Slow phosphorylation of a tyrosine residue in LAT optimizes T cell ligand discrimination

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Self-non-self discrimination is central to T cell-mediated immunity. The kinetic proofreading model can explain T cell antigen receptor (TCR) ligand discrimination; however, the rate-limiting steps have not been identified. Here, we show that tyrosine phosphorylation of the T cell adapter protein LAT at position Y132 is a critical kinetic bottleneck for ligand discrimination. LAT phosphorylation at Y132, mediated by the kinase ZAP-70, leads to the recruitment and activation of phospholipase C-γ1 (PLC-γ1), an important effector molecule for T cell activation. The slow phosphorylation of Y132, relative to other phosphosites on LAT, is governed by a preceding glycine residue (G131) but can be accelerated by substituting this glycine with aspartate or glutamate. Acceleration of Y132 phosphorylation increases the speed and magnitude of PLC-γ1 activation and enhances T cell sensitivity to weaker stimuli, including weak agonists and self-peptides. These observations suggest that the slow phosphorylation of Y132 acts as a proofreading step to facilitate T cell ligand discrimination.

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T cell responses, mediated by T cell antigen receptors (TCRs), are remarkable for their high sensitivity, exquisite specificity, and rapidity1. T cells can be activated in response to very few foreign peptide major histocompatibility complex (pMHC) ligands (one to ten)2–4, with a small error rate ($10^{-6}$)5,6 and rapid response time (seconds to a few minutes)7. This rapid and highly accurate responsiveness allows T cells to detect peptides derived from foreign pathogens or abnormal cells early and efficiently without reacting to self-tissues. Several factors have been proposed to affect T cell discrimination and correlate with responsiveness, including subtle differences in TCR–pMHC off-rates, on-rates, affinities, and catch-bond formation. However, differences in these factors for agonist and non-agonist ligands are not always sufficient to explain the actual T cell error rate8.

The remarkable selectivity of T cells may be explained by a kinetic proofreading model9,10. Following ligand binding, TCR proximal signaling molecules undergo a series of biochemical reactions, such as phosphorylation, and these multiple steps create a time delay between the input signal (pMHC recognition) and the output response (T cell activation)11. If these signaling steps are rapidly reversible following removal of the stimulus (for example, through dephosphorylation by phosphatases), the TCR–pMHC interaction would have to persist for a sufficient duration to initiate successful activation. By this mechanism, small differences in TCR–pMHC affinities or off-rates could lead to vastly different cellular outcomes, with each signaling step functioning as a ‘proofreader’ to allow only a bona fide activation signal to propagate downstream. Thus far, most efforts to assess the importance of kinetic proofreading in TCR signaling have been restricted to biochemical or mathematical models, and have failed to account for the role of endogenous self-peptides and changes in ligand discrimination during development12,13.

LAT (the linker for activation of T cells) is an important scaffold that coordinates TCR proximal signals in a phosphorylation-dependent manner following receptor stimulation14. Although there are several phosphorylation sites in LAT that have a role in signal transduction, Y132 is the only residue in LAT that recruits phospholipase C-γ1 (PLC-γ1) following its phosphorylation by ZAP-70. Binding of PLC-γ1 to phosphorylated Y132 (p-Y132) in LAT leads to the Tec family kinase ITK-mediated PLC-γ1 phosphorylation and activation15. This activation of PLC-γ1 ultimately leads to calcium mobilization, ERK and protein kinase C (PKC) activation, and eventually cellular effector and transcriptional responses16. Interestingly, despite the importance of LAT p-Y132, the presence of a glycine at position 131 in LAT makes Y132 a particularly poor substrate for ZAP-70 because the kinase domain of ZAP-70 strongly favors an acidic residue (aspartate or glutamate) at the −1 position relative to substrate tyrosine residues17.

Here, we show that substitution of the glycine residue at the −1 position with aspartate or glutamate markedly increases the phosphorylation rate of LAT Y132 in T cells. This focused amino acid substitution in a signaling scaffold protein is sufficient to enhance T cell responsiveness to weak antigens or self-peptides. We demonstrate that slow phosphorylation of Y132 in LAT serves as an essential rate-limiting step in TCR signaling to enable ligand discrimination. Although a glycine at position 131 in LAT is highly conserved in tetrapods, some fish have other residues preceding the homologous tyrosine residue, including aspartate and glutamate, that are more optimal for phosphorylation by ZAP-70. Our results suggest that the slow phosphorylation of LAT Y132 is an important...
regulatory mechanism that contributes to T cell ligand discrimination in most jawed vertebrates and might underlie the remarkable selectivity of T cells.

**Results**

Mammalian LAT Y132 has a glycine at the −1 position, unlike other ZAP-70 substrates. LAT and SLP-76 are two adaptors in T cells that rely on their phosphorylation by ZAP-70 to link initial TCR signals to many downstream cellular events required for full T cell activation (Fig. 1a)\(^1\). ZAP-70 has a strong preference for its substrate tyrosine residues to be surrounded by acidic residues (that is, aspartate and glutamate)\(^15,16\). These acidic residues facilitate substrate tyrosine residues to be surrounded by acidic residues (that then phosphorylate TCR ITAMs and ZAP-70, allowing ZAP-70 to phosphor- ylate Y132 in the in vitro kinase assay, suggesting that this nearby site of phosphorylation does not have a priming effect. To extend this analysis to cells, we used Csk-deficient Jurkat cells reconstituted with a PP1 analog-sensitive Csk mutant (J.CskAS), to rapidly activate Lck by inhibiting Csk-dependent phosphorylation of an inhibitory tyrosine in Lck (data not shown)\(^17\). Activated Lck could then phosphorylate TCR ITAMs and ZAP-70, allowing ZAP-70 to initiate its kinase activities in its native cellular environment without triggering the TCR. Such treatment showed slower tyrosine phosphorylation of Y132 than of Y171, and the phosphorylation of PLC-γ1 exhibited similar time-dependent phosphorylation to Y132 (Supplementary Fig. 1a,b).

Scanning mutagenesis screens with LAT-derived peptides suggested that the substitution of G131 with almost any other amino acid should enhance Y132 phosphorylation, with aspartate and glutamate substitutions showing the greatest enhancement (Supplementary Fig. 1c)\(^18\). In a colorimetric in vitro kinase assay, in which ATP consumption is coupled to NADH oxidation, the ZAP-70 kinase domain showed negligible activity towards a wild-type peptide encompassing Y132 (Fig. 1e,f). Replacement of the glycine at the −1 position with aspartate or glutamate greatly increased the phosphorylation efficiency of Y132 (Fig. 1e,f). Despite its slow phosphorylation by ZAP-70, LAT Y132 is a bona fide well-established ZAP-70 substrate, as shown in the kinase assay (Fig. 1d) and in the experiments in which ZAP-70 deficiency but not ITK deficiency eliminated Y132 phosphorylation (Supplementary Fig. 1d), and as reported in the literature\(^19,20\).

Since p-Y132 in LAT is directly upstream of PLC-γ1, we examined the possibility that the glycine at position 131 might have been selected to promote a better PLC-γ1 interaction with the p-Y132 site. Phosphorylated Y132 interacts with the N-terminal SH2 domain of PLC-γ1 (refs. \(^19,20\)). A peptide with glycine preceding Y132 had similar binding affinity to the PLC-γ1 N-SH2 domain, as did a peptide with aspartate preceding Y132 (Supplementary Fig. 2a,b). A scanning mutagenesis screen for PLC-γ1 N-terminal SH2 binding to LAT p-Y132 peptide mutants confirmed that most substitutions at G131 do not affect SH2 binding, whereas polar substitutions at Y135 (the +3 position) in the p-Y132-containing peptide impaired SH2 binding, consistent with the known binding motif preference for PLC-γ1 N-terminal SH2 (Supplementary Fig. 2c)\(^14\). It is also unlikely that the G131D and G131E mutations would change which kinase phosphorylates this site, because the aspartate or glutamate residue at the −1 position is disfavored by Src family kinases and Tec family kinases\(^13,16\). Therefore, this highly conserved glycine at the −1 position impedes the efficiency of LAT Y132 phosphorylation by ZAP-70.

Mutation of LAT G131 to an aspartate or glutamate enhances calcium responses. To determine how the G131 residue preceding Y132 affects PLC-γ1-dependent signal transduction (for example, calcium responses), we substituted G131 with aspartate or glutamate in T cells. LAT and its G131 variants were used to reconstitute CRISPR–Cas9-generated LAT-deficient human Jurkat cells (J.LAT), hereafter termed J.LAT.WT, J.LAT.G131D, and J.LAT.G131E. When these cells were stimulated with anti-CD3 mAb (OKT3), the G131D and G131E LAT variants markedly augmented the magnitude of maximal calcium peaks (Fig. 2a,b). Particularly evident at lower doses of anti-CD3, expression of G131D and G131E mutant LAT molecules endowed cells with faster and larger calcium responses than did wild-type LAT (Fig. 2a,c). Thus, by mutating G131 to an aspartate or glutamate, the reconstituted J.LAT cells had increased sensitivity and decreased response times to weak anti-CD3 stimuli.

We examined whether the elevated calcium mobilization in J.LAT.G131D and J.LAT.G131E cells resulted from alteration of LAT Y132 and PLC-γ1 phosphorylation. J.LAT.G131D and J.LAT. G131E cells responded to lower anti-CD3 concentrations than did wild-type cells (Fig. 2d and Supplementary Fig. 3a). The mutation at G131 did not influence the activation of Lck and ZAP-70 (Supplementary Fig. 3a,b), but LAT Y132 and PLC-γ1 phosphorylation were greatly enhanced in G131D- and G131E-expressing cells (Fig. 2d and Supplementary Fig. 3a,c). Notably, we sometimes observed an enhancement in the phosphorylation of the distal tyrosine residues in cells expressing LAT G131D; however, this effect was not consistently observed (Fig. 2d and Supplementary Fig. 3b). In time-course experiments, G131D and G131E LAT also accelerated the phosphorylation of LAT Y132 and PLC-γ1 (Fig. 2c). These results suggest that LAT G131D and G131E lowered the TCR response threshold and amplified T cell responsiveness by promoting the phosphorylation of LAT Y132 and PLC-γ1.

Enhanced Y132−PLC-γ1-derived signals allow T cells to respond to low-affinity ligands. The OT-I TCR recognizes a peptide spanning residues 257−264 from chicken ovalbumin (OVA) presented by the H-2K\(^{\beta}\) MHC molecules. Substitutions of one or two amino acids convert the full agonist OVA peptide into partial or weak agonists, all termed altered peptide ligands (APLs), which provide a sensitive and specific system to examine the capability of T cell ligand discrimination.

We reconstituted LAT-deficient OT-I+CD8\(^{+}\) Jurkat cells with wild-type LAT or the G131D or G131E variants (termed JOT-I-LAT. WT, JOT-I-LAT.G131D, and JOT-I-LAT.G131E, respectively). Each cell clone was stimulated with OVA APL-pulsed H-2K\(^{\beta}\)-expressing T2 cells (T2-K\(^{\beta}\))\(^21\). Cells upregulated the activation marker CD69 in response to OVA stimulation but remained unresponsive towards an unrelated peptide, VSV (Fig. 3a). When the cells were stimulated with OVA or partial agonists Q4R7 or T4, G131D-LAT- and G131E-LAT-expressing JOT-I-LAT cells were alternatively tenfold more sensitive to the peptide stimulus. Q4H7 is a very weak agonist and activated only approximately 30% of the JOT-I-LAT.WT cells, but approximately 60% of JOT-I-LAT.G131D and 50% of JOT-I-LAT. G131E cells were able to respond to Q4H7, as measured by CD69.
upregulation (Fig. 3a,b). Another weak OVA APL peptide, G4, did not stimulate JOT-LLAT-WT cells to upregulate CD69, but was able to activate about 40% of G131D-expressing JOT-1+ T cells and 20% of G131E-expressing cells (Fig. 3a,b). The responses to Q4H7 and G4 peptides are noteworthy because both peptides can promote positive selection of OT-1+ T cells in fetal thymic organ cultures and, as such, are considered to have an affinity in the range of positively selecting self-peptides.23,24
G131D- or G131E-expressing JOT-I+ cells were also weakly activated by the naturally occurring positively selecting self-peptide for OT-I TCR, Catnb (derived from β-catenin residues 329–336)\(^{25}\), whereas wild-type LAT-expressing cells were not (Fig. 3a,b). We compared the potency of each OVA APL peptide and the self-peptide Catnb using JOT-I+ G131D and G131E versus wild-type LAT-expressing cells (Table 1). Peptide potencies were quantified by calculating EC\(_{50}\) (half maximal responses) in the CD69 upregulation assays and normalizing these values to the percentage of maximal CD69 responses\(^{26}\). These results suggest that
augmented LAT Y132 phosphorylation disrupted the ability of the cells to accurately discriminate ligands with different potencies. G131D-expressing cells exhibited a lower ligand responsiveness threshold than did wild-type LAT-expressing cells.

CD69 upregulation is a prominent feature of T cell activation, but its expression can also be induced by exposure to cytokines. Therefore, we examined TCR-induced proximal signals, including phosphorylation of ERK and calcium flux, in response to OVA APL peptides. LAT-deficient J.OT-I cells acquired the ability to upregulate ERK phosphorylation following stimulation with the self-peptide Catnb, whereas wild-type-expressing J.OT-I cells were unresponsive (Fig. 4c).

Next, we used OVA- or APL-loaded biotinylated pMHC monomers and streptavidin to analyze antigen-specific calcium responses. Expression of G131D or G131E LAT augmented calcium mobilization

**Table 1** | EC_{50} (pM) and ligand potency analysis of CD69 upregulation

| Peptide   | Selecting effect on thymocytes | K_{D} by SPR (μM) | EC_{50} WT | EC_{50} G131D | EC_{50} G131E | 1/potency WT | 1/potency G131D | 1/potency G131E |
|-----------|--------------------------------|-------------------|-----------|---------------|---------------|---------------|---------------|---------------|
| Q4R7      | Partial                        | 288               | 14.92     | 4.23          | 4.27          | 2.7           | 0.6           | 0.7           |
| T4        | Partial/boarder                | 444               | 82.67     | 4.89          | 9.34          | 13.8          | 0.7           | 1.5           |
| G4        | Positive selection             | >1000             | 87.89     | 15.56         | 18.11         | 34.5          | 3.5           | 4.5           |
| Catnb     | Positive selection             | N.A.              | 231.4     | 39.04         | 97.24         | 588.0         | 19.2          | 60.6          |

The table shows EC_{50} (pM) and ligand potency analysis of CD69 upregulation from assays as in Fig. 3a. K_{D} values, determined by surface plasmon resonance (SPR), were obtained from the literature. N.A., not applicable. WT, wild type.
Fig. 4 | Substitution of G131D or G131E in LAT promotes ERK activation and calcium increase in response to weak ligand or self-peptide stimulation.

a, c, Wild-type (WT), and G131D- (D) and G131E-expressing (E) LAT-deficient J.OT-I+H2Db+ Jurkat variants were first individually labeled with CellTrace Violet dye at different concentrations. Cells were washed and pooled together for the experiments. T2-K+ cells were pulsed with OVA peptides, APL ligands, the self-peptide Catnb, or VSV control peptide. Pooled J.OT-I+H2Db+ Jurkat variants and peptide-pulsed T2-K+ cells were mixed on ice, and quickly centrifuged. Cells were then stimulated by moving them to 37°C for 5 min, and then fixed with 4% PFA to terminate the stimulation. Cells were then subjected to flow cytometry-based p-ERK analysis. Data are representative of four independent experiments. 

b, Representative flow cytometry plot of J.OT-I+H2Db+ Jurkat variants barcoded with titrated amounts of CellTrace Violet dye. FSC, forward scatter.

Representative histograms for ERK phosphorylation responses in G131D, G131E, and wild-type LAT-expressing J.OT-I+H2Db+ Jurkat variants stimulated with T2-K+ cells pulsed with 1000 pM of each peptide as indicated. The black bar in the first panel depicts the gate used to define the p-ERK-induced population in c. Percentages of positive cells are indicated. Ligands used for stimulation are indicated above the plots.

c, Analysis of the p-ERK+ population of G131D, G131E, or wild-type LAT-expressing J.OT-I+H2Db+ Jurkat variants stimulated with the indicated concentrations of peptide-pulsed T2-K+ cells. Data were pooled from four independent experiments (mean ± s.d., n = 4 in four independent experiments). Ligands used for stimulation are indicated above the plots. 

d, Wild-type, and G131D- and G131E-expressing LAT-deficient J.OT-I+H2Db+ Jurkat variants were loaded with the calcium-sensitive dye Indo-1, and labeled with 1:100 biotinylated OVA/H-2Kb, T4/H-2Kb, G4/H-2Kb, or VSV/H-2Kb monomers. Cells were then subjected to calcium mobilization assays on Flex Station II. Indo-1 ratios were first recorded for 30 s to determine a relative baseline calcium level, followed by streptavidin addition to trigger the TCR-induced calcium response. Ionomycin was added at 240 s as a positive control. Representative calcium traces are shown. Each stimulus is as indicated. Data are representative of three independent experiments (mean ± s.d., n = 3 technical replicates). e, Bar graphs depict the statistical analysis of the fold change of the peak. Each symbol represents a technical replicate (mean ± s.d., n = 8 samples in three independent experiments). **P = 0.0002 (OVA), ***P = 0.0006 (T4), ****P = 0.0003 (G4), **P = 0.0047 (OVA), **P = 0.0070 (G4); ns, not significant; P = 0.3823 (VSV, left), P = 0.0830 (VSV, right), two-tailed Mann–Whitney U test.

f, Bar graphs depict the statistical analysis of the response time to reach the peak (mean ± s.d., n = 8 samples in three independent experiments). Each symbol represents a technical replicate. **P = 0.0002 (OVA), *P = 0.0011 (OVA), **P = 0.0054 (T4, top), **P = 0.0023 (T4, bottom) **P = 0.0059 (G4, top), **P = 0.0076 (G4, bottom), two-tailed Mann–Whitney U test.

(Murine T cells rely on slow phosphorylation kinetics of Y136 in LAT to allow self–non-self antigen discrimination. The mutations at LAT residue 131 elicited similar functional consequences in primary mouse T cells. Ectopic over-expression of the G135D mutant LAT (Y136 is the murine ortholog of human Y132) in the presence of endogenous LAT was sufficient to endow OT-I+CD8 and OT-II+CD4 T cells with a gain-of-function ability to respond to low-affinity ligands (Supplementary Fig. 5). OT-I+CD8 or OT-II+CD4 T cells transduced with a retrovirus encoding wild-type LAT-P2A-BFP or G135D LAT-P2A-BFP were stimulated with various peptide-pulsed T cell-deficient splenocytes. In response to stimulation with G4 peptide-pulsed splenocytes, the expression of G135D LAT enabled OT-I+CD8 T cells to increase the expression of the key transcriptional factor IRF4 and activation marker CD69 (Supplementary Fig. 5a), augmented the activation of ERK phosphorylation (Supplementary Fig. 5b), and...
promoted the mobilization of calcium (Supplementary Fig. 5c). Similar gain of function was observed in G135D LAT-expressing OT-II+ CD4 T cells when stimulated with the E336Q peptide, a partial agonist of OVA peptide (residues 329–336) specific for OT-II+ TCR (Supplementary Fig. 5a,b).

Our data suggest that the human G131–Y132 and the homologous mouse G135–Y136 LAT sequences may place an important regulatory constraint on TCR signaling that enables ligand discrimination. To further test this hypothesis in the absence of endogenous LAT, we used a mouse expressing a floxed Lat allele in which germline Lat could be deleted by tamoxifen treatment28. Endogenous, wild-type LAT is expressed during thymic selection28.

The diagram shows the experimental flow. Naive ERCre+OT-I+ LATf/– CD8+ T cells were isolated and lentivirally transduced to express wild-type LAT or G135D LAT and tamoxifen-treated for 4 d to delete endogenous LAT. Mouse G135D is homologous to human G131D. The wild-type or G135D LAT was conjugated to mCherry fluorescent protein through a self-cleaving P2A peptide. Cells with successful tamoxifen-induced deletion of endogenous LAT are GFP+ (efficiency >90%). Cells were rested for 1 d and stimulated with peptide-pulsed TCR Ca-deficient splenocytes overnight. TCR Ca-deficient splenocytes were pulsed with 1 μM of OVA, T4, or G4 peptide, 10 μM of Catnb peptide, or 10 μM of VSV peptide. Cells were then analyzed for their ability to upregulate CD69 and produce IFN-γ.

Fig. 5 | The G135D mutation in LAT promotes primary mouse T cells to respond to low-affinity antigen or self-peptide stimulation. a, The diagram shows the experimental flow. Naive ERCre+OT-I+ LATf/– CD8+ T cells were isolated and lentivirally transduced to express wild-type LAT or G135D LAT and tamoxifen-treated for 4 d to delete endogenous LAT. Mouse G135D is homologous to human G131D. The wild-type or G135D LAT was conjugated to mCherry fluorescent protein through a self-cleaving P2A peptide. Cells with successful tamoxifen-induced deletion of endogenous LAT are GFP+ (efficiency >90%). Cells were rested for 1 d and stimulated with peptide-pulsed TCR Ca-deficient splenocytes overnight. TCR Ca-deficient splenocytes were pulsed with 1 μM of OVA, T4, or G4 peptide, 10 μM of Catnb peptide, or 10 μM of VSV peptide. Cells were then analyzed for their ability to upregulate CD69 and produce IFN-γ. Numbers in all flow cytometry plots indicate the percentages of positive cells. b, Representative contour plots depict the expression of Vα2 (OT-I TCR α chain) and mCherry after cells were transduced with lentivirus expressing wild-type LAT-P2A-mCherry or G135D LAT-P2A-mCherry. Data are representative of three experiments. c, Flow cytometric analysis of CD69 expression in mCherry+ subpopulations of GFP+Vα2+CD8+ T cells, pulsed with OVA peptide, G4 peptide, or Catnb self-peptide. d, Bar graphs represent the mean of CD69+ cells in the peptide stimulation assay. Each symbol represents one technical replicate (mean ± s.d., n = 9 samples in three independent experiments). **P = 0.0043 (Q4H7), \( P = 0.0022 \) (Q4H7), ***P < 0.0001 (Catnb), \( P = 0.0294 \) (VSV). ns, not significant (\( P = 0.1359 \)); two-tailed Mann–Whitney U test. e, Flow cytometric analysis of IFN-γ-producing ability in mCherry+ subpopulations of GFP+Vα2+CD8+ T cells, pulsed with various peptides. f, Bar graph demonstrates the mean of IFN-γ-producing cells in the peptide-stimulation assay. Each symbol represents one technical replicate (mean ± s.d., n = 4 samples in two independent experiments). \( P = 0.0286 \); ns, not significant (\( P = 0.8857 \)); two-tailed Mann–Whitney U test.
ERCre\textsuperscript{OT-1}\textsuperscript{+}Lat\textsuperscript{wt}– CD8 T cells transduced with wild-type or G135D-P2A-mCherry lentivirus showed similar transduction frequencies and expression (Fig. 5b). The P2A sequence allows the mCherry fluorescence to function as a marker for successful transduction\textsuperscript{29} and its fluorescence intensity can serve as a surrogate marker for protein expression without interfering with LAT function. The mCherry\textsuperscript{+} ERCre\textsuperscript{OT-1}\textsuperscript{+}Lat\textsuperscript{wt}– CD8 T cells regained the ability to respond to stimulation with OVA and the partial agonist Q4R7, as shown by the upregulation of CD69, but the non-transduced mCherry-negative cells did not (Supplementary Fig. 6). Expression of the G135D LAT mutant lowered the reactivity threshold to allow for an increased percentage of cells to be activated compared with that of wild-type LAT. The difference was particularly noteworthy when cells were stimulated with low-affinity peptides, such as the G4 peptide or the natural self-peptide Catnb (Fig. 5c,d). A small but increased response to the VSV peptide-pulsed antigen-presenting cells was also seen in the LAT-G135D-expressing cells. This could reflect responses to endogenous self-peptides that the VSV-pulsed antigen-presenting cells also expressed. The percentages of IFN-\(\gamma\)-producing cells in G135D\textsuperscript{+} groups also increased compared with those in the wild-type groups (Fig. 5e,f). Thus, an aspartate residue preceding Y136 endowed mature T cells with the ability to be activated by low-affinity antigens and allowed at least one relevant self-peptide to become an agonist. Our data suggest that ZAP-70-mediated phosphorylation of LAT Y136 in primary mouse T cells may function as a critically important node involved in kinetic proofreading to enforce T cell ligand discrimination.

T cell ligand discrimination is uniquely susceptible to the phosphorylation kinetics of LAT Y132. The kinetic proofreading model predicts that proper ligand discrimination can be achieved by a series of gated biochemical events. In addition to the proofreading node involving LAT Y132 shown here, co-receptor scanning and delivery of Lck can also influence T cell ligand discrimination\textsuperscript{26}. To experimentally compare the relative contributions of co-receptor scanning and G131–Y132 of LAT in TCR signaling, we again used the OT-I\textsuperscript{+} Jurkat cells and OVA APL systems. We used OT-I\textsuperscript{+} Lat\textsuperscript{f/–} CD8 T cells transduced with wild-type or G131D mutant (termed J.OT-I.hCD8\textsuperscript{neg.LAT.WT} or J.OT-I.hCD8\textsuperscript{neg.LAT.G131D}, respectively). With these cells, we performed experiments as we did in Fig. 3. The presence of hCD8 seemed to enhance the number of cells responding towards OVA peptide or the partial agonists Q4R7, T4, or Q4H7, compared with cells lacking hCD8, but did not alter the EC\textsubscript{50} value of cell responses towards the low-affinity peptide G4 or self-peptide Catnb (Supplementary Fig. 7b,c). Thus, G131–Y132 of LAT creates an important TCR signaling bottleneck that is distinct from the previously reported time delay generated by co-receptor scanning\textsuperscript{26}.

To investigate whether the slow phosphorylation of Y132 in LAT is a unique rate-limiting step to control ligand discrimination, we explored the possibility that any of the other four individual tyrosine residues in LAT (that are phosphorylated more efficiently than Y132) could similarly function as ‘artificial’ TCR signaling bottlenecks to further improve T cell ligand discrimination. We expressed ‘GY series’ LAT mutants in JOT-I\textsuperscript{+}.hCD8– LAT-deficient cells (termed JOT-I-LAT.G126Y127, JOT-I-LAT.G170Y171, JOT-I-LAT.G190Y191, or JOT-I-LAT.G225Y226, respectively). Interestingly, these mutants and wild-type LAT showed similar CD69 expression levels after stimulation with OVA or the partial agonists Q4R7 or T4-pulsed T2-K\textsuperscript{+} cells (Supplementary Fig. 7d). The artificial attenuation of phosphorylation at LAT tyrosines other than Y132 did not influence OT-I TCR sensitivity or specificity, although there is probably redundancy in binding interactions with other SH2-containing proteins at the other phosphorylation sites. Thus, T cell ligand discrimination is preferentially controlled by the slow phosphorylation of LAT Y132 and probably depends on the importance of events downstream of PLC-\(\gamma\).1.

Divergence at the LAT Y132 phosphosite in fish lineages may reflect temperature-sensitive signaling. The slow phosphorylation kinetics of Y132 can be largely attributed to a –1 glycine residue, which cannot form critical interactions with a conserved lysine residue (K338) in the substrate-binding site of the ZAP-70 catalytic domain\textsuperscript{15,16}. K338 and other positively charged residues in the ZAP-70 active site are conserved across all jawed vertebrates (Fig. 6a)\textsuperscript{5}, suggesting that the substrate-binding mode of ZAP-70 is conserved in these organisms. The glycine residue at position 131 in LAT is also highly conserved across jawed vertebrates, consistent with a conserved regulatory role for slow LAT Y132 phosphorylation (Fig. 6b,c). Interestingly, a ‘better neighbor’ preceding Y132, such as asparagine, aspartate, or glutamate, is present in some fish (Fig. 6b). Among the other key tyrosine residues in LAT that get phosphorylated, there is also a relative lack of sequence conservation at the –1 positions among fish (Fig. 6d). However, throughout jawed vertebrate evolution, the preceding sequence is dominated by a negatively charged residue and lacking in any positively charged residues, consistent with the features preferred by ZAP-70 for tyrosine phosphorylation (Fig. 6d).

Given our data on the critical role of the slow kinetics of Y132 phosphorylation for T cell ligand discrimination, we wondered why G131 is not completely conserved in fish. Based on our data in mammalian T cells, the other three amino acids observed at position 131 in fish LAT sequences (asparagine, glutamate, and asparagine) should speed up the phosphorylation of Y132 and enhance calcium responses towards low concentrations of anti-CD3 stimuli (data for G131N not shown). We therefore questioned why some fish would tolerate faster phosphorylation of this tyrosine if it might impair ligand discrimination.

![Fig. 6 | LAT Y132 probably represents a conserved kinetic proofreading step in tetrapodal T cells. a. Sequence conservation between the human and zebrafish ZAP-70 kinase domains. The kinase domains of human and zebrafish ZAP-70 are 65% identical. Conservation is mapped onto a model of the human ZAP-70 kinase domain bound to a peptide surrounding LAT Y226 (ref. 15). The surface of the kinase domain is colored based on which residues are identical (blue), have physiochemical similarity (light purple), or are unconserved (purple), between the human and zebrafish sequences. The peptide is shown in ball and stick representation, with LAT Y226 and the lysine residue (K538) in the substrate-binding site of the ZAP-70 catalytic domain\textsuperscript{15,16}. K538 and other positively charged residues in the ZAP-70 active site are conserved across all jawed vertebrates (Fig. 6a). Interestingly, a ‘better neighbor’ preceding Y132, such as asparagine, aspartate, or glutamate, is present in some fish (Fig. 6b). Among the other key tyrosine residues in LAT that get phosphorylated, there is also a relative lack of sequence conservation at the –1 positions among fish (Fig. 6d). However, throughout jawed vertebrate evolution, the preceding sequence is dominated by a negatively charged residue and lacking in any positively charged residues, consistent with the features preferred by ZAP-70 for tyrosine phosphorylation (Fig. 6d).](https://www.nature.com/natureimmunology)
Fish have a more limited antigen receptor repertoire than do mammals\(^3\), and are also generally cold-blooded\(^3\). We considered the possibility that better Y132 phosphorylation kinetics might provide certain immune fitness advantages for these fish, as their body temperatures are probably lower than those of mammals.

To test the effect of temperature on the ability of T cells to induce...
calcium mobilization, we compared mouse and zebrafish thymocytes. Zebrafish have a naturally occurring aspartate preceding the tyrosine residue in LAT that is homologous to human Y132 and mouse Y136 (Fig. 6b). We used zebrafish that carry an Lck-eGFP transgene to harvest the zebrafish thymi and identify thymocytes (Fig. 7a). Since no monoclonal antibodies are currently available to stimulate zebrafish TCRs or CD3 (ref. 33), we stimulated zebrafish or mouse thymocytes with concanavalin A, a plant lectin that depends on TCR expression to induce calcium increases in Jurkat T cells33,34. Interestingly, temperature change had little impact on concanavalin A responses of Lck-eGFP35 zebrafish thymocytes (Fig. 7b). By contrast, mouse thymocytes, which have a glycine preceding Y136, did not respond to concanavalin A stimulation at lower temperatures (Fig. 7c and Supplementary Fig 8). Importantly, when the cells were treated with ionomycin, zebrafish and mouse thymocytes had similar maximal calcium responses, although responses were delayed in mouse thymocytes incubated at a reduced concentration (Fig. 7c).

Next, we tested whether G131D-expressing Jurkat cells may gain the temperature resistance properties seen in zebrafish thymocytes. We used biotinylated OVA H-2K\(^{\alpha}\) monomers followed by streptavidin to stimulate J.OT-I.LAT.G131D cells. Notably, J.OT-I.LAT.WT cells were more vulnerable to the change in temperature than were J.OT-I.LAT.G131D cells (Fig. 8a,b). At room temperature, crosslinking of OVA H2-K\(^{\alpha}\) was unable to induce calcium flux in wild-type LAT-expressing cells, whereas the change in temperature had minimal impairment on G131D-expressing JOT-I\(^{+}\) cells. Next, we used immunoblots to examine LAT phosphorylation of J.OT-I.LAT.WT or J.OT-I.LAT.G131D. G131D cells stimulated with biotin-labeled OVA monomers, followed by streptavidin crosslinking, across different temperatures. Although wild-type LAT was phosphorylated in response to stimulation at temperatures close to 37 °C, we could not detect the phosphorylation of Y132 at 25 °C or 28 °C (Fig. 8c). By contrast, at 25 °C stimulation, we could detect phosphorylation at LAT tyrosine residues that have aspartate at the −1 position (Y171 or G131D preceding Y132), although this was much weaker than under stimulation at 37 °C (Fig. 8c). Interestingly, we also observed an increase in basal phosphorylation of LAT Y132 and PLC-\(\gamma\) Y783 in J.OT-I.LAT.G131D cells, compared with those in J.OT-I.LAT.WT cells (Fig. 8c). Since we did not observe a similar increase in basal phosphorylation of Y132 in experiments that used anti-CD3 as the stimulus, the increase in basal phosphorylation here could be due to the prelabeling of cells with biotinylated OVA monomers at room temperature.

Our sequence analyses and comparison of temperature effects on calcium responses in mouse versus zebrafish thymocytes, or wild-type LAT versus G131D-expressing OT-I\(^{+}\) Jurkat cells suggest that slow LAT Y132 phosphorylation has been selected for in most jawed vertebrate lineages, and is almost fixed in tetrapods. Some fish species, such as zebrafish, seem to have evolved to have faster phosphorylation of Y132, which may be required to enable signaling at the range of water temperatures in which these animals live in the wild (Supplementary Fig 9).

### Discussion

The balance of T cell sensitivity and specificity requires TCR discrimination of agonist pMHC from self-MHC, which can differ by as little as a factor of ten in their affinities for the TCR\(^{36}\). The kinetic proofreading model proposes that signal accuracy can be achieved by accepting some tolerable time delay through a series of reversible biochemical modifications before the commitment step that triggers a response. Here, we have shown that LAT functions as a critical time-keeper through constraints placed by a single residue, G131, which attenuates ZAP-70-mediated phosphorylation of Y132. The phosphorylation of Y132 is required for the recruitment, phosphorylation, and activation of PLC-\(\gamma\). The activation of PLC-\(\gamma\) is critical for the subsequent calcium increase and PKC and Ras activation, which mediate T cell cytokine production, proliferation, and effector responses. The molecular time delay at LAT Y132 is necessary for proper self–non-self discrimination, and our observations support the importance of slow Y132 phosphorylation in TCR kinetic proofreading.

Mutating G131 to aspartate leads to faster phosphorylation kinetics for Y132, but with negative consequences for ligand discrimination. Y132 is unique among the LAT phosphorylation sites in its ability to influence ligand discrimination, most likely because it is the only phosphorylation site that directly recruits PLC-\(\gamma\). The other four phosphorylation sites in LAT all have a YXXN motif, consistent with Grb2 or Gads recruitment sites\(^{37,38,39}\). Mutation of Y171, Y191, or Y226 has a minimal effect on T cell development and maturation\(^{39}\). By contrast, replacement of Y132 with phenylalanine disrupts thymic development\(^{40,41}\), and also leads to TCR-independent lymphoproliferation of T cells, suggesting that PLC-\(\gamma\) signals may maintain ‘balanced’ cell signaling to ensure an appropriate T cell response\(^{42}\). Thus, Y132 in LAT is a potentially unique and important bottleneck, to temporally regulate the recruitment of PLC-\(\gamma\), with help from other tyrosine residues (probably through Gads–SLP-76 preassembly on LAT to stabilize PLC-\(\gamma\) binding\(^{37,38,43,44}\).
Although artificial attenuation of LAT phosphorylation at individual tyrosine residues other than Y132 did not influence signaling sensitivity, we expect that combinations of −1 glycine substitutions at LAT Y171, Y191, or Y226 might perturb T cell sensitivity. There may be several events involved in kinetic proofreading, each of which could contribute to the time delay required for ligand discrimination. Very recently, using optogenetic methods, two groups implicated immediate downstream events of PLCγ1 activation, calcium and diacylglycerol increases, in kinetic proofreading. Notably, our study has identified PLCγ1 (p-Y783) as a very poor substrate for ZAP-70 in tetrapods, which might reflect the need for optimal ligand discrimination.

We therefore questioned why some fish have not adopted this same strategy for ligand discrimination. One major difference between tetrapods and fish is the maintenance of their body temperatures. Mammals are warm-blooded, whereas fish are cold-blooded, and the temperature of amphibians and reptiles can vary greatly depending on the environment. Various enzymes, including kinases, are sensitive to the temperature. Thus, some fish may require a more optimal ZAP-70 substrate to phosphorylate their LAT Y132 homologous sequences to activate PLCγ1 when in cold water. Zebrafish T cells have a limited TCR repertoire, and they are more cross-reactive to self-antigens than are mammalian T cells. This may be one strategy to compensate for their limited and self-cross-reactive TCR repertoire, but allow for protective T cell immunity to foreign antigens. This increased self-reactivity of zebrafish T cells may be constrained by the prominence of regulatory T cells to prevent auto-reactivity. T and B cell-mediated adaptive immunity evolved approximately 500 million years ago, striking that almost all tetrapodal LAT molecules examined have a glycine at this position. This could be the result of a strong selective pressure that was more relaxed in fish. Interestingly, other tyrosine residues of LAT have been under strong selection pressure in tetrapods, which might reflect the need for optimal ligand discrimination.

As for the combination of glycine substitutions at LAT Y171, Y191, and Y226, we speculate that their LAT Y132 homologous sequences to activate PLCγ1, there may be some synergy among the glycine substitutions at LAT Y171, Y191, and Y226, which might be beneficial to T cell kinetic proofreading.
with the emergence of jawed vertebrates. Since then, different organisms may have adapted slight variations on this system, as our analyses suggest for LAT phosphorylation. More comparisons of the sequences and activities of T cell signaling molecules across the animal kingdom are likely to reveal new mechanisms for the control of T cell activation.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0502-2.

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References
1. Feinerman, O., Germain, R. N. & Altman-Bonnet, G. Quantitative challenges in understanding ligand discrimination by αβ T cells. Mol. Immunol. 45, 619–631 (2008).
2. Brameshuber, M. et al. Monomeric TCRs drive T cell antigen recognition. Nat. Immunol. 19, 487–496 (2018).
3. Chakraborty, A. K. & Weiss, A. Insights into the initiation of TCR signaling. Nat. Immunol. 15, 798–807 (2014).
4. Huang, I. et al. A single peptide-major histocompatibility complex ligand triggers digital cytokine secretion in CD4+ T cells. Immunity 39, 846–857 (2013).
5. Cui, W. & Mehta, P. Identifying feasible operating regimes for early T-cell recognition: the spectrum of efficiency trade-off in kinetic proofreading and adaptive sorting. PLoS One 13, e0202331 (2018).
6. McKeithan, T. W. Kinetic proofreading in T-cell receptor signal transduction. Proc. Natl Acad. Sci. USA 92, 5042–5046 (1995).
7. Dustin, M. L. Stop and go traffic to tune T cell responses. Curr. Top. Microbiol. Immunol. 373, 49–67 (2014).
8. Siller-Farfan, J. A. & Dushek, O. Molecular mechanisms of T cell sensitivity to antigen. Immunol. Rev. 285, 194–205 (2018).
9. Germain, R. N. Computational analysis of T cell receptor signaling and ligand discrimination—past, present, and future. FEBS Lett. 584, 4814–4822 (2010).
10. Gaud, G., Lesourne, R. & Love, P. E. Regulatory mechanisms in T-cell receptor-mediated signaling pathways. J. Biol. Chem. 290, 26422–26429 (2015).
11. Andreotti, A. H., Schwartzberg, P. L., Joseph, R. E. & Berg, L. J. T-cell specificity screen. J. Immunol. 175, 2449–2458 (2005).
12. Schoenborn, J. R., Tan, Y. X., Zhang, C., Shokat, K. M. & Weiss, A. Feedback circuits monitor and adjust basal Lck-dependent events in T cell receptor signaling. Sci. Signal. 4, ra9 (2011).
13. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Tribble, R. P. & Samelson, L. E. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell 92, 83–92 (1998).
14. Stoisca, B. et al. The amino-terminal Src homology 2 domain of phospholipase Cγ1 is the potent activator of LAT tyrosine phosphorylation of phospholipase Cγ1. J. Immunol. 160, 1059–1066 (1998).
15. Songyang, Z. et al. SH2 domains recognize specific phosphopeptide sequences. Cell 72, 767–778 (1993).
16. Lo, W. L. et al. Lck promotes Zap70-dependent LAT phosphorylation by bridging Zap70 to LAT. Nat. Immunol. 19, 733–741 (2018).
17. Rosette, C. et al. The impact of duration versus extent of TCR occupancy on T cell activation: a revision of the kinetic proofreading model. Immunity 15, 59–70 (2001).
18. Houtman, J. C. et al. Binding specificity of multiprotein signaling complexes is determined by both cooperative interactions and affinity preferences. Biochem. (Mosc.) 43, 4170–4178 (2004).
19. Zhu, M., Janssen, E. & Zhang, W. Minimal requirement of tyrosine residues of linker for activation of T cells in TCR signaling and thymocyte development. J. Immunol. 170, 325–333 (2003).
20. Aguado, E. et al. Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. Science 296, 2036–2040 (2002).
21. Sommers, C. L. et al. A LAT mutation that inhibits T cell development yet induces lymphoproliferation. Science 296, 2040–2043 (2002).
22. Cortum, R. L. et al. A phosphophosphopeptide Y121-independent, RasGRP1-ERK-dependent pathway drives lymphoproliferative disease in linker for activation of T cells-Y136P mutant mice. J. Immunol. 190, 147–158 (2013).
23. Miyaji, M. et al. Genetic evidence for the role of Erk activation in a lymphoproliferative disease of mice. Proc. Natl Acad. Sci. USA 106, 14582–14587 (2009).
24. Lin, J. & Weiss, A. Identification of the minimal tyrosine residues required for linker for activation of T cell function. J. Biol. Chem. 276, 29588–29595 (2001).
25. Zhang, W. et al. Association of Grb2, Gads, and phospholipase C-γ1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. J. Biol. Chem. 275, 23355–23361 (2000).
26. Yousefi, O. S. et al. Optogenetic control shows that kinetic proofreading regulates the activity of the T cell receptor. Elife 8, e42475 (2019).
27. Fischer, D. K. & Weis, A. Dynamic allostery: a putative ribosomal ‘skip’. Nature 458, 47–59 (2010).
28. Tang, M. A., Motshiona, H. & Watanabe, K. Cold adaptation: structural and functional characterizations of psychrophilic and mesophilic acetate kinase. Protein J. 33, 313–322 (2014).
29. Roudier, J. M., Lebrun, J. A. & Hlad, M. J. Dynamic allostery can drive a cold-adaptive enzyme. Nature 558, 324–328 (2018).
30. Flajnik, M. F. & Kasahara, M. Origin and evolution of the adaptive immune system: genetic events and selective pressures. Nat. Rev. Genet. 11, 47–59 (2010).
31. Hollar, M., Das, S., Guo, P. & Cooper, M. D. The evolution of adaptive immunity in vertebrates. Adv. Immunol. 109, 125–157 (2011).
32. Polos, M. et al. Tyrosine 319 in the interdomain B of ZAP-70 is a key gatekeeper of T cell receptor signaling in vivo. Mol. Cell. Biol. 36, 2396–2402 (2016).
53. Lo, W. L., Solomon, B. D., Donermeyer, D. L., Hsieh, C. S. & Allen, P. M. T cell immunodominance is dictated by the positively selecting self-peptide. Elife 3, e01457 (2014).

54. Sayers, E. W. et al. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 37, D5–D15 (2009).

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Author contributions
W.-L.L, N.H.S., J.K., and A.W were responsible for conceptualization; W.-L.L, N.H.S., S.A.R., L.I.Z., J.K., and A.W were responsible for the methodology; W.-L.L, N.H.S, S.A.R., and L.I.Z. carried out the investigations; W.-L.L, N.H.S., S.A.R., and A.W wrote the original draft; W.-L.L, N.H.S., S.A.R., V.H, I.R.F., W.Z., O.S., L.I.Z., J.K., and A.W reviewed and edited the manuscript; W.Z., V.H., and O.S. provided resources; L.I.Z., J.K., and A.W supervised the study; and W.-L.L, N.H.S., W.Z., O.S., L.I.Z., J.K., and A.W acquired funding.

Competing interests
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Methods

Experimental models. Animals. The C57BL/6 mice were housed in the specified pathogen-free facilities at the University of California, San Francisco. The E8Cre/LAT\(^{−}\)/OT-I mice were maintained at Duke University. Mice were treated according to protocols that were approved by the University of California, San Francisco veterinary committee, or by Duke University animal care ethics committee, and are in accordance with National Institutes of Health (NIH) guidelines. Both male and female mice, 6–12 weeks of age, were used in the studies. Zebrafish were maintained in accordance with Boston Children's Hospital Institutional Animal Care and Use Committee protocols and in line with Animal Resources at Children's Hospital (ARCH) guidelines. Tg(Ick-eGFP) was previously described\(^{22}\). Male and female zebrafish between 2 and 4 months post-fertilization were used in all studies.

Cell lines. The human leukemic Jurkat T cell line, Jurkat variants with LAT deficiency, ZAP-70 deficiency, or ITK deficiency, and T2-K\(^{+}\) cells were maintained in RPMI culture medium supplemented (Thermo Fisher Scientific) with 5% FBS (Omega Scientific) and 2 mM glutamine (Thermo Fisher Scientific). For additional drug selection, LAT-deficient Jurkat variants that were reconstituted with wild-type LAT or various mutant LAT constructs were maintained in 0.5 mg ml\(^{-1}\) of the aminoglycoside gentamicin (G418, Santa Cruz Biotech), the C-AS Jurkat variant was maintained in 10 μg ml\(^{-1}\) blasticidin (Thermo Fisher Scientific).

Antibodies. Antibodies are listed in Reporting Summary and Supplementary Note. Sequence alignments and analysis. Sequences of LAT orthologs from various jawed vertebrates were identified as described previously\(^{17}\). Briefly, human LAT was used as a query sequence for an initial protein–protein BLAST search using the NCBI non-redundant protein database\(^{47,48}\). This initial search yielded mostly mammalian sequences, as well as a few fish, amphibian, and reptile sequences that were annotated to be LAT orthologs. These non-mammalian LAT sequences were used as queries in subsequent BLAST searches. Putative orthologous tyrosine phosphosites were identified in the C-terminal approximately 100 amino acids of each LAT sequence based on two criteria: they matched the SH2 binding motifs, and they occurred in the order seen for known human and mouse LAT phosphosites. The sequences surrounding individual putative phosphosites across all orthologs were manually aligned, and this local alignment was visualized using the online tool WebLogo\(^{58}\).

Intracellular calcium measurements by flow cytometry. Mouse CD8\(^{+}\) T cells stimulated by OVA- or APL-loaded biotinylated pMH C monomers. Naïve OT-3\(^{+}\) CD8\(^{+}\) T cells were isolated and transduced with retrovirus expressing wild-type LAT-P2A-HP or G135D LAT-P2A-BFP. Cells were loaded with 1 μM of the calcium indicator dye Indo-1 AM calcium indicator dye (Thermo Fisher Scientific) and 0.02% Pluronic F-127 (Thermo Fisher Scientific) at 37°C in RPMI medium for 30 min, washed twice with PBS, and then labeled with 1:100 dilution of biotinylated OVA/H-2K\(^{b}\), T4/H-2K\(^{b}\), G4/H-2K\(^{b}\), or VSV/H-2K\(^{b}\) monomers (NIST Tetramer Core Facility) at 37°C for 30 min. Cells were then fixed and treated to flow cytometry with calcium assays. Indo-1 cell-associated fluorescence was first recorded for 30 s to obtain a baseline calcium level, followed by the addition of concanavalin A at 30 s, and the calcium–fluorescence changes were recorded for another 4.5 min. Data were analyzed into GraphPad Prism software (version 7) for analysis and production of graphs.

Reconstituted LAT-deficient Jurkat with LAT mutants. LAT-deficient Jurkat cells were generated in our previous study\(^{28}\) and were used for reconstitution with PEF-vectors that express wild-type LAT, G131D, or G131E mutants by electroporation (Bio-Rad Laboratories).

Zebrafish thymocytes stimulated by concanavalin A at different temperatures. Ice water immersion was used to euthanize the zebrafish before dissection of the thymi. Thymi were dissected from 15 or 16 zebrafish bilaterally into 800 μl of dissection solution: HBSS (no Ca\(^{2+}\), no Mg\(^{2+}\)) with 0.2% FBS. After all of the thymi were collected, the solution was pipetted 15–20 times and filtered, followed by filtration through a pre-moistened 40 μm cell strainer. Next, the cells were centrifuged at 400 × g for 5 min at 25°C. The cell pellet was washed with 800 μl of dissection solution, centrifuged at 400 × g for 5 min, and resuspended at 2.2 × 10\(^{6}\) cells per ml in assay buffer (HBSS with Ca\(^{2+}\) and Mg\(^{2+}\), 20 mM HEPES at pH 7.4). Indo-1 AM (Millipore Sigma) was pre-mixed 1:1 with Pluronic F-127 (Thermo Fisher Scientific) and added to the cell suspension at a final concentration of 1 μM Indo-1, 0.02% Pluronic F-127, and 0.18% DMSO and vortexed immediately. After incubating in the dark for 30 min at 28.5°C, the cells were washed twice with assay buffer, centrifuged at 400 × g for 5 min at room temperature, and then resuspended at a final concentration between 1.6 × 10\(^{6}\) and 3.5 × 10\(^{6}\) cells per ml in assay buffer. The cells were incubated at 15°C, 25°C, and 37°C using Eppendorf thermomixers (Eppendorf) for 10–15 min before running on an LSRFortessa (BD Biosciences). Baseline calcium-dependent fluorescence was determined over a 30 s interval before the addition of stimulus: concanavalin A (final concentration 10 μg ml\(^{-1}\), Millipore Sigma) followed by the addition of ionomycin at 240 s to a final concentration of 1 μM. Mouse thymocytes stimulated with concanavalin A at different temperatures. Thymi from C57BL/6 mice were harvested and prepared as single cell suspension in RPMI. Cells were labeled with 1 μM Indo-1 at 37°C for 30 min, washed twice with PBS, and then resuspended in HBSS at the concentration of 1 × 10\(^{6}\) cells per ml. The cells were incubated at 37°C in a water bath, at room temperature, or on ice in a pre-cooled benchtop centrifuge for 15 min before running the experiment started. The calcium–dependent Indo-1 fluorescence was recorded on an LSRFortessa (BD Biosciences). The fluorescence was first recorded for 30 s to obtain a baseline level, and then cells were stimulated with concanavalin A at 30 s, followed by stimulation with ionomycin at 240 s. The concanavalin A was purchased through the same vendor and lot number as the calcium experiments performed with zebrafish thymocytes.

Immunoblot analysis. Immunoblot analysis was performed as previously described\(^{15}\) and more details could be found in the Supplementary Note.

C690 activation assay. The JOT-1 hCD8\(^{+}\) or JOT-1 hCD8\(^{+}\) series of Jurkat derivative cells expressing wild-type LAT or G131D or G131E variants were used in the experiments. A series of titrated concentrations of OVA or APL peptides were incubated with T2-K\(^{+}\) cells (2.5 × 10\(^{5}\) cells per well) in flat-bottom 96-well plates at 37°C for 1 h. 2.5 × 10\(^{5}\) cells of JOT-1 hCD8\(^{+}\) or JOT-1 hCD8\(^{+}\) series of Jurkat derivative cells were added into each well of the 96-well plate that contained the
peptide-pulsed T2-K⁺ cells. The plates were incubated at 37 °C for around 16 h. For mouse OT-I⁺ CD8 or OT-II⁺ CD4 cells, cells were prepared and transduced to express wild-type or G135D LAT. Splenocytes from TCR Co-deficient mice were used as antigen-presenting cells. 5 × 10⁴ cells per well of TCR Co-deficient splenocytes were pulsed with titrated concentrations of OVA or APL peptides, and cultured with 5 × 10⁴ cells of the transduced mouse T cells at 37 °C for around 16 h. Upregulation of CD69 was analyzed on an LSRFortessa (BD Biosciences) the next day.

**Phospho-ERK activation assay.** T2-K⁺ cells (2.5 × 10⁴ cells per well) were pulsed with titrated concentrations of OVA or APL peptides in U-bottom 96-well plates and incubated at 37 °C for 1 h. Individual clones of Jurkat derivative cells were barcoded by labeling each clone with differently titrated concentrations of CellTrace Violet (Thermo Fisher Scientific). Jurkat derivative cells expressing wild-type LAT were labeled with 2.5 μM CellTrace Violet in PBS at 37 °C for 20 min in the dark, whereas G131D- or G131E-expressing cells were labeled with 0.1 μM or 0.5 μM of CellTrace Violet, respectively. Complete medium was then added to the cells for another 5 min of incubation at 37 °C, and cells were washed with ice-cold PBS, resuspended in PBS to a concentration of 2.5 × 10⁵ cells per ml, and kept on ice. 2.5 × 10⁵ cells of the barcoded Jurkat derivatives were added into each well that contained the peptide-pulsed T2-K⁺ cells. During the whole process the plates remained on ice. Cells were mixed well, and the plate was quickly centrifuged at 2,200 r.p.m. at 4 °C for 30 s. The plate was then moved to a 37 °C water bath for 5 min to start the stimulation. The stimulation was stopped by the direct addition of 4% formaldehyde (final concentration of 2%). The plate was incubated at 25 °C for 30 min for formaldehyde fixation. Cells were washed once with FACS buffer (2% FBS, 1 mM EDTA in PBS buffer), and permeabilized in 90% ice-cold methanol overnight at 4 °C. Cells were washed once with FACS buffer, rested in FACS buffer at 25 °C for 30 min, and stained with anti-phospho-ERK (Cell Signaling Technology) for LSFRFortessa analysis (BD Biosciences). For mouse T cells stimulated with OVA or APL-pulsed Co-deficient splenocytes, the procedures were similar to the experiments in Jurkat derivative cells, except that TCR Co-deficient splenocytes were used as antigen-presenting cells.

**IRF4 upregulation assay.** Mouse OT-I⁺ CD8 or OT-II⁺ CD4 cells were prepared and transduced to express wild-type or G135D LAT. Cells were rested for 1 d before being used in the experiments. Splenocytes from Co-deficient mice (2.5 × 10⁵ cells per well) were mixed with titrated concentrations of OVA or APL peptides and mouse T cells, and incubated at 37 °C for approximately 16 h at 37 °C. Cells were washed once with FACS buffer (2% FBS, 1 mM EDTA in PBS buffer), stained for CD4 or CD8, washed in FACS buffer, and then fixed and permeabilized in ebioscience Transcription Factor Staining Buffer (Thermo Fisher Scientific). Cells were stained with anti-IRF4 (BioLegend) and analyzed by LSFRFortessa (BD Biosciences).

**IFN-γ secretion assay.** Mouse OT-I⁺ CD8 or OT-II⁺ CD4 cells were prepared and transduced to express wild-type or G135D LAT. A 48-well plate was coated with a 1:100 dilution of OVA- or APL-loaded biotinylated monomeric the day before. Cells were washed and rested for 1 d in complete media without IL-2 before being used in the experiments. Cells were stimulated overnight at 37 °C and harvested for IFN-γ secretion analysis using an IFN-γ secretion assay (Miltenyi Biotec) and assayed by LSFRFortessa (BD Biosciences).

**Production of lentivirus or retrovirus expressing wild-type or G135D LAT.** Murine wild-type Lat was cloned into the pHR backbone under the expression of the Ef1α promoter. The murine G131D-LAT mutant was generated using a QuickChange Lightning site-directed mutagenesis kit (Agilent Technologies). A C-terminal P2A self-cleaving peptide followed by mCherry was incorporated to assess transduction efficiency and expression levels. Packaging vector pCMV dR8.91, envelope vector pMD2.G, and pHR.WT-LAT.P2AmCherry or pHR.G135D-LAT.P2AmCherry constructs were transiently co-transfected into LX-293T cells using TransIT-LT1 reagent (Mirus Bio). Supernatants containing virus particles were collected 48 h after transfection, filtered, and concentrated by PEG 8000 precipitation. The virus particles were resuspended in PBS and stored at −80°C. For retrovirus-transduced experiments, murine WT-LAT or G135D-LAT were cloned into the mPCI vectors individually, along with a C-terminal P2A self-cleaving peptide followed by BFP to help determine transduction efficiency and monitor expression level. The Phoenix-Eco packaging cell line was transduced using Lipofectamine 2000 (Thermo Fisher Scientific). 48 h after transduction, the supernatants were harvested for experimental use. The viral supernatants were prepared freshly for each experiment.

**Retroviral transduction of mouse peripheral CD8⁺ or CD4⁺ T cells.** Naïve mouse CD8 or CD4 T cells were isolated using biotinylated antibody cocktails (a mixture of anti-CD4 or anti-CD8, together with anti-CD19, anti-B220, anti-CD11b, anti-CD11c, anti-DX5, anti-TER119, and anti-CD24) and magnetic bead-mediated negative selection (anti-biotin Miltenyi Beads, Miltenyi Biotec). The retroviral supernatants (prepared freshly for every experiment) were first mixed with Lipofectamine (final concentration of 8 μg/ml⁻¹) and IL-2 (final concentration of 50 U/ml⁻¹), and incubated at 37 °C for 20–30 min. In a 24-well plate, 1 × 10⁵ T cells were incubated with 1 ml retroviral supernatants, lipofectamine, and IL-2 per well. The plate was wrapped in saran wrap and centrifuged at 460 g for 1 h at 25 °C. The plate was then moved to a 37 °C incubator. BFP expression was monitored by LSFRFortessa (BD Biosciences) and can be seen 24 h after transduction.

**Tamoxifen treatment and lentiviral transduction of mouse peripheral CD8⁺ T cells.** CD8⁺ T cells from spleens of ERT²Cre⁺ OT-I⁺LATf⁻ mice were prepared, and naïve CD4⁺CD62L⁺Vα2⁺CD8⁺ cells were sorted using a FACSArria II (BD Biosciences). naïve CD8⁺ T cells were cultured in a 24-well plate with 5 μg/ml⁻¹ plate-bound anti-CD3 (clone 2C11, Weiss Lab) and 5 μg/ml⁻¹ soluble anti-CD28 (clone 37.51, Weiss Lab) overnight at 37 °C. A non-tissue culture-treated 24-well plate was coated with 3 μg RetroNectin (Takara Bio) in 250 μl PBS/Well per well for 25 °C for 2 h, blocked with 2% BSA at 25 °C for 30 min, and then bound with concentrated lentivirus particles by centrifuging at 460 g for 1 h at 25 °C and washed with PBS. The next day, activated CD8⁺ T cells were added to the RetroNectin-coated, lentivirus-bound plates and centrifuged at 460 g for 5 min at 25 °C, and incubated at 37 °C overnight in the presence of mouse IL-2 (10 ng/ml⁻¹) and 50 nM 4-hydroxytamoxifen (Millipore Sigma) for 4 d. Tamoxifen-mediated deletion of endogenous LAT can be monitored by the expression of GFP, and the transduction efficiency can be monitored by the expression of mCherry. Cells were rested in the complete medium without IL-2 a day before being used in experiments.

**Quantification and statistical analysis.** Statistical analysis was applied to technical replicates, or biologically independent mice for each experiment. All experiments described in this study have been performed at least twice, and the exact numbers of independent experiments with similar results are indicated in the figure legends. All statistical analyses of experiments were performed using non-parametric, two-tailed Mann–Whitney U tests. GraphPad Prism 6 Software (GraphPad Software) was used for data analysis and representation. All bar graphs show means with overlaid scatter dots, or error bars (indicating s.d.), to show the distribution of the data, as indicated in each figure legend. P values for comparisons are provided as exact values or as P < 0.0001. 95% confident intervals were used to determine statistically significant P values.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the corresponding author.

**References.**

55. Langenau, D.M. et al. In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish. *Proc. Natl Acad. Sci. USA* 101, 7369–7374 (2004).

56. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410 (1990).

57. Pruitt, K.D., Tatusova, T. & Maglott, D.R. NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res.* 33, D501–D504 (2005).

58. Crooks, G.E., Hon, G., Chandonia, J.M. & Brenner, S.E. WebLogo: a sequence logo generator. *Genome Res.* 14, 1188–1190 (2004).
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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  *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐ A description of all covariates tested
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☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
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☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- Image Lab [Bio-Rad] V5.2.1 built 11 was used to acquire immunoblot data; BD FACSDiva v8.0.1 software was used for flow cytometry.

Data analysis
- Prism (GraphPad) Version 7 was used to data analysis; FlowJo, Version 9.9.3 or Version 10 was used for flow cytometry data analysis. SnapGene software v4.0.8 was used to analyze DNA sequences or protein sequences.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
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All studies must disclose on these points even when the disclosure is negative.

**Sample size**
For cell line-related experiments, in general 5 x 10⁶ cells per sample were used for immunoblot analysis, calcium analysis, or flow cytometry analysis. For primary mouse cells experiments, cell numbers were as indicated in each method section. Cell numbers were decided upon empirically, and were routinely used in the lab that were known to be sufficient to allow reliable detection of immunoblot analysis, and each experiment was repeated at least three times. Relevant data for accurate power calculation was unavailable.

**Data exclusions**
We did not exclude any sample.

**Replication**
The findings were reliably reproducible. Each set of experiments was repeated at least three times, and different experiments were designed to test the hypothesis from orthogonal perspectives. Regarding CRISPR/Cas9 generated cell lines, multiple clones were tested where possible to limit off target effect. We obtained 12 J.IAT (LAT deficient) clones, 5 1.2ap70 (ZAP-70 deficient) and 2 J.I.TK (TK deficient) and randomly chose two different J.IAT clones for reconstitution studies, and similar results were found with each. For primary mouse T cell experiments, we utilized two different approaches (retrovirally overexpression studies in Supplementary Figure 4 and lentivirally expression in Fig 5) and obtained similar results.

**Randomization**
Experimental allocations were not randomized. This is because we analyzed global effect of the mutant protein of our interest, and analyzed on the population levels, and therefore, there was no individual cell-to-cell variations to be controlled of.

**Blinding**
The bar coded experimental set up allowed us to examine WT and mutant samples under the same experimental conditions. Otherwise, no blinding was used. However, Internal negative controls were used to obtain unbiased data (as provided in Supplementary Figure 3 and 5).

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**Reporting for specific materials, systems and methods**

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study, if you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

| System | Involved in the study |
|--------|------------------------|
| Antibodies | ☒ |
| Eukaryotic cell lines | ☒ |
| Palaeontology | ☐ |
| Animals and other organisms | ☐ |
| Human research participants | ☐ |
| Clinical data | ☐ |

**Methods**

| Method | Involved in the study |
|--------|------------------------|
| ChIP-seq | ☒ |
| Flow cytometry | ☒ |
| MRI-based neuroimaging | ☒ |

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**Antibodies**

**Antibodies used**

- Rabbit monoclonal anti-human Itk (clone Y402; Abcam; Cat # ab32507; RRID:AB_2296410).
- BV711 rat monoclonal anti-mouse CD4 (clone RM4-4; BD Biosciences; Cat # 740651; RRID:AB_2740340).
- BV805 rat monoclonal anti-mouse CD8 (clone 53.6.7; BD Biosciences; Cat # 564920; RRID:AB_2718856).
- BV786 American monoclonal anti-mouse CD69 (clone H1.2F3; BD Biosciences; Cat # 564683; RRID:AB_2738890).
- APC Armenian monoclonal anti-mouse CD69 (clone H1.2F3; BD Biosciences; Cat # 556889; RRID:AB_1727506).
- PE-Cy7 mouse monoclonal anti-human CD69 (clone FN50; BD Biosciences; Cat # 557745; RRID:AB_396581).
- Mouse monoclonal anti-mouse/human phospho-LAT [Yyr171] (clone IS8-1169; BD Biosciences; Cat # 558399; RRID:AB_647174).
- Biotin rat monoclonal anti-mouse/human CD45R/B220 (clone RA3-6B2; BioLegend; Cat # 103204; RRID:AB_312989).
- Biotin rat monoclonal anti-mouse CD49b (pan-NK cells) (clone DX5; BioLegend; Cat # 108904; RRID:AB_313411).
- Biotin rat monoclonal anti-mouse TER-119/Erythroid Cells (clone TER-119; BioLegend; Cat # 116204; RRID:AB_313705).
- Biotin rat monoclonal anti-mouse CD24 (clone M1/69; BioLegend; Cat # 101804; RRID:AB_312837).
- Biotin rat monoclonal anti-mouse CD4 (clone GK1.5; BioLegend; Cat # 100404; RRID:AB_312629).
- PE mouse monoclonal anti-human CD3e (clone OKT3; BioLegend; Cat # 317308; RRID:AB_571913).
- Alexa Fluor 488 rat monoclonal anti-mouse IRF4 (clone IRF4.3E4; BioLegend; Cat # 646406; RRID:AB_2563267).
- PE-Cy7 rabbit monoclonal anti-mouse/human phospho-p44/p42 MAPK [Erk1/2] (Thr202/Tyr204) (clone 17G2; Cell Signaling Technology; Cat # 9101S).
- Rabbit polyclonal anti-mouse/human phospho-LAT [Yyr171] (clone 5197G2; Cell Signaling Technology; Cat # 35845; RRID:AB_2157728).
- Rabbit polyclonal anti-mouse/human LAT (clone 9166; RRID:AB_2283298).
- Mouse monoclonal anti-myc tag (clone 9B11; Cell Signaling Technology; Cat # 2765S; RRID:AB_331783).
- Anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology; Cat # 7074; RRID:AB_2099233).
- Anti-Phosphotyrosine Antibody (clone 4G10; EMD Millipore; Cat # 05-321; RRID:AB_309678).
- Mouse monoclonal anti-alpha tubulin (clone B-5-1-2; Sigma-Aldrich; Cat # T5168; RRID:AB_477579).
- Rabbit polyclonal anti-mouse/human phospho-LAT [Yyr132] (Thermo Fisher Scientific; Cat # 44-224; RRID:AB_2553608).
- Biotin rat monoclonal anti-mouse/human CD11b (clone M1/70; [Tonbo Biosciences; Cat # 30-0112-U500; RRID:AB_2621639].
- Biotin Armenian hamster monoclonal anti-mouse CD11c (clone N418) [Tonbo Biosciences; Cat # 30-0114-U100; RRID:AB_2621640].
- Biotin rat monoclonal anti-mouse CD6a (clone 53.6.7; Tonbo Biosciences; Cat # 30-0081-U500; RRID:AB_2621638).
- Biotin rat anti-mouse CD19 (clone 1D3; Tonbo Biosciences; Cat # 30-0193-U500; RRID:AB_2621641).
Validation

All the antibodies are from commercial sources and have been validated by the vendors. Validation data are available on the manufacturer’s website. The LAT phospho-Y132, phospho-Y171 antibodies were validated using the Y132F mutant or Y171F mutant expressing Jurkat cells and J LAT deficient Jurkat cells.

Eukaryotic cell lines

Policy information about cell lines

Jurkat cell lines and related variants were generated in Weiss lab or in Stepanek lab. HK293 cells were obtained from UCSF Cell Culture Facility. The Weiss Lab has deposited the original parental Jurkat E6-1 in the ATCC. Phoenix-ECO cells were obtained from ATCC, Cat # CRL-3214; RRID:CVCL_H717. Lenti-X 293T cells were obtained from Clontech, Cat # 632180.

Authentication

Identity of Jurkat is routinely validated using an anti-TCR Vbeta mAb (C305) generated by Dr. Weiss. LAT deficient and ZAP-70 deficient Jurkat cells have been routinely checked the deficiency of protein by immunoblot analysis.

Mycoplasma contamination

The parental Jurkat line has been tested for mycoplasma negative in past years. But derivative lines used here were not.

Commonly misidentified lines (See ICLAC register)

The Jurkat cell line was not among the misidentified cell lines published from the most recent version 8.0 of the database. HEK293 cell line was not listed in ICLAC database.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

The C57BL/6 mice were housed in the specific pathogen-free facilities at the University of California, San Francisco. The ERCre +LAT1/OT-I mice were maintained at Duke University. Mice were treated according to protocols that were approved by University of California, San Francisco veterinary committees, or by Duke University animal care ethics committee, and are in accordance with NIH guidelines. Both males and females, 6-12 weeks of ages, were used in the studies. Zebrafish were maintained in accordance with Boston Children's Hospital Institutional Animal Care and Use Committee protocols and in line with Animal Resources at Children's Hospital (ARCH) guidelines. Tg(lck:egFP) was previously described (Langenau et al., 2004). Male and female zebrafish between 2 and 4 months post-fertilization were used in all studies.

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected from the field.

Ethics oversight

Animals were maintained in accordance with Boston Children's Hospital Institutional Animal Care and Use Committee protocols, University of California, or approved by San Francisco veterinary committees, or by Duke University animal care ethics committee, and are in accordance with NIH guidelines.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☐ All plots are contour plots with outliers or pseudocolor plots.
☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Spleens and lymph node single cell suspensions were prepared by gently tweezing samples in cold PBS buffer containing 0.5% FBS and 0.2% EDTA.

Instrument

BD LSR Fortessa was used to collect flow cytometry data. BD FACSaria III was used for cell sorting.

Software

BD FACSDiva v8.0.1 software was used to collect samples. FlowJo v9.9.3 or v10 was used to analyze flow cytometry data.

Cell population abundance

Sorted naive CD25-CD44lowCD62Li OT-II CD4 or OT-I CD8 population was about 98% purity. The tamoxifen-induced GFP+ (LAT-deleted) population was >90%-95% after 96 hour of tamoxifen treatment. mCherry+ population (as an indicator of successful viral transduction) was about 50%-60%.

monoclonal anti-mouse/human ZAP-70 (clone IE7; Laboratory of Arthur Weiss, N/A). All primary antibodies were used at 1:1000 dilutions unless specified otherwise; secondary HRP conjugated antibodies were used at 1:10000 dilution.
Doublets were excluded using forward light-scatter gating followed by gating on lymphocytes based on FSC-SSC. Additional gating strategies were provided in the figures (Fig 4a, 5b, 6e).

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.