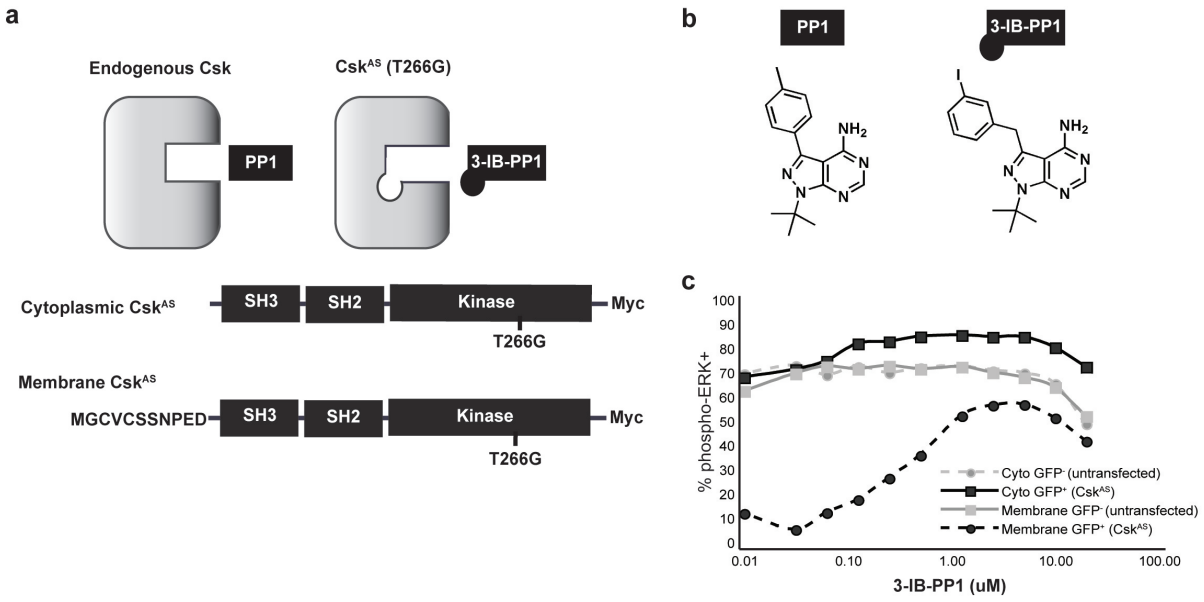
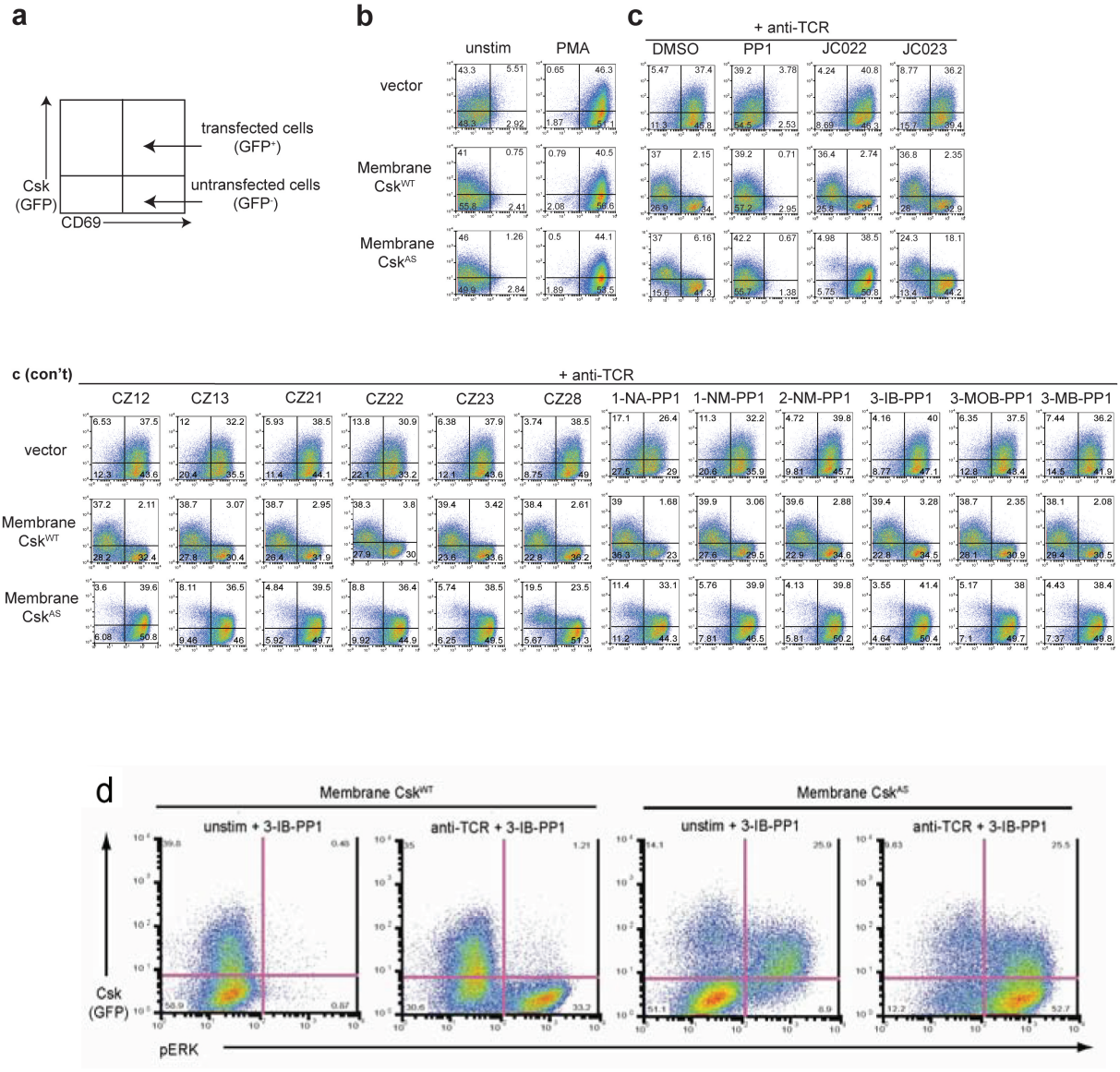

a
Endogenous Csk
Csk^AS (T266G)
PP1
3-IB-PP1
Cytoplasmic Csk^AS
SH3
SH2
Kinase
Myc
T266G
Membrane Csk^AS
MGCVCSSNPED
SH3
SH2
Kinase
Myc
T266G
b
PP1
3-IB-PP1
NH2
NH2
c
% phospho-ERK+
100
90
80
70
60
50
40
30
20
10
0
0.01
0.10
1.00
10.00
100.00
3-IB-PP1 (uM)
Cyto GFP- (untransfected)
Cyto GFP+ (Csk^AS)
Membrane GFP- (untransfected)
Membrane GFP+ (Csk^AS)

Figure 2.2. Screen of PP1 analogs for a specific inhibitor of Csk^{AS}. Jurkat cells were transiently cotransfected with plasmid encoding GFP together with control vector, plasmid encoding wild-type (WT) membrane-Csk, or plasmid encoding analog-sensitive (AS) membrane-Csk. Cells were stimulated through the TCR in the presence of PP1 and its analogs for 16 hours as denoted, and then analyzed by flow cytometry for surface CD69. (a) Flow cytometry schematic of transfected cells. (b) Unstimulated cells were all $CD69^{lo}$, whereas PMA-treated cells were all $CD69^{hi}$. (c) Cells were treated with antibody against the TCR and inhibitors (all at 10 μM). Note the ability of 3-IB-PP1 to revert GFP+ transfected cells to CD69+ cells where cells contained membrane-Csk^{AS} but not membrane-Csk^{WT}. (d) Cells containing membrane-targeted WT or AS Csk were treated with 3-IB-PP1 and were either left unstimulated or were stimulated through the TCR for 5 min, after which they were analyzed by flow cytometry for ERK phosphorylation. 3-IB-PP1 impaired the inhibitory effect of Csk^{AS}, but not Csk^{WT}, on TCR stimulation; furthermore, 3-IB-PP1 had no stimulatory effect on cells transfected with plasmid encoding Csk^{WT}.

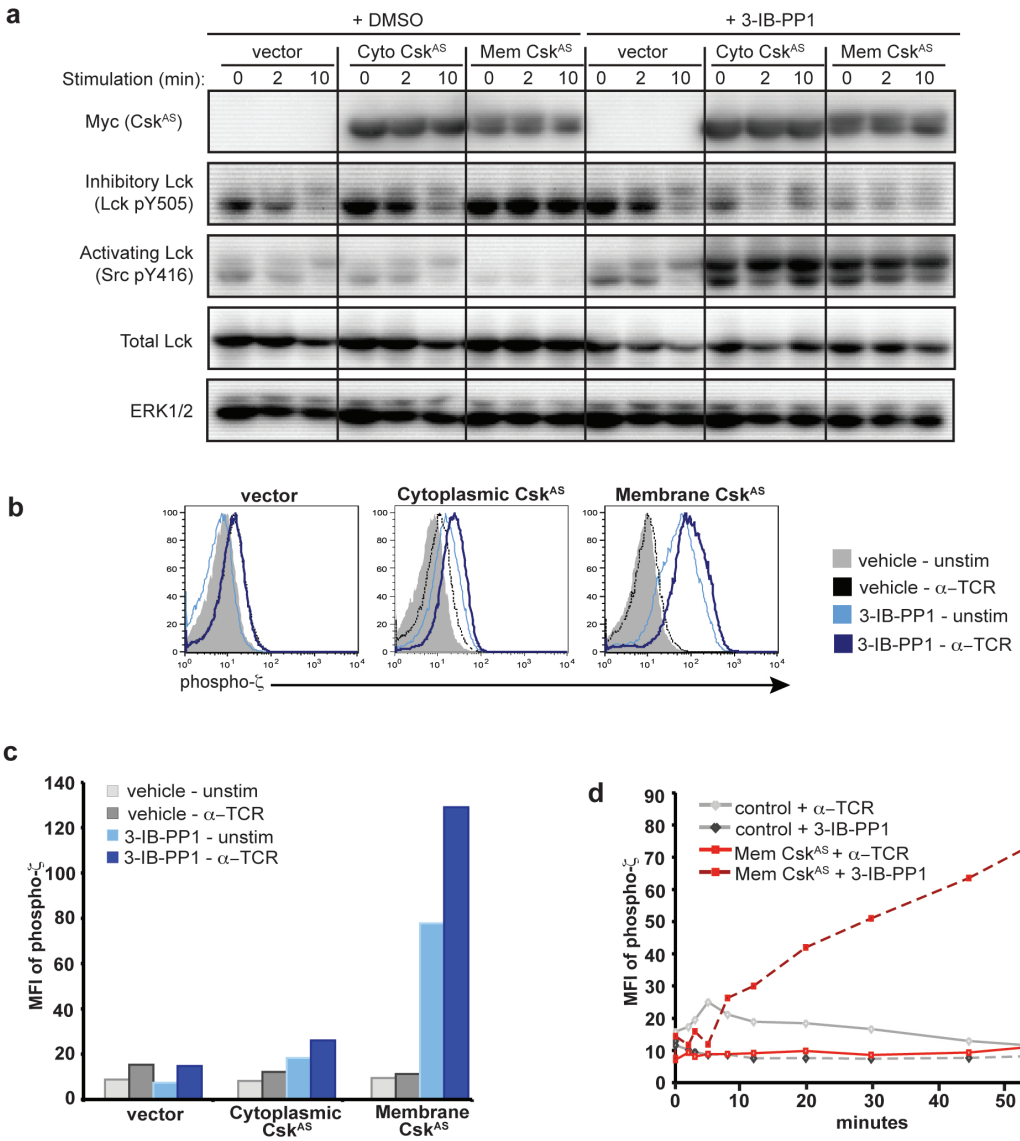

a
transfected cells (GFP+)
untransfected cells (GFP-)
Csk (GFP)
CD69
b
unstim
PMA
vector
Membrane Csk^WT
Membrane Csk^AS
c
+ anti-TCR
DMSO
PP1
JC022
JC023
c (con't)
+ anti-TCR
CZ12
CZ13
CZ21
CZ22
CZ23
CZ28
1-NA-PP1
1-NM-PP1
2-NM-PP1
3-IB-PP1
3-MOB-PP1
3-MB-PP1
d
Membrane Csk^WT
Membrane Csk^AS
unstim + 3-IB-PP1
anti-TCR + 3-IB-PP1
Csk (GFP)
pERK

tert-butyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine] as a selective inhibitor of Csk^{AS}, but not Csk^{WT} (Fig. 2.1b and Fig. 2.2, c and d). Treatment of transfected cells with 3-IB-PP1 blocked the inhibitory function of membrane Csk^{AS}, as assessed by detection of the phosphorylation of extracellular signal–regulated kinase (ERK), with EC_{50} (median effective concentration) values of ~0.5 μM (Fig. 2.1c); however, when present in similar abundance to that of membrane Csk^{AS}, cytoplasmic Csk^{AS} did not inhibit TCR-induced activation of ERK.

Inhibition of Csk^{AS} catalytic function activates proximal TCR signaling pathways

To characterize the effects of 3-IB-PP1 on Csk^{AS} activity, we examined the phosphorylation of the activating (Tyr394) and inhibitory (Tyr505) tyrosines of Lck after inhibition of Csk^{AS} with 3-IB-PP1. In control cells, TCR stimulation resulted in decreased phosphorylation of the inhibitory Tyr505 residue within 2 min, with further decreases seen by 10 min (Fig. 2.3a). In contrast, total phosphorylation of the activating Tyr394 residue was not substantially changed in response to TCR stimulation, consistent with a previous study (Nika et al., 2010); however, one band corresponding to Lck migrated more slowly when analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) because of ERK-mediated phosphorylation of Ser57 in Lck (Stefanova et al., 2003). This pattern of Lck phosphorylation was similar in control cells pretreated with 3-IB-PP1, supporting the insensitivity of endogenous Csk to 3-IB-PP1. Vehicle-treated cells containing cytoplasmic Csk^{AS} exhibited a moderate increase in the phosphorylation of Lck Tyr505 in the basal state compared to that in control cells, but they lost Tyr505 phosphorylation and showed increased phosphorylated Lck Ser57 in response to TCR stimulation (Fig. 2.3a). These data are consistent with the notion that only minimal amounts of Csk^{AS} and endogenous Csk are localized to the plasma membrane of resting

Figure 2.3. Effect of Csk^{AS} on Lck activity. (a) Jurkat cells transfected with empty plasmid, plasmid encoding cytoplasmic Csk^{AS}, or plasmid encoding membrane Csk^{AS} were starved of serum and pretreated with DMSO or 3-IB-PP1 for 25 min, and then lysates were prepared directly or after TCR stimulation for the indicated times. Lysates were analyzed by western blotting for the indicated proteins. Data are representative of three independent experiments. (b) Cells cotransfected with plasmid encoding GFP and with empty plasmid, plasmid encoding cytoplasmic Csk^{AS}, or plasmid encoding membrane Csk^{AS} were starved of serum and then pretreated with DMSO or 3-IB-PP1 (10 μM) for 20 min. Cells were then stimulated with antibody against TCR (α-TCR) or treated with vehicle (unstim) for 2 min before fixation and flow cytometric analysis for ζ chain phosphorylation. Histograms show ζ chain phosphorylation in live transfected cells. (c) Bar graphs of the data in (b) representing the mean fluorescence intensity (MFI) of phospho– ζ chain in GFP+ cells. (d) Cells cotransfected with plasmid encoding GFP and either empty plasmid or plasmid encoding membrane Csk^{AS} were starved of serum and were fixed directly or after stimulation with antibody against the TCR or after treatment with 3-IB-PP1. Data represent the MFI of phospho– ζ chain in GFP+ cells and are representative of at least three independent experiments.

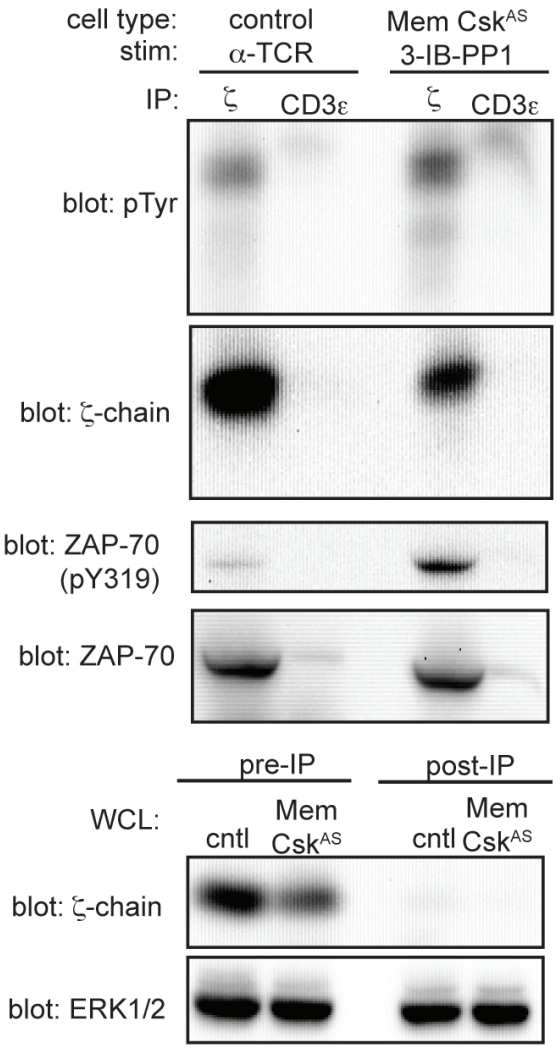

a
+ DMSO
+ 3-IB-PP1
vector
Cyto Csk^AS
Mem Csk^AS
Stimulation (min):
0 2 10
Myc (Csk^AS)
Inhibitory Lck (Lck pY505)
Activating Lck (Src pY416)
Total Lck
ERK1/2
b
vector
Cytoplasmic Csk^AS
Membrane Csk^AS
phospho-ζ
vehicle - unstim
vehicle - α–TCR
3-IB-PP1 - unstim
3-IB-PP1 - α–TCR
c
MFI of phospho-ζ
vector
Cytoplasmic Csk^AS
Membrane Csk^AS
d
control + α–TCR
control + 3-IB-PP1
Mem Csk^AS + α–TCR
Mem Csk^AS + 3-IB-PP1
minutes

cells and that both are under normal regulatory control mechanisms. In contrast, resting cells containing membrane Csk^{AS} showed increased Lck Tyr505 phosphorylation and only minimally detectable Lck Tyr394 phosphorylation, which did not change after TCR stimulation. Thus, as previously described (Cloutier et al., 1995), membrane Csk^{AS} potently inhibited Lck.

The effects of Csk^{AS} inhibition by 3-IB-PP1 on the activation of Lck were unanticipated. Whereas pretreatment of control cells with 3-IB-PP1 did not affect the phosphorylation of Lck, pretreatment of cells containing cytoplasmic Csk^{AS} or membrane Csk^{AS} resulted in reduced phosphorylation of Lck Tyr505 and increased phosphorylation of Lck Tyr394. The activation of Lck in response to Csk^{AS} inhibition occurred in the absence of TCR stimulation. These data indicate that inhibition of the catalytic activity of Csk^{AS} was sufficient to result in ligand-independent Lck activation, even when only a small amount of Csk^{AS} was present at the membrane, as was the case for cytoplasmic Csk^{AS}.

Lck phosphorylates the ITAMs of the TCR complex and initiates TCR-mediated signaling. We found that TCR stimulation of control cells induced phosphorylation of the ζ and CD3ε chains, as did treatment of cells containing membrane Csk^{AS} with 3-IB-PP1 (Fig. 2.4). Relative to TCR-stimulated control cells, 3-IB-PP1–treated cells containing Csk^{AS} showed enhanced phosphorylation of the ζ chain, which correlated with the increased recruitment and phosphorylation of the cytoplasmic tyrosine kinase ζ chain–associated protein kinase of 70 kD (ZAP-70) (Fig. 2.4). To quantify the amount of ζ chain phosphorylation, we used a flow cytometry–based approach. After TCR stimulation, control cells or cells containing cytoplasmic Csk^{AS} exhibited a two- to fourfold increase in ζ chain phosphorylation compared to that in unstimulated cells (Fig. 2.3, b and c). In contrast, TCR stimulation of vehicle-treated cells containing membrane Csk^{AS} did not exhibit increased ζ chain phosphorylation. Although the

Figure 2.4. Phosphorylation of ζ chain and its association with ZAP-70 upon inhibition of Csk^{AS}. Jurkat cells transfected with vector control plasmid or with plasmid encoding membrane-Csk^{AS} were starved of serum and then stimulated with antibody against the TCR or 3-IB-PP1 for 5 min. Whole-cell lysates (WCLs) were sequentially subjected to immunoprecipitation with antibodies against the ζ-chain and CD3ε (top); pre- and post-immunoprecipitation (IP) WCLs were 1/10 the volume of input (bottom). Data represent at least two experiments.

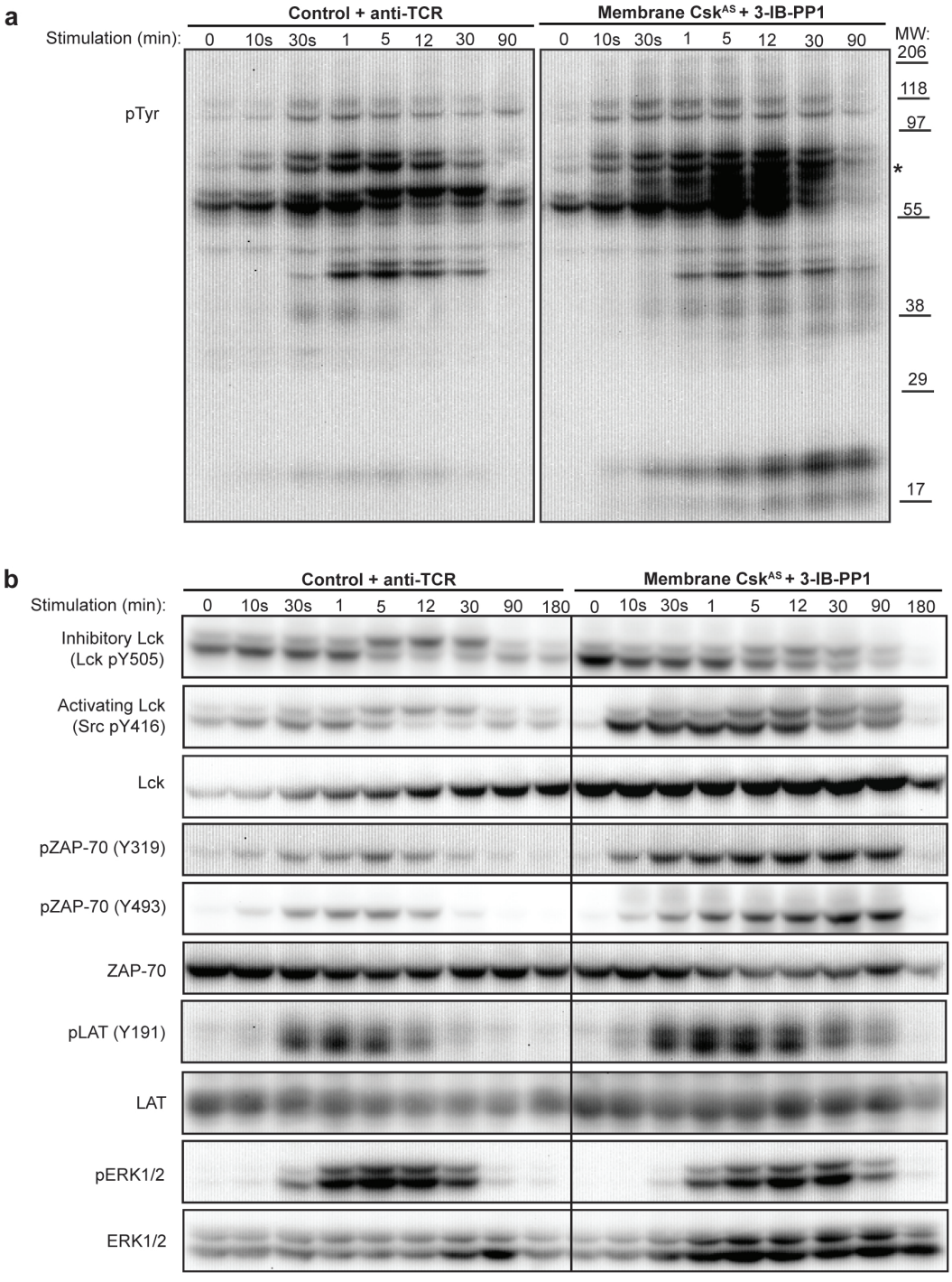

cell type:
stim:
control
α-TCR
Mem Csk^AS
3-IB-PP1
IP:
ζ
CD3ε
ζ
CD3ε
blot: pTyr
blot: ζ-chain
blot: ZAP-70 (pY319)
blot: ZAP-70
pre-IP
post-IP
WCL:
cntl
Mem Csk^AS
cntl
Mem Csk^AS
blot: ζ-chain
blot: ERK1/2

phosphorylation of Lck Tyr394 was increased to a similar extent in 3-IB-PP1–pretreated cells containing either cytoplasmic Csk^{AS} or membrane Csk^{AS} (Fig. 2.3a), the extent of ζ chain phosphorylation was much greater (5- to 10-fold higher) in the cells containing membrane Csk^{AS}. In both cell types, ζ chain phosphorylation was further increased by TCR stimulation. Control cells responding to TCR stimulation had peak ζ chain phosphorylation at 5 min, which returned to the basal state by 45 min (Fig. 2.3d). In contrast, cells containing membrane Csk^{AS} continued to exhibit increased ζ chain phosphorylation over the course of 1 hour. Thus, phosphorylation of proximal signaling proteins was augmented and sustained in response to inhibition of Csk^{AS}, relative to that in TCR-stimulated control cells. This suggests that rather than returning to basal conditions, enhanced activation of Lck activity occurs when the function of Csk^{AS} is inhibited. This effect was amplified in cells that contained the more inhibitory form of Csk^{AS} that is targeted to the membrane.

TCR signaling is augmented and prolonged after inhibition of CskAS activity

TCR stimulation results in the sudden dephosphorylation of PAG in T cells, causing the rapid dissociation of Csk from the plasma membrane and its movement to the cytoplasm. Csk reappears in membrane lipid rafts within 5 to 10 min of TCR stimulation (Davidson et al., 2007; Torgersen et al., 2001). The increased and sustained ζ chain phosphorylation that occurred after inhibition of membrane Csk^{AS} suggested that normal mechanisms to control TCR signaling were altered. After treatment with 3-IB-PP1, cells containing membrane Csk^{AS} exhibited rapid and prolonged tyrosine phosphorylation of several proteins, including the 21- to 23-kD ζ chain (Fig. 2.5a). The overall pattern of tyrosine phosphorylation was similar to that observed in TCR-stimulated control cells, with the exception of a 65-kD phosphorylated protein seen in the

Figure 2.5. Sustained signaling in the context of Csk^{AS} inhibition. (a and b) Jurkat cells transfected with empty plasmid or plasmid encoding membrane Csk^{AS} were starved of serum and then were lysed directly or after treatment for the indicated times with antibody against TCR (left, anti-TCR) or 3-IB-PP1 (right), respectively. (a) Total tyrosine phosphorylation and (b) phosphorylation of Lck, ZAP-70, LAT, and ERK were assessed by Western blotting analysis with the appropriate antibodies. Data are representative of at least three independent experiments.


a
Control + anti-TCR
Membrane Csk^AS + 3-IB-PP1
Stimulation (min):
0 10s 30s 1 5 12 30 90
0 10s 30s 1 5 12 30 90
MW:
206
118
97
*
55
38
29
17
pTyr
b
Control + anti-TCR
Membrane Csk^AS + 3-IB-PP1
Stimulation (min):
0 10s 30s 1 5 12 30 90 180
0 10s 30s 1 5 12 30 90 180
Inhibitory Lck (Lck pY505)
Activating Lck (Src pY416)
Lck
pZAP-70 (Y319)
pZAP-70 (Y493)
ZAP-70
pLAT (Y191)
LAT
pERK1/2
ERK1/2

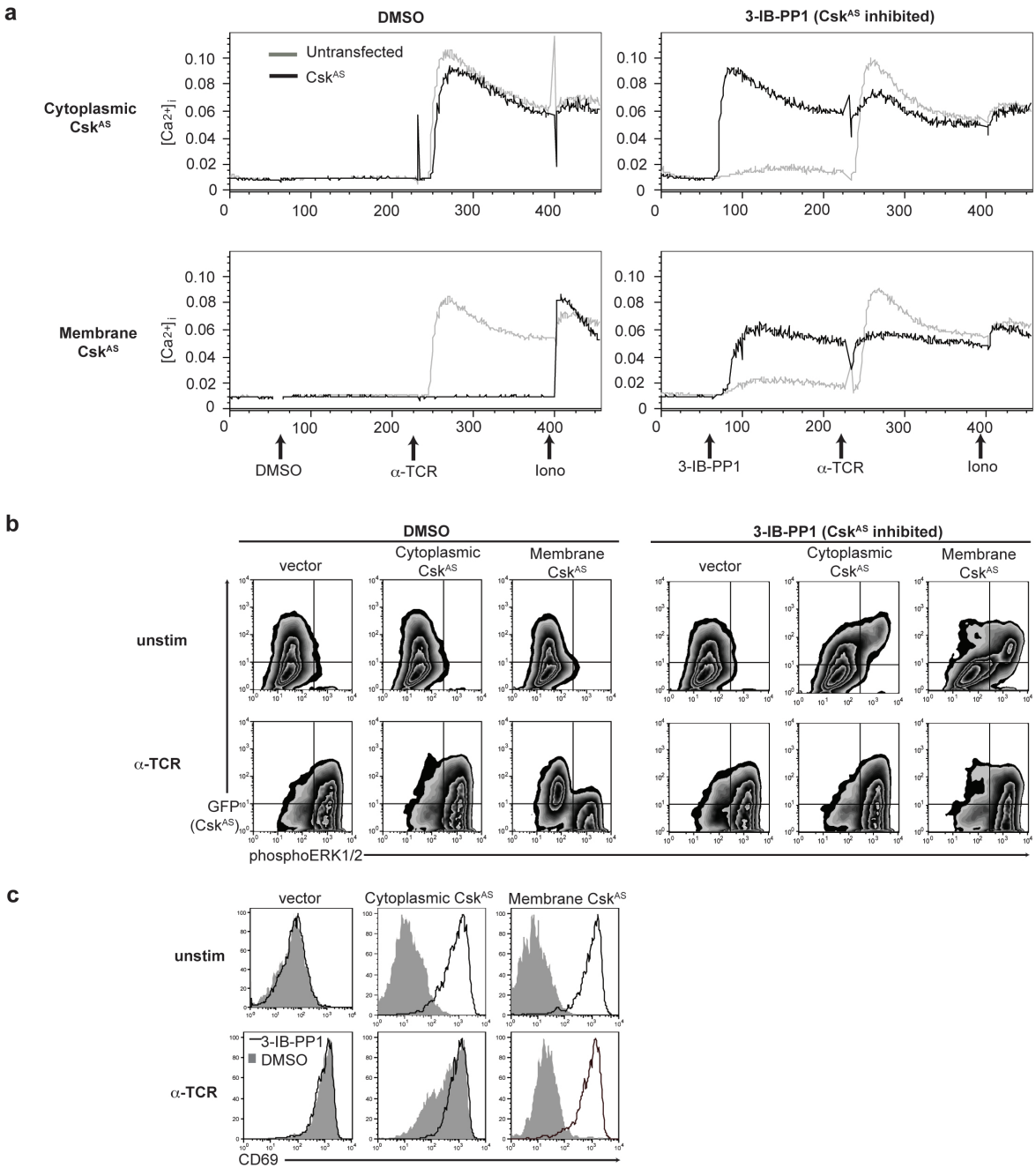
3-IB-PP1–stimulated cells (Fig. 2.5a, marked with an asterisk). This suggests that the inhibition of membrane Csk^{AS} activates signaling events similar to those driven by TCR stimulation of control cells.

We next compared the phosphorylation states of specific signaling proteins in control cells stimulated through the TCR with those in cells expressing membrane Csk^{AS} treated with 3-IB-PP1 (Fig. 2.5b). The kinase ZAP-70 binds to the Lck-phosphorylated ζ chains and is phosphorylated by Lck on Tyr315 and Tyr319 to maintain an active conformation (Au-Yeung et al., 2009). Tyr493 is located in the activation loop of ZAP-70, and it is phosphorylated by either Lck or ZAP-70. We found that both Tyr319 and Tyr493 exhibited enhanced and sustained phosphorylation after the inhibition of membrane Csk^{AS} (Fig. 2.5b). Active ZAP-70 is necessary for the phosphorylation of two critical adaptor proteins: linker of activated T cells (LAT) and SH2 domain–containing leukocyte phosphoprotein of 76 kD (SLP-76). Together, phosphorylated LAT and SLP-76 create a platform for the recruitment of numerous other molecules necessary for the signaling events downstream of the TCR that direct transcriptional responses and induce cell proliferation. Similar to ZAP-70, enhanced and more prolonged LAT phosphorylation was observed in response to 3-IB-PP1–treated membrane Csk^{AS} cells than was seen in control cells stimulated through the TCR, which correlated with sustained signaling downstream of the TCR, including the phosphorylation of ERK. Thus, inhibition of membrane Csk^{AS} was sufficient to rapidly activate and induce sustained signaling events that are associated with the canonical TCR signaling pathway, but in a ligand-independent manner.

Activation of distal signaling events and cellular activation are induced by inhibition of Csk^{AS}

Lck-mediated phosphorylation of TCR ITAMs and ZAP-70 is necessary to stimulate increases in the concentration of intracellular calcium ions ([Ca2+]i) and the activation of mitogen-activated protein kinases (MAPKs), which coordinate transcriptional responses, including expression of the genes encoding CD69 and interleukin-2 (IL-2). To examine whether the phosphorylation events described earlier were sufficient to activate downstream signaling and induce cellular activation, we examined Ca2+ flux, phosphorylated ERK (pERK) abundance, and increases in CD69 surface expression. Similar to control cells, cells containing cytoplasmic Csk^{AS} exhibited increased $[Ca2+]_i$ in response to TCR stimulation or ionomycin (Fig. 2.6a), whereas cells containing membrane Csk^{AS} did not exhibit increased $[Ca2+]_i$ in response to TCR stimulation, consistent with their inability to activate Lck or phosphorylate ITAMs. However, cells containing either cytoplasmic Csk^{AS} or membrane Csk^{AS} exhibited increased $[Ca2+]_i$ in response to Csk^{AS} inhibition alone, independently of engagement of the TCR. Similarly, vehicle-treated control cells and cells containing cytoplasmic Csk^{AS}, but not membrane Csk^{AS}, showed increased pERK abundance in response to TCR stimulation (Fig. 2.6b). A small portion of cells that contained a large amount of cytoplasmic Csk^{AS} exhibited increased pERK abundance in response to Csk^{AS} inhibition; however, most cells containing membrane Csk^{AS} exhibited pERK in response to 3-IB-PP1. Only those cells that contained the greatest amounts of membrane Csk^{AS} were unable to exhibit ERK phosphorylation or Ca^{2+} flux (as was evident by a moderate reduction in Ca^{2+} flux) upon treatment with 3-IB-PP1. Consistent with a role for sustained Ras-MAPK signaling in driving expression of the gene encoding CD69, cells containing membrane Csk^{AS} that were treated with 3-IB-PP1 alone, but not vehicle, nor cells stimulated through the

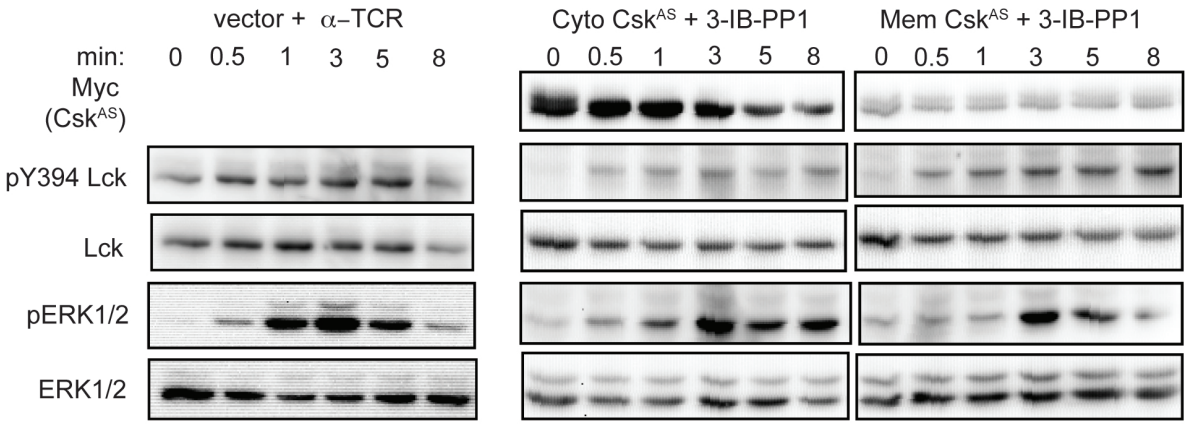
Figure 2.6. Distal TCR signaling and cell activation are induced by the inhibition of Csk^{AS}. (a) Ca^{2+} release is triggered by Csk^{AS} inhibition alone in cells containing cytoplasmic Csk^{AS} (top) or membrane Csk^{AS} (bottom). Jurkat cells were transiently cotransfected with plasmids encoding the indicated Csk^{AS} constructs and CD16. The ratios of Fluo-3 and Fura Red fluorescence are shown for CD16– (untransfected) and CD16+ (transfected, Csk^{AS}) cells in response to DMSO, 3-IB-PP1, antibody against TCR (α-TCR), and ionomycin (Iono). (b) Jurkat cells containing membrane Csk^{AS} have impaired ERK phosphorylation that is overcome by inhibition of Csk^{AS} alone. Cells transiently cotransfected with plasmid encoding GFP and with empty plasmid or plasmids encoding the indicated Csk^{AS} constructs were starved of serum and pretreated with DMSO or 3-IB-PP1 for 15 min. Cells were then harvested directly (Unstim) or after 2 min of stimulation with an antibody against TCR. Plots show total live cells, with GFP– (untransfected) cells in the bottom quadrants and GFP+ (transfected) cells in the upper quadrants. (c) The increase in the abundance of CD69 is impaired in TCR-stimulated cells containing membrane Csk^{AS} compared to that in control cells and is induced in response to inhibition of Csk^{AS} alone. Transiently transfected Jurkat cells were pretreated with either 3-IB-PP1 or DMSO and were then incubated with antibody against TCR for 18 hours before undergoing flow cytometric analysis for CD69 at the cell surface. Data are representative of two (a) or three (b and c) independent experiments.


a
DMSO
3-IB-PP1 (Csk^AS inhibited)
Untransfected
Csk^AS
Cytoplasmic Csk^AS
Membrane Csk^AS
[Ca2+]i
DMSO
α-TCR
Iono
3-IB-PP1
b
vector
Cytoplasmic Csk^AS
Membrane Csk^AS
unstim
α-TCR
GFP (Csk^AS)
phosphoERK1/2
c
CD69
3-IB-PP1
DMSO

TCR alone, showed an increased abundance of CD69 (Fig. 2.6c). Cells that contained cytoplasmic Csk^{AS} induced CD69 expression in response to either inhibition of Csk^{AS} or stimulation of the TCR.

We verified that these inducible signaling events in response to inhibition of Csk^{AS} also occurred in primary T cells by introducing cytoplasmic Csk^{AS} and membrane Csk^{AS} into primary mouse T cells. Mouse T cells transfected with empty plasmid alone or with plasmid encoding either Csk^{AS} construct exhibited low basal activation of Lck and ERK (Fig. 2.7). TCR stimulation of cells transfected with empty plasmid resulted in a moderate increase in the extent of Lck Tyr394 phosphorylation and robust ERK activation. Consistent with the data observed in Jurkat cells, inhibition of Csk^{AS} with 3-IB-PP1 induced Lck activation and ERK phosphorylation. Thus, we demonstrated that membrane Csk^{AS} was a potent inhibitor of TCR stimulation; however, blockade of Csk^{AS} catalytic function not only released the Csk-mediated inhibition of Lck, but was also sufficient to activate T cells independently of TCR stimulation. In the presence of 3-IB-PP1, cells containing Csk^{AS} showed an enhanced and sustained phosphorylation of signaling proteins relative to the response to TCR stimulation in control cells. These phosphorylation events were most robust when Csk^{AS} was targeted to the membrane. Rather than returning to the normal pattern and signaling that requires TCR stimulation, after Csk^{AS} inhibition, an enhanced phosphorylation of signaling proteins was observed. Together, these data suggest that ectopic expression of Csk^{AS}, particularly when targeted to the membrane, dampens basal signaling in resting cells, which may alter the signaling network as a mechanism to compensate for this impaired basal signaling state. Upon inhibition of Csk^{AS}, we hypothesize that this altered basal circuitry is responsible for the unexpected increases in the extent of

Figure 2.7. Inhibition of Csk^{AS} in primary mouse T cells activates Lck and ERK. Mouse CD4+ T cells were transduced with control retrovirus or with retrovirus expressing the indicated form of Csk^{AS}. Cells were rested in the absence of exogenous IL-2 for 16 hours and were starved of serum in the final 30 min. Cells were harvested directly or after stimulation with antibody against TCR or treatment with 3-IB-PP1 for the indicated times. Cell lysates were analyzed by western blotting for the phosphorylation of Lck and ERK proteins. Data are representative of at least three experiments.


vector + α–TCR
Cyto Csk^AS + 3-IB-PP1
Mem Csk^AS + 3-IB-PP1
min: 0 0.5 1 3 5 8
0 0.5 1 3 5 8
0 0.5 1 3 5 8
Myc (Csk^AS)
pY394 Lck
Lck
pERK1/2
ERK1/2

phosphorylation of signaling proteins. The more prolonged response seen with Csk^{AS} inhibition also suggests that Csk might play a role in terminating TCR signaling.

The enhanced signaling mechanism requires TCR, CD45, and Lck

In resting T cells, the TCR is constitutively internalized and rapidly recycled back to the cell surface. Strong antigenic stimulation induces degradation of the ζ chain through Lck-dependent mechanisms. Consistent with the inhibition of basal Lck activity, T cells containing membrane Csk^{AS} had decreased basal TCR internalization compared to that in control cells, which resulted in their having enhanced amounts of TCR complexes at the cell surface (Fig. 2.8). After inhibition of Csk^{AS}, the amount of TCR at the cell surface was decreased to an abundance similar to that of TCR-stimulated control cells. We found that the presence of the TCR or other ITAM-containing receptors at the cell surface was required for the induction of signaling upon inhibition of Csk^{AS}, because no response was observed in TCRβ-deficient cells transfected with plasmid encoding membrane Csk^{AS} and treated with 3-IB-PP1 (Fig. 2.9). However, the increased abundance of the TCR or ITAM-containing receptors was not responsible for the enhanced response observed when Csk^{AS} was inhibited, because cell lines that had either low amounts of surface TCR or a fixed amount of a CD8-ζ chimeric receptor in the absence of the TCR were activated in a manner similar to that of wild-type Jurkat cells (Fig. 2.9 and 2.10).

Experiments with mutant Jurkat cells deficient in CD45, Lck, ZAP-70, or SLP-76 have revealed the roles of these molecules in the initiation of proximal TCR signaling pathways. We found that signaling downstream of the TCR was blocked in cells deficient in either CD45 (J45 cells) or Lck (J.CaM1 cells) (Fig. 2.11a). As expected, inhibition of membrane Csk^{AS} in these cell lines did not induce downstream signaling events (Fig. 2.11b). Thus, Lck was required for

Figure 2.8. Csk^{AS} activity regulates the amount of the TCR at the cell surface. (a and b) Membrane-Csk^{AS} protects cells from TCR degradation, even in a basal state. Jurkat cells were transiently cotransfected plasmid encoding GFP and with vector control or plasmid encoding the indicated form of Csk^{AS}. Cells were treated with 3-IB-PP1 or DMSO and were stimulated through the TCR for 18 hours before flow cytometric analysis for the surface expression of CD3ε. (a) Flow cytometry plots show the surface expression of CD3 in transfected (upper quadrants) and untransfected (bottom quadrants) cells in a representative experiment. (b) MFIs of CD3 in GFP+ (transfected) cells from three independent experiments. (c) Cells containing membrane-Csk^{AS} cells do not exhibit basal internalization of CD3. Cells were with plasmid encoding GFP and with vector control or with plasmid encoding membrane-Csk^{AS}. Cells were starved of serum in the presence of vehicle or cytochalasin D for 30 min, after which receptor internalization was allowed to occur at 37°C for the indicated times. Cells were then washed and the amount of internalized CD3 was measured as described in the Materials and Methods. Data are the means of three experiments.

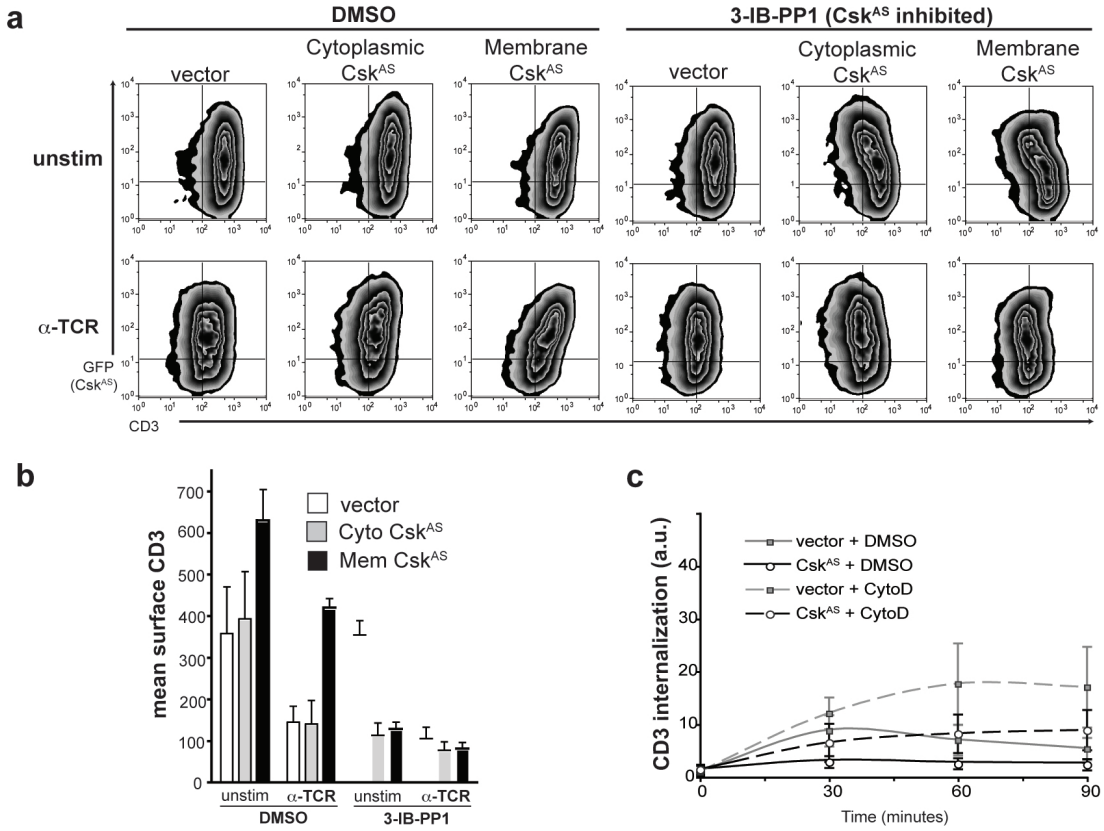

a
DMSO
3-IB-PP1 (Csk^AS inhibited)
vector
Cytoplasmic Csk^AS
Membrane Csk^AS
unstim
α-TCR
GFP (Csk^AS)
CD3
b
mean surface CD3
vector
Cyto Csk^AS
Mem Csk^AS
unstim
α-TCR
DMSO
3-IB-PP1
c
CD3 internalization (a.u.)
vector + DMSO
Csk^AS + DMSO
vector + CytoD
Csk^AS + CytoD
Time (minutes)

Figure 2.9. Cellular activation induced by Csk^{AS} inhibition requires TCR at the cell surface, but is not dependent on the amount of TCR. WT Jurkat cells, TCRβ-deficient JRT.T3-5 cells, and stable cell lines reconstituted to have 10% (JRT.T3-5 PF2.8) or 60% (JRT.T3-5 PF2.4) of the amount of TCR on the surface of WT Jurkat cells, were transiently transfected with plasmid encoding GFP together with vector control or plasmid encoding membrane-Csk^{AS}. (Top) Cells were starved of serum and then pretreated with DMSO or 3-IB-PP1 (10 μM) for 15 min before undergoing TCR stimulation for 5 min and analysis for the presence of pERK. (Bottom) Cells were starved of serum and then pretreated with DMSO or 3-IB-PP1 (10 μM) for 18 hours before being analyzed for surface CD69 expression. Plots show the percentages of total live GFP+ (transfected) cells that contained pERK or surface CD69. Data are the averages of three experiments.

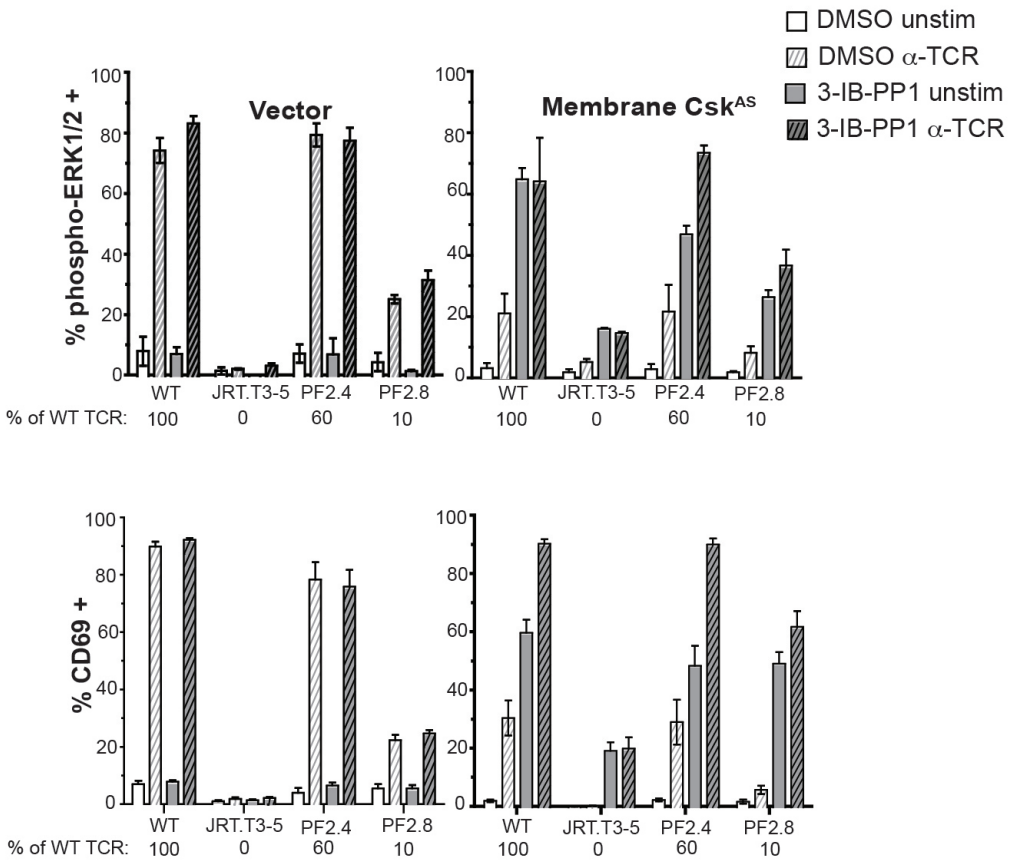

DMSO unstim
DMSO α-TCR
3-IB-PP1 unstim
3-IB-PP1 α-TCR
% phospho-ERK1/2 +
Vector
Membrane Csk^AS
% CD69 +
WT
JRT.T3-5
PF2.4
PF2.8
% of WT TCR:
100
0
60
10

Figure 2.10. Increase in TCR abundance in response to Csk^{AS} is not required for cellular activation upon inhibition of Csk^{AS}. WT Jurkat cells and TCRβ-deficient JRT.T3-5 cells stably expressing a chimeric CD8-ζ molecule containing the intracellular ITAM motifs of the ζ-chain were transiently cotransfected with vector control or with plasmid encoding membrane-Csk^{AS}. Cells were treated with vehicle and left unstimulated or were treated with antibody against the TCR (WT Jurkat cells) or with antibody against CD8 (Jβ/CD8-ζ cells), or were treated with 3-IB-PP1 (10 μM) for 18 hours. Cells were then analyzed by flow cytometry for the surface expression of CD3ε, the CD8-ζ chain, and CD69. (a) Flow cytometry plots from one representative experiment. (b) Bar graphs show the average mean surface receptor expression in GFP+ (transfected) cells from three experiments. (c) Histograms show the increase in CD69 abundance in GFP+ (transfected) cells expressing the indicated receptors. Gray, filled histograms indicate unstimulated cells; the dark gray dashed line indicates cells whose receptors (either TCR or CD8) had been stimulated; whereas the solid black line indicates cells that were treated with 3-IBPP1. Data are representative of two independent experiments.

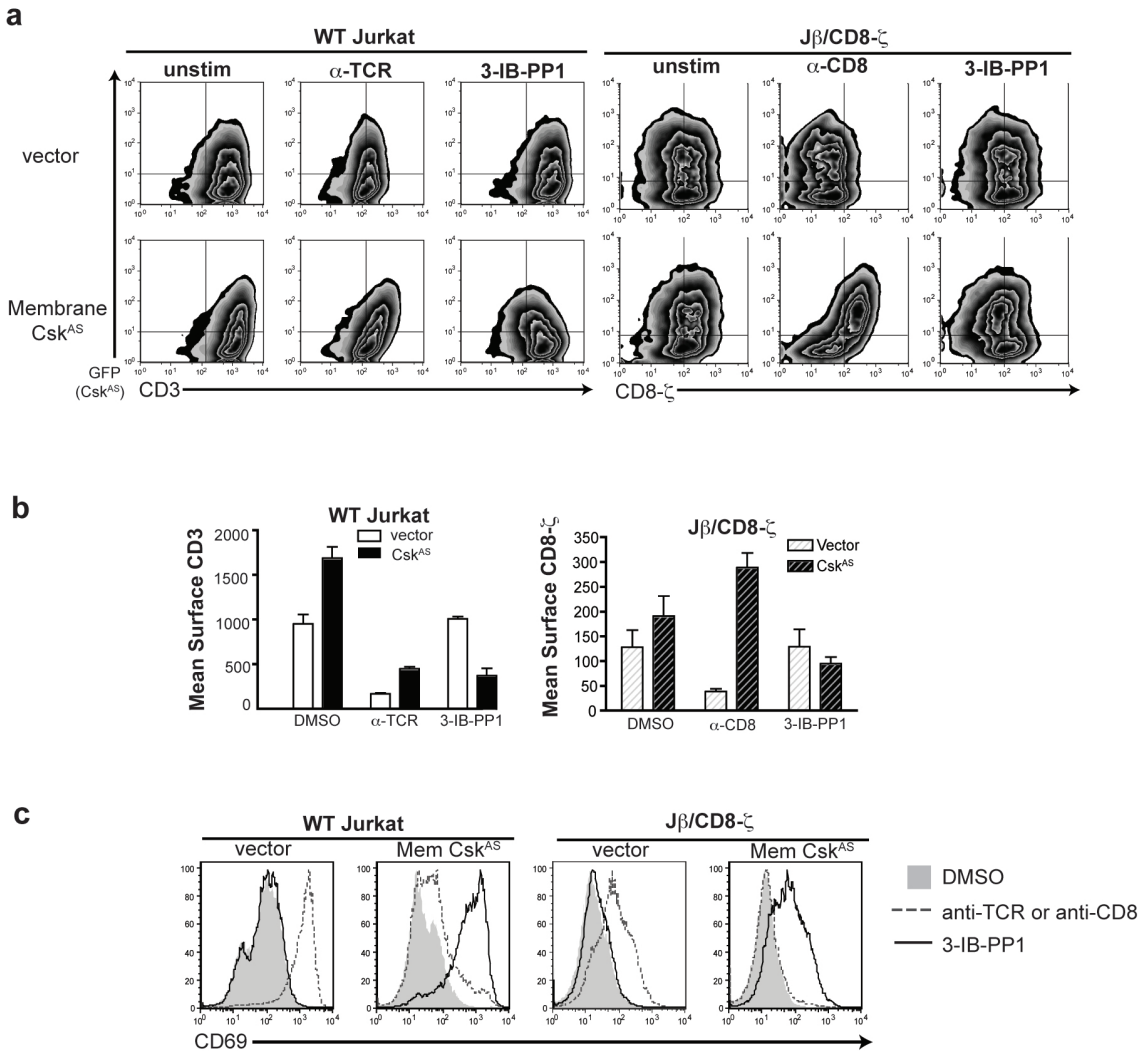
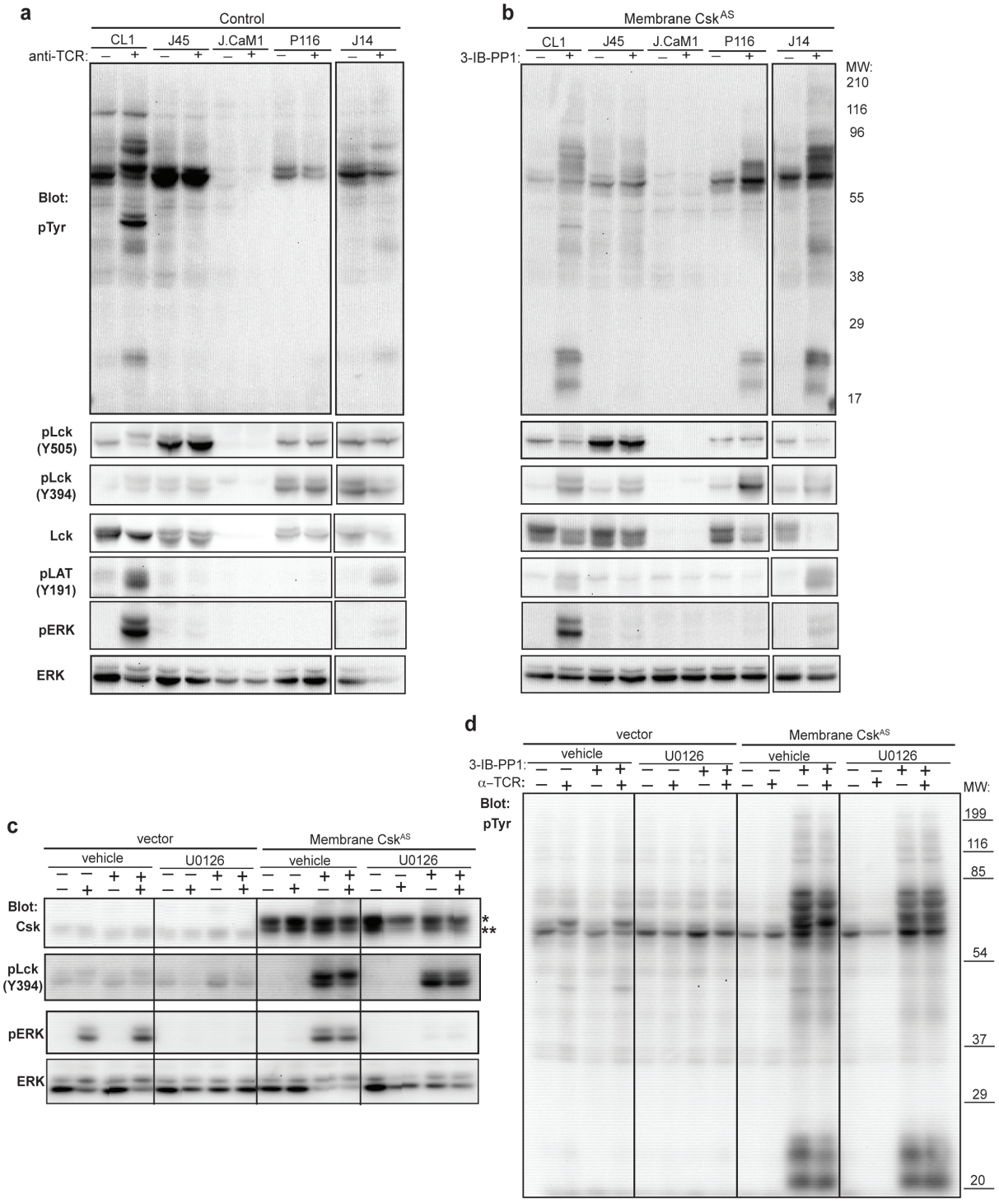

a
WT Jurkat
Jβ/CD8-ζ
unstim
α-TCR
3-IB-PP1
unstim
α-CD8
3-IB-PP1
vector
Membrane Csk^AS
GFP (Csk^AS)
CD3
CD8-ζ
b
WT Jurkat
vector
Csk^AS
Mean Surface CD3
2000
1500
1000
500
0
DMSO
α-TCR
3-IB-PP1
Jβ/CD8-ζ
Vector
Csk^AS
Mean Surface CD8-ζ
350
300
250
200
150
100
50
0
DMSO
α-CD8
3-IB-PP1
c
WT Jurkat
Jβ/CD8-ζ
vector
Mem Csk^AS
vector
Mem Csk^AS
CD69
DMSO
anti-TCR or anti-CD8
3-IB-PP1

Figure 2.11. TCR signaling components are required for T cell activation upon inhibition of Csk^{AS}. (a and b) (a) Untransfected Jurkat cells or (b) Jurkat cell signaling mutants transfected with plasmid encoding membrane Csk^{AS} were purified, starved of serum, and treated with antibody against TCR (anti-TCR) or with 3-IB-PP1 for 10 min and then lysed. Total tyrosine phosphorylation and phosphorylation of Lck, LAT, and ERK were assessed by Western blotting. (c and d) Jurkat cells containing membrane Csk^{AS} were treated for 2 hours with either vehicle or the MEK inhibitor U0126. Cells were then starved of serum for 30 min in the presence of vehicle or U0126 and then treated with antibody against the TCR or with 3-IB-PP1 for 5 min, as indicated. Cell lysates were analyzed by Western blotting for total tyrosine phosphorylation, total Csk, and phosphorylation of Lck and ERK. The antibody against Csk recognizes both endogenous Csk (**) and Csk^{AS} (*), which has a larger molecular mass than that of endogenous Csk because of the Myc tag, linker sequences, and the membrane-targeting motif. Data are representative of at least three independent experiments.


a
Control
CL1
J45
J.CaM1
P116
J14
anti-TCR:
Blot:
pTyr
pLck (Y505)
pLck (Y394)
Lck
pLAT (Y191)
pERK
ERK
b
Membrane Csk^AS
3-IB-PP1:
MW:
210
116
96
55
38
29
17
c
vector
Membrane Csk^AS
vehicle
U0126
Blot:
Csk
pLck (Y394)
pERK
ERK
d
3-IB-PP1:
α-TCR:
199
116
85
54
37
29
20

phosphorylation of the ζ chain, and Lck required CD45 for its activation in response to TCR stimulation and activation of signaling in response to Csk^{AS} inhibition. ZAP-70–deficient P116 cells were also defective in their ability to respond to TCR stimulation; however, inhibition of membrane Csk^{AS} was still sufficient to induce phosphorylation of ζ chain and Lck Tyr394. The ERK-dependent upper band corresponding to Lck Ser57 phosphorylation was largely absent after inhibition of Csk^{AS}, presumably because of the inability of P116 cells to signal downstream of ZAP-70. SLP-76–deficient J14 cells exhibited activation of proximal signaling events when transfected with either empty plasmid or plasmid encoding membrane Csk^{AS} in response to TCR stimulation or 3-IB-PP1 treatment, respectively; however, distal signaling events, such as phosphorylation of ERK, were impaired in both settings. These findings suggest that the mechanism for the phosphorylation of Lck and the ζ chain did not require signaling events dependent on ZAP-70 and SLP-76, but required more receptor-proximal components.

Positive and negative feedback loops are implicated in the regulation of Lck to explain signaling differences related to the strength of TCR engagement (Stefanova et al., 2003). In one model, strong TCR ligands activate a positive feedback loop that involves ERK-mediated phosphorylation of Lck Ser57, which prevents recruitment of the inhibitory SH2 domain–containing tyrosine phosphatase 1 (SHP-1) to Lck to inactivate TCR signaling. Given the importance of Lck activation for the sustained enhanced activation of TCR-dependent signaling in response to Csk^{AS} inhibition, we examined the requirement for ERK-mediated phosphorylation of Lck in 3-IB-PP1–induced activation. As predicted, inhibiting the activation of mitogen-activated and extracellular signal–regulated kinase kinase (MEK) with U0126 before treatment with 3-IB-PP1 led to a block in ERK activation after Csk^{AS} inhibition (Fig. 2.11c). In addition, the upper band corresponding to Ser57-phosphorylated Lck was reduced in abundance

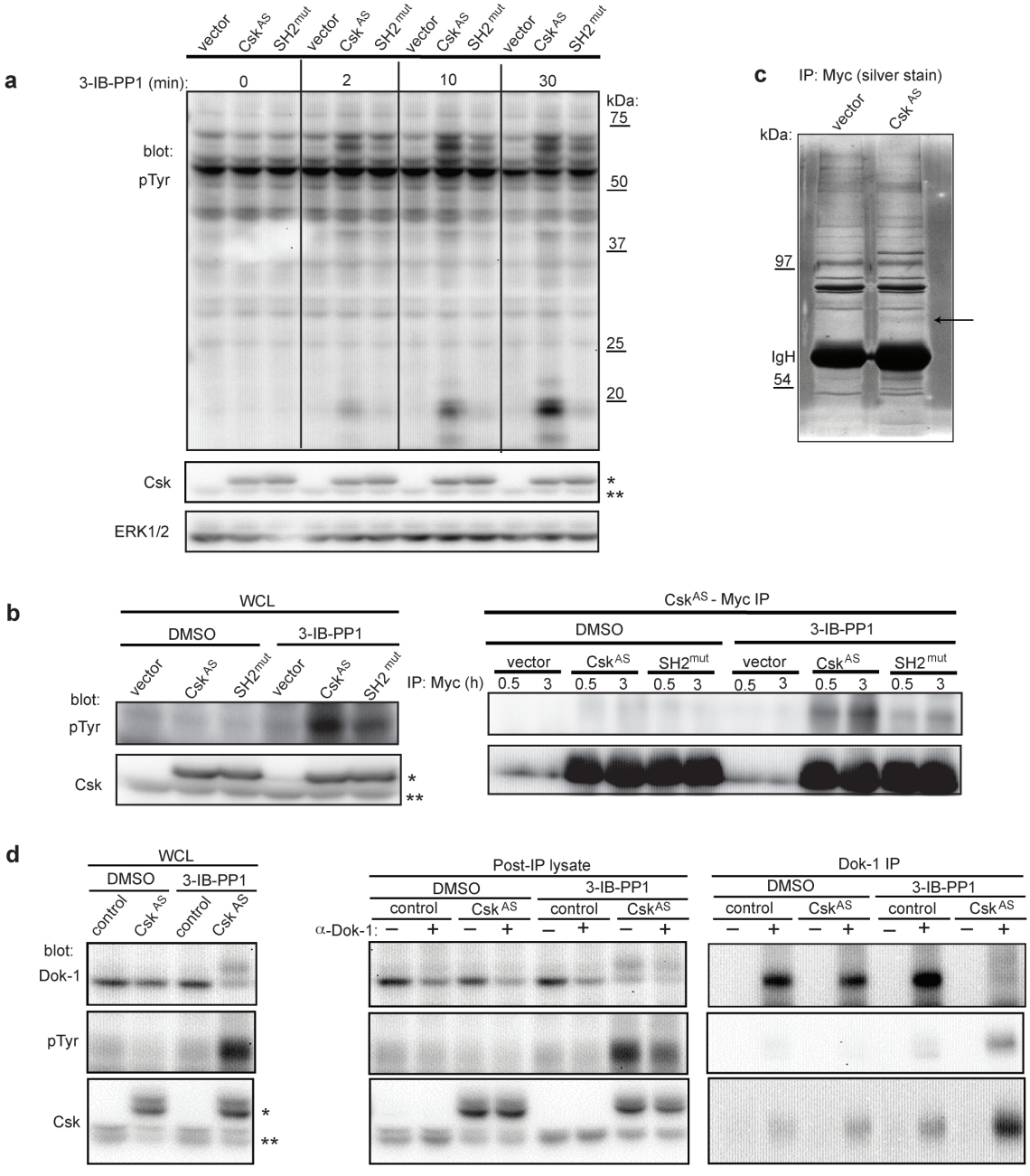
in U0126-pretreated cells compared to that in untreated cells. However, the extent of TCR-proximal signaling (that is, phosphorylation of Lck Tyr394 and the ζ chain) was unaffected by MEK inhibition (Fig. 2.11, c and d), suggesting that the previously reported ERK-mediated phosphorylation feedback mechanism was not required for enhanced signaling as a result of Csk^{AS} inhibition. Thus, we conclude that the enhanced signaling mechanism that occurs in response to Csk^{AS} requires the TCR, CD45, and Lck and involves molecules upstream of ZAP-70, SLP-76, and ERK.

Dok-1 is phosphorylated and interacts with Csk after inhibition of Csk^{AS}

Endogenous Csk is dynamically recruited to the cell membrane because of interactions between its SH2 domain and phosphorylated tyrosines in membrane-anchored adaptor molecules; however, membrane Csk^{AS} is constitutively enriched in lipid rafts of the plasma membrane. Despite this, the SH2 domain may still interact with phosphorylated proteins that are important for the regulation of signaling. We postulated that if this were true, then a variant membrane Csk^{AS} with a mutation in its SH2 domain would alter signaling differently after treatment with 3-IB-PP1. Indeed, mutation of the SH2 domain of Csk^{AS} resulted in a clear diminution in the extent of ζ chain phosphorylation as well as that of other proteins after Csk^{AS} inhibition (Fig. 2.12a).

We further observed a tyrosine-phosphorylated band of ~65 kD that was less phosphorylated in cells containing the variant of Csk^{AS} with the SH2 mutation than in cells containing Csk^{AS}. We were particularly interested in identifying this 65-kD protein because it was uniquely phosphorylated in cells in which either cytoplasmic Csk^{AS} or membrane Csk^{AS} had been inhibited by 3-IB-PP1 (Figs. 2.5a and 2.11b). Phosphorylation of this band occurred within 30 s of Csk^{AS} inhibition and was detectable for at least 30 min; however, this 65-kD

Figure 2.12. Dok-1 is uniquely phosphorylated upon Csk^{AS} inhibition and interacts with Csk. (a) Jurkat cells transfected with empty plasmid, plasmid encoding membrane Csk^{AS}, or plasmid encoding the SH2 domain R107K mutant (SH2^{mut}) of membrane Csk^{AS} were starved of serum and then lysed directly or after treatment with 3-IB-PP1 for the indicated times. Total tyrosine phosphorylation and the amounts of Csk and ERK were assessed by Western blotting analysis. (b) Immunoprecipitation (IP) of WT and SH2^{mut} membrane Csk^{AS} demonstrates that p65 interacts with Csk in an SH2-dependent manner. Cells transfected with empty plasmid or plasmid encoding WT or the SH2 domain mutant of membrane Csk^{AS} cells were treated with 3-IB-PP1 or DMSO for 10 min. Lysates were subjected to immunoprecipitation for the indicated times with antibody against Myc and analyzed by Western blotting for the presence of pTyr and Csk. (c) Silver staining of samples immunoprecipitated from control cells or cells containing membrane Csk^{AS}. Dok-1 is denoted with an arrow. (d) Immunoprecipitation of control cells or cells containing membrane Csk^{AS} with antibody against Dok-1. Cells were treated with 3-IB-PP1 or DMSO for 10 min. Lysates were then subjected to immunoprecipitation with antibody against Dok-1 and analyzed by Western blotting for the presence of Dok-1, pTyr, and Csk. Whole-cell lysates (WCL) and lysates after immunoprecipitation were analyzed by Western blotting with an antibody against Csk that recognizes WT (**) and membrane Csk^{AS} (*), whereas the samples immunoprecipitated with the antibody against Dok-1 were analyzed with an antibody against Myc, which recognizes only membrane Csk^{AS}. Data are representative of at least two (c) or three (a and b) independent experiments.


a
vector
Csk AS
SH2 mut
3-IB-PP1 (min):
0
2
10
30
kDa:
75
50
37
25
20
blot:
pTyr
Csk
ERK1/2
*
**
c
IP: Myc (silver stain)
kDa:
97
IgH
54
b
WCL
DMSO
3-IB-PP1
blot:
pTyr
Csk
Csk AS - Myc IP
IP: Myc (h)
0.5
3
d
WCL
control
Post-IP lysate
Dok-1 IP
α-Dok-1:
blot:
Dok-1
pTyr
Csk

phosphorylated protein was undetectable before inhibition of Csk or in response to TCR stimulation in control cells. Furthermore, immunoprecipitates of membrane Csk^{AS} showed a strong interaction with a tyrosine-phosphorylated protein of 65 kD after inhibition of Csk^{AS} by 3-IB-PP1, which was reduced when the SH2 domain of Csk^{AS} was mutated (Fig. 2.12b). We hypothesized that the 65-kD protein might interact with Csk^{AS} through its SH2 domain and might play a role in the enhanced signaling response. Mass spectrometric analysis of the 65-kD band that coimmunoprecipitated with Myc-tagged Csk^{AS} identified various peptides, but only a single peptide that was from the adaptor molecule p62dok1 (Dok-1) (Fig. 2.12c and 2.13). The Dok-1 peptide that we identified was tyrosine-phosphorylated and encompassed Tyr449, a known binding site for the SH2 domain of Csk.

Dok-1 is a scaffolding protein that has an inhibitory role in receptor signaling and cell activation in several cell types, including T cells (Mashima et al., 2009). The inhibitory effect of Dok-1 requires its localization to the membrane through the binding of its pleckstrin homology (PH) domain to phospholipids (Guittard et al., 2009). Phosphorylation of Dok-1 on Tyr449 by SFKs creates the Csk-binding site (Shah and Shokat, 2002), which was identified by mass spectrometry (MS), and is a likely mechanism for the membrane recruitment of Csk after TCR stimulation. Indeed, we found that Dok-1 phosphorylation was increased when Csk^{AS} was inhibited (Fig. 2.12d). Immunoprecipitation and partial depletion of Dok-1 from cells containing membrane Csk^{AS} further confirmed its identity as at least a component of the 65-kD protein band that we observed in lysates. Inhibition of Csk^{AS} and phosphorylation of Dok-1 induced an interaction between the two proteins. We suggest that Dok-1 is phosphorylated by Lck and may serve as a regulator that normally senses Lck activity and interacts with Csk at the plasma membrane, particularly after TCR stimulation. The enhanced phosphorylation of Dok-1 by Lck

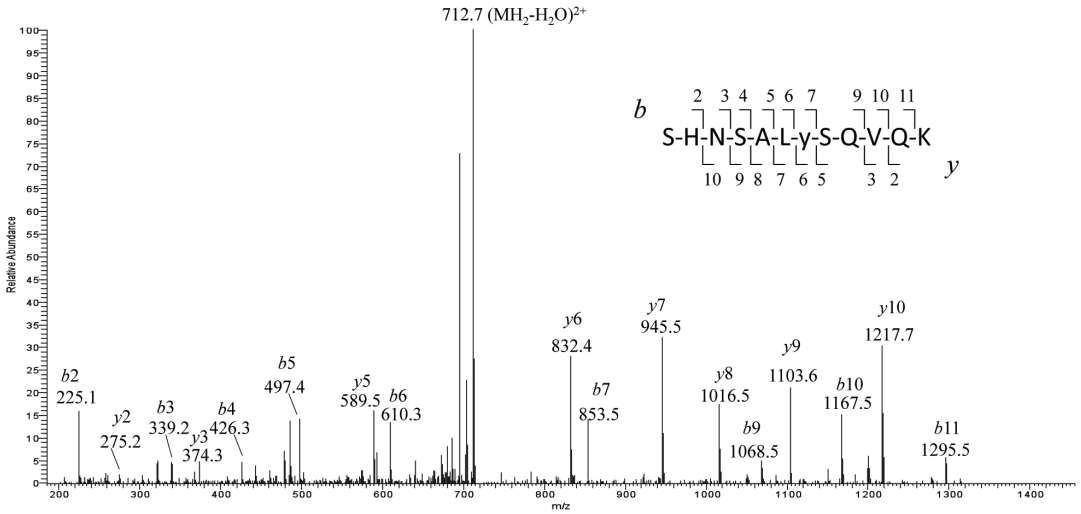
and its association with Csk^{AS} may play a role in the enhanced signaling mechanism that we have observed as a result of inhibition of membrane Csk^{AS}.

Figure 2.13. Identification of Dok-1 as p65 by mass spectrometric analysis of Csk^{AS} immunoprecipitates. (a) Table of proteins identified by mass spectrometric analysis of Csk^{AS} immunoprecipitates. [1]Protein scores are derived from ions scores as a nonprobabilistic basis for ranking protein hits. [2]Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 40 indicate identity or extensive homology ($P < 0.05$). [3]Expectation value denotes the number of times that the peptide score was as good as or better than the one observed could be expected by chance. *MS/MS spectrum was manually inspected. (b) MS/MS spectrum of the tryptic peptide ^{443}SHNSALYSQVQ^{454}K derived from Dok1_Human protein. The spectrum was generated by collision-induced dissociation with a linear ion trap mass spectrometer (LTQ, Thermo Scientific). The observed b and y fragment ion series are annotated on the spectrum and shown on a peptide sequence drawing (top right). Fragment ion pairs b6:b7 and y5:y6 confirm the presence of phosphorylation at Tyr449. (c) Table of theoretical m/z values of b and y fragment ions. The observed fragment ions are shown in red. Fragment ion pairs that are diagnostic for phosphotyrosine are shown in bold.

a

Accession #	Entry Name	Protein Name	Protein Score [1]	# Peptides (p <0.05)	Ion Score of the Best Peptide [2]	Expectation Value of the Best Peptide [3]	% Protein Sequence Coverage	Protein Mass (Da)
P10809	CH60_HUMAN	60 kDa heat shock protein, mitochondrial	612	10	93	4.2E-07	18.5	61187
P38646	GRP75_HUMAN	Stress-70 protein, mitochondrial	456	7	88	1.1E-06	13.1	73920
P41240	CSK_HUMAN	Tyrosine-protein kinase CSK	452	7	83	1.4E-06	17.3	51242
P31146	COR1A_HUMAN	Coronin-1A	273	5	73	3.7E-05	9.8	51678
P40227	TCPZ_HUMAN	T-complex protein 1 subunit zeta	149	3	70	1.0E-04	6	58444
P17987	TCPA_HUMAN	T-complex protein 1 subunit alpha	138	2	83	2.9E-06	4.5	60819
P48643	TCPE_HUMAN	T-complex protein 1 subunit epsilon	83	1*	85	1.5E-06	1.8	60089
P61978	HNRPK_HUMAN	Heterogeneous nuclear ribonucleoprotein K	71	1*	71	3.7E-05	2.6	51230
P61626	LYSC_HUMAN	Lysozyme C	65	1*	68	1.2E-04	8.1	16982
Q5D862	FILA2_HUMAN	Filaggrin-2	58	1*	58	6.5E-04	0.5	249296
P62988	UBIQ_HUMAN	Ubiquitin	58	1*	60	8.6E-04	21.1	8560
Q99704	**DOK1_HUMAN**	**Docking protein 1 (p62^{Dok1})**	**57**	**1***	**63**	**3.9E-04**	**2.5**	**52815**
P52294	IMA1_HUMAN	Importin subunit alpha-1	46	1*	49	9.0E-03	2	60952

b



c

b				*y*
---	1	S	12	---
225.10	2	H	11	1354.62
339.14	3	N	10	1217.56
426.17	4	S	9	1103.51
497.21	5	A	8	1016.48
610.29	6	L	7	945.44
853.32	7	**y**	6	**832.36**
940.36	8	S	5	**589.33**
1068.41	9	Q	4	502.30
1167.48	10	V	3	374.24
1295.54	11	Q	2	275.17
---	12	K	1	147.11

Discussion

We have generated an analog-sensitive variant of Csk, whose catalytic function can be rapidly and dynamically controlled, which we used to investigate the role of Csk in controlling basal and ligand-induced signaling downstream of the TCR. Because Csk is a ubiquitous regulator of SFK activity, these findings may be applicable to other receptor systems that are dependent on SFK activity. In our studies, constitutive association of Csk^{AS} with the plasma membrane potently inhibited SFK activity and TCR signaling, whereas a cytoplasmic Csk^{AS} had no such effect, consistent with previous data (Cloutier et al., 1995). Inhibition of either form of Csk^{AS} led to the unanticipated activation of the canonical TCR signaling pathway in the complete absence of ligand binding to the receptor. Upon inhibition of membrane Csk^{AS}, the enhanced phosphorylation of proximal signaling molecules indicated that ectopic expression of Csk^{AS} may have reset the basal signaling tone in the cell. Furthermore, signaling induced by inhibition of Csk^{AS} was more sustained than was signaling as a result of direct stimulation of the TCR. These findings suggest the possible involvement of a compensatory adaptive mechanism that monitors and controls basal signaling, at least by SFKs.

These studies suggest that an important function of basal signaling is to monitor and respond to changes in the tonic activity of key signaling components. Direct evidence for basal signaling in T cells has been limited, but some studies suggest a role for basal signaling in survival, transcription, and effector function: Inducible deletion of surface TCR results in a shortened half-life of mature T cells (Polic et al., 2001), ablation of TCR signaling perturbs gene expression profiles and sensitivity to foreign antigens (Roose et al., 2003; Stefanova et al., 2002), and expression of the tandem SH2 domains of ZAP-70 increases ITAM phosphorylation in the absence of TCR stimulation (Qian et al., 1996). A substantial amount of the ZAP-70 tandem SH2

domains associate with ITAMs in resting cells, suggesting that ongoing Lck phosphorylation of the ζ chain is protected from phosphatases by the binding of ZAP-70 in the basal state. Our data imply that basal signaling is monitored and regulated by feedback circuitry.

The ability of Csk^{AS} inhibition to rapidly activate Lck in the absence of TCR ligands demonstrates the plasticity in the Lck activation state in resting T cells. A previous study focused on the substantial amount of Lck that is phosphorylated on its activation loop in resting T cells (Nika et al., 2010). Furthermore, a substantial fraction of Lck molecules contained both pTyr394 and inhibitory phosphorylation on Tyr505. On the basis of these results and our own data, we propose that in resting T cells, a dynamic equilibrium is imposed on Lck by its interactions with Csk and CD45. These interactions lead to a continuous turnover of Tyr394 and Tyr505 phosphorylation of Lck and its activity in a basal state and enable rapid and efficient phosphorylation of ITAMs by Lck in response to TCR stimulation. We further propose that this dynamic equilibrium of Lck phosphorylation and activity in resting T cells is monitored and adjusted to signaling perturbations during the inactive basal state.

One possible mechanism to prevent increased phosphorylation of ITAMs by Lck in resting cells would be a requirement for ligand-induced structural changes in the TCR to enable an increased amount of signaling above the basal state to occur (Aivazian and Stern, 2000; Sigalov et al., 2007; Xu et al., 2008). In this model, the cytoplasmic portion of TCR ITAM tyrosines may bind to the plasma membrane in resting cells. Upon TCR engagement, allosteric or other changes cause the ITAM-containing tyrosines to dissociate from the membrane and become phosphorylated, driving an increased amount of signaling. An alternative model has suggested that allosteric changes in CD3ε result in accessibility to a polyproline region that binds Nck (Gil et al., 2002); however, this model has been challenged by knock-in of the CD3ε

proline-rich motif (Mingueneau et al., 2008). Because TCR signaling pathways are activated by inhibiting Csk^{AS} activity in the absence of TCR engagement, neither of these models is complete. Our results suggest that either the CD3ε chains or the ζ chains do not associate with the membrane or that the interactions are highly dynamic and transient. We favor a model in which a low amount of basal signaling is continuously occurring, but is held in check by the balanced action of numerous phosphatases and other inhibitory mechanisms.

Our studies suggest the possibility that adaptation or an alteration in cellular circuitry may have occurred in cells containing the membrane Csk^{AS} construct because of decreased basal signaling. Alternatively, the effects of this construct could reflect dominant-negative functions of the 3-IB-PP1–inhibited kinase, whose SH2 and SH3 domains could block the function of endogenous Csk. Although such a dominant-negative effect may contribute to the enhanced signaling response, we favor some amount of cellular adaptation in signaling circuitry to the altered basal signaling. First in support of an adaptation or alteration in basal cellular circuitry is the rapid appearance of the 65-kD phosphoprotein, representing Dok-1, that was not observed in the context of TCR signaling. Second, the amplitude and kinetics of signaling were substantially altered and did not simply mimic the loss of Csk activity from the plasma membrane that was observed after TCR stimulation. Third, in preliminary studies, we have been unable to detect an alteration in the distribution of endogenous Csk by the presence of membrane Csk^{AS}. Together, these data suggest that the cellular circuitry in cells containing membrane Csk^{AS} has been altered by (or adapted to) the change in basal signaling.

The mechanisms that modulate Csk activity during T cell activation are incompletely understood; however, we suggest that Dok proteins, specifically Dok-1, contribute to the regulation of Csk. During T cell activation, removal of Csk from the immune synapse occurs in

response to acute dephosphorylation of Csk-recruiting adaptor proteins, such as PAG; however, this release of Csk is transient, and genetic ablation of PAG has no effect on T cell signaling, suggesting that Csk is likely to interact with additional proteins at the membrane. We suggest that one candidate may be the inhibitory scaffolding molecule Dok-1, because it is rapidly phosphorylated and associates with Csk after 3-IB-PP1–mediated inhibition of Csk^{AS}. Dok proteins bind to phospholipids in the plasma membrane and can interact with target proteins through a protein tyrosine–binding domain. Furthermore, several conserved tyrosines can be phosphorylated by Abl and SFKs after SFK activation (Shah and Shokat, 2002), including the Csk-binding site Tyr449. Indeed, we found that a peptide encompassing this site was tyrosine-phosphorylated and associated with Csk^{AS} after treatment with 3-IB-PP1. In fibroblasts, the interaction of Dok-1 pTyr449 with the SH2 domain of Csk is necessary for the recruitment of Csk to the plasma membrane and the control of Src activity (Zhao et al., 2006). Dok-1 also directly abrogates MAPK signaling by recruiting Ras guanosine triphosphatase–activating proteins (GAPs) to the signalosome. In our studies, membrane Csk^{AS} may have caused an alteration in the localization of Dok-1 in the basal state and also competed more effectively than endogenous Csk to interact with Dok-1. Thus, endogenous Csk may not have been able to effectively inhibit or regulate Lck function, which would explain the enhanced signaling seen as a result of 3-IB-PP1–mediated inhibition of Csk^{AS} function.

T cells contain Dok-1 and the related family member Dok-2, which together play a critical role in establishing negative feedback loops in TCR signaling. TCR-stimulated T cells deficient in both Dok-1 and Dok-2 exhibit enhanced proliferation and increased cytokine secretion and have prolonged and enhanced phosphorylation of various signaling molecules, including the ζ chain, ZAP-70, LAT, and ERK (Dong et al., 2006; Yasuda et al., 2007), which is

reminiscent of our findings in the context of Csk^{AS} inhibition. Our attempts to alter the abundance or function of Dok-1 yielded inconsistent results, suggesting that Dok-2 may functionally compensate for Dok-1, as has been previously suggested (Di Cristofano et al., 2001; Yasuda et al., 2007). Future studies will need to clarify the role of Dok proteins as part of a proximal TCR feedback loop to regulate proximal signaling through the recruitment of Csk to the plasma membrane. Moreover, together with Csk, Dok-1 may play an important role in regulating the intensity and termination of the TCR response.

In conclusion, we have used an analog-sensitive variant of Csk to demonstrate that dynamic control of SFK activity downstream of the TCR is necessary for setting basal signal tone and preventing aberrant cellular activation. Because Csk-mediated regulation of SFKs is ubiquitous, these results may be broadly relevant toward understanding how other SFK-associated pathways are regulated in other cell types. Introduction of the Csk^{AS} allele into mice will further enable an examination of the regulatory function of Csk in controlling basal signaling tone, and of regulation of TCR-stimulated signaling, in primary cells and during lymphocyte development in the context of endogenous Csk-null T cells. Moreover, the Csk^{AS} allele holds potential for being able to exogenously control T cell activity independently of ligand binding.

Materials and Methods

Cell lines, transfections, and stimulations

The Jurkat T cell line E6-1 was cultured and transfected as described before (Phee et al., 2005). J.CaM1, J45, P116, and J14 T cell lines have been previously described (Koretzky et al., 1991; Sieh et al., 1993; Straus and Weiss, 1992; Williams et al., 1998; Yablonski et al., 1998). Plasmids encoding green fluorescent protein (GFP) or human CD16 were used as cotransfection controls. Before stimulation, cells were starved of serum at 37°C for at least 25 min. Stimulations were performed in serum-free RPMI at 37°C with a 1:1000 final dilution of C305, an antibody against the Vβ8 chain of the TCR. 3-IB-PP1 was used at 10 μM unless otherwise noted. Before cell lysate preparation, transfected CD16+ cells were purified with Miltenyi human CD16 microbeads. Cells were treated with the MEK inhibitor U0126 (20 μM) for 45 to 120 min.

Western blotting and flow cytometric analyses

To analyze the phosphorylation of ERK and the ζ chain by flow cytometry, we serum-starved and stimulated Jurkat cells as described earlier. The addition of an equivalent volume of Cytofix (BD Pharmingen) was used to stop the stimulations. Cells were collected by centrifugation, washed in fluorescence-activated cell sorting (FACS) buffer, and then resuspended in ice-cold 90% methanol. Cells were incubated on ice for 30 min after which they were washed three times in FACS buffer. Cells were incubated with antibody against phosphorylated p44/42 MAPK (pERK1/2), followed by allophycocyanin (APC)– or phycoerythrin (PE)–conjugated donkey antibody against rabbit immunoglobulin (Ig) (Jackson) or with antibody against CD3ζ-pY142 used at a 1:5 dilution. For the analysis of Ca2+ flux, cells were loaded with the Ca2+-sensitive dyes Fura Red and Fluo-3 (Invitrogen) for 30 min at 37°C in RPMI and 5% fetal bovine serum

(FBS), washed, and incubated on ice with Alexa Fluor 647–conjugated antibody against CD16 (BD Pharmingen) to detect transfected cells. Cells were then resuspended in RPMI and warmed to 37°C for 5 min before stimulation. Basal Ca2+ concentrations were measured for 1 min before the addition of vehicle or 3-IB-PP1 (10 μM) for 3 min. Cells were then stimulated through the TCR for 3 min and treated with ionomycin (1 μM) as a positive control for 1 min. Ca2+ increase was measured as the ratio of Fluo-3 to Fura Red fluorescence and was displayed as a function of time for CD16+ (transfected) cells and CD16– (untransfected) cells.

Antibodies

Antibodies used in this study were against the following targets: LAT-pY191 (Invitrogen/BioSource); ZAP-70–pY319 (van Oers et al., 1995), ZAP-70–pY493, Src pY416, p44/42 MAPK pThr202/Tyr204, Myc 9B11 (Cell Signaling); Lck (1F6 from J. B. Bolen); pY (4G10, Upstate Biotechnology); LAT, Dok-1 (Abcam); ERK1/2 (Santa Cruz Biotechnology); CD3ε(clone UCHT.1), CD69 (BD Pharmingen); human CD16, Lck pY505, and CD3ζ-pY142 (BD Pharmingen). Horseradish peroxidase–conjugated goat antibody against rabbit IgG (H+L) and mouse IgG (H+L) (Southern Biotech) were used as secondary antibodies. The following antibodies have been described previously: 2F3.2 (ZAP-70) (Iwashima et al., 1994) and 6B10.2 (CD3ζ) (van Oers et al., 1995).

Generation of the Csk^{AS} allele

The Csk^{AS} allele was generated by cloning mouse Csk from C57BL/6 splenocyte complementary DNA (cDNA) with SuperScript II (Invitrogen) and subcloning it into the plasmid pCR2.1-TOPO (Invitrogen). The sequences encoding the 11 N-terminal amino acid residues of Lck (MGCVCSSNPED) and the Myc tag were added onto the cDNA for the membrane Csk^{AS} allele

through flexible linker regions. We used the QuikChange kit (Stratagene) to generate the Thr266->Gly (T266G) mutation of the gatekeeper residue of Csk. Correctly targeted CskT266G mutations were verified by sequencing and ligated into pEF6/myc-His A (Invitrogen) by digestion with Bam HI and Eco RV. The final construct can be described as 5'-Bam HI–(Lck11-SAGGSAGG)-Csk^{AS}-SAGGSAGG-Myc-Eco RV-3', where SAGGSAGG is the amino acid sequence of the flexible linker region. Jurkat cells were transfected with plasmids encoding either cytoplasmic Csk^{AS} or membrane Csk^{AS}, and the cellular localization of these constructs was confirmed by fluorescence microscopy and Western blotting analysis of membrane and cytoplasmic cellular fractions. Whereas only a small portion of cytoplasmic Csk^{AS} was recruited to the membrane, which likely represented interactions with endogenous membrane adaptor proteins, membrane Csk^{AS} was found almost exclusively at the plasma cell membrane and enriched in lipid rafts. Primers for generation of the Csk^{AS} allele were as follows: Cyto-Csk forward: 5'-ggatccatcatgtcggcaatacaggccgcct-3'; Lck11-Csk forward (external): 5'-ggatccatcatgggctgtgtctgcagctcaaaccctgaagatagtgctggtggtagtgctggtggttc-3'; Lck11-Csk forward (internal): 5'-agtgctggtggtagtgctggtggttcggcaatacaggccgcctggccat-3'; Csk-Myc reverse (external): 5'-gatatctacagatcctcttctgagatgagtttttgttcaccaccagcactaccaccagcactcaggtg-3'; Csk-Myc reverse (internal): 5'-accaccagcactaccaccagcactcaggtgcagctcgtgggttttgatgt-3'; mutagenesis forward: 5'-ggctctacatcgtcggagagtacatggccaaggg-3'; and mutagenesis reverse: 5'-cccttggccatgtactctccgacgatgtagagcc-3'.

Description of 3-IB-PP1

A white powder; 1H NMR ($CDCl_3$, 400 MHz) δ 1.79 (s, 9H), 4.24 (s, 2H), 4.90 (s, 2H), 7.04 (t, 1H), 7.13 (d, 1H), 7.58 (s, 1H), 7.60 (d, 1H), 8.26 (s, 1H); 13C NMR ($CDCl_3$, 100 MHz) δ 29.4, 34.9, 60.3, 95.4, 100.8, 127.8, 131.0, 136.6, 137.5, 140.0, 140.8, 154.8, 154.9, 157.7; high-

resolution MS (electron ionization) molecular ion calculated for $C_{16}H_{18}IN_5$ is 407.0685; found, 407.0705. Stock solutions of 3-IB-PP1 (10 mM) were prepared in dimethyl sulfoxide (DMSO), and aliquots for individual use were stored at –80°C.

In-gel digestion, peptide separation, MS, and protein identification

Immunoprecipitates from 1 x 10^9 cells transfected with plasmid expressing membrane CskAS were eluted from protein G beads with 0.1 M glycine (pH 2.5) and concentrated to 60 µl by centrifugal filtration (Vivaspin 500, Sartorius). The concentrates were resolved by SDS-PAGE, and protein bands were visualized with SYPRO Ruby stain (Invitrogen) according to the manufacturer's protocol. The 65-kD band of interest was excised and subjected to in-gel trypsin digestion together with that of the appropriate negative control band (Jimenez et al., 2001). Modified porcine trypsin (Promega) was used at a final concentration of 12.5 ng/µl. Mixtures of proteolytically generated peptides were analyzed by nano-liquid chromatography (nanoLC) tandem MS (MS/MS) with a 2DLC nanoHPLC System (Eksigent) interfaced with an LTQ XL mass spectrometer (Thermo Fisher Scientific) equipped with an Advance ion source (Michrom Bioresources). An LC Phenomenex Onyx monolithic column (100-µm internal diameter, 15 cm in length) was used for both desalting and reversed-phase peptide separation. A 20-min linear gradient from 2% solvent B to 40% solvent B was run at a flow rate of 750 nl/min with solvent A (2% acetonitrile, 0.1% formic acid) and solvent B (90% acetonitrile, 0.1% formic acid). External calibration of the LTQ XL mass spectrometer was performed in MS/MS mode with fragment ions of angiotensin I as references. Peptide fragment ion spectra were obtained by precursor ion selection, which used an automated routine that consisted of a series of one survey MS scan [mass/charge ratio (m/z) 400 to 1700], followed by six MS/MS scans (m/z 60 to 1500) where helium served as the trap gas and the collision energy was set to 35. Protein identification was

accomplished with the MASCOT 2.2 (Matrix Science) search engine. Mammalia taxonomy was searched within the UniProt database (11/27/2009: 513,877 sequences; 180,750,753 residues; taxonomy, Homo sapiens; 20,401 sequences) with the following parameters: precursor ion mass tolerance: 0.8 dalton; fragment mass tolerance: 0.8 dalton; tryptic digestion with three missed cleavages, fixed modifications: S-carboxyamidomethyl, variable modifications: deamidation (Asn and Gln); Met sulfoxide; and Pyro-Glu (from N-terminal Gln). In-gel digestion of candidate proteins was performed according to the established protocol.

Cell lines, primary mouse T cell culture, retroviral transduction, and stimulations

A TCRβ-deficient Jurkat cell line stably reconstituted to express either 10% or 60% of the amount of surface TCR in wild-type cells was previously described (Graber et al., 1991; Ohashi et al., 1985). TCRβ-deficient cells reconstituted with a chimeric CD8-ζ receptor containing the extracellular and transmembrane portions of the CD8 co-receptor linked to the cytoplasmic domain of the ζ chain were previously described (Irving and Weiss, 1991). Mouse CD4+ T cells were purified with the CD4 T Cell Isolation Kit (Miltenyi Biotech) according to the manufacturer's instructions. Purified T cells were stimulated on plates coated with antibodies against CD3 and CD28 in the presence of IL-2 (100 U/ml) for 24 to 32 hours before retroviral transduction. CskAS was cloned into pMIGR plasmid upstream of the internal ribosomal entry site (IRES) for GFP coexpression, which was used to infect Phoenix packaging cells to generate supernatants for transduction, as previously described by G. Nolan (Swift et al., 2001). For transductions, medium was gently aspirated from cells and replaced with 1 ml of viral supernatant containing polybrene (8 μg/ml, Sigma). Cells were centrifuged at 1100g for 2 hours at 30°C. Retroviral supernatants were left on cultured cells overnight. Cell culture medium was supplemented with IL-2 for a period of 12 to 24 hours, during which time ~80 to 90% of cells

were GFP+. Cells were rested from IL-2 for 12 hours and starved of serum for 30 min before stimulation. Cells were stimulated with antibody against CD3 (2C11, 10 μg/ml) and goat antibody against Armenian hamster Ig (Jackson Immunolabs, 50 μg/ml) as indicated. Lysates were prepared by directly lysing cells in an equivalent volume of SDS-PAGE sample buffer. For immunoprecipitations, cells were stimulated as described earlier and immediately washed in ice-cold phosphate-buffered saline. Cells were resuspended in NP-40 lysis buffer with protease and phosphatase inhibitors, and immunoprecipitations were performed according to Abcam's protocol. Dithiothreitol was added to a final concentration of 1% in all lysates, and samples were boiled before running on SDS-PAGE. Proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) by standard Western blotting techniques, and proteins were visualized with SuperSignal ECL reagent (Pierce Biotechnology) and a Kodak Imaging Station.

TCR internalization assay

For quantification of TCR internalization, Jurkat cells were starved of serum for 30 min at 37°C and then incubated with an antibody against CD3ε (BD Pharmingen, clone UCHT1) on ice in serum-free RPMI for 30 min. Cells were washed in FACS buffer and warmed up to 37°C for stimulation or treatment with 3-IB-PP1 for the indicated times. TCR internalization was stopped by incubating the cells in ice-cold FACS buffer. Cells were divided into two groups to analyze the amount of total CD3ε and that of internalized CD3ε. Cells in the former group were washed in FACS buffer and fixed immediately, whereas cells in the latter group were washed in FACS buffer and had any surface-bound antibody against CD3ε acid-stripped by resuspension in RPMI + 3% FBS (pH 2.0) for 30 s at room temperature. Cells were washed immediately in ice-cold FACS buffer and fixed in 2% paraformaldehyde before flow cytometric analysis of the mean fluorescence intensity (MFI) of CD3ε. To calculate the amount of internalized TCR, we used the

following equation: (Stx – St0)/(Tt0 – St0) x 100, where Stx is the MFI of cells that were acid-stripped after stimulation, St0 is the MFI of cells that were acid-stripped but not stimulated, and Tt0 is the total MFI of cells without stripping. Controls to verify cell viability and other surface markers were used to verify quality of data.

Chapter 3

Inhibition of Csk in thymocytes reveals a requirement for actin remodeling in the initiation of full T cell receptor signaling

Summary

T cell receptor (TCR) signaling is initiated by Src-family kinases (SFKs). To understand how C-terminal Src kinase (Csk), the negative regulator of SFKs, controls the basal state and the initiation of TCR signaling, we generated mice expressing a PP1-analog inhibitor-sensitive Csk allele (Csk^{AS}). Inhibition of Csk^{AS} in thymocytes, without TCR engagement, induced potent SFK activation and proximal TCR signaling up to phospholipase C gamma-1 (PLCγ1). Surprisingly, increases in inositol phosphates (IP), intracellular calcium and ERK phosphorylation were impaired. Altering the actin cytoskeleton pharmacologically or providing CD28 costimulation rescues these defects. Thus, Csk plays a critical role in preventing TCR signaling. However, our studies also reveal a requirement for actin remodeling, initiated by costimulation, for full TCR signaling.

Introduction

Signals transduced by the TCR are critical for thymocyte selection and maturation, peripheral T cell homeostasis and activation, as well as specification of effector and memory cell fates. Hence the initiation of TCR signaling in response to antigens of diverse affinities at different stages of T cell development must be tightly regulated. This ensures the selection of a protective T cell repertoire and the mounting of efficacious immune responses against foreign pathogens while preventing aberrant immune activation. The TCR complex has no intrinsic kinase activity but instead possesses two spatially separated tyrosines within immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic tails of its non-ligand binding CD3 and ζ subunits (Smith-Garvin et al., 2009). Phosphorylation of these ITAMs is mediated by the T cell SFKs Lck and Fyn T, thus creating docking sites for the recruitment of the cytoplasmic kinase ZAP-70 via its tandem SH2 domains. The autoinhibited conformation of ZAP-70 is relieved by ITAM binding as well as by its phosphorylation by Lck or Fyn T. ZAP-70 activation is critical for downstream signaling events leading to cellular responses.

In freshly isolated resting thymocytes and T cells, non-phosphorylated ZAP-70 is bound to constitutively phosphorylated ITAMs (van Oers et al., 1993; van Oers et al., 1994). Following prolonged cell culture, the constitutively phosphorylated state of the ITAMs in primary cells is lost but is reinduced by TCR stimulation, as it is in T cell lines. Various mechanisms have been proposed for how ITAM and/or ZAP-70 phosphorylation by SFKs is initiated during TCR stimulation. They include: 1) colligation of the CD4/CD8 coreceptors with the TCR by peptide-bound major histocompatibility complex (pMHC), which redistributes the coreceptor-associated SFK Lck into proximity with TCR ITAMs/ZAP-70; 2) TCR conformational change induced by pMHC binding that permits increased ITAM accessibility to SFKs; and, 3) redistribution of

bulky transmembrane phosphatases that inhibit signaling away from the narrow TCR-pMHC cell-cell interface due to size exclusion (i.e. kinetic segregation model) (Choudhuri et al., 2005; James and Vale, 2012; van der Merwe and Dushek, 2011; Xu et al., 2008). The relative importance of these mechanisms is unresolved because the experimental evidence available is conflicting or incomplete. It is also uncertain if any of these mechanisms alone is sufficient to trigger full TCR downstream signaling.

Since SFKs phosphorylate TCR ITAMs, the control of their activities represents a key regulatory node in the initiation of TCR signaling. Trans-autophosphorylation of a conserved activation loop tyrosine within the SFK catalytic domain increases catalytic activity (Palacios and Weiss, 2004; Salmond et al., 2009). Phosphorylation of the conserved C-terminal inhibitory tyrosine of SFKs by the tyrosine kinase Csk promotes their closed, inactive conformation (Bergman et al., 1992). In T cells, the receptor-like tyrosine phosphatase CD45 opposes the action of Csk and dephosphorylates the inhibitory tyrosine. Thus, the equilibrium between Csk and CD45 may set the threshold for activation of TCR signaling (Hermiston et al., 2002; Hermiston et al., 2009). In resting T cells, there are multiple phosphorylation states of Lck, which consists of unphosphorylated, each of the singly phosphorylated and the doubly phosphorylated species (Nika et al., 2010). It is unclear if this basal equilibrium has a fixed state or is a dynamic, ongoing process in unstimulated primary T cells.

Ubiquitously expressed, Csk is a cytosolic protein. Since Csk deficient mice are embryonic lethal due to excessive SFK activity and conditional deletion of Csk in thymocytes results in TCR- and MHC-independent development of abnormal CD4+ T cells, understanding the importance of Csk regulation in the T cell lineage has been challenging (Imamoto and Soriano, 1993; Nada et al., 1993; Schmedt et al., 1998). Positioning Csk at the plasma

membrane, proximal to the membrane-localized SFKs, is thought to be regulated through protein-protein interactions that may mediate its dynamic translocation between the cytosol and the cell membrane (Chong et al., 2005; Cloutier et al., 1995). The lipid raft-localized adaptor phosphoprotein associated with glycosphinogolipid-enriched microdomains (PAG) is believed to be involved in the recruitment of Csk to lipid rafts where some SFK molecules are localized and initiation of TCR signaling may occur (Brdicka et al., 2000; Davidson et al., 2003; Kawabuchi et al., 2000). However, in sharp contrast to Csk deficiency, PAG deficient mice develop normally without defect in T cell development or signaling, indicating the existence of alternative mechanisms for Csk regulation (Dobenecker et al., 2005; Xu et al., 2005).

To interrogate the role of Csk activity in TCR signaling, we recently generated a novel allele of Csk (Csk^{AS}) by mutating the conserved bulky gatekeeper residue in its ATP-binding pocket, thereby enlarging the pocket. Csk^{AS} kinase activity is specifically and rapidly inhibited by 3-iodo-benzyl-PP1 (3-IB-PP1), a bulky analog of the common kinase inhibitor, PP1. Unexpectedly, in Jurkat T cells, inhibiting the kinase activity of a dominant inhibitory membrane-targeted Csk^{AS} induced potent SFK activation and ligand-independent TCR signaling (Schoenborn et al., 2011). This suggests that perturbing the finely tuned Csk-CD45 kinase-phosphatase activity equilibrium can lead to SFK activation and trigger TCR signal initiation. However, Jurkat T cells express endogenous Csk; so, the expression of membrane-targeted Csk^{AS} may have induced compensatory rewiring of the signaling circuitry or activation could have reflected a dominant negative effect mediated by the protein interaction domains of the inhibited Csk^{AS}.

To determine the role of Csk in TCR signal initiation in primary T lineage cells, we generated mice expressing only the normally regulated cytosolic form of Csk^{AS}. We found that

inhibition of Csk^{AS} alone in thymocytes led to strong activation of SFKs and proximal signaling, but did not result in full downstream TCR signaling in the absence of additional actin cytoskeletal remodeling. Our data suggests that Csk plays a major role in restraining SFK activation in the basal state and that SFK activation alone is sufficient for the initiation of proximal TCR signaling. However, our studies also indicate that in addition to SFK activity, the actin cytoskeleton must be remodeled in thymocytes for downstream signal propagation. This required cytoskeletal remodeling is most likely mediated by CD28 costimulation.

Results

Generation of Csk^{AS} mice

We introduced Csk^{AS} into mice by bacterial artificial chromosome (BAC) transgenesis. The Csk^{AS} allele is 25% as active as wildtype Csk (Fig. 3.1a, b). We obtained three independent founders and crossed them with $Csk^{+/-}$ mice, ultimately removing expression of endogenous wildtype Csk. Two of the lines expressed less than half the wildtype levels of Csk and displayed defects in T cell development (data not shown). The third line had approximately 2.5 fold as much expression of Csk as wildtype mice, but displayed normal T cell development (Fig. 3.2a-c). Importantly, Csk^{AS} thymocytes had TCR responses to stimulation with both high and low doses of anti-CD3 mAb comparable to wildtype (Fig. 3.2d). We therefore conducted further studies with this third Csk^{AS} transgenic line.

Inhibition of Csk^{AS} activates SFKs and proximal TCR signaling, but not calcium or Ras-ERK signaling

At 10 μM, 3-IB-PP1 strongly inhibited recombinant Csk^{AS} but not wildtype Csk (Fig. 3.1c, d). Treatment of thymocytes or peripheral CD4+ T cells with 3-IB-PP1 rapidly led to sustained hyperphosphorylation of the activating loop tyrosine of both Lck and Fyn as well as slower progressive dephosphorylation of the inhibitory tyrosine of Lck, in Csk^{AS} but not wildtype cells (Fig. 3.3a, b). These data reaffirm the role of Csk as the primary negative regulator of SFKs. They also indicate that in mouse T cells, the basal phosphorylation status of Lck is in a dynamic equilibrium which requires constant restraint by Csk activity. Removal of this constraint drives rapid ligand-independent autoactivation of SFKs, presumably by trans-autophosphorylation. The incomplete dephosphorylation of the inhibitory tyrosine of Lck may

Figure 3.1. The Csk^{AS} allele is active and can be specifically inhibited by the PP1 analog, 3-IB-PP1. (a) Substituting glycine for threonine at the gatekeeper position in the active site of Csk to generate analog sensitive Csk (Csk^{AS}) impairs but does not abrogate kinase activity in purified Csk^{AS}, as monitored by decreasing absorbance in an assay coupling ATP hydrolysis to NADH oxidation. (b) Csk^{AS} phosphorylates peptide at a rate in between that of Csk^{WT} and the kinase-impaired variant Csk^{K222R}. Error bars represent 95% confidence intervals (CI), n=4. The Csk variants are significantly different from each other ($p<0.0001$) in a one-way ANOVA test (or $p=0.0041$ (Csk^{AS}, Csk^{K222R}) in a two-tailed t test). (c) A bulky analog of kinase inhibitor PP1, 3-IB-PP1, specifically targets Csk^{AS} over Csk^{WT}. Data points are independent samples from at least four separate experiments, and lines represent fit dose-response curves. (d) 3-IB-PP1 has a 27-fold lower IC_{50} for Csk^{AS} than for Csk^{WT}. Error bars represent 95% CI of a global fit.

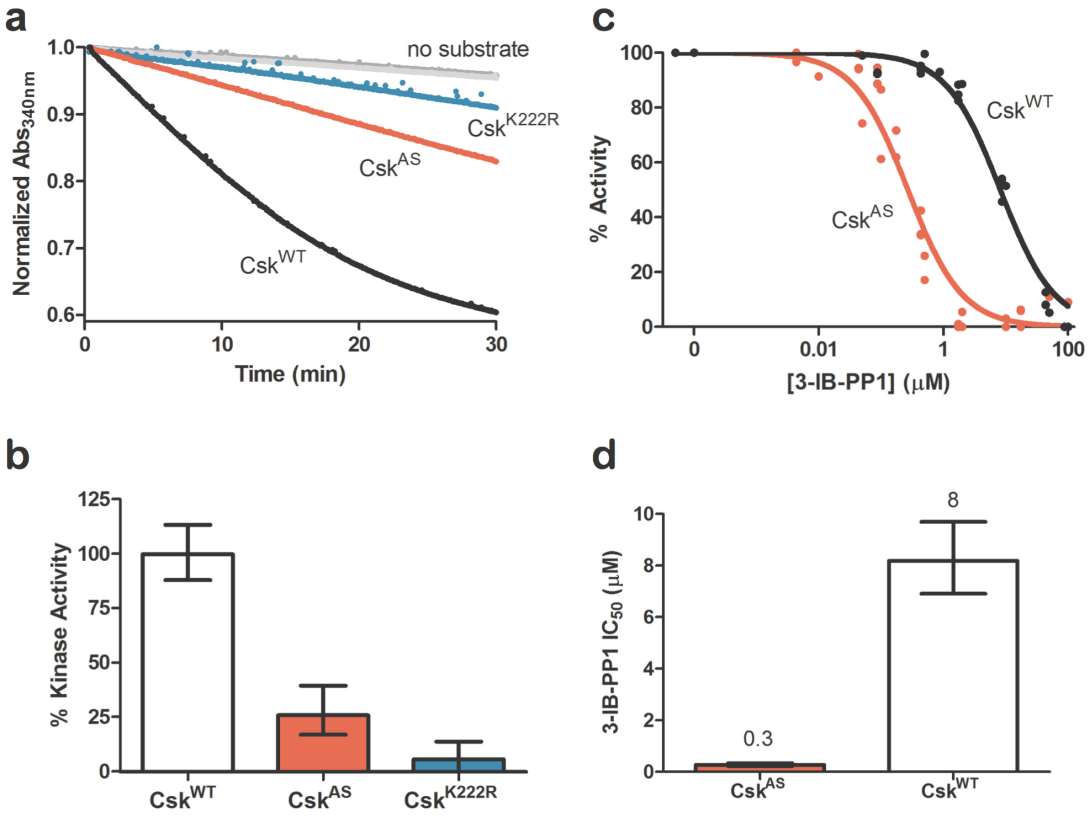

a
Normalized Abs340nm
1.0
0.9
0.8
0.7
0.6
0
10
20
30
Time (min)
no substrate
CskK222R
CskAS
CskWT
c
% Activity
100
80
60
40
20
0
0
0.01
1
100
[3-IB-PP1] (μM)
CskWT
CskAS
b
% Kinase Activity
125
100
75
50
25
0
CskWT
CskAS
CskK222R
d
3-IB-PP1 IC50 (μM)
10
8
6
4
2
0
0.3
8
CskAS
CskWT

Figure 3.2. T cell develop normally and respond normally to TCR stimulation in Csk^{AS} BAC transgenic mice. (a**)** Immunoblot and densitometric analysis of Csk expression in wildtype (WT) and Csk^{AS}(AS) thymocytes. (b) Thymocytes or splenocytes from wildtype (WT) and Csk^{AS}(AS) thymi were surface stained for the indicated markers and analysed by flow cytometry. Data representative of at least five littermate pairs. (c) Mean thymic cellularity of ten wildtype (WT) and ten Csk^{AS}(AS) littermate thymi with means and S.E.M indicated. (d) Thymocytes from wildtype (WT:thin lines) or Csk^{AS} (AS:thick lines) mice loaded with Indo-1AM dye were stimulated with high dose (red lines: 20mg/mL) or low dose (blue lines: 5mg/mL) anti-CD3ε crosslinking. Ratiometric assessment of intracellular calcium of CD4+CD8+ thymocytes over time is shown. Data is representative of three independent experiments.

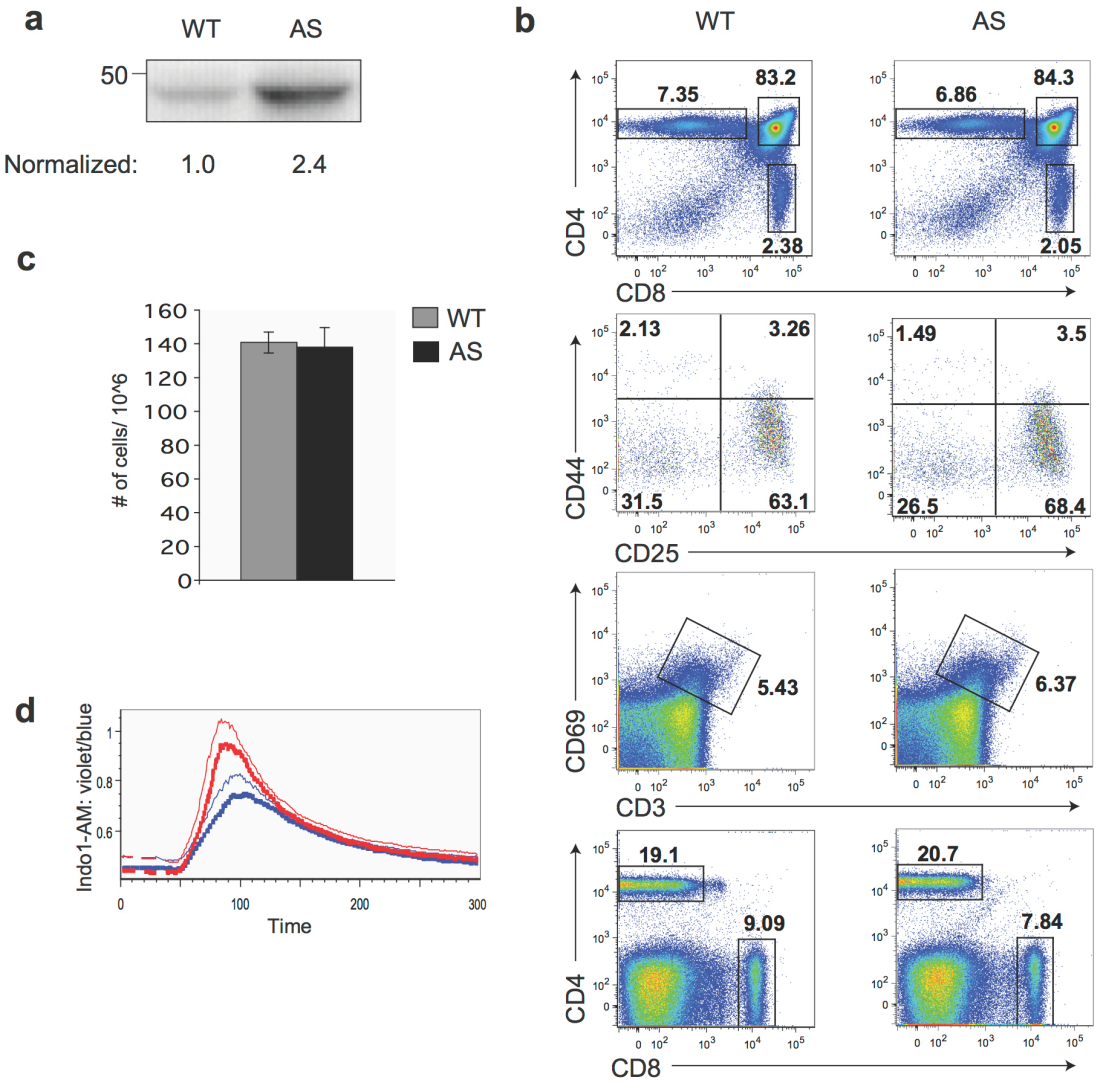
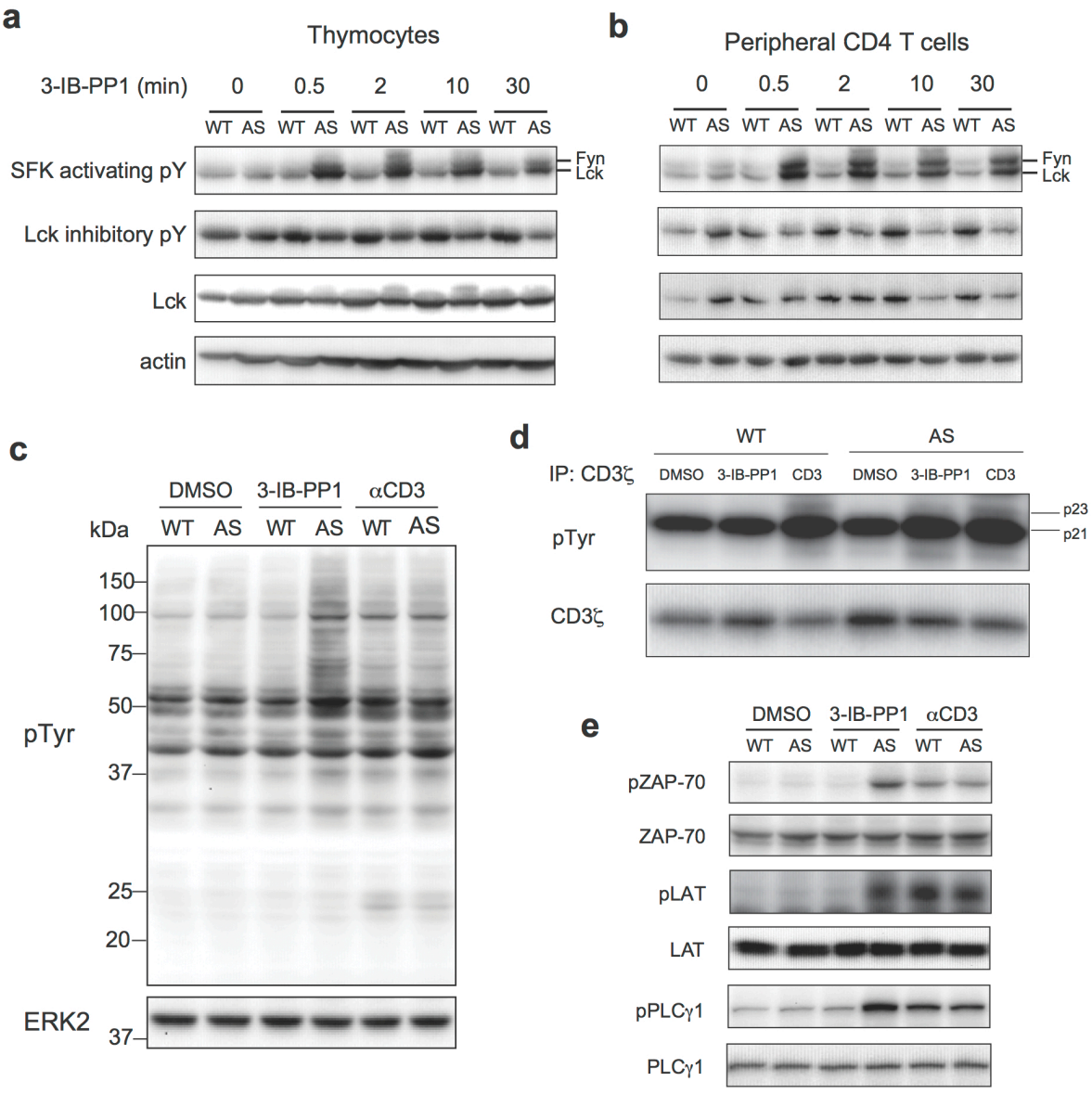

a
WT
AS
50
Normalized:
1.0
2.4
b
WT
AS
7.35
83.2
2.38
6.86
84.3
2.05
CD4
CD8
2.13
3.26
31.5
63.1
1.49
3.5
26.5
68.4
CD44
CD25
5.43
6.37
CD69
CD3
19.1
9.09
20.7
7.84
CD4
CD8
c
160
140
120
100
80
60
40
20
0
# of cells/ 10^6
WT
AS
d
Indo1-AM: violet/blue
1
0.8
0.6
0
100
200
300
Time

Figure 3.3. Inhibition of Csk^{AS} in primary murine T cells induces hyperactivation of SFKs and phosphorylation of TCR proximal signaling molecules. (a) Wildtype (WT) or Csk^{AS} (AS) thymocytes or (b**)** peripheral CD4+ T cells were treated for the indicated times with 10 mM 3-IB-PP1 and analyzed by immunoblotting. (c) Wildtype (WT) or Csk^{AS} (AS) thymocytes were treated for 3 minutes as indicated and analyzed by immunoblotting. (d) Wildtype (WT) or Csk^{AS} (AS) thymocytes were treated for 3min as indicated, then lysed and immunoprecipitated with anti-ζ antibody. Immunoprecipitates were analyzed by immunoblotting. (e) Wildtype (WT) or Csk^{AS} (AS) thymocytes were treated for 3 minutes as indicated and analyzed by immunoblotting. All data representative of at least 3 independent experiments.

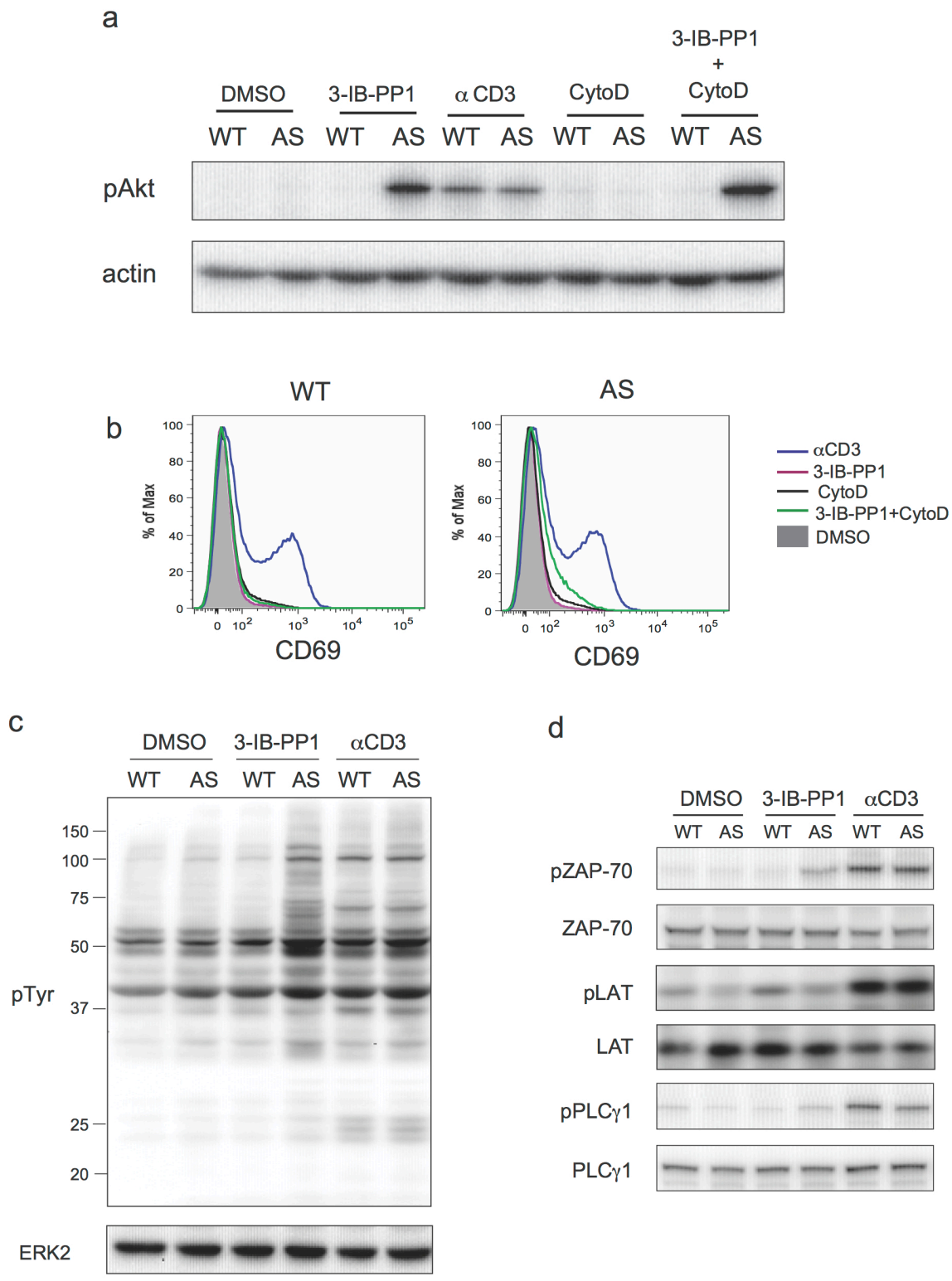

a
Thymocytes
3-IB-PP1 (min)
0
0.5
2
10
30
WT AS WT AS WT AS WT AS WT AS
SFK activating pY
Fyn
Lck
Lck inhibitory pY
Lck
actin
b
Peripheral CD4 T cells
0
0.5
2
10
30
WT AS WT AS WT AS WT AS WT AS
Fyn
Lck
c
DMSO
3-IB-PP1
αCD3
kDa
WT AS WT AS WT AS
150
100
75
50
37
25
20
pTyr
ERK2
37
d
WT
AS
IP: CD3ζ
DMSO 3-IB-PP1 CD3 DMSO 3-IB-PP1 CD3
p23
p21
pTyr
CD3ζ
e
DMSO
3-IB-PP1
αCD3
WT AS WT AS WT AS
pZAP-70
ZAP-70
pLAT
LAT
pPLCγ1
PLCγ1

imply that a subset of Lck molecules is shielded from or is inaccessible to CD45 phosphatase activity.

The activation of Lck could lead to TCR signaling initiation. Lck phosphorylates TCR CD3 and ζ-chain ITAMs, allowing for the recruitment, phosphorylation and activation of ZAP-70. Active ZAP-70 phosphorylates LAT and SLP-76, which form a supramolecular signalosome that recruits PLCγ1. Subsequent phosphorylation of PLCγ1 on tyrosine 783 by Tec family kinases leads to its activation and the hydrolysis of phosphatidylinositol-(4,5)-bisphosphate (PIP_2) (Smith-Garvin et al., 2009). The Class I Phosphoinositide-3-kinase-δ (PI3K-δ) is also activated upon TCR stimulation, and phosphorylates PIP_2 to phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) that regulates Akt phosphorylation (Gamper and Powell, 2012). In thymocytes, Lck activation in response to Csk^{AS} inhibition led to increased global tyrosine phosphorylation, including CD3ζ-p23 phosphorylation (Fig. 3.3c, d). Csk^{AS} inhibition also induced phosphorylation of ZAP-70, LAT, PLCγ1 and Akt in thymocytes, comparably to that resulting from CD3 crosslinking (Fig. 3.3e and Fig. 3.4a). The phosphorylation of both PLCγ1 and Akt is consistent with Tec kinase activation in response to Csk inhibition. Overall, our data implies that inhibiting Csk increases activation of SFKs, leading to the proximal phosphorylation events associated with TCR signaling even without receptor engagement. In contrast to the conformational change and kinetic segregation models of TCR triggering, our findings suggest that by activating SFKs, proximal signaling can be initiated even without inducing TCR conformational change or segregating the TCR from abundant large transmembrane phosphatases such as CD45.

Active PLCγ1 hydrolyses PIP_2 into diacylglycerol (DAG) and inositol triphosphate (IP_3)

Figure 3.4. Inhibition of Csk^{AS} induces tyrosine phosphorylation in primary murine T cells. (a) Thymocytes from wildtype (WT) or Csk^{AS} (AS) mice were starved of serum, treated for 3min at 37^0C as indicated, then lysed and analysed by western blot with the indicated antibodies. (b) Thymocytes from wildtype (WT) or Csk^{AS} (AS) mice were treated as indicated for 12 hours, stained for cell surface CD69 and analyzed by flow cytometry. Histograms are gated on CD4+CD8+ thymocytes. (c, d) Purified peripheral CD4+ T cells from wildtype (WT) or Csk^{AS} (AS) mice were starved of serum, treated for 3min at 37^0C as indicated, then lysed and analyzed by western blot with the indicated antibodies. (a, b and d) Data is representative of at least three independent experiments. (c) Data is representative of 2 independent experiments.

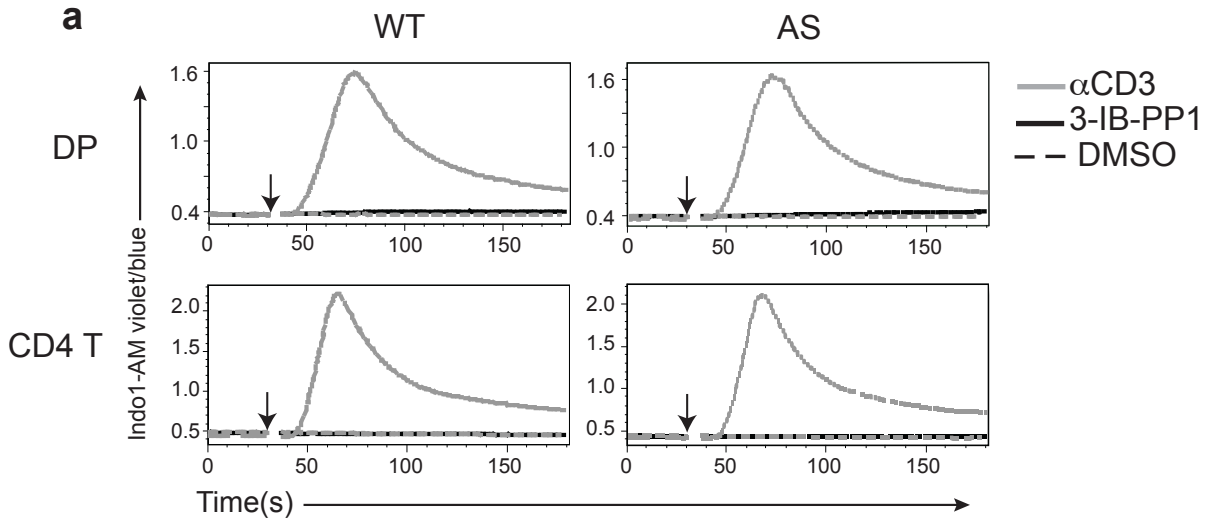

a
DMSO
3-IB-PP1
α CD3
CytoD
3-IB-PP1 + CytoD
WT AS WT AS WT AS WT AS WT AS
pAkt
actin
b
WT
AS
% of Max
CD69
αCD3
3-IB-PP1
CytoD
3-IB-PP1+CytoD
DMSO
c
DMSO
3-IB-PP1
αCD3
WT AS WT AS WT AS
150
100
75
50
37
25
20
pTyr
ERK2
d
DMSO
3-IB-PP1
αCD3
WT AS WT AS WT AS
pZAP-70
ZAP-70
pLAT
LAT
pPLCγ1
PLCγ1

that activate the Ras-ERK and the calcium signaling pathways, respectively (Smith-Garvin et al., 2009). Surprisingly, in contrast to TCR stimulation with anti-CD3, the inhibition of Csk^{AS} that led to similar increases in PLCγ1 phosphorylation in thymocytes was not associated with robust ERK phosphorylation or increased intracellular calcium (Fig. 3.5a, b). The low-level ERK phosphorylation seen in DP thymocytes was not sufficient to drive CD69 upregulation (Fig. 3.4b). The defect in ERK phosphorylation was more severe in CD4+ T cells, and this correlated with reduced ZAP-70, LAT and PLCγ1 phosphorylation relative to CD3 crosslinking (Fig. 3.5a, b and Fig. 3.4d). IP_3 is rapidly metabolized to inositol bisphosphate (IP_2) and inositol phosphate (IP_1) (Berridge, 2009). Therefore, measuring total IP production in the presence of lithium chloride, an inhibitor of IP_1 phosphatases, reflects overall hydrolysis of PIP_2 by PLCγ1. We found that total IP production in thymocytes was severely impaired following Csk^{AS} inhibition relative to TCR stimulation (Fig. 3.6a). The small increase in DAG may be sufficient to activate the Ras-GEF RasGRP1 but may not generate enough Ras-GTP to drive the positive feedback loop for ERK activation by SOS, hence resulting in the very low-level ERK phosphorylation we observed. Alternatively, the recently described PLCγ1-independent RasGRP1-ERK pathway may be involved (Kortum et al., 2013).

Altering the actin cytoskeleton restores full TCR signaling following Csk^{AS} inhibition

The cortical actin cytoskeleton restricts lateral diffusion of transmembrane molecules and actin-binding proteins can sequester PIP_2 (Kusumi et al., 2005; McLaughlin and Murray, 2005). Altering the actin cytoskeleton alone in primary B cells can activate calcium and ERK pathways (Treanor et al., 2010). We speculated that the cortical actin cytoskeleton might limit the PLCγ1 activity induced downstream of Csk^{AS} inhibition. Indeed, Csk^{AS} thymocytes treated simultaneously with 3-IB-PP1 and the actin depolymerizing agent, cytochalasin D, had increased

Figure 3.5. Increases in intracellular calcium and ERK phosphorylation are impaired following Csk^{AS} inhibition. (a) Wildtype (WT) or Csk^{AS} (AS) thymocytes or purified peripheral CD4+ T cells loaded with Indo-1AM dye were stimulated as indicated. Ratiometric assessment of intracellular calcium of CD4+CD8+ thymocytes or CD4+ T cells over time is shown. (b) Wildtype (WT) or Csk^{AS} (AS) thymocytes or splenocytes were stimulated for 2 minutes as indicated, then analyzed for pERK content. Histograms are gated on CD4+CD8+ thymocytes or CD4+ splenocytes. Shaded histogram is DMSO control. All data are representative of at least three independent experiments.


a
WT
AS
DP
CD4 T
Indo1-AM violet/blue
Time(s)
αCD3
3-IB-PP1
DMSO

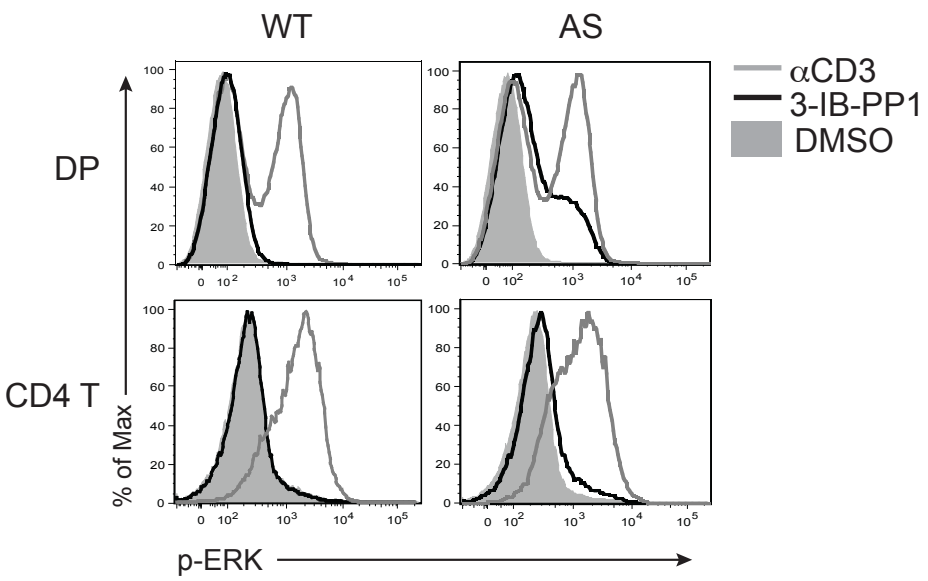
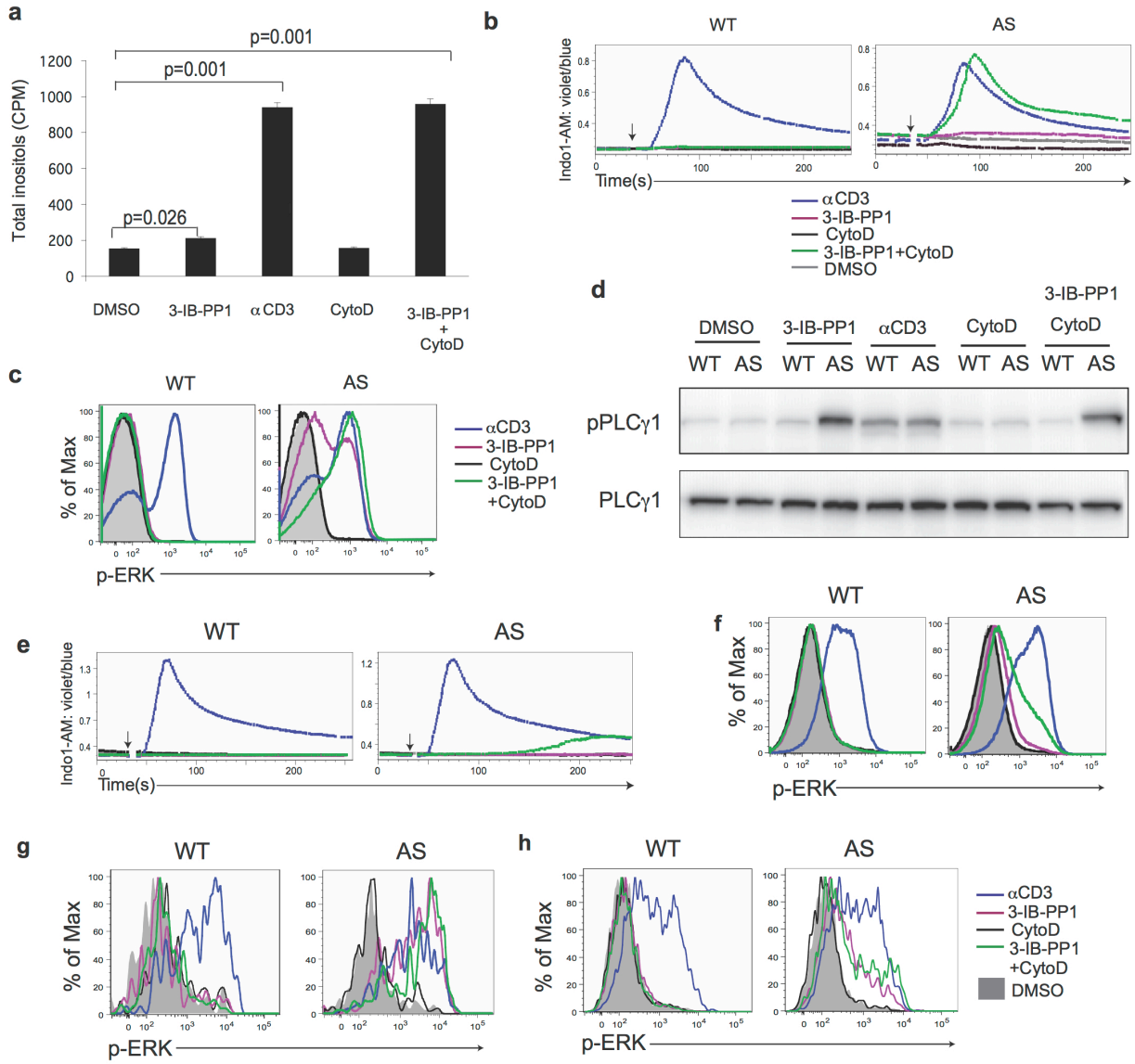

b
WT
AS
DP
CD4 T
% of Max
p-ERK
αCD3
3-IB-PP1
DMSO

Figure 3.6. Calcium flux and ERK phosphorylation are restored by altering the actin cytoskeleton following Csk^{AS} inhibition. (a) Csk^{AS}(AS) thymocytes were stimulated as indicated for 5 minutes, and total inositol phosphate content was measured (mean, ± S.E.M., n=3, p values from paired two-tailed student's t test). (b) Wildtype (WT) or Csk^{AS} (AS) thymocytes were stimulated as indicated. Ratiometric assessment of intracellular calcium of CD4+CD8+ thymocytes over time is shown. (c) Wildtype (WT) or Csk^{AS} (AS) thymocytes were stimulated for 2 minutes as indicated, then analyzed for pERK content. Histograms are gated on CD4+CD8+ thymocytes. Shaded histogram is DMSO control. (d) Wildtype (WT) or Csk^{AS} (AS) thymocytes were treated for 3 minutes as indicated and analyzed by immunoblotting. (e) Wildtype (WT) or Csk^{AS} (AS) splenocytes were stimulated as indicated. Ratiometric assessment of intracellular calcium of CD4+ splenocytes over time is shown. (f) Wildtype (WT) or Csk^{AS} (AS) splenocytes were stimulated for 2 minutes as indicated, then analyzed for pERK content. Histograms are gated on CD4+ splenocytes. Shaded histogram is DMSO control. (g) Wildtype (WT) or Csk^{AS} (AS) thymocytes or (h) purified splenic T cells were stimulated for 2 minutes as indicated, then analyzed for pERK content. Histograms are gated on CD4- CD8- γδ TCR+ cells. Shaded histogram is DMSO control. All data are representative of at least three independent experiments.


a
Total inositols (CPM)
p=0.001
p=0.001
p=0.026
DMSO
3-IB-PP1
αCD3
CytoD
3-IB-PP1 + CytoD
b
WT
AS
Indo1-AM: violet/blue
Time(s)
αCD3
3-IB-PP1
CytoD
3-IB-PP1+CytoD
DMSO
c
WT
AS
% of Max
p-ERK
αCD3
3-IB-PP1
CytoD
3-IB-PP1 +CytoD
d
DMSO
3-IB-PP1
αCD3
CytoD
3-IB-PP1
CytoD
WT AS WT AS WT AS WT AS WT AS
pPLCγ1
PLCγ1
e
WT
AS
Indo1-AM: violet/blue
Time(s)
f
WT
AS
% of Max
p-ERK
g
WT
AS
% of Max
p-ERK
h
WT
AS
% of Max
p-ERK
αCD3
3-IB-PP1
CytoD
3-IB-PP1 +CytoD
DMSO

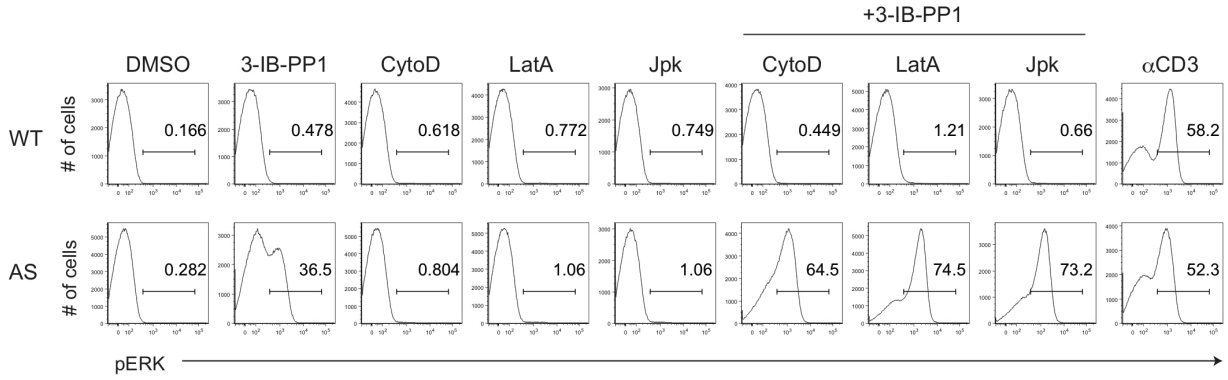
total IP, intracellular calcium and ERK phosphorylation that was comparable to that seen with CD3 crosslinking (Fig. 3.6a-c). In contrast, treatment of Csk^{AS} thymocytes with cytochalasin D alone did not result in an increase in intracellular calcium or ERK phosphorylation. Similar responses were observed with other actin-modifying agents (Fig. 3.7). The striking increase in PIP_2 hydrolysis and downstream responses induced by altering the actin cytoskeleton was not accounted for by changes in PLCγ1 phosphorylation (Fig. 3.6d). These data suggest that actin cytoskeletal remodeling plays a crucial role in downstream TCR signaling events in thymocytes by controlling the access of PLCγ1 to PIP_2. In contrast, Akt phosphorylation was induced by Csk^{AS} inhibition alone, indicating that PI3K access to PIP_2 and Akt access to PIP_3 were not as strongly restricted by the actin cytoskeleton.

On the other hand, altering the actin cytoskeleton in peripheral CD4+ T cells only partially restored the defect in intracellular calcium increase and ERK phosphorylation following Csk^{AS} inhibition (Fig. 3.6e, f). Combined with our earlier observation that proximal phosphorylation induced by Csk^{AS} inhibition in peripheral CD4+ T cells was reduced (Fig. 3.4d), it appears that additional regulatory mechanisms for signal initiation develop as T cells mature. In support of this possibility, we found that although Csk^{AS} inhibition alone induced strong ERK phosphorylation in immature thymic γδ T cells, this responsiveness was greatly reduced in mature splenic γδ T cells, which behaved similarly to peripheral CD4+ T cells (Fig. 3.6g, h).

Mature dendritic cells restore full TCR signaling following Csk^{AS} inhibition

We sought to determine if there was a physiologic costimulus that could induce the requisite cytoskeletal remodeling in thymocytes that was not achieved in response to SFK

Figure 3.7. Simultaneous alteration of the actin cytoskeleton enhances ERK phosphorylation in thymocytes during inhibition of Csk^{AS}. Thymocytes from wildtype (WT) or Csk^{AS} (AS) mice were serum-starved, stimulated for 2 min at 37^0C as indicated, then analysed for pERK content by phosphoflow. Histograms are gated on CD4+CD8+ thymocytes. Data is representative of two independent experiments. CytoD, cytochalasin D; LatA, latrunculinA; Jpk, jasplakinolide.


+3-IB-PP1
DMSO
3-IB-PP1
CytoD
LatA
Jpk
CytoD
LatA
Jpk
αCD3
WT
AS
# of cells
0.166
0.478
0.618
0.772
0.749
0.449
1.21
0.66
58.2
0.282
36.5
0.804
1.06
1.06
64.5
74.5
73.2
52.3
pERK

activation by Csk^{AS} inhibition alone. In vivo, thymocytes initiate TCR signaling when they recognize pMHC molecules on antigen presenting cells (APCs) such as thymic epithelial cells or dendritic cells (DCs). APCs also express numerous costimulatory molecules that can provide additional signals. The TCR signal together with costimulatory signals dictate thymocyte developmental fate (Klein et al., 2009). Remarkably, interaction of the polyclonal thymocytes with activated but not naïve splenic DCs, in thymocyte-DC conjugates, enhanced ERK phosphorylation and increased intracellular calcium in Csk^{AS} thymocytes when Csk^{AS} was inhibited (Fig. 3.8a, b and Fig. 3.9a). Since in vitro activated splenic DCs may differ from thymic DCs (Proietto et al., 2008), the physiologic APCs for thymocytes, we tested the ability of thymic DCs to enhance ERK phosphorylation induced by Csk^{AS} inhibition and they did, albeit to a lesser extent (Fig. 3.9b).

B7 and MHC on mature DCs are required to restore full TCR signaling following Csk^{AS} inhibition

We postulated that DCs restored full activation of TCR signaling leading to calcium increase and ERK activation in response to Csk^{AS} inhibition by providing a costimulatory signal that initiated cytoskeletal remodeling. Using a candidate approach, we tested the role of several pathways known to influence actin remodeling during T cell-APC interactions (Burkhardt et al., 2008). The diminished ability of activated B7.1/B7.2 doubly deficient DCs to enhance 3-IB-PP1-induced ERK phosphorylation implicated the importance of their receptor CD28 (Fig. 3.10a). In contrast, activated intercellular adhesion molecule-1 (ICAM-1)-deficient DCs enhanced 3-IB-PP1-induced ERK phosphorylation as potently as wildtype DCs, suggesting the T cell integrin

Figure 3.8. Mature DCs restore calcium flux and ERK phosphorylation following Csk^{AS} inhibition. (a) DCs enriched from wildtype splenocytes were activated by overnight culture. Thymocytes from wildtype (WT) or Csk^{AS}(AS) mice were pelleted with or without activated DCs at a 1:1 ratio and stimulated as indicated for 3 minutes. Cells were then analyzed for pERK content. Histograms shown are gated on CD4+CD8+ thymocytes. (b) Wildtype (WT) or Csk^{AS} (AS) thymocytes were loaded with Indo1-AM dye and surface stained for CD4 and CD8. Activated DCs were prepared as in (a), and surface stained for $CD11^c$. Thymocytes and activated DCs were mixed at a 1:1 ratio and stimulated for 5 minutes with 3-IB-PP1. Cells collected were first gated for CD4+CD8+ multiplets, then further separated into thymocyte-thymocyte conjugates ($CD11^c$-) and thymocyte-DC conjugates ($CD11^c$+) before ratiometric assessment of intracellular calcium over time. All data are representative of at least three independent experiments.

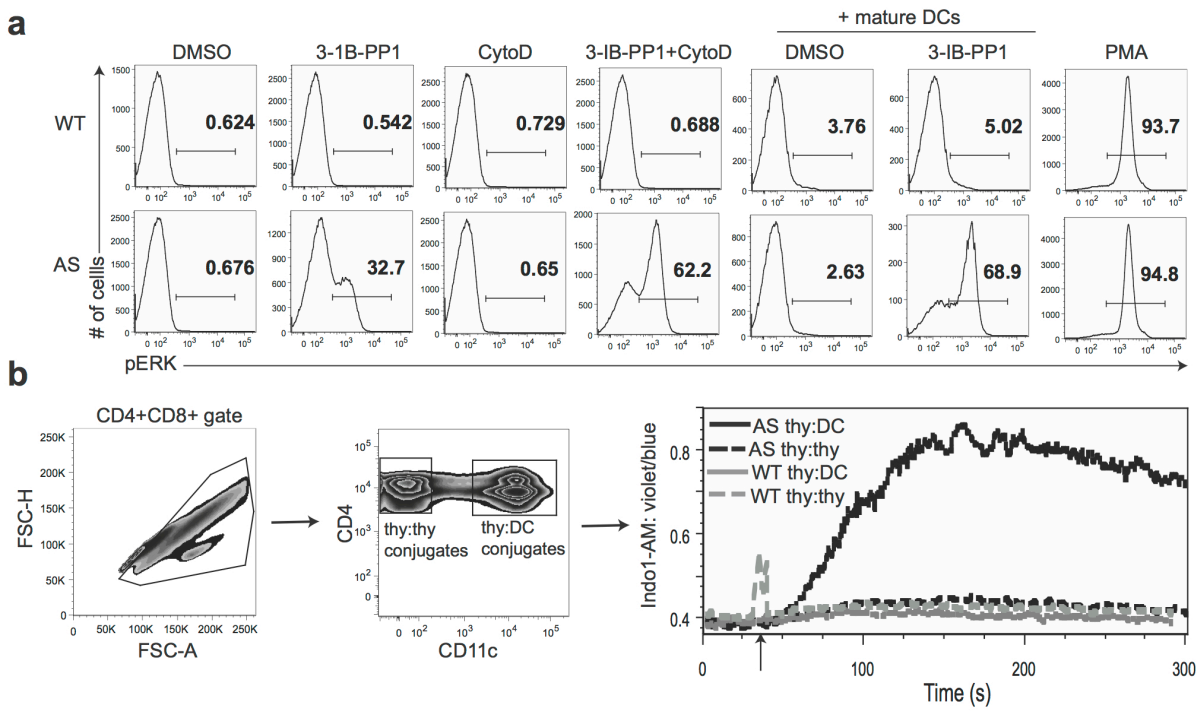

a
+ mature DCs
DMSO
3-1B-PP1
CytoD
3-IB-PP1+CytoD
DMSO
3-IB-PP1
PMA
WT
AS
0.624
0.542
0.729
0.688
3.76
5.02
93.7
0.676
32.7
0.65
62.2
2.63
68.9
94.8
# of cells
pERK
b
CD4+CD8+ gate
FSC-H
FSC-A
CD4
CD11c
thy:thy conjugates
thy:DC conjugates
Indo1-AM: violet/blue
AS thy:DC
AS thy:thy
WT thy:DC
WT thy:thy
Time (s)

Figure 3.9. Thymic DCs, but not naïve splenic DCs, ICAM-1 deficient DCs or chemokines secreted by mature splenic DCs, enhance ERK phosphorylation in DP thymocytes during Csk^{AS} inhibition. (a) Naïve DCs were enriched from wildtype splenocytes by $CD11^c$ positive selection and used immediately. Data is representative of two independent experiments. (b) Thymic DCs were sorted from wildtype thymi as described in methods. Data is representative of three independent experiments. (c) DCs were enriched from ICAM-1 deficient splenocytes and activated by overnight culture. (d) Thymocytes from Csk^{AS} mice were treated with DMSO or 50ng/mL pertussis toxin (PTx) for 1h. Data is representative of two independent experiments. (a,b and d) Thymocytes from Csk^{AS} mice or (c) wildtype (WT) and Csk^{AS} (AS) mice were pelleted with or without DCs at a 1:1 ratio and stimulated as indicated at 37^0C for 3min. Cells were then analyzed for pERK content by phosphoflow. Histograms shown are gated on CD4+CD8+ thymocytes.

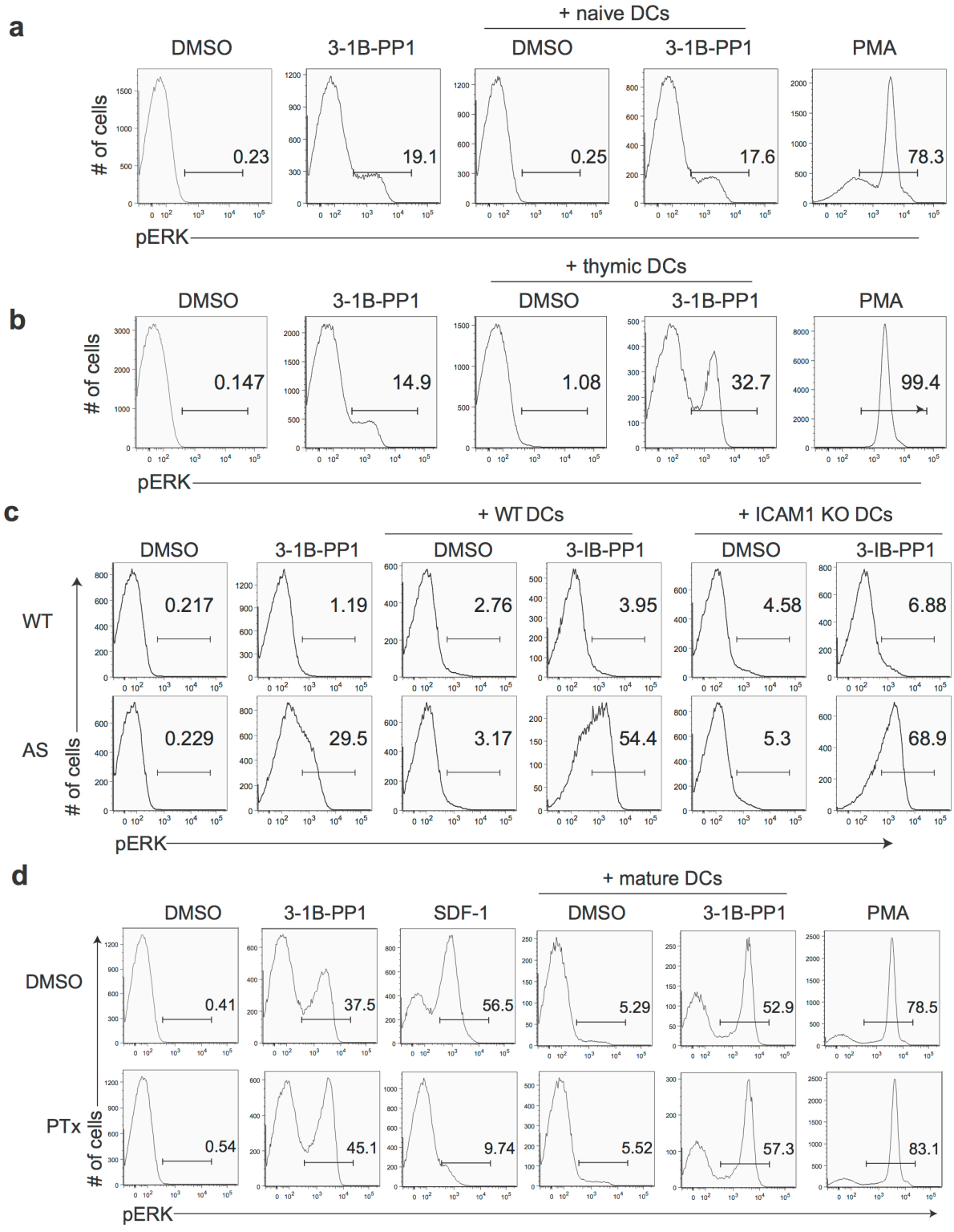
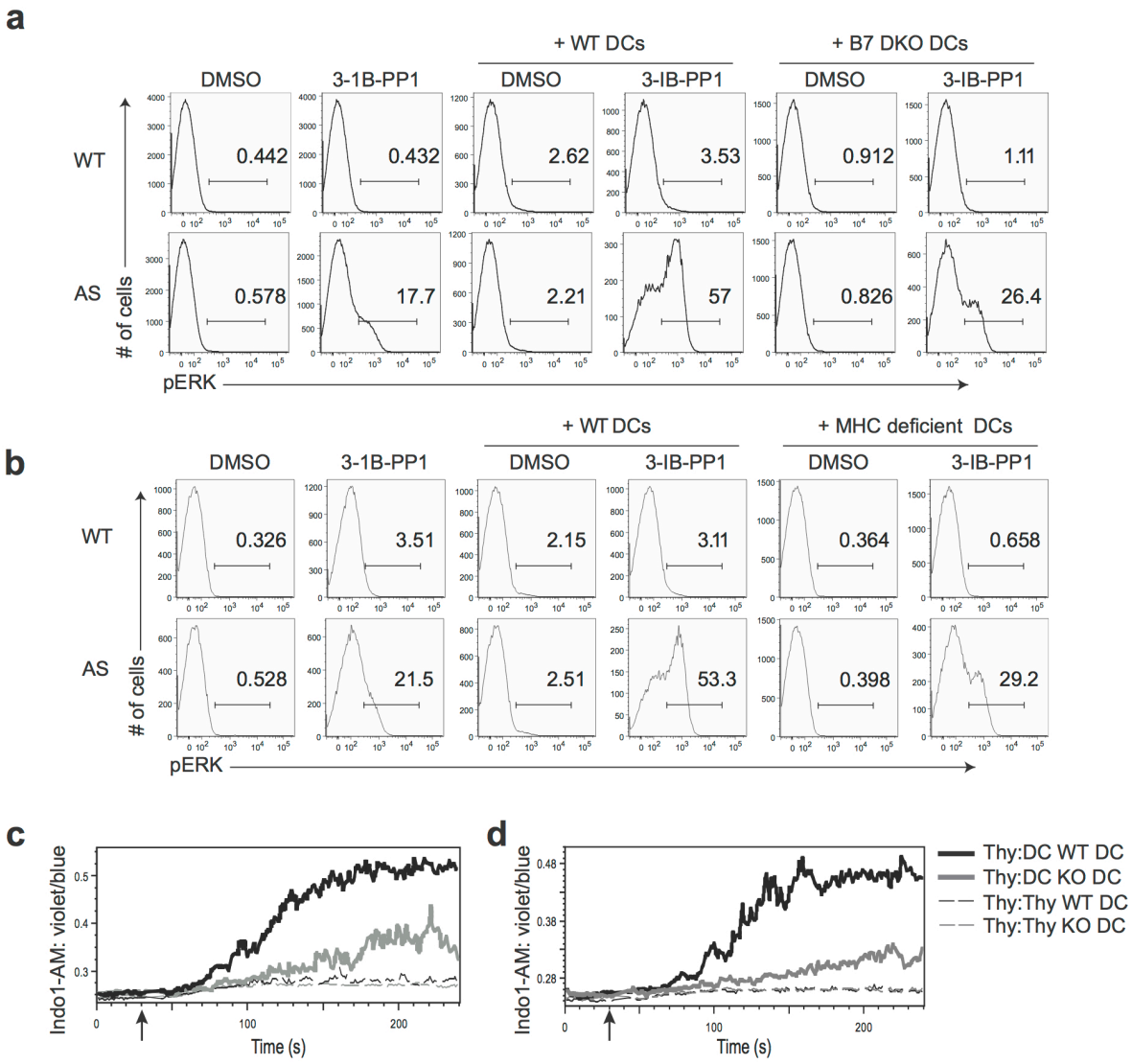

a
+ naive DCs
DMSO
3-1B-PP1
DMSO
3-1B-PP1
PMA
# of cells
0.23
19.1
0.25
17.6
78.3
pERK
b
+ thymic DCs
DMSO
3-1B-PP1
DMSO
3-1B-PP1
PMA
# of cells
0.147
14.9
1.08
32.7
99.4
pERK
c
+ WT DCs
+ ICAM1 KO DCs
DMSO
3-1B-PP1
DMSO
3-IB-PP1
DMSO
3-IB-PP1
WT
AS
# of cells
0.217
1.19
2.76
3.95
4.58
6.88
0.229
29.5
3.17
54.4
5.3
68.9
pERK
d
+ mature DCs
DMSO
3-1B-PP1
SDF-1
DMSO
3-1B-PP1
PMA
DMSO
PTx
# of cells
0.41
37.5
56.5
5.29
52.9
78.5
0.54
45.1
9.74
5.52
57.3
83.1
pERK

Figure 3.10. Restoration of full TCR signalling upon Csk^{AS} inhibition requires both MHC and B7 on DCs. DCs enriched from wildtype or (a) B7 double knockout or (b) MHCI and MHCII deficient splenocytes were activated by overnight culture. Wildtype (WT) or Csk^{AS} (AS) thymocytes were pelleted with or without activated DCs at a 1:1 ratio and stimulated as indicated for 3 minutes. Cells were then analyzed for pERK content. Histograms shown are gated on CD4+CD8+ thymocytes. (c, d**)** Wildtype (WT) or Csk^{AS} (AS) thymocytes were loaded with Indo1-AM dye and surface stained for CD4 and CD8. Activated DCs from (c) B7 double knockout or (d) MHCI and MHCII deficient splenocytes were surface stained for $CD11^c$. Thymocytes and activated DCs were mixed at a 1:1 ratio and stimulated with 3-IB-PP1. Cells collected were first gated for CD4+CD8+ multiplets, then further separated into thymocyte-thymocyte conjugates ($CD11^c$-) and thymocyte-DC conjugates ($CD11^c$+) before ratiometric assessment of intracellular calcium over time. All data are representative of at least three independent experiments.


a
+ WT DCs
+ B7 DKO DCs
DMSO
3-1B-PP1
DMSO
3-IB-PP1
DMSO
3-IB-PP1
WT
AS
0.442
0.432
2.62
3.53
0.912
1.11
0.578
17.7
2.21
57
0.826
26.4
# of cells
pERK
b
+ WT DCs
+ MHC deficient DCs
DMSO
3-1B-PP1
DMSO
3-IB-PP1
DMSO
3-IB-PP1
WT
AS
0.326
3.51
2.15
3.11
0.364
0.658
0.528
21.5
2.51
53.3
0.398
29.2
# of cells
pERK
c
Indo1-AM: violet/blue
Time (s)
d
Indo1-AM: violet/blue
Time (s)
Thy:DC WT DC
Thy:DC KO DC
Thy:Thy WT DC
Thy:Thy KO DC

lymphocyte function-associated antigen 1 (LFA-1) was dispensable (Fig. 3.9c). Likewise, inhibition of Gi-protein coupled receptor signaling in thymocytes by using pertussis toxin had no effect, arguing against the involvement of chemokine receptors (Fig. 3.9d). Most Lck in thymocytes is associated with CD4 or CD8 coreceptors and by docking with MHC, they recruit coreceptor-bound Lck to the TCR (Veillette et al., 1988). We found that MHCI and MHCII doubly deficient activated DCs had diminished ability to enhance ERK phosphorylation upon 3-IB-PP1 treatment (Fig. 3.10b). In agreement with their reduced ability to enhance ERK phosphorylation, we observed that activated B7.1/B7.2 doubly deficient or MHCI and MHCII doubly deficient DCs had reduced ability to induce intracellular calcium increase in Csk^{AS} thymocytes treated with 3-IB-PP1 (Fig 3.10c, d). Thus, our data reveal that activated DCs require both B7 and MHC to restore full TCR signaling in thymocytes leading to calcium and ERK responses when Csk^{AS} is inhibited. This is consistent with our observation that naïve DCs could not restore full TCR signaling (Fig. 3.9a), since B7 and MHC molecules are upregulated during DC maturation (Steinman, 2012).

CD28 engagement induces actin remodeling and is sufficient to restore robust ERK phosphorylation following Csk^{AS} inhibition

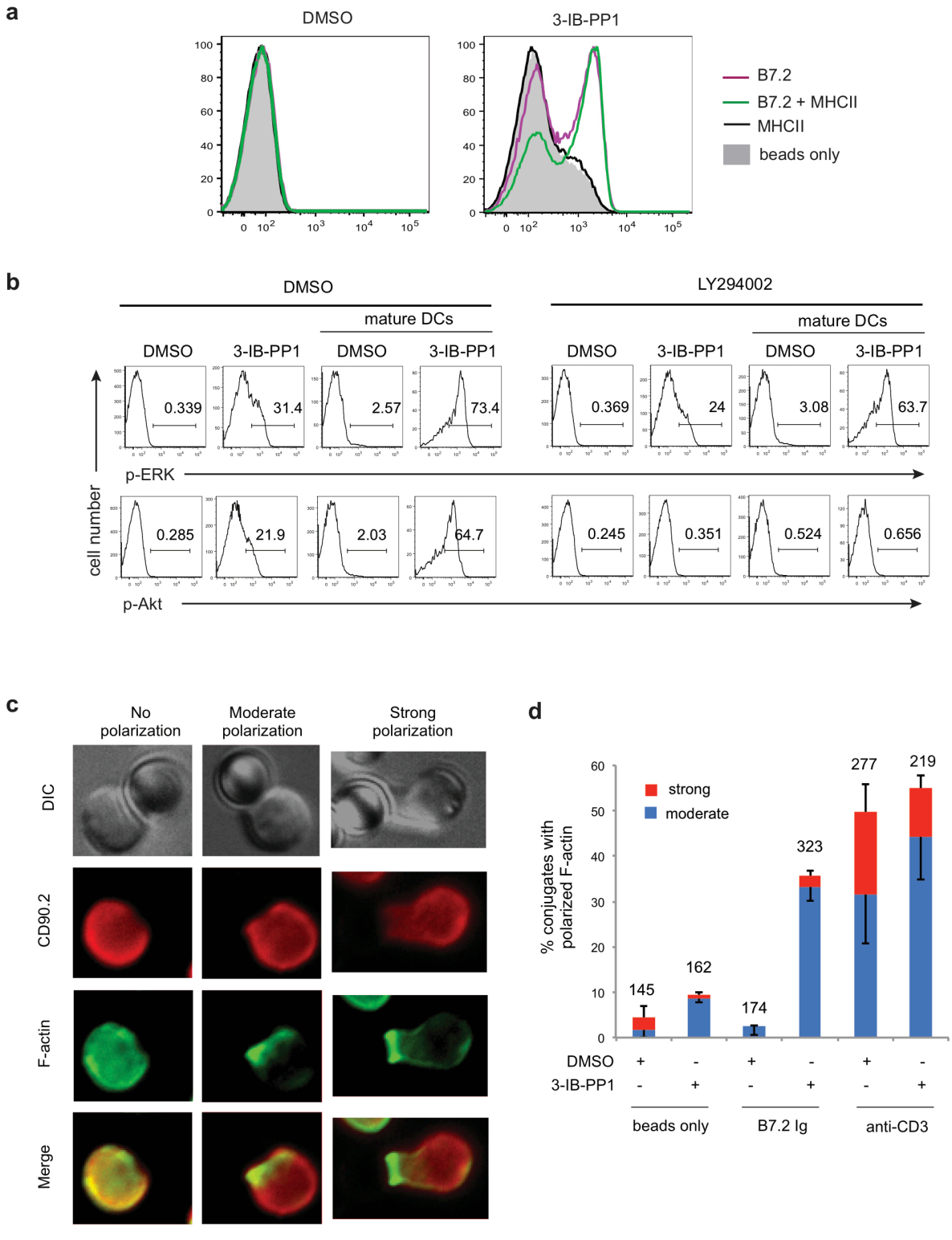
We next examined whether providing CD28/B7 interaction alone is sufficient to enhance ERK phosphorylation following Csk^{AS} inhibition. Indeed, inhibition of Csk^{AS} in thymocytes in the presence of B7.2 Ig coated beads increased ERK phosphorylation. Moreover, coating the beads with both B7.2 and class II MHC tetramer further increased the level of ERK phosphorylation, even though class II MHC tetramer alone did not boost ERK phosphorylation (Fig. 3.11a). This provides evidence that CD28/B7 induced signals are sufficient to restore robust

ERK signaling following Csk^{AS} inhibition, particularly when MHC-mediated interactions are also present.

We hypothesized that the B7/CD28 interaction provided by activated DCs restored full TCR signaling by inducing actin remodeling in thymocytes. CD28 signaling has been shown to induce both PI3K activation and actin remodeling (Boomer and Green, 2010). The YMNM motif in its cytoplasmic tail recruits the p85 subunit of PI3K. In addition, the actin cytoskeletal regulators Vav-1, cofilin-1 and Rltpr are activated downstream of CD28 (Liang et al., 2013). To determine if PI3K signaling plays a role in the restoration of full TCR signaling by activated DCs, we pre-treated thymocytes with the PI3K inhibitor LY294002 before Csk^{AS} inhibition in the presence of activated DCs. We found that although Akt phosphorylation was completely abrogated as expected, the enhancement in ERK phosphorylation was unaffected, suggesting that PI3K signaling was not important for this effect (Fig. 3.11b).

We then explored the role of CD28 signaling in actin remodeling by imaging the F-actin distribution of thymocytes conjugated to B7.2 Ig coated beads and treated with 3-IB-PP1. We observed F-actin polarization and accumulation at the thymocytes-B7.2 Ig bead interface only when Csk^{AS} was inhibited (Fig. 3.11c,d). In contrast, 3-IB-PP1 alone induced only a negligible amount of F actin polarization, indicating that this actin remodeling required both CD28 engagement and SFK activation.

Figure 3.11. CD28 signaling induces actin remodeling and restores robust ERK phosphorylation following Csk^{AS} inhibition. (a) Csk^{AS} thymocytes were pelleted with styrene beads coated with B7.2 Ig (B7.2) and/or I-A^{b} tetramer (MHCII) and treated for 3 minutes with DMSO or 10μM 3-IB-PP1 and then analyzed for pERK content. Histograms shown are gated on CD4+CD8+ thymocytes. (b) Csk^{AS} thymocytes were pretreated for 15 minutes with DMSO or 10μM LY294002, then pelleted with or without activated WT DCs at a 1:1 ratio and stimulated as indicated for 3 minutes. Cells were then analyzed for pERK and pAkt content. Histograms shown are gated on CD4+CD8+ thymocytes. (c, d) Csk^{AS} thymocytes were pelleted with styrene beads coated as indicated and treated for 3 minutes with DMSO or 10μM 3-IB-PP1. Bead-thymocyte conjugates were imaged for F-actin distribution. (c) Representative images show the different F-actin distribution patterns observed. (d) Graph shows the percentage of conjugates with moderate and strong F- actin polarization at the bead-thymocyte interface (means, ± S.E.M., n=3). Total number of conjugates analyzed for each condition is indicated on top of the bars.

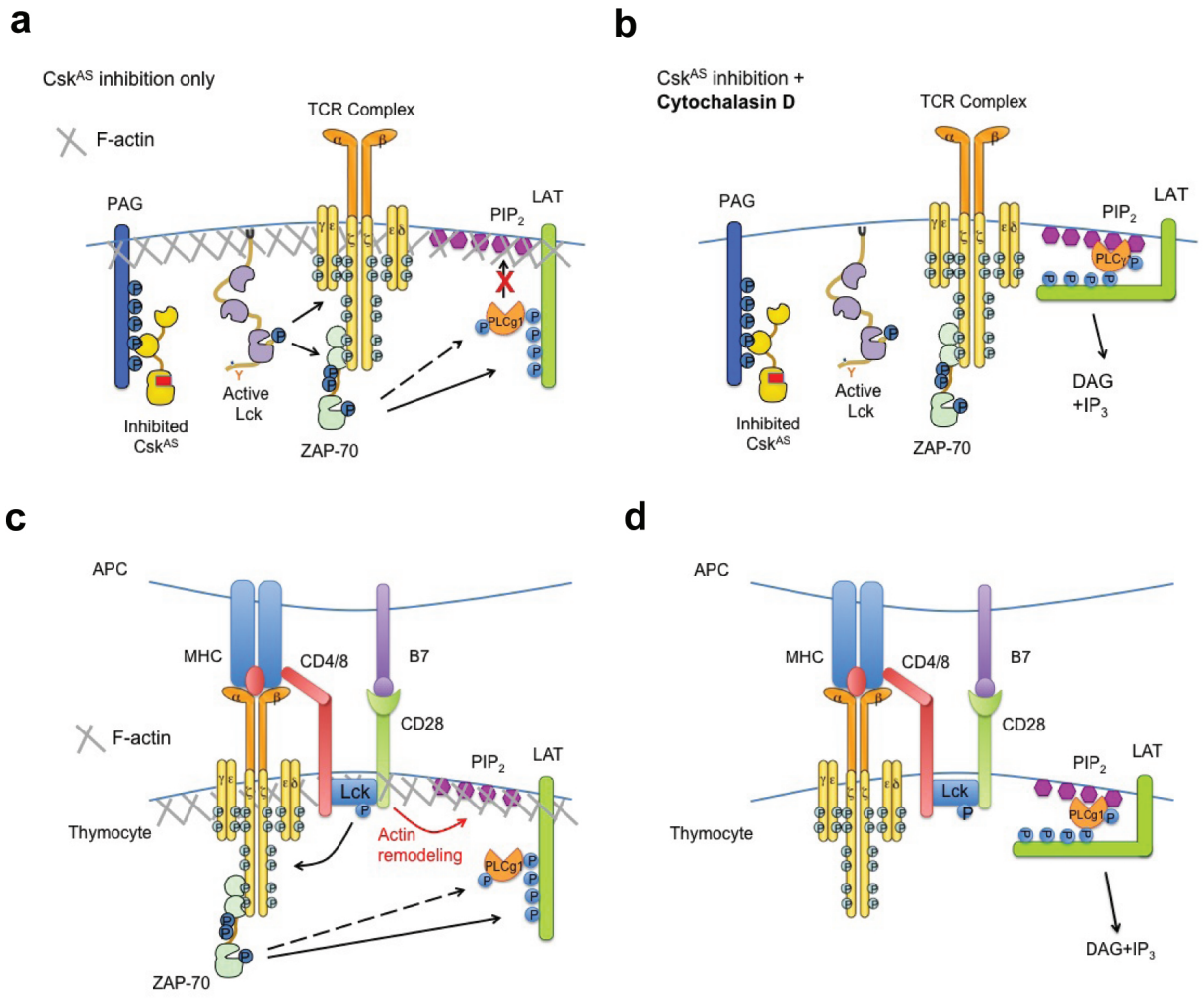

a
DMSO
3-IB-PP1
B7.2
B7.2 + MHCII
MHCII
beads only
b
DMSO
LY294002
mature DCs
DMSO
3-IB-PP1
0.339
31.4
2.57
73.4
0.369
24
3.08
63.7
p-ERK
cell number
0.285
21.9
2.03
64.7
0.245
0.351
0.524
0.656
p-Akt
c
No polarization
Moderate polarization
Strong polarization
DIC
CD90.2
F-actin
Merge
d
% conjugates with polarized F-actin
strong
moderate
145
162
174
323
277
219
DMSO
3-IB-PP1
beads only
B7.2 Ig
anti-CD3

Discussion

In this study, we have generated mice expressing Csk^{AS}, allowing for genetically specific and rapid inhibition of Csk kinase activity in cells. This has enabled us to elucidate for the first time, the function of Csk kinase activity in the initiation of TCR signaling in unmanipulated primary T cells. Following Csk^{AS} inhibition in thymocytes, we observed rapid hyperactivation of Lck and Fyn T, which in turn induced the phosphorylation of proximal TCR signaling componenents up to and including PLCγ1. We propose that a dynamic equilibrium of Csk and CD45 activity controls SFK activity, and is responsible for maintaining the basal unstimulated state of the most proximal components of the TCR signaling pathway. Such an ongoing equilibrium may confer rapid plasticity of Lck activation status, allowing for sensitive and efficient TCR triggering in response to receptor engagement. Our results also suggest that Csk plays the dominant inhibitory role in controlling the basal state and may largely be responsible for setting the threshold for TCR signaling. Inhibition of Csk function without CD45 perturbation or induction of TCR-CD3 conformational change can therefore play a prominent physiologic role in allowing SFKs to be activated and initiate downstream TCR-dependent signaling (Fig. 3.12a). Physiologically, local changes induced by ligating the TCR can be envisioned to influence this equilibrium through various mechanisms that might influence Csk, CD45 or Lck (or Fyn) localization. ITAM accessibility seems to be less of a concern since the TCRζ chain is already largely constitutively phosphorylated in vivo in the basal state, albeit there is a modest increase in its phosphorylation following receptor stimulation.

Notably, our results also suggest that activation of SFK activity alone is insufficient to initiate the full downstream signaling pathway leading to calcium and ERK responses, events critical for cellular activation. In thymocytes, downstream pathway activation requires events

Figure 3.12. Model for TCR signal initiation and propagation in thymocytes. (a) Perturbing the Csk-CD45 equilibrium regulating Lck by inhibiting Csk^{AS} results in potent Lck activation, thereby initiating phosphorylation of CD3 and ζ ITAMS and ZAP-70, and eventually activation of PLCγ1 bound to the LAT signalosome. However, active PLCγ1 bound to LAT cannot access plasma membrane PIP_2 to hydrolyze it because the dense cortical actin meshwork acts as a barrier. (b) Disrupting the cortical actin with cytochalasin D allows active PLCγ1 bound to LAT to access and hydrolyze PIP_2 to generate DAG and IP_3. (c) During a thymocyte-APC interaction, TCR engagement by pMHC recruits CD4/8 coreceptor bound active Lck. Association of B7 engaged CD28 with Lck allows for activation of local actin cytoskeletal remodeling. (d) This enables active PLCγ1 bound to LAT to hydrolyse PIP_2.


a
Csk^AS inhibition only
F-actin
TCR Complex
PAG
PIP2
LAT
Inhibited Csk^AS
Active Lck
ZAP-70
PLCg1
b
Csk^AS inhibition +
Cytochalasin D
TCR Complex
PAG
PIP2
LAT
PLCγ
Inhibited Csk^AS
Active Lck
ZAP-70
DAG +IP3
c
APC
MHC
CD4/8
B7
CD28
F-actin
PIP2
LAT
Lck
Thymocyte
Actin remodeling
PLCg1
ZAP-70
d
APC
MHC
CD4/8
B7
CD28
PIP2
LAT
Lck
Thymocyte
PLCg1
DAG+IP3

that lead to actin cytoskeletal remodeling to allow PLCγ1 access to PIP_2 (Fig. 3.12b). Thus, the actin cytoskeleton can provide a barrier to control the transmission of downstream signaling events. The required actin remodeling to overcome this barrier may be accomplished experimentally ex-vivo by ligation of TCR with very high concentrations of mAbs and pMHC complexes. Under physiologic conditions where TCR stimuli is limiting, the function of other coreceptors or costimulatory molecules, such as CD28, that influence actin cytoskeletal remodeling, is likely necessary for PLCγ1 to access its substrate.

The requirement for actin remodeling in primary thymocytes to initiate full TCR signaling is in contrast to our previous findings in Jurkat T cells (Schoenborn et al., 2011). Inhibiting Csk in Jurkat T cells, in the absence of TCR engagement and additional actin cytoskeletal manipulation, resulted in substantial intracellular calcium increases, ERK phosphorylation and the upregulation of CD69. One reason may be that the cortical actin cytoskeletal dynamics of Jurkat T cells and primary T cells are different. Jurkat T cells lack expression of PTEN and SHIP-1 phosphatases that dephosphorylate PIP_3, and most likely have an abnormal phosphatidlyinositol composition in their plasma membrane (Abraham and Weiss, 2004). This could in turn have a profound impact on their cortical actin cytoskeleton, since PIP_2 and PIP_3 regulate F-actin assembly in cells by binding and influencing the activity of numerous actin regulatory proteins (Di Paolo and De Camilli, 2006).

Another notable difference we observed in response to Csk inhibition was between thymocytes and mature CD4+ T cells. Similar to thymocytes, mature CD4+ T cells displayed strong SFK activation after 3-IB-PP1 treatment, but in contrast to thymocytes, the phosphorylation of ZAP-70, LAT and PLCγ1 in was reduced compared to anti-CD3 crosslinking. Furthermore, altering the actin cytoskeleton in mature CD4+ T cells only partially

restores the calcium and MAPK signaling following Csk^{AS} inhibition. We observed similar differences in the response to Csk^{AS} inhibition between immature thymic γδ T cells and mature splenic γδ T cells. There are likely additional negative regulatory pathways acquired during development, perhaps involving phosphatases or inhibitory receptors, which have to be overcome in mature T cells. Alternatively, mature T cells may have differential regulation of their cortical actin cytoskeletons. Interestingly, thymic γδ T cells were able to phosphorylate ERK robustly without the need for additional actin remodeling stimulus following Csk^{AS} inhibition, further underscoring the fact that different T cell subsets have distinct requirements for signal initiation, most likely due to differences in the wiring of their signaling networks.

Actin cytoskeletal dynamics play multiple important roles in T cell activation and have been best studied in the context of the immunological synapse (IS), a supramolecular structure found at the contact site between a T cell and an APC, and is proposed to be important for prolonging and modulating TCR signaling. F-actin first accumulates in the T cell-APC interface and is then followed by a central clearing. Disruption of the actin cytoskeleton with cytochalasin D impairs formation of TCR microclusters and the IS (Burkhardt et al., 2008). Numerous pathways downstream of TCR or costimulation that are involved in actin remodeling and important for IS formation have been identified. On the other hand, exactly how actin dynamics influence TCR signaling remains elusive. Stimulating cytochalasin D treated T cells with superantigen-pulsed APCs leads to reduced calcium flux but stimulating the same T cells with anti-CD3 leads to prolonged calcium flux (Valitutti et al., 1995). Our results clearly reveal a previously unappreciated direct role for actin cytoskeletal dynamics in the initiation of calcium and MAPK signaling in thymocytes.

Recently, Treanor et al. demonstrated that merely altering the actin cytoskeleton in primary naïve B cells results in a spontaneous increase in intracellular calcium that is dependent on antigen receptor signaling (Treanor et al., 2010). In contrast, in primary thymocytes, we have shown that altering the actin cytoskeleton alone did not initiate any TCR signaling events and that additional activation of SFKs was required for full TCR signaling. This difference between T and B cells may reflect their differing dependence on basal signaling for peripheral homeostasis and suggests that initiation of TCR signaling is more tightly regulated (Kraus et al., 2004; Polic et al., 2001). Treanor et al. proposes that in B cells, actin cytoskeletal remodeling allows for greater BCR mobility and increases its interactions with downstream signaling molecules such as CD19. This is unlikely to be the case in thymocytes, since SFK activation alone was sufficient to induce proximal phosphorylation events. In thymocytes, it appears that the predominant role of the cortical actin cytoskeleton is in preventing PLCγ1 from accessing PIP_2.

Lending physiologic significance to our finding that actin cytoskeletal remodeling was required for full TCR signaling was our observation that activated DCs could restore calcium and Ras-ERK signaling upon Csk^{AS} inhibition in thymocytes. This required the presence of B7 costimulatory molecules on the DCs. Indeed, providing CD28 costimulation alone during Csk^{AS} inhibition was sufficient to enhance ERK phosphorylation. Surface levels of CD28 are highest on thymocytes and loss of CD28 results in impaired negative selection (Buhlmann et al., 2003; Gross et al., 1992). CD28 signaling induces pathways including PI3K activation and actin cytoskeletal remodeling (Boomer and Green, 2010). Our results demonstrate that the CD28-dependent rescue of Ras-ERK signaling does not require PI3K activation. Moreover, we show that CD28 signaling induces actin remodeling and polarization of the cortical actin cytoskeleton

in thymocytes upon Csk^{AS} inhibition. Whether the function of CD28 costimulation is to provide a qualitatively different input to complement TCR signals or serves to quantitatively enhance TCR signal strength has remained an open question. Our data provides evidence supporting the idea that CD28 costimulation delivers a unique actin cytoskeletal remodeling signal that is required for the initiation of complete TCR signaling.

Finally, we propose the following model for TCR signal propagation in thymocytes (Supplementary Fig. 3.12c, d): When a thymocyte encounters an APC bearing its cognate pMHC, active Lck initiates a tyrosine phosphorylation cascade that leads to the phosphorylation and activation of PLCγ1. Simultaneously, because Lck interacts with and phosphorylates CD28 (Holdorf et al., 1999), the costimulatory CD28:B7 interaction delivers local actin remodeling signals at the site of active TCR signaling. This enables LAT-bound PLCγ1 to come into sufficient proximity to PIP_2, perhaps because the LAT cytoplasmic tail associated with PLCγ1 can now access the plasma membrane or because PIP_2 is released from binding with the numerous actin regulatory proteins it associates with (Di Paolo and De Camilli, 2006; Yin and Janmey, 2003). Alternatively, it has been shown that TCR stimulation results in PLCγ1 translocation to the cortical actin cytoskeleton (Patsoukis et al., 2009). Actin turnover may therefore enhance PLCγ1 recruitment to the membrane, and thus position it close to PIP_2. PIP_2 hydrolysis ensures further propagation of the proximal TCR signal to the calcium signaling and Ras-ERK signaling modules via the generation of the critical second messengers IP_3 and DAG. We suggest that the role of the cortical actin cytoskeleton in regulating access of signaling enzymes to substrates may apply to other membrane receptor signaling pathways in other cell types.

Material and Methods

Generation of Csk^{AS} mice

BAC RP24-400B5 containing the region of chromosome 9 with the Csk locus was from BACPAC Resources at the Children's Hospital Oakland Resource Institute. The following modifications were engineered by bacterial recombination-mediated genetic engineering (Tischer et al., 2006): 1- Mutation of the sequence encoding the mouse Csk threonine residue at position 266 to sequence encoding glycine. 2-Deletion on both ends of the BAC to leave Chr9: 57,602,462-57,676,881 (GRCm38.p1 C57BL/6J assembly). 3- Introduction of an in-frame stop codon in exon 2 of Lman1L gene. The modified BAC was injected into fertilized C57BL/6 embryos by the UCSF Transgenic and Targeted Mutagenesis Core Facility. Transgenic founders were crossed to Csk-/- mice[14]. Mice were genotyped with the following PCR primers: transgenic allele, 5′-AATAGGGAAGGGGGAGTTTG-3′ and 5′-CTTGGCCATGTACTCTCC-3′; wild-type allele, 5′-AATAGGGAAGGGGGAGTTTG -3′ and 5′-CTTGGCCATGTACTCTGT-3′

Mice

Mice used for these studies were 4-12 weeks of age. MHCI/MHCII deficient Abb/B2m-/- mice were from Taconic. ICAM-1 -/- and B7.1/B7.2 -/- mice were previously described (Sligh et al., 1993; Borriello et al., 1997). 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 (Schoenborn et al., 2011) and was used at 10μM. Cytochalasin D, Latrunculin A and Jasplakinolide (Sigma) were used at 10μM, 0.5μM and 1μM respectively.

Antibodies and Reagents

Murine CD3, CD4, CD5, CD8, CD11b, CD11c, CD19, CD25, CD44, CD69, CD90.2, B220, γδ, NK1.1, Gr1, Siglec H antibodies conjugated to FITC, PE, PerCP-Cy5.5, PE-Cy7, Pacific Blue, Pacific orange, APC, or Alexa 647 (eBiosciences or BD Biosciences); p44/42 MAPK pThr202/Tyr204 (197G2), Src 416, ZAP-70–pY319, LAT, pAkt-Ser473 (Cell Signaling); LAT-pY132, PLCγ1-pY783 (Invitrogen/BioSource); Phalloidin-Alexa 488 (Life Technologies); Csk (C20, Santa Cruz); Phospho-tyrosine (4G10, Upstate); Phospho-tyrosine PY20, PLCγ1 (Millipore); actin (A2066, Sigma Aldrich); Lck (1F6 from J. B. Bolen); ζ (H146 from H. Wang); CD3ε (2C11), ZAP-70 (1E7.2) (Harlan); LckpY505 (BD Biosciences); Goat anti-armenian hamster IgG(H+L) and goat anti-rabbit IgG Ab conjugated to APC (Jackson Immunoresearch); Horseradish peroxidase (HRP)–conjugated goat antibody against rabbit IgG (H+L) and mouse IgG (H+L), Rabbit anti-hamster IgG (H+L) HRP (Southern Biotech); B7.2 Ig (R&D Systems).

Flow Cytometry and data analysis

Stained cells were analyzed on a BD Fortessa (BD Biosciences). Data analysis was performed using FlowJo software (Treestar Incorporated, Ashland, OR). Statistical analyses with paired t-tests were performed using Microsoft Excel (Microsoft).

Cell stimulations and intracellular phosphoflow

Before stimulations, cells were serum-starved at 37°C for at least 15 min. Stimulations were performed in serum-free RPMI at 37°C. CD3ε crosslinking was induced by addition of 20mg/mL anti- CD3ε followed by goat anti-armenian hamster IgG (H+L) to a final concentration of 50mg/mL. For bead-based stimulations, 4.5 μm styrene beads (Polyscience) were coated overnight with B7.2 Ig (5mg/ml) and/or I-A^b tetramer loaded with human class II-associated

invariant chain peptide 103-117 (5ug/mL; NIH tetramer core facility) in PBS under continuous rotation at 4^0C. Beads were then saturated with 1% BSA PBS under continuous rotation for at least 1 h at RT, and washed with PBS before use. Intracellular phospho-ERK and phospho-Akt staining was performed as previously described[26].

Calcium flux assays

Cells were loaded with the Indo1-AM (1.5mM, Invitrogen) for 30 min at 37°C in RPMI with 5% fetal bovine serum, washed, surface stained and kept on ice in 5% FBS-RPMI. Cells were warmed to 37°C for 5 min before stimulation.

Immunoblotting and Immunoprecipitation

Cells were lysed directly in 6x SDS-PAGE sample buffer after stimulation. Proteins were separated on SDS-PAGE gels, transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) by standard Western blotting techniques, and visualized with SuperSignal ECL reagent or SuperSignal West Femto maximum sensitivity substrate (Pierce Biotechnology) on a Kodak Imaging Station. For immunoprecipitations, cells were lysed in 1% NP-40 lysis buffer with protease and phosphatase inhibitors, and lysates were incubated overnight at 4 °C with protein G-sepharose beads preincubated with anti-ζ antibody. Immunoprecitates were washed with 3x1mL lysis buffer before elution from beads.

Cell enrichments

Enriched populations of CD4+ T cells or pan T cells were obtained by negative selection according to manufacturer's protocol (STEMCELL Technologies, 19752, 19751). Enriched populations of DCs were obtained by $CD11^c$ positive selection selection according to

manufacturer's protocol (STEMCELL Technologies, 18758 or 18780). Thymic DCs were obtained by grinding thymi in gentleMACS C tubes (Miltenyi Biotec) with Spleen 2 program, digesting at 37^0C for 20 min with 62.4mg/mL DNaseI (Roche) and 1.25mg/mL collagenase D (Worthington), then further grinding with the Spleen 1 program. The single cell suspension obtained was surface stained and then sorted for $CD11^c$ positive cells after dumping B220, CD3, Siglec H and side scatter high cells.

Total inositol phosphates measurement

Thymocytes ($4X10^7$ cells/mL) were labeled with 40mCi/mL of [^{3}H]-myo-inositol (Perkin Elmer) for 6 h at 37°C, washed and cultured overnight at 37°C in complete RPMI. Labelled thymocytes were treated with 20mM lithium chloride for 20 min at 37°C and stimulated as appropriate. Cells were lysed in 0.75 mL of chloroform-methanol-12M hydrochloric acid (100:200:2) and the phases were separated by the addition of 0.25 mL H_2O and 0.25 mL chloroform. The upper aqueous phase was mixed with 2.3 mL H_2O and the precipitate formed pelleted. The aqueous phase was then applied to columns made from 1.2 ml of a 0.5 g/ml aqueous slurry of AG 1-X8 (Biorad 140-1444). [^{3}H]-Inositol and [^{3}H]-glycerophosphorylinositol were washed off with 18ml of 60 mM sodium formate and 5 mM disodium tetraborate. Total inositol phosphates were eluted with 10 mL of 1 M ammonium formate plus 0.1 M formic acid. Radioactivity was determined by scintillation counting in Ecoscint (National Diagnostics).

Microscopy

Cell-bead conjugates were fixed in 4 % paraformaldehyde and 0.2% glutaraldehyde for 30 minutes at room temperarure. After spin-washes in PBS and staining buffer (PBS with 2% FCS), cells are permeabilized in 0.05% Triton X-100 for 5 min and washed with staining buffer. After

30 min in staining buffer, conjugates were stained with CD90.2-APC (BD Biosciences) and phalloidin-Alexa488 (Life technologies). Z- stacks of conjugates were imaged unmounted in Ibidi 96 well plates with 40x air objective on Zeiss Axiovert 200M. Best focus of cell-bead interface was scored for polarized F-actin towards bead, while stimulus identity was blinded.

Protein Purification

Full-length mouse Csk (Csk^{WT}), the PP1-analog-sensitive mutant T266G (Csk^{AS}), and the kinase-impaired mutant K222R (Csk^{K222R}) (Chow et al., 1993) were subcloned into a pGEX-6P-3 vector containing an N-terminal glutathione S-transferase (GST) affinity tag. Each was transformed into BL21(DE3) cells (Agilent), and expression was induced with 0.2 mM isopropyl-β-D-thio-galactoside (IPTG) at 18°C overnight. The bacterial pellet was resuspended in GST binding buffer, pH 7.4 (phosphate-buffered saline (PBS) with 5 mM EDTA and 5 mM dithiothreitol (DTT)) and lysed by freeze/thaw, lysozyme treatment, and sonication (Branson 450). All purification steps were performed on ice or at 4°C, and all columns and proteases were obtained from GE Healthcare. Clarified lysate was affinity purified by binding to a GST Gravitrap column and elution at pH 8.0 with 10 mM reduced glutathione, 25 mM Tris, 50 mM NaCl, and 1 mM DTT. The GST tag was cleaved with PreScission Protease overnight. After buffer exchange by concentration and dilution, Csk was further purified by HiTrap Q anion exchange chromatography at pH 8.0 (50 mM Tris, 50 - 1000 mM NaCl, and 1 mM DTT) followed by a Superdex 200 gel filtration column in 100 mM NaCl, 10% Glycerol, and 50 mM Tris, pH 8.0. Purified Csk was flash frozen in liquid nitrogen and stored at -70°C. Homogeneity and molecular weight of purified proteins were verified by SDS PAGE and mass spectrometry. The concentration of purified Csk was determined from its absorbance at 280 nm using a molar absorptivity of 73800 M^{-1} cm^{-1} calculated by ExPASy ProtParam (Gasteiger et al, 2005).

Kinase Activity Assays

The activity of purified Csk was obtained from a continuous spectrometric assay in which ATP hydrolysis is coupled via pyruvate kinase and lactate dehydrogenase to NADH oxidation, which results in a decrease in absorbance at 340 nm (Barker et al., 1995). Kinase activity was measured at 30°C with a Molecular Devices Spectramax 340PC spectrophotometer in a 75 µl reaction volume with final concentrations of 2.5 µM Csk, 55.7 U/ml pyruvate kinase, 78 U/ml lactate dehydrogenase, 0.6 mg/ml NADH, 1 mM phosphoenolpyruvate, 250 µM ATP, 10 mM Tris pH 7.5, 1 mM $MgCl_2$, and 1 mM tris(2-carboxyethyl)phosphine (TCEP). The reaction was initiated by adding a final concentration of 250 µM Csk optimal peptide substrate (KKKKEEIYFFF) (Sondhi et al., 1998) synthesized by Elim Biosciences. Negligible background activity was observed if substrate or kinase was omitted from the reaction. Kinase activity was obtained by fitting the initial segment of the decay curve to a linear function. Reported data represent four separate experiments. For IC_{50} curves, independent samples were mixed with DMSO containing varying concentrations of 3-IB-PP1 just prior to the initiation of the reaction. Linear velocities were obtained at each concentration of 3-IB-PP1 and normalized between 0 and 100% within each data set. Five independent data sets with Csk^{AS} and four with Csk^{WT} were fit globally to the function y=100/(1+10^((x-logIC_{50}))) to determine the IC_{50} value. All curve fitting and statistical analysis was performed using Graphpad Prism.

Chapter 4

Discussion

The lack of a Csk deficient T cell line and the aberrant thymic development in mice deficient in Csk in the T lineage hampered progress in understanding the function of Csk in T cells. To overcome this limitation, we generated the Csk^{AS} allele and introduced it into both cell lines and mice. The data presented in chapter 2 and 3 demonstrate the utility of the Csk^{AS} allele in probing the role of Csk in T cell receptor signaling and raises interesting questions about the initiation of TCR signaling: 1) How is PLCγ1 hydrolysis of PIP_2 regulated by the actin cytoskeleton? 2) How and why do the requirements for the initiation of TCR signaling differ in different T cell subsets (e.g. mature versus immature; αβ T versus γδ T)? 3) Is the actin remodeling pathway downstream of CD28 that is required for initiating full TCR signaling in thymocytes unique to CD28? If so, what are the components? If not, why are the TCR-derived actin remodeling signals insufficient? In this chapter, I discuss future directions for this work as well as other potential applications of the Csk^{AS} mice.

Regulation of PLCγ1 and PIP_2 by the actin cytoskeleton

In chapter 3, we showed that actin cytoskeleton remodeling was necessary for phosphorylated and activated PLCγ1 to hydrolyze PIP_2. There are several possible reasons for this requirement. First, the dense cortical actin may act as a physical barrier that limits the ability of phosphorylated PLCγ1 that is bound to LAT to contact the plasma membrane, since the LAT signalosome is bulky and would likely clash with the cortical actin meshwork. Second, PIP_2 is bound by a wide variety of actin regulatory/binding proteins, such as myristoylated alanine-rich C-kinase substrate (MARCKS), profilin, ezrin and dynamin (Saarikangas et al., 2010). As such, in the absence of actin turnover, it may be preferentially sequestered by these proteins from PLCγ1. Work from McLaughlin and coworkers suggests that proteins that bind to PIP_2 may

control the level of "free" PIP_2 that might be available in the plasma membrane. For instance, it was shown that binding of MARCKS to PIP_2 on vesicles reduces the ability of PLCδ to hydrolyze PIP_2 (Carvalho et al., 2008; Gambhir et al., 2004; McLaughlin and Murray, 2005). Third, PLCγ has been reported to be associated with the actin cytoskeleton in various cell types including T cells (Bar-Sagi et al., 1993; Yang et al., 1994). In macrophages and T cells, PLCγ1 colocalization with actin is correlated with signal activation. Moreover, pharmacologic inhibition of actin turnover in macrophages prevented growth factor induced increase in PLCγ1 colocalization with actin. In T cells, PLCγ1 colocalization with actin after TCR stimulation required the presence of the actin regulator Rap1–guanosine triphosphate (GTP)–interacting adaptor molecule (RIAM) (Dearden-Badet and Mouchiroud, 2005; Patsoukis et al., 2009). Perhaps actin remodeling enables recruitment of PLCγ1 to cortical actin and positions it proximal to PIP_2.

In order to distinguish between these possibilities and gain insight into the regulation of PLCγ1 activity, it will be important to determine the relative localization of PLCγ1 with respect to that of LAT, PIP_2, F-actin and the plasma membrane. For the first and third possibilities discussed above, one prediction would be that PLCγ1 fails to approach the plasma membrane close enough. The fact that PLCγ1 is phosphorylated in thymocytes following Csk^{AS} inhibition implies that it is recruited to LAT and is already near the membrane. Therefore in order to test the hypothesis that PLCγ1 fails to access PIP_2 because it is not able to get into direct contact with the plasma membrane, the experimental approach adopted should allow one to visualize differences in proximity to the plasma membrane in the nanometer range. Three approaches can

achieve such resolution: electron microscopy (EM), super resolution microscopy or fluorescent resonance energy transfer (FRET).

Electron microscopy can achieve a resolution of a few nanometers, but it can only be performed on fixed cells and fixation artifacts due to antigen loss and cell structural changes are a major concern of this approach. Super resolution microscopy such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) have rapidly gained popularity in recent years and have been used to characterize TCR signaling nanoclusters (Lillemeier et al., 2010; Sherman et al., 2011; Williamson et al., 2011). These optical methods involve tagging your protein-of-interest with a photoswitchable or photoactivatable probe so that only a few molecules fluoresce at a time, thereby allowing you to precisely pinpoint their location in the cell. However, the resolution achieved is lower, in the 20-50nm range, and the statistical analyses involved in data interpretation remain controversial and challenging. Moreover imaging in live cells or using more than two different probes in the same cell remains difficult due to current instrument limitations. FRET has been in use for the past few decades and has been well characterized in both live and fixed cells. Depending on the donor-acceptor pair chosen, it generally has a resolution of between 2-8 nm (Padilla-Parra and Tramier, 2012). In recent years, analyzing FRET by fluorescence lifetime imaging microscopy (FLIM) has further enabled quantitative characterization of protein-protein interactions in live cells and allows one to determine the proportion of donor molecules undergoing FRET at any given time.

To test the various hypotheses addressing why actin remodeling is required for PIP_2 hydrolysis by PLCγ1, I propose using a PLCγ1-plasma membrane fluorophore FRET pair to determine if there is any change in the proximity of PLCγ1 to the plasma membrane before and after Csk^{AS} inhibition in the presence or absence of an actin remodeling stimulus. A PLCγ1-

PLCδ-PH domain FRET pair, a PLCδ-PH domain-membrane fluorophore FRET pair and a PLCγ1-actin FRET pair could also be used to further characterize the interaction of PLCγ1 with PIP_2 and F-actin and monitor the pool of free PIP_2. Since thymocytes are resistant to conventional transfection methods, the fluorescent protein-tagged proteins could be introduced into Csk^{AS} bone marrow cells by retroviral transduction and the resultant cells then used to generate bone marrow chimeras that will produce Csk^{AS} thymocytes expressing these proteins. The FRET experiments are best carried out using FLIM to also allow for quantitation of the interactions being studied. The use of TIRFM in combination may allow for increased sensitivity for the relevant FRET signal by eliminating background. Indeed, using a FRET strategy, Wucherpfennig and coworkers have been able to successfully detect changes in the association of the CD3ε cytoplasmic domain with the plasma membrane (Xu et al., 2008).

Differential regulation of the initiation of TCR signaling in different T cell subsets

Our data reveal that activation of Lck and Fyn by Csk^{AS} inhibition in T cells induces stronger TCR signaling in immature T cells than in mature peripheral T cells. A particularly striking observation was that thymic γδ T cells were able to activate downstream signaling pathways, including calcium increases and ERK activation, with Csk^{AS} inhibition alone, further indicating that αβ and γδ T cells have different requirements and controls regulating their signaling pathways. Pursuing the molecular explanation behind these differences promises to yield insight into the changes in signaling circuitry acquired during maturation and to shed light on the differences between αβ and γδ T cells. The knowledge acquired is likely to be relevant in designing strategies to either activate or inactivate T cells in the clinical setting, depending on disease context.

There is substantial evidence in the literature supporting differences in TCR signaling between thymocytes and peripheral T cells. Thymocytes respond to low affinity ligands and are positively selected by them; high affinity ligands induce thymocyte apoptosis. In contrast, mature T cells lose sensitivity to low affinity ligands and are activated by high affinity ligands to proliferate and differentiate into effector and memory cells in response (Davey et al., 1998). This difference may stem from developmentally regulated changes in gene expression that affect the levels of kinases and phosphatases in the TCR signaling pathway, the accessibility of signaling proteins due to changes in adapter or cytoskeletal proteins, or the quality of interactions with APCs by affecting costimulatory or adhesion molecules. For example, the expression of the SFK Fyn T is low in DP thymocytes but is upregulated in peripheral T cells (Olszowy et al., 1995). Cell surface sialylation is higher on mature T cells and was shown to be partially responsible for reducing sensitivity of mature CD8 T cells to low-affinity ligands (Starr et al., 2003a). CD28 is expressed most highly on DP thymocytes compared to single positive thymocytes and mature T cells, and impaired negative selection has been observed in CD28 deficient mice (Gross et al., 1992; Buhlmann et al., 2003). Given the vast number of changes that occur during development, whether these or other changes might individually or collectively account for the observed differences in sensitivity to Csk^{AS} inhibition is not known. Moreover, how each of these changes account for the developmental differences in TCR signaling remains poorly elucidated.

In terms of specific differences in TCR signaling between thymocytes and mature T cells, it has been shown that TCR/CD28 stimulation induces much less Akt phosphorylation in thymocytes than in mature T cells (Cunningham et al., 2006). Studies of T cell-APC conjugates formed using high affinity ligands have also revealed that SMACs formed by thymocytes are rather different from those formed by mature T cells. The classical SMAC formed by mature T

cells consists of a cSMAC enriched with TCR surrounded by a pSMAC of LFA-1. One study describes that unlike the case in mature T cells, TCR ζ does not accumulate in the cSMAC though it is recruited to the cell-cell interface (Richie et al., 2002). Another study found that in thymocytes, the SMAC formed was multifocal, with numerous spots of TCR enrichment where phosphotyrosine was enriched and LFA-1 was excluded (Hailman et al., 2002).

In the context of our findings in the Csk^{AS} T cells, there are a few clues that may help define our search for relevant differences between mature and immature T cells and between αβ and γδ T cells. First, the reduced ZAP-70 phosphorylation induced by Csk^{AS} inhibition in mature T cells compared to thymocytes implies a more proximal block in activation in mature T cells or more potent negative regulation. This could be due to failure of activated Lck to increase phosphorylation of CD3 ITAMs and ZAP-70 due to segregation of Lck from the TCR or the presence of a negative feedback pathway that acts proximally, likely involving phosphatases. Determining whether phosphorylation of CD3 ITAMs is increased in mature T cells will therefore be an important next step. Lack of increase in ITAM phosphorylation supports either possibility, but strong upregulation of ITAM phosphorylation would support the latter hypothesis.

To test the possibility that Lck is sequestered from its substrates, one could examine the relative localization of Lck, TCR and ZAP-70 using immunofluorescence TIRF microscopy. However, the small size of primary naïve lymphocytes makes imaging them challenging and it may be difficult to detect a clear difference between thymocytes and mature T cells. Moreover, DP thymocytes have 80-90% less surface TCR than mature T cells, so directly comparing and interpreting the relative spatial distribution of TCR and Lck in these two population may not be straightforward. To test the involvement of phosphatases, one could first examine the

phosphorylation/activation status of candidate phosphatases that have been implicated in the negative regulation of TCR signaling, such as SHP-1, PEP, Sts-1 and Sts-2, during Csk^{AS} inhibition alone (Brockdorff et al., 1999; Mikhailik et al., 2007; San Luis et al., 2011; Wu et al., 2006). The relevance of candidate phosphatases that behave differentially in immature versus mature T cells could then be verified using RNAi-mediated transient knockdown or by introducing their null alleles into the Csk^{AS} mice.

In thinking about the difference in response to Csk^{AS} inhibition between thymic αβ T cells and γδ T cells, one possible explanation is the absence of CD4/CD8 coreceptors that associate with Lck and may therefore sequester Lck from the TCR when physiologic pMHC is not used for stimulation. However this is unlikely the reason, since proximal phosphorylation does occurs in αβ thymocytes and the observed block in activation is at the level of PIP_2 hydrolysis. A more likely explanation would be that PIP_2 access by PLCγ1 following SFK activation is less restricted in γδ T cells, perhaps due to differential regulation of the actin cytoskeleton or maybe SFK activation alone is sufficient to drive co-stimulation independent actin remodeling in γδ T cells. Thus an examination of the actin cytoskeletal dynamics using photoactivatable actin before and after Csk^{AS} inhibition in both αβ DP thymocytes and γδ thymic T cells could be informative (Galbraith et al., 2007). Likewise, an in silico assessment of differences in the expression of actin cytoskeletal regulators using databases such as Immgen might provide invaluable clues.

CD28-dependent actin remodeling

It is well established that optimal T cell activation in response to agonist pMHC requires CD28 costimulation. CD28 signals synergize with TCR signals to promote IL-2 production and

proliferation. In fact, TCR engagement in the absence of CD28 coligation leads to anergy or cell death (Bluestone, 1995; Rudd et al., 2009). Similar to the TCR, CD28 does not have intrinsic catalytic activity but instead recruits signaling proteins to its cytoplasmic tail which bears two key interaction motifs. One of them is the N-terminal YNMN motif that when phosphorylated on the tyrosine, associates with the p85 subunit of PI3K. Recruitment of PI3K induces PIP_3 production and the activation of PDK-1/Akt signaling that regulates cellular metabolism and survival (Pages et al., 1994; Prasad et al., 1994; Truitt et al., 1994). However the significance of this pathway downstream of CD28 signaling has been called into question, since mice expressing the FMNM mutant of CD28 do not show substantial defects in T-cell proliferation or function (Dodson et al., 2009; Okkenhaug et al., 2001). The second CD28 interaction motif is the C-terminal PYAPP proline rich region that recruits Lck, Grb2, PKCθ and filamin A (Holdorf et al., 1999; Okkenhaug and Rottapel, 1998; Tavano et al., 2006; Yokosuka et al., 2008). The function of Lck interaction with CD28 is uncertain, although it is thought that interaction with CD28 enhances Lck activation (Holdorf et al., 1999). Grb 2 association can recruit many proteins including the Rho-GEF Vav-1 to CD28 and initiate actin remodeling events. Filamin A, an actin-binding scaffolding protein, is important for the recruitment of CD28 and PKCθ to the IS (Hayashi and Altman, 2006). Importantly, mutation of the PYAPP motif results in severe impairment in IL-2 secretion and T cell activation, suggesting that this is a physiologically relevant signaling motif of CD28.

In addition to the signaling effectors that are associated with the two interaction motifs, CD28 engagement is also linked to the dephosphorylation and activation of the actin severing protein, cofilin, by a pathway that involves Ras and PI3K, though the exact mechanism is not well defined (Lee et al., 2000; Wabnitz et al., 2006). Recently, an actin-uncapping protein that

promotes actin polymerization, Rltpr, was identified as a critical player of CD28 signaling that is required for CD28 association with PKCθ (Liang et al., 2013).

Since CD28 signaling involves many of the same effectors as TCR signaling including PI3K, Vav and PKCθ, whether the synergy of CD28 signals with TCR signals is the result of a quantitative amplification of signal strength or whether unique pathways downstream of CD28 play a more important role is controversial. However, as discussed above, unique mediators of CD28 signaling such as filamin-A, cofilin and Rltpr have been identified. It is immediately apparent that all of these unique effectors are involved in the regulation of the actin cytoskeleton, suggesting that CD28 may provide a unique and essential actin remodeling signal for TCR activation. CD28 engagement alone can indeed induce actin polymerization and the formation of actin-rich filopodia or focal-adhesion like contacts with APCs via the recruitment and activation of Rho-GTPases (Kaga et al., 1998; Salazar-Fontana et al., 2003). However, many unknowns remain. Are the actin remodeling pathways utilized by CD28 distinct from those of the TCR? What are the key effectors? When and how is actin remodeling downstream of CD28 important during T cell activation? Is it important during signal initiation and/or signal maintenance?

Using the Csk^{AS} mice, we were able to activate SFKs in the absence of actin remodeling induced by antibody-mediated crosslinking or costimulation. This has enabled us to uncover a role for CD28 in the initiation of full TCR signaling by inducing actin remodeling that in turn facilitates access of PLCγ1 to its substrate PIP_2. We believe that the Csk^{AS} system will also be useful for identifying CD28-specific actin remodeling pathways. For instance, we can compare the activation of various actin-regulatory proteins upon Csk^{AS} inhibition in the absence or presence of B7 costimulus. Examples of candidate proteins to examine would be Vav-1, Rac, Cdc42, cofilin and Rtplr. For an unbiased approach to identify novel actin regulators downstream

of CD28 signaling, one could envision a forward genetic screen using ENU mutagenesis of Csk^{AS} mice to identify mutant strains that activate full TCR signaling with Csk^{AS} inhibition alone. Alternatively, a reverse genetic screen using RNAi knockdown of actin regulatory proteins in bone marrow chimeras could be carried out to identify proteins that are required for B7-mediated restoration of full TCR signaling following Csk^{AS} inhibition.

Role of Csk in regulating T cell basal state, TCR signaling thresholds and signal termination

Prior efforts to understand Csk's role in primary mature T cells have been hampered by the loss of normal T cell development when Csk is ablated in the T-lineage (Schmedt et al., 1998), hence the precise role of Csk in the regulation of basal and inducible TCR signaling is unclear. The Csk^{AS} BAC transgenic mouse line we have developed will be an excellent tool for addressing this. Introducing TCR transgenes such as OT-I or OT-II into the Csk^{AS} mice will also allow us to characterize how Csk activity influences responses to physiologic pMHC ligands. Specifically, does Csk activity set the basal signaling tone and TCR responsiveness to different levels of stimuli? Is Csk activity required for TCR signal propagation and termination following TCR engagement?

The balance of the opposing activities of Csk and CD45 creates a basal equilibrium in resting T cells that likely sets the threshold for TCR activation. It follows that one requirement for TCR activation is the perturbation of this basal equilibrium. However, the mechanisms that govern this equilibrium remain poorly defined, in part due to the lack of a system where the equilibrium can be rapidly and reversibly perturbed to varying extents. Important remaining questions regarding this equilibrium include 1) whether the balance can dynamically readjust in

response to minor perturbations that fail to surpass the activation threshold and 2) what determines how easily this balance is disrupted (i.e. the threshold of activation). To examine the role of Csk in the control of this basal equilibrium and how that tunes the TCR signaling threshold, one can subject mature Csk^{AS} T cells to a careful dose titration of 3-IB-PP1 for a prolonged period of time to alter the basal signaling state. Lck phosphorylation status as well as TCRζ phosphorylation can be evaluated for changes in basal signaling strength. This can then be correlated to changes in responsiveness to varying doses of anti-CD3 to determine whether TCR signaling thresholds have been altered.

Although there is strong evidence for Csk's role in basal negative regulation of TCR signaling, its role in ligand-induced signaling has been largely unstudied. This is because in order to do so effectively, one needs to achieve precise temporal control of Csk activity and until the Csk^{AS} allele was established, a system that allows that has not been available. It is therefore unknown whether Csk kinase activity is necessary at all stages during TCR signaling. To investigate whether Csk activity influences the initiation, propagation and termination of ligand-induced signaling, one can stimulate naive Csk^{AS} T cells ex vivo with anti-CD3 or in antigen-specific systems and inhibit Csk^{AS} activity either simultaneously or at various time points after addition of anti-CD3. The progress of signaling events from initiation to termination can then be monitored by looking at phosphorylation of proximal TCR signaling effectors, increases in intracellular calcium, as well as ERK phosphorylation. If Csk activity influences signal initiation or propagation, one would expect a change in the magnitude or quality of signaling. If Csk activity is important for signal termination, then one would expect prolonged signaling.

Regulation of Csk localization and the identification of novel Csk adaptors

Changing the subcellular localization of kinases and phosphatases is an important means of regulating protein tyrosine phosphorylation in many signal transduction pathways. The shuttling of Csk between the cytosol and the plasma membrane has been proposed to play an important role in TCR activation, but how it is regulated dynamically before and during TCR stimulation remains ambiguous. Several Csk membrane adaptors have been identified and the most prominent of these is PAG. Brdicka et al. first demonstrated that following TCR stimulation, PAG undergoes extensive dephosphorylation (Brdicka et al., 2000). This finding was reaffirmed by others, and it was further observed that Csk association with PAG is correspondingly reduced (Davidson et al., 2003; Torgersen et al., 2001). In chapter 2, our work in Jurkat T cells indicates that Dok-1 could also regulate Csk localization. We found that TCR activation in response to Csk^{AS} inhibition induced Dok-1 hyperphosphorylation and increased Csk recruitment. However, since neither PAG deficient nor Dok-1 and Dok-2 doubly deficient T cells phenocopy Csk deficient T cells, the mechanisms for Csk recruitment to the membrane in T cells remain poorly understood (Yasuda et al., 2007). It is likely that multiple adaptors are involved and that additional Csk adaptors remain to be identified.

The Csk^{AS} mice can be used to investigate whether Csk activity regulates its own relocalization to the plasma membrane. One hypothesis is that since Csk activity influences SFK activity and SFKs phosphorylate Csk membrane adaptors, decreased Csk activity may in turn lead to more Csk recruitment to the plasma membrane. One can determine Csk^{AS} levels at the plasma membrane at varying doses of 3-IB-PP1, using biochemical fractionation or immunofluorescence microscopy. Preliminary P100/S100 fractionation experiments show an increased amount of Csk in the P100 membrane fraction in the presence of Csk^{AS} inhibition that

is associated with increased SFK activity and PAG phosphorylation (data not shown). This finding suggests that Csk^{AS} inhibition leads to increased Csk association with its membrane adaptors. It is therefore possible to identify novel adaptors of Csk by determining what Csk is associated with at the membrane following Csk^{AS} inhibition. One could immunoprecipitate Csk from the p100 fraction and then subject the immunoprecipitate to mass spectrometric analyses. These experiments could be carried out on a PAG-deficient and/or Dok-1 and Dok-2 deficient background to increase the likelihood of identifying novel Csk interactors.

Role of Csk in thymic development and the immune response

With the generation of the Csk^{AS} BAC transgenic mouse, it is now possible to study the role of Csk in vivo. Attempts to inhibit ASKAs in vivo can be foiled by the lack of a suitable analog inhibitor that is potent enough and has good pharmacokinetics. Fortunately, Csk^{AS} is sensitive to the analog-inhibitor, 1-NM-PP1, which has previously been used in the study of neurotrophin signaling in vivo (Chen et al., 2005). Before using 1-NM-PP1 for in vivo experiments, it will be important to determine the dosing route and dosing regime required for maximal inhibition of Csk^{AS} activity. The inhibition of Csk^{AS} in CD4+ T cells leads to rapid downregulation of surface CD4 levels (data not shown), thus one can use that as a convenient readout to assess efficacy of dosing (i.e. to assess pharmacodynamics effects). Since Csk^{AS} is expressed in all of the cells in the Csk^{AS} BAC transgenic mouse, inhibiting its activity in the mouse will most likely result in lethality. Hence, to study Csk^{AS} T cells in vivo, it will be necessary to either use an adoptive transfer strategy or a bone marrow chimera strategy.

Thymocyte maturation requires activation of TCR signals at two distinct stages, namely the DN3-DN4 beta-selection checkpoint and the DP-SP positive selection checkpoint (Palacios

and Weiss, 2004). As mentioned earlier, conditional deletion of Csk in thymocytes using Lck-Cre leads to TCR-independent passage of thymocytes through both selection checkpoints. With the Csk^{AS} inhibitor, one way to add on to these findings is to titrate the level of Csk activity and ask whether the development of different T cell subsets, such γδ T cells, natural regulatory T cells or natural Th17 cells, is differentially sensitive to Csk inhibition and therefore the level of SFK activity. These studies can also be carried out using reaggregate thymic organ cultures or micron-thin thymic slices to better control the input thymic population. A reaggregate culture or thymic slice is also necessary to avoid confounding of the results by effects of Csk inhibition on the thymic stromal cells and other APCs. To study the beta-selection checkpoint, thymocytes from early E14 fetal thymi containing mainly DN thymocytes can be used. On the other hand, to study positive selection, synchronized preselection DP thymocytes from MHC deficient mice can be used.

The contribution of Csk activity in T cells to the regulation of the peripheral immune response can also be probed in mice adoptively transferred with Csk^{AS} T cells. These mice can be immunized with model antigen or infected with model pathogens while being treated with different amounts of the analog inhibitor. The inhibitor can be added at different stages before and during the mounting of the immune response and the generation of effector and memory T cells can be monitored. Of particular interest would be models of T cell anergy or exhaustion, such as the chronic strains of lymphocytic choriomeningitis virus (LCMV) or certain tumor models (Ahmed et al., 1984; Kim and Ahmed, 2010). One can use these models to determine if inhibiting Csk activity and increasing SFK activity at any stage could prevent anergy/exhaustion or reactivate anergic/exhausted T cells. These findings may be clinically relevant.

Role of Csk and SFKs in other signal transduction pathways in other cell lineages

Csk is a ubiquitous protein and negatively regulates all SFK family members. SFKs are involved in numerous signaling pathways downstream of receptor tyrosine kinases, non-receptor tyrosine kinases, integrins and GPCRs in many different cell lineages, controlling diverse cellular processes including metabolism, viability, proliferation, differentiation, adhesison and migration (Parsons and Parsons, 2004). The Csk^{AS} allele serves as an elegant tool to manipulate SFKs activity in a tunable and temporally controlled manner, thereby allowing one to carefully dissect the role of SFKs downstream of other signaling pathways in other cells types.

Conclusion

The work described here demonstrates the power of the Csk^{AS} allele and mice in providing novel insights into TCR signal transduction. Future studies in T cells using the Csk^{AS} mice will likely deepen our understanding of the role of costimulation in actin remodeling and TCR signal initiation as well as the regulation of basal and inducible TCR signaling by Csk. The use of these novel tools in the study of other signal transduction networks and their associated diseases may also uncover previously unappreciated roles for Csk and SFKs and inspire new therapeutic strategies.

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