Supplementary Information for

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

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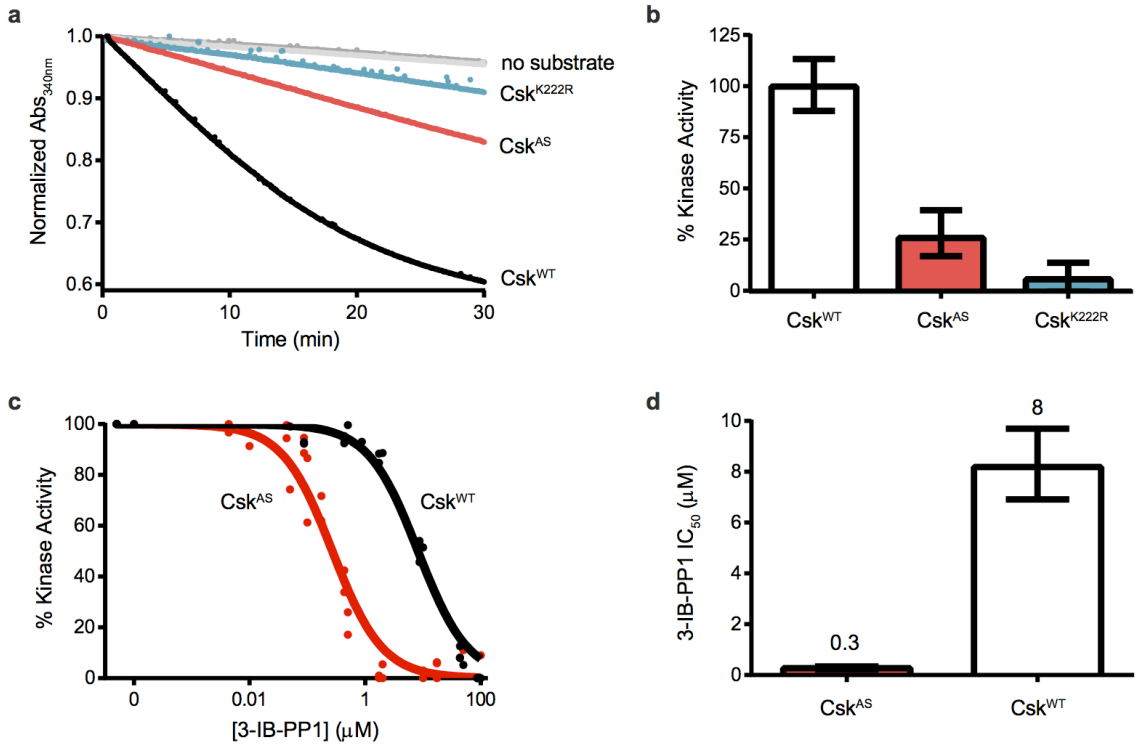
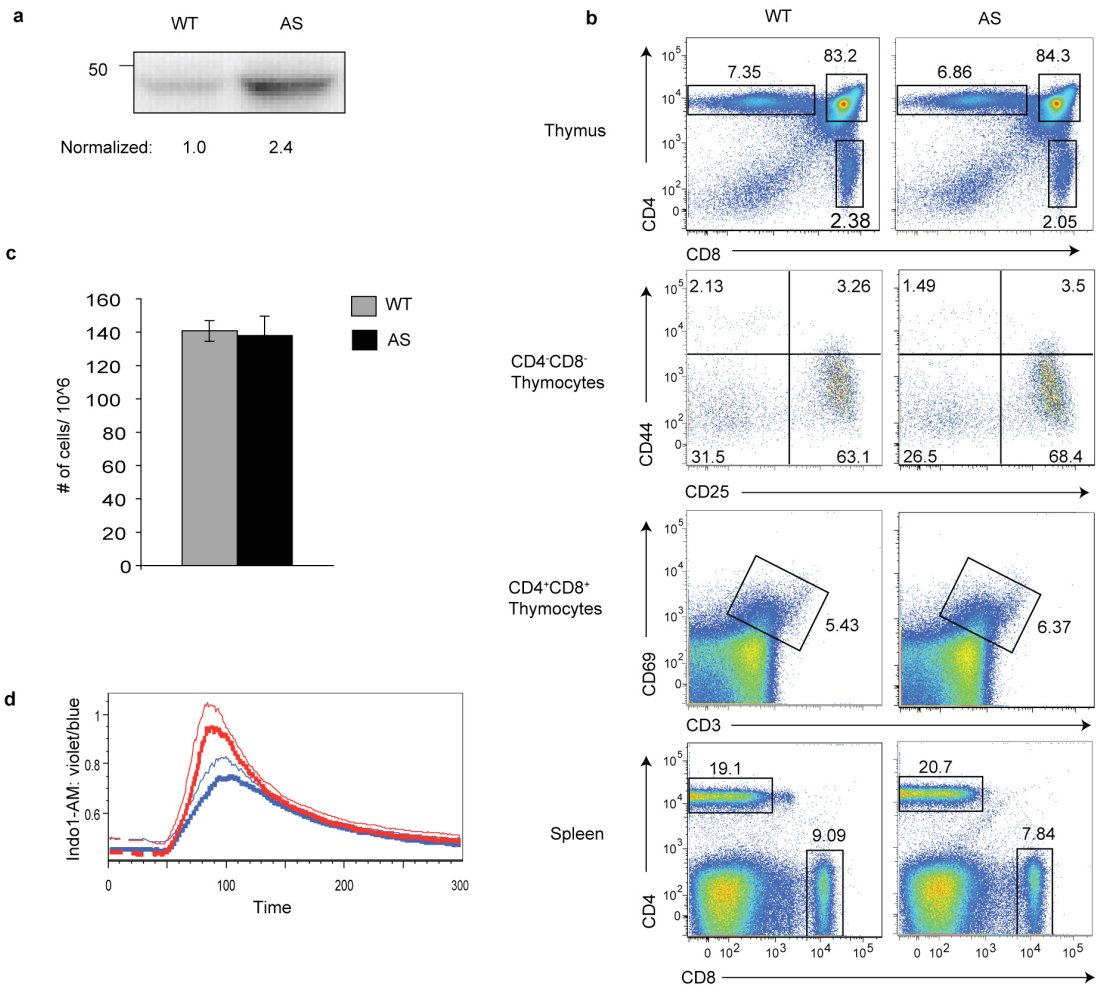
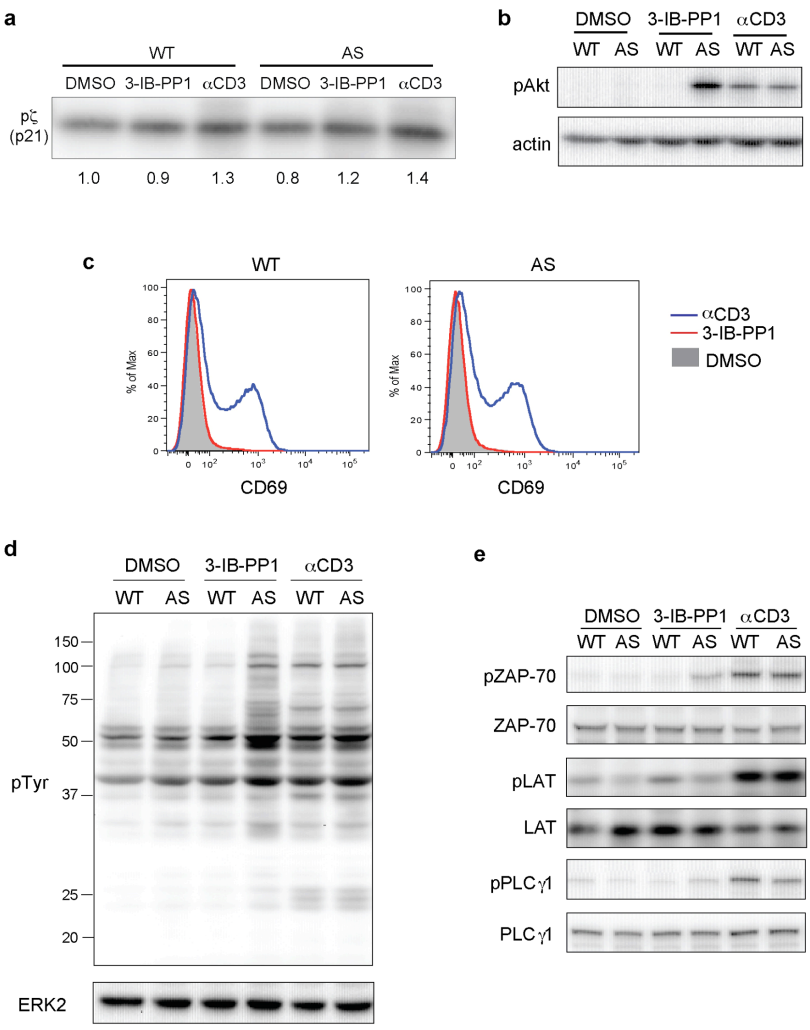


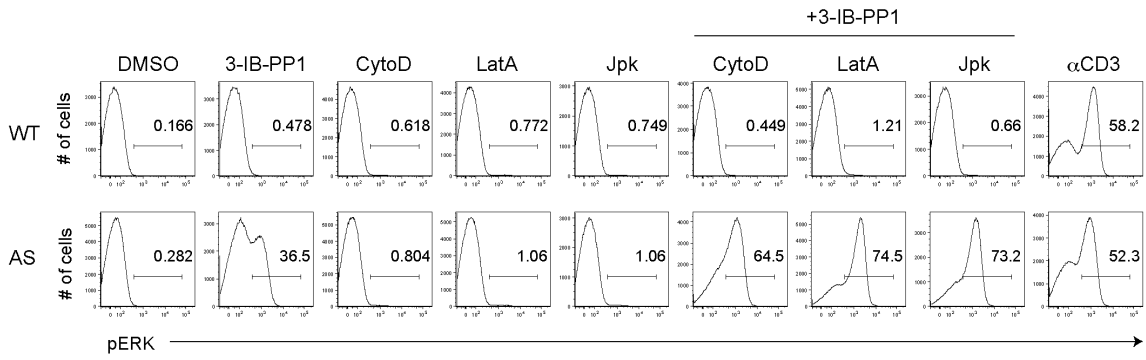
Supplementary Figure 1. The Csk^{AS} allele is active and can be specifically inhibited by the PP1 analog, 3-IB-PP1. (**a**) Substituting glycine for threonine at the gatekeeper position in the active site of Csk to generate analog sensitive Csk (Csk^{AS}) impairs but does not abrogate kinase activity 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 four (Csk^{WT}) or five (Csk^{AS}) 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.



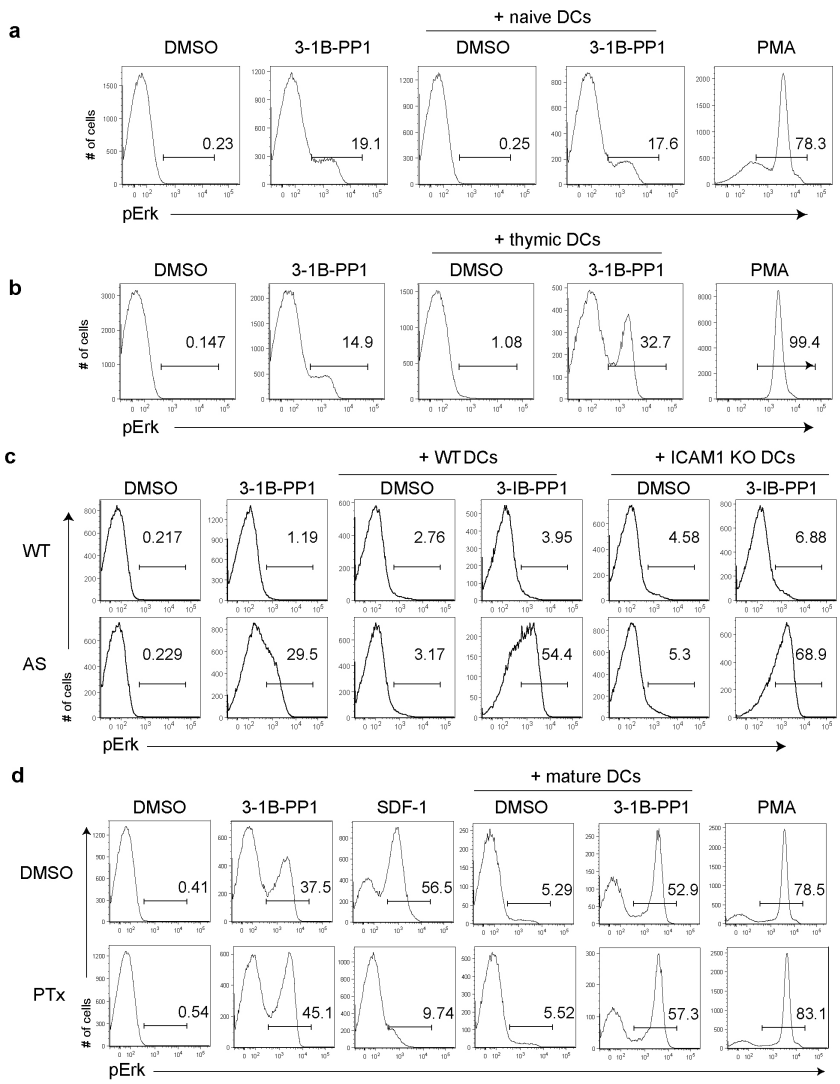
Supplementary Figure 2. T cell develop normally and respond normally to TCR stimulation in Csk^{AS} BAC transgenic mice. (**a**) Immunoblot and densitometric analysis of Csk expression in wild-type (WT) and Csk^{AS}(AS) thymocytes. (**b**) Thymocytes or splenocytes from wild-type (WT) and Csk^{AS}(AS) thymi were surface stained for the indicated markers and analyzed by flow cytometry. Data representative of five littermate pairs. (**c**) Mean thymic cellularity of ten wild-type (WT) and ten Csk^{AS}(AS) littermate thymi with means and S.E.M indicated. (**d**) Thymocytes from wild-type (WT:thin lines) or Csk^{AS} (AS:thick lines) mice loaded with Indo-1AM dye were stimulated with high dose (red lines: 20μg/mL) or low dose (blue lines: 5μg/mL) anti-CD3ε. Ratiometric assessment of intracellular calcium of CD4^{+}CD8^{+} thymocytes over time is shown. Data is representative of three independent experiments.



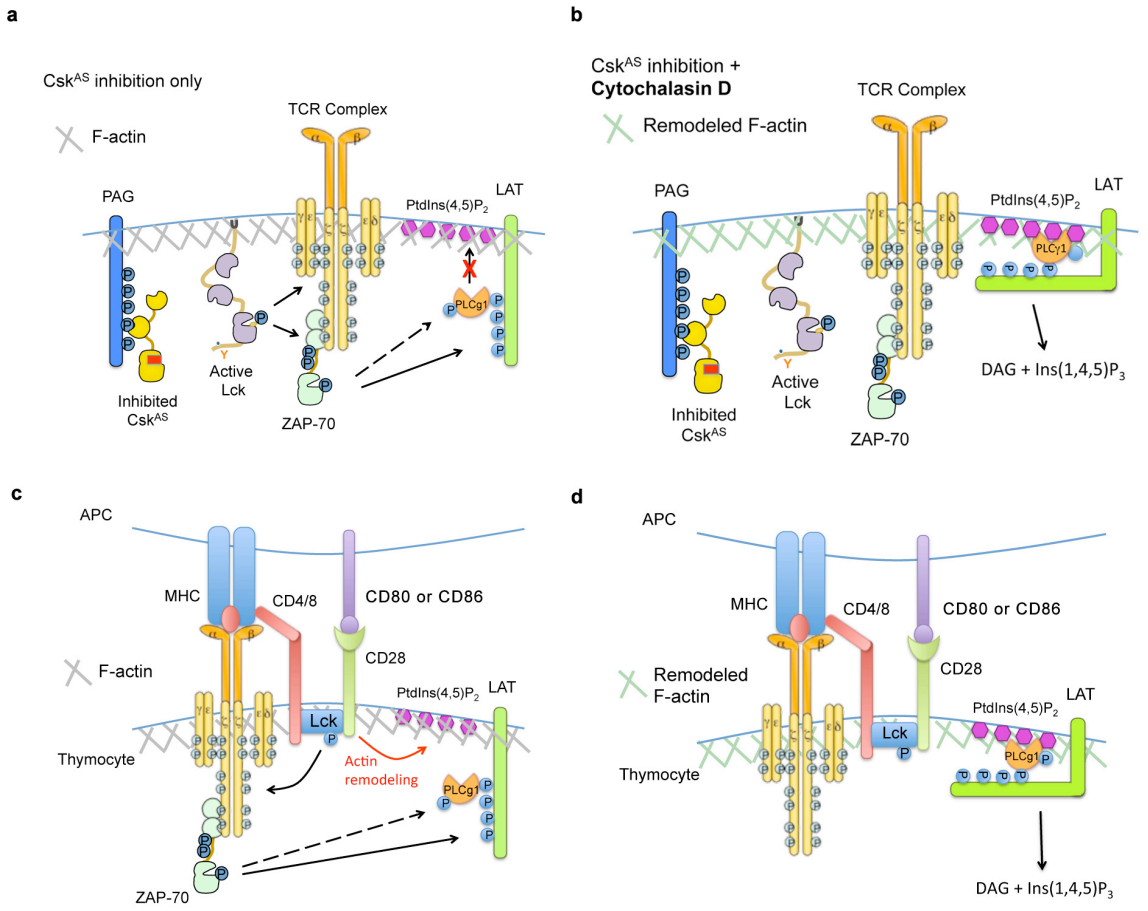
Supplementary Figure 3. Inhibition of Csk^{AS} induces tyrosine phosphorylation but not CD69 upregulation in primary murine T cells. (**a**) Lighter exposure of pTyr blot from Figure 1d with quantification of p21 normalized to total ζ immunoprecipitated. (**b**) Thymocytes from wild-type (WT) or Csk^{AS} (AS) mice were treated for 3min with DMSO, 10 μM 3-IB-PP1 and 20 μg/mL anti-CD3ε then lysed and analyzed by immunoblot with the indicated antibodies. (**c**) Thymocytes from wild-type (WT) or Csk^{AS} (AS) mice were treated with DMSO, 10 μM 3-IB-PP1 or 10 μg/mL platebound anti-CD3ε for 12 hours, stained for cell surface CD69 and analyzed by flow cytometry. Histograms are gated on $CD4^{+}CD8^{+}$ thymocytes. (**d, e**) Purified peripheral $CD4^{+}$ T cells from wild-type (WT) or Csk^{AS} (AS) mice were treated for 3min as indicated, then lysed and analyzed by immunoblot with the indicated antibodies. (**a-c and e**) Data is representative of three independent experiments. (**d**) Data is representative of 2 independent experiments.



Supplementary Figure 4. Simultaneous alteration of the actin cytoskeleton enhances Erk phosphorylation in thymocytes during inhibition of Csk^{AS}. Thymocytes from wild-type (WT) or Csk^{AS} (AS) mice were stimulated for 2 min as indicated (10 μM 3-IB-PP1; CytoD, 10 μM cytochalasin D; LatA, 0.5 μM latrunculin A; Jpk, 1 μM jasplakinolide; 20 μg/mL anti-CD3ε), then analyzed for pErk content by phosphoflow. Histograms are gated on $CD4^+CD8^+$ thymocytes. Data is representative of two independent experiments. Numbers within histograms indicate percentage of $pErk^+$ cells.



Supplementary Figure 5. Thymic DCs, but not naïve splenic DCs, ICAM-1 deficient DCs or chemokines secreted by mature splenic DCs, enhance ERK phosphorylation in thymocytes during Csk^{AS} inhibition. (**a**) Naïve DCs were enriched from wild-type splenocytes by CD11c positive selection and used immediately. Data is representative of two independent experiments. (**b**) Thymic DCs were sorted from wild-type 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 50 ng/mL pertussis toxin (PTx) for 1h. Data is representative of two independent experiments. (**a, b** and **d**) Thymocytes from Csk^{AS} mice or (**c**) wild-type (WT) and Csk^{AS} (AS) mice were pelleted with or without DCs at a 1:1 ratio and stimulated with vehicle (DMSO), 10 μM 3-IB-PP1, 10 ng/mL stromal cell-derived factor (SDF-1) or 50 ng/mL phorbol myristate acetate (PMA) for 3min. Cells were then analyzed for pErk content by phosphoflow. Histograms shown are gated on $CD4^+CD8^+$ thymocytes. Numbers within histograms indicate percentage of $pErk^+$ cells.



Supplementary Figure 6. 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 PLC-γ1 bound to the LAT signalosome. However, in the absence of actin turnover, active PLC-γ1 cannot access plasma membrane PtdIns(4,5)P_2 to hydrolyze it because the dense cortical actin meshwork and its associated proteins may act as a barrier or because PLC-γ1 fails to be recruited to the actin cytoskeleton. (**b**) Inducing remodeling of the cortical actin cytoskeleton with cytochalasin D allows active PLC-γ1 to access and hydrolyze PtdIns(4,5)P_2 to generate DAG and Ins(1,4,5)P_3. (**c**) During a thymocyte-APC interaction, TCR engagement by pMHC recruits CD4 or CD8 coreceptor bound active Lck. Association of CD80 or CD86 engaged CD28 with Lck allows for activation of local actin cytoskeletal remodeling. (**d**) This enables active PLC-γ1 to hydrolyze PtdIns(4,5)P_2.