

Quantitative and temporal requirements revealed for Zap70 catalytic activity during T cell development

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The catalytic activity of Zap70 is crucial for T cell antigen receptor (TCR) signaling, but the quantitative and temporal requirements for its function in thymocyte development are not known. Using a chemical-genetic system to selectively and reversibly inhibit Zap70 catalytic activity in a model of synchronized thymic selection, we showed that CD4⁺CD8⁺ thymocytes integrate multiple, transient, Zap70-dependent signals over more than 36 h to reach a cumulative threshold for positive selection, whereas 1 h of signaling was sufficient for negative selection. Titration of Zap70 activity resulted in graded reductions in positive and negative selection but did not decrease the cumulative TCR signals integrated by positively selected OT-I cells, which revealed heterogeneity, even among CD4⁺CD8⁺ thymocytes expressing identical TCRs undergoing positive selection.

The Syk family tyrosine kinases, Zap70 and Syk, are activated upon engagement of TCRs and promote downstream signal transduction essential for T cell development¹⁻³. Expression of Zap70 and Syk varies throughout T cell development: Syk is expressed in high amounts during β selection, whereas Zap70 is the dominant kinase in CD4⁺CD8⁺ cells (double-positive (DP) cells)⁴. In mice, Zap70 has a nonredundant role in positive selection; deficiency causes a complete block at the DP stage, and expression of hypomorphic *Zap70* alleles impairs positive selection⁵⁻⁹.

Different experimental models have manipulated Zap70 expression as a means of limiting TCR signals during positive selection or to synchronize positive selection^{10,11}. Although genetic systems are useful for inducible or developmental stage-specific expression, it is difficult to titrate or temporally halt Zap70 expression with precision. We reasoned that a cell-permeant, reversible pharmacologic inhibitor would enable titration and temporal control of Zap70 activity to study the requirements for magnitude and duration of TCR signaling for thymic selection. Such control over TCR-derived Zap70-dependent signal strength was not previously possible. To inhibit Zap70 activity, we had previously developed a chemical-genetic approach in which bulky analogs of the kinase inhibitor PP1 selectively inhibit an 'analog-sensitive' variant of Zap70 (referred to as Zap70(AS)), but not wild-type Zap70 (ref. 12). Activation of primary mouse T cells expressing a *Zap70(AS)* transgene is sensitive to Zap70(AS)-specific inhibitor 3-MB-PP1 in a rapid, reversible and dose-dependent manner¹³.

Here we used catalytic inhibition of Zap70 as a method to manipulate the strength of TCR signaling during T cell development. Our studies focused on the timing and dose of Zap70 inhibition. These data provide unanticipated insights regarding the thresholds for the duration and magnitude of Zap70 activity required for positive and negative selection.

RESULTS

Zap70 and Syk-specific inhibition

We first confirmed the specificity of inhibitors of Zap70(AS) and Syk. Consistent with previous studies on mature T cells¹³, treatment of thymocytes with 3-MB-PP1 impaired anti-CD3 cross-linking-induced increases in cytosolic free Ca²⁺ (here referred to as [Ca²⁺]_i) and Erk phosphorylation in a dose-dependent manner in B6.Cg-Tg(*Zap70*^{M413A}*)2Weis (here referred to as *Zap70(AS)*) thymocytes, but not control *Zap70*^{+/−} thymocytes that express the wild-type kinase (Supplementary Fig. 1a,b). When we simultaneously stimulated splenic T cells (expressing Zap70(AS)) with anti-CD3 monoclonal antibody and B cells (expressing Syk) with anti-IgM, we detected antigen receptor-induced increases in [Ca²⁺]_i. Indeed, treatment with 3-MB-PP1 impaired increases in [Ca²⁺]_i induced upon CD3 cross-linking in CD4⁺ T cells, but not IgM cross-linking in B cells, which suggests that 3-MB-PP1 specifically inhibits Zap70(AS) but not Syk (Supplementary Fig. 1c). Conversely, treatment with BAY61-3606, a Syk selective kinase inhibitor (ref. 14), impaired anti-IgM-induced but not anti-CD3-induced [Ca²⁺]_i increases, which demonstrated the specificity of BAY61-3606 for Syk and not Zap70(AS).

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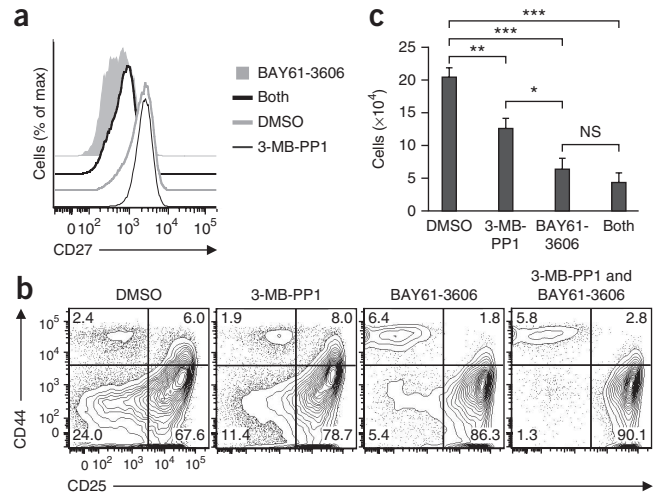
Figure 1 Greater dependence on catalytic activity of Syk versus Zap70 for β selection. (a) CD27 expression on gated CD25⁺CD44⁻DN3 cells from FTOC of e15.5 *Zap70*(AS) thymic lobes cultured for 4 d with vehicle alone (DMSO), 5 μ M 3-MB-PP1, 1 μ M BAY61-3606 or both. (b) Flow cytometry analyses of FTOC treated as in a, gated on total CD4⁻CD8⁻ and TCR $\gamma\delta$ -negative cells. Numbers indicate percentage of cells in each quadrant. (c) Total cell numbers for a single fetal thymic lobe cultured under the indicated inhibitor conditions on day 3. Data are representative of three independent experiments (a,b) and cumulative of three experiments (c; mean \pm s.e.m.; $n = 3$ lobes). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; NS, not significant (unpaired two-tailed Student's t -test).

Differential importance of Zap70 versus Syk

One caveat to studying gene-knockout models is the possibility of compensatory mechanisms or artifacts introduced at earlier stages of T cell development in the absence of Zap70. Furthermore, catalytic inhibitors enable the interrogation of noncatalytic functions of Zap70 to T cell development. Therefore, we revisited the relative functions of Syk and Zap70 during β selection. We performed fetal thymic organ culture (FTOC) of thymic lobes from embryonic day 15.5 (e15.5) *Zap70*^{+/-} and *Zap70*(AS) mice in the presence of 3-MB-PP1 or BAY61-3606. Inhibition of Syk, but not of Zap70, robustly impaired expression of CD27, a marker associated with the CD4⁻CD8⁻CD44⁻CD25⁺ subset b (DN3b) postselection population¹⁵ (Fig. 1a). Syk inhibition also profoundly inhibited the transition from CD4⁻CD8⁻CD44⁻CD25⁺ (DN3) to CD4⁻CD8⁻CD25⁻CD44⁻ (DN4) cells and total thymocyte numbers after 4 d of culture (Fig. 1b,c). After 4 d of 3-MB-PP1 treatment in FTOC, there was an about twofold impairment in the proportion of DN4 cells in 3-MB-PP1-treated versus DMSO (vehicle control)-treated FTOCs (Fig. 1b). Total FTOC cell numbers decreased in the presence of 3-MB-PP1 but less than with Syk inhibition (Fig. 1c). The effects of both inhibitors were additive, such that simultaneous addition resulted in a near complete block in generation and/or maintenance of DN4 and DP cells (Fig. 1c and Supplementary Fig. 1d).

Zap70 activity is required for positive selection

To determine the effect of titrating Zap70 activity on positive selection, we performed FTOC of e15.5 *Zap70*(AS) thymic lobes for 5 d with various concentrations of 3-MB-PP1. Analysis of total thymocytes showed little apparent inhibitory effect of 3-MB-PP1 on the frequency of CD4⁺CD8⁻ (CD4⁺ single positive (SP)) and CD4⁻CD8⁺ (CD8⁺SP) cells. However, gating on TCR β ^{hi} cells revealed dose-dependent impairment in development of both populations (Fig. 2a and Supplementary Fig. 2a,b). High concentrations of 3-MB-PP1 also resulted in an overall decrease in CD5 expression on the DP population and resulted in the absence of the TCR β ^{hi}CD5^{hi} population that includes cells undergoing positive selection (Fig. 2a). The relative impairment in CD5 expression on DP cells is consistent with an attenuated magnitude of TCR signaling¹⁶, and coincided with a block in the generation of CD4⁺SP and CD8⁺SP cells with a mature TCR β ^{hi}CD24^{lo} phenotype. Titration of 3-MB-PP1 revealed dose-dependent reductions in the percentages of TCR β ^{hi}CD5^{hi} DP cells as well as mature TCR β ^{hi}CD24^{lo} CD4⁺SP and CD8⁺SP cells, which suggested that the capacity of DP cells to complete positive selection is proportional to the magnitude of Zap70-dependent signals (Supplementary Fig. 2b,c). Analysis of only the TCR β ^{hi} cells revealed that 3-MB-PP1 inhibited the development of mature CD4⁺SP and CD8⁺SP cells to a comparable dose-dependent extent, which suggested that Zap70 catalytic activity is similarly required for the differentiation of both lineages (Supplementary Fig. 2b,c).



One advantage of small molecule-mediated inhibition over genetic models is the capacity to rapidly block catalytic activity of Zap70(AS) independently of the rate of Zap70 protein turnover. Previous work demonstrated that addition of 3-MB-PP1 to activated T cells could decrease $[Ca^{2+}]_i$ to baseline levels within 1 min of addition¹³. We next determined how acute inhibition of Zap70 activity, after normal T cell development has been initiated, affects positive selection. We performed FTOC of e15.5 *Zap70*(AS) thymic lobes for 4 d in the absence of inhibitor, followed by continuous treatment with a high concentration of 3-MB-PP1 or with DMSO alone, for 48 h (Fig. 2b). After 4 d without inhibitor, there was a population of TCR β ^{hi}CD5^{hi} DP cells but few mature single-positive cells. On day 6, after a pulse with vehicle alone, there was a threefold increase in the percentage of DP cells with a TCR β ^{hi}CD5^{hi} phenotype and a marked accumulation of CD24^{lo} CD4⁺SP and CD8⁺SP cells. In contrast, FTOC pulsed with 3-MB-PP1 from days 4 to 6 had significantly fewer TCR β ^{hi}CD5^{hi} DP cells ($P = 0.004$, unpaired two-tailed Student's t -test), and decreased percentages of mature CD24^{lo} CD4⁺SP and CD8⁺SP cells, which suggested that abrupt inhibition of Zap70 catalytic activity interrupts positive selection (Supplementary Fig. 2d).

Positive selection in thymic slices requires Zap70

To more precisely define the temporal signal threshold required for positive selection, the ability to monitor a cohort of DP cells undergoing relatively synchronous positive selection is required. We adapted an experimental system in which thymocytes expressing the OT-I TCR transgene (specific for ovalbumin peptide (residues 257–264) in the context of H-2K^b) from a nonselecting background (preselection thymocytes) are added to thymic tissue slices containing endogenous positive selecting ligands, which allows for a relatively synchronous wave of positive selection over 2–3 d of culture^{17,18}. To generate preselection thymocytes expressing Zap70(AS), we reconstituted irradiated β_2 -microglobulin-deficient (*B2m*^{-/-}) recipients with *Zap70*^{+/-} OT-I or *Zap70*(AS) OT-I TCR transgenic bone marrow. Then we introduced preselection DP cells from the reconstituted mice onto thymic slices from congenic CD45.1⁺ *B2m*^{-/-} or wild-type mice and incubated for up to 72 h. As previously reported¹⁸, mature CD8⁺SP thymocytes appeared at 24–72 h of culture, whereas no detectable CD8⁺SP cells arose on nonselecting (*B2m*^{-/-}) slices (Fig. 3a). Addition of 3-MB-PP1 completely suppressed the appearance of mature CD8⁺SP cells. In addition, upregulation of CD5 and CD69 on DP cells normally observed at 24 h was blocked by Zap70 inhibition (Fig. 3b).

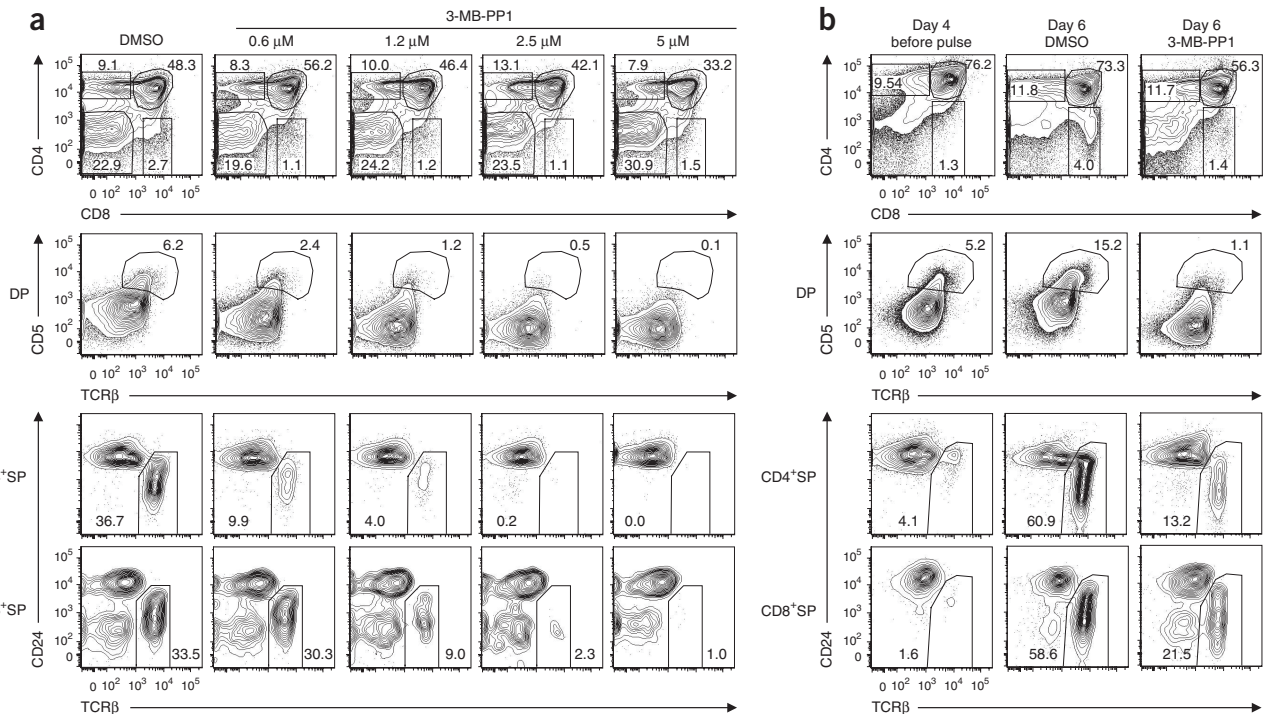


Figure 2 Positive selection requires continuous Zap70 catalytic activity. **(a)** Flow cytometry analysis of *Zap70(AS)* FTOC after 5 d in the presence of the indicated concentrations of 3-MB-PP1. Flow cytometry plots are gated on total viable cells (top), DP cells, CD4⁺SP cells and CD8⁺SP cells. Numbers indicate percentage of cells in each gate. **(b)** Flow cytometry analysis of fetal thymic lobes cultured in the absence of inhibitor for 4 d (before pulse), and cultured for two additional days with vehicle only (day 6 DMSO) or 5 μM 3-MB-PP1 (day 6 3-MB-PP1). Data are representative of two **(a)** and three **(b)** independent experiments.

Previously we have shown that OT-I thymocytes in thymic slices undergo transient signaling events characterized by transient elevations in $[Ca^{2+}]_i$ and migratory pauses¹⁸. To examine the impact of Zap70 inhibition on calcium signals during positive selection, we overlaid OT-I *Zap70(AS)* DP thymocytes loaded with a ratiometric Ca^{2+} indicator dye on thymic slices and added inhibitor while imaging the cells by two-photon microscopy (**Supplementary Video 1**). In the absence of inhibitor, thymocytes in wild-type thymic slices displayed brief (1–10 min) elevations in $[Ca^{2+}]_i$ that coincided with migratory pauses (**Fig. 3c,d**). Plots of calcium ratios for individual time points showed that these events corresponded to occasional elevations in $[Ca^{2+}]_i$, with the majority (>70%) of time points remaining at background levels (**Fig. 3e**). Within 10 min of inhibitor addition, we observed a sharp decrease in the frequency of elevations in $[Ca^{2+}]_i$, which corresponded to the decreased number of time points that displayed elevated $[Ca^{2+}]_i$ (**Fig. 3d,e**). This confirmed that addition of inhibitor to thymic slices effectively and rapidly abrogates the TCR signaling events associated with positive selection. To confirm the reversibility of Zap70 inhibition, we also performed inhibitor ‘wash-out’ experiments, in which *Zap70(AS)* thymocytes migrated into the slice in the presence of 3-MB-PP1, and then samples were placed in medium without inhibitor just before imaging. Calcium signaling events were detectable 7 min after removal of inhibitor, and by 2 h, the frequency of cells signaling was comparable to that in noninhibited cells (**Supplementary Fig. 3a,b**).

To more precisely define the temporal requirements for Zap70 signaling during positive selection, we added 3-MB-PP1 at various times after initiating the culture (**Fig. 4a**). We detected appreciable accumulation of CD8⁺SP cells only when we added inhibitor after at least 36 h of culture, which is in good agreement with the timing of the appearance of CD8⁺SP cells (**Fig. 3a**) and consistent with data from

studies using inducible Zap70 expression¹¹. This indicates that Zap70 catalytic activity is required at late stages of positive selection, up to or just before the DP to CD8⁺SP transition.

However, it was not clear whether the lengthy requirement of signaling for DP cells to complete positive selection reflected a requirement for continuous Zap70-dependent signals. To determine whether a transient interruption of Zap70 activity would affect the completion of positive selection, we inhibited Zap70 catalytic function for 12-h time periods with a high concentration of 3-MB-PP1 (2.5 μM) followed by a chase with vehicle alone to reverse inhibition of Zap70 (**Fig. 4b**). We performed the inhibitor pulses in consecutive 12-h blocks covering 0 h to 72 h. Such an experiment is feasible because the effects of 3-MB-PP1 on TCR signaling are rapidly reversible¹³ (**Supplementary Fig. 3a,b**). Addition of 3-MB-PP1 between 0 h and 12 h or between 60 h and 72 h had moderate inhibitory effects on the appearance of CD8⁺SP cells, which correlated with the two conditions that had the longest intervals (60 h) of uninterrupted Zap70 activity. In contrast, 12-h periods of Zap70 catalytic inhibition, especially between 12 h and 48 h, all resulted in markedly impaired generation of CD8⁺SP cells, despite an identical aggregate of 60 h of uninhibited Zap70 activity. Additionally, CD5 expression on DP cells was comparably reduced among all samples that were exposed to 3-MB-PP1 for 12-h intervals (**Fig. 4c**). These results strongly imply that robust positive selection has a temporal TCR-Zap70 signaling requirement that consists of a minimum of 36 h of continuous Zap70 activity.

In addition to the temporal requirement for Zap70-dependent TCR signaling, we probed the strength of TCR signaling required for efficient positive selection. To do so, we exposed thymic slices containing OT-I *Zap70(AS)* DP thymocytes to various concentrations of 3-MB-PP1. Titration of 3-MB-PP1 concentration resulted in a graded, dose-dependent reduction in the percentage of OT-I CD8⁺SP cells,

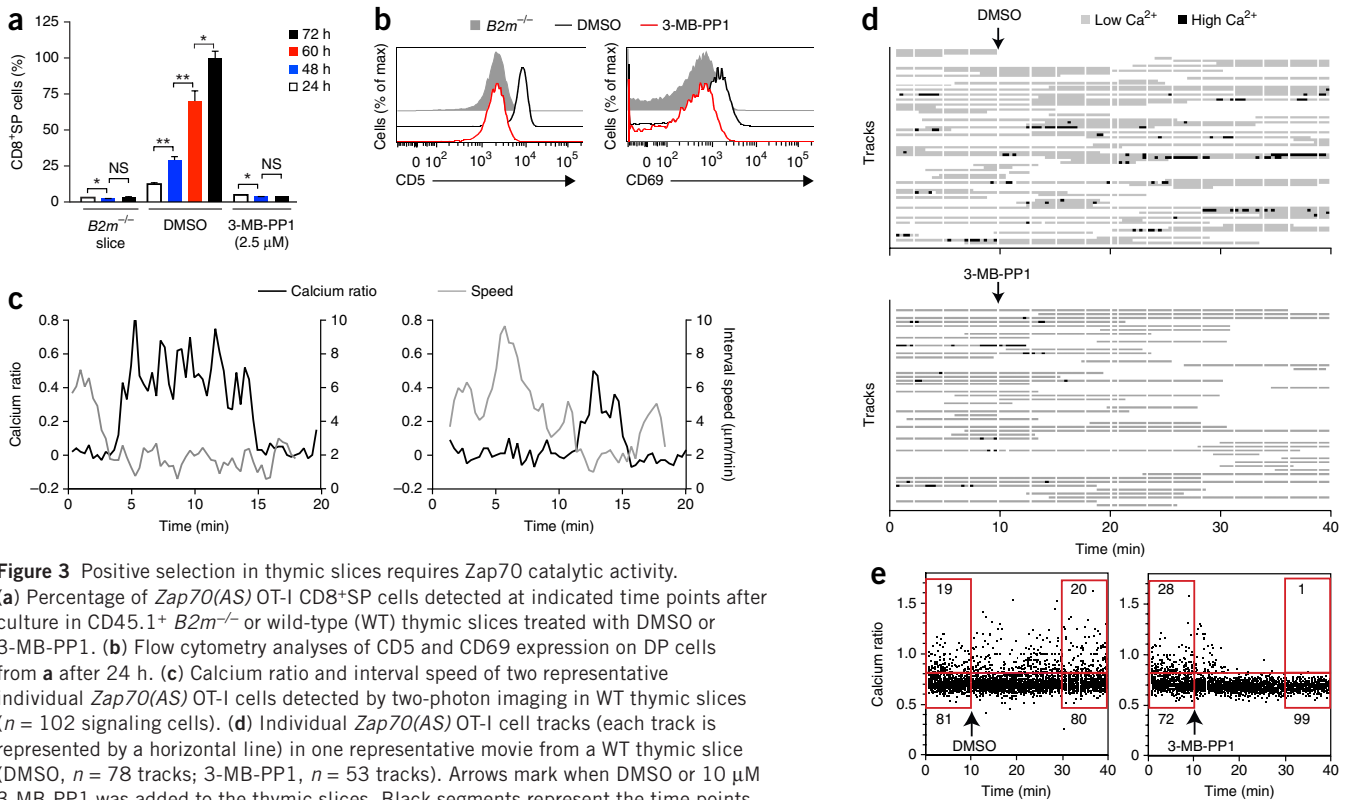


Figure 3 Positive selection in thymic slices requires Zap70 catalytic activity.

(a) Percentage of *Zap70(AS)* OT-I CD8⁺SP cells detected at indicated time points after culture in CD45.1⁺ *B2m*^{-/-} or wild-type (WT) thymic slices treated with DMSO or 3-MB-PP1. (b) Flow cytometry analyses of CD5 and CD69 expression on DP cells from a after 24 h. (c) Calcium ratio and interval speed of two representative individual *Zap70(AS)* OT-I cells detected by two-photon imaging in WT thymic slices ($n = 102$ signaling cells). (d) Individual *Zap70(AS)* OT-I cell tracks (each track is represented by a horizontal line) in one representative movie from a WT thymic slice (DMSO, $n = 78$ tracks; 3-MB-PP1, $n = 53$ tracks). Arrows mark when DMSO or 10 μ M 3-MB-PP1 was added to the thymic slices. Black segments represent the time points during which elevated [Ca²⁺]_i was detected. (e) Imaging data from one representative movie, displaying the calcium ratio versus time. Each dot represents a single time point for each cell (DMSO, $n = 3,474$ time points; 3-MB-PP1, $n = 2,512$ time points). The horizontal red line delineates signaling from nonsignaling cells. Numbers represent percentage of events in each gate. Data are representative of three independent experiments (a,b; in a, mean \pm s.e.m., $n = 3$ samples), and three movies from two independent experiments (c–e). * $P < 0.05$; ** $P < 0.005$; NS, not significant (unpaired two-tailed Student's *t*-test).

with concomitant shifts in CD5 expression (Fig. 4d,e). These data reveal that even modest inhibition of TCR signal strength significantly impaired positive selection. Moreover, DP cells with the same TCR behaved heterogeneously in response to varying the strength of TCR-dependent and Zap70-dependent signals.

Integration of TCR signaling during positive selection

The requirement for TCR signaling over an extended time period, together with the brief duration of TCR signaling events in individual cells undergoing positive selection, implied that thymocytes might be summing TCR signals from transient serial encounters over a period of days to reach a cumulative TCR signaling threshold for positive selection. To visualize the cumulative TCR signals associated with positive selection, we took advantage of a *nur77*-GFP transgene, which reports on the relative 'strength' of antigen receptor signaling perceived at a single-cell level¹⁹. By combining both the Zap70(AS) and the *nur77*-GFP systems we simultaneously titrated Zap70 catalytic activity with an inhibitor and indirectly measured the accumulation of downstream signals in response to TCR stimulation by the accumulation of GFP fluorescence intensity.

To test this capacity, we stimulated OT-I preselection *Zap70(AS)* *nur77*-GFP DP thymocytes with varying concentrations of anti-CD3 or with a single concentration of anti-CD3 plus various concentrations of 3-MB-PP1 (Supplementary Fig. 4a). As expected, titration of anti-CD3 or 3-MB-PP1 resulted in dose-dependent reductions in the proportion of cells that induced GFP expression. For anti-CD3 stimulation, the fluorescence intensity of the responding cells was not dependent on the magnitude of stimulus, which is consistent with

other studies showing that mature T cells make a digital response to limited TCR triggering²⁰. In contrast, the GFP mean fluorescence intensity of the responding cells was dependent on the concentration of 3-MB-PP1. These data demonstrate that although varying TCR triggering at the membrane leads to a digital 'off-on' response in thymocytes, titration of Zap70 catalytic activity provides a means to control the level of TCR signaling experienced by individual cells in an analog fashion.

To determine whether the GFP fluorescence intensity induced during positive selection was at 'saturating' levels, we analyzed DP thymocytes from wild-type and *Bim*-deficient *nur77*-GFP mice. Comparison of preselection (TCR β^{lo} CD69 $^{\text{lo}}$) and postselection (TCR β^{hi} CD69 $^{\text{hi}}$) DP cells in each genotype revealed elevated GFP fluorescence in the postselection population (Supplementary Fig. 4b). Further comparison of wild-type and *Bim*^{-/-} postselection DP cells showed skewing toward a higher GFP fluorescence intensity in the absence of *Bim*, which is consistent with stronger TCR signals experienced by cells rescued from negative selection²¹ (Supplementary Fig. 4c). These results indicate that GFP fluorescence associated with positive selection is within the dynamic range of the *nur77*-GFP reporter and faithfully reports TCR signal strength.

To determine the effects of titration of Zap70 catalytic activity on TCR signal integration during positive selection, we added various concentrations of the 3-MB-PP1 to OT-I *Zap70(AS)* *nur77*-GFP DP cells under positive selection conditions. Although titration of 3-MB-PP1 revealed a dose-dependent reduction in CD8⁺SP cell frequency, it had little effect on GFP fluorescence intensity among the CD8⁺SP populations generated (Fig. 5a,b). Given evidence that 3-MB-PP1

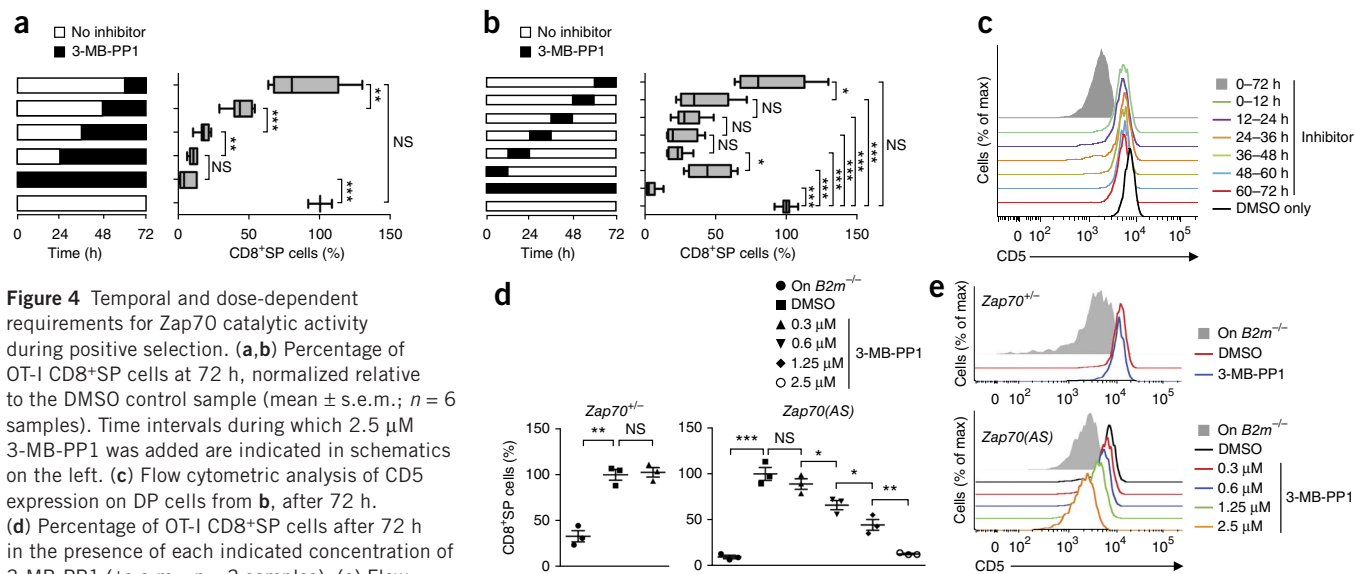


Figure 4 Temporal and dose-dependent requirements for Zap70 catalytic activity during positive selection. (a,b) Percentage of OT-I CD8⁺SP cells at 72 h, normalized relative to the DMSO control sample (mean \pm s.e.m.; $n = 6$ samples). Time intervals during which 2.5 μ M 3-MB-PP1 was added are indicated in schematics on the left. (c) Flow cytometric analysis of CD5 expression on DP cells from b, after 72 h. (d) Percentage of OT-I CD8⁺SP cells after 72 h in the presence of each indicated concentration of 3-MB-PP1 (\pm s.e.m.; $n = 3$ samples). (e) Flow cytometric analysis of CD5 expression on DP cells after 48 h. One of three technical replicates is shown. Data are cumulative of five experiments (a,b), representative of five experiments (c) and representative of three experiments (d,e). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; NS, not significant (unpaired two-tailed Student's t -test).

titration leads to an analog reduction in TCR signaling in individual thymocytes (Supplementary Fig. 4a), these data imply that dampening the magnitude of Zap70-dependent signals reduces the proportion of thymocytes that reach a cumulative TCR signal threshold for positive selection but does not shift the TCR signal threshold itself. Moreover, these results point to heterogeneity of thymocytes in their ability to accumulate TCR signals, even when all cells express the same TCR.

To explore the impact of Zap70 activity titration on positive selection, we cultured polyclonal e15.5 *Zap70(AS) nur77-GFP* fetal thymic lobes in the constant presence of various concentrations of 3-MB-PP1. Consistent with results of the *Zap70(AS)* FTOC and thymic slice experiments, titration of 3-MB-PP1 resulted in a dose-dependent reduction in the percentage of TCR β^{hi} CD69⁺ DP cells (Fig. 5c). Comparison of the CD69⁻ and CD69⁺ DP populations in

FTOC treated with DMSO revealed elevated GFP fluorescence only in the CD69⁺ cells (Fig. 5c). Further analysis of the TCR β^{hi} CD69⁺ cell subpopulation showed that the mean fluorescence intensity of GFP was not influenced by the concentration of 3-MB-PP1 (Fig. 5d). To support this observation, we reexamined DP cells based on CD4⁺CD8⁺TCR β -CD5^{lo} (DP1), CD4⁺CD8⁺TCR β^{int} CD5^{hi} (DP2) and CD4⁺CD8⁺TCR β^{hi} CD5^{hi} (DP3) subsets, transitional stages through which DP cells pass during positive selection¹¹. Consistent with the effects of Zap70 inhibition on CD69 expression, the presence of 3-MB-PP1 resulted in fewer DP2 and DP3 cells; however, DP3 cells exposed to different concentrations of 3-MB-PP1 had comparable GFP fluorescence (Supplementary Fig. 4e). These data imply that titration of Zap70 activity reduces the proportion of thymocytes that receive sufficient signals to undergo positive selection. However, those few cells that still undergo positive selection have attained a level of

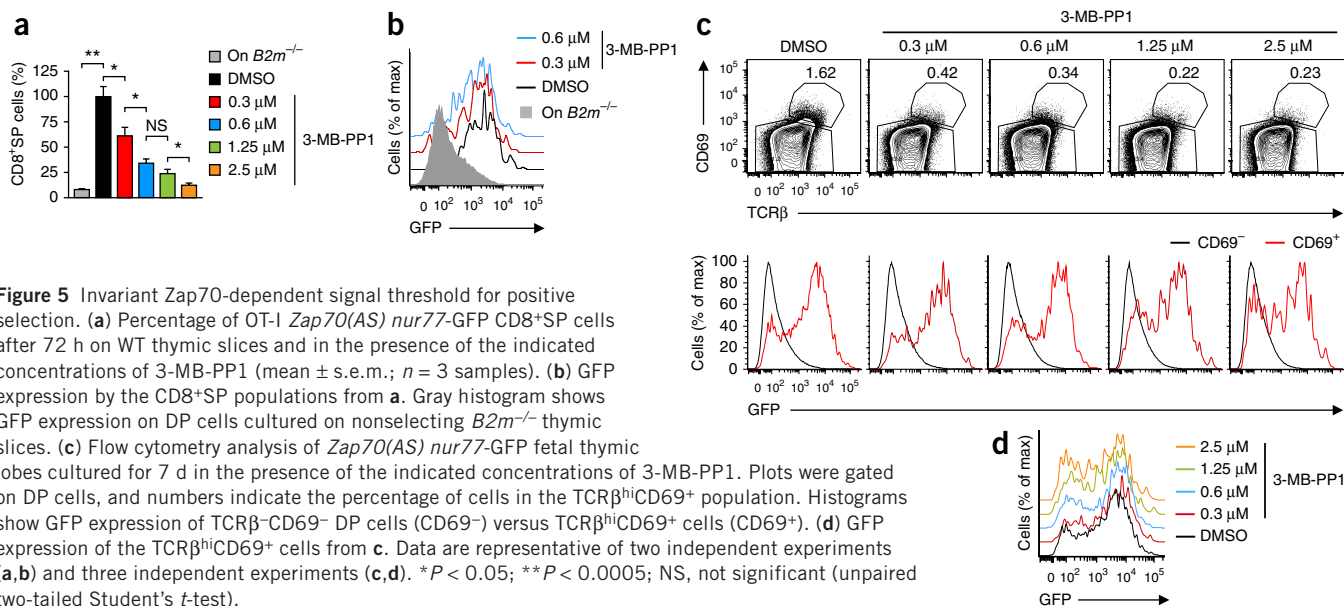


Figure 5 Invariant Zap70-dependent signal threshold for positive selection. (a) Percentage of OT-I *Zap70(AS) nur77-GFP* CD8⁺SP cells after 72 h on WT thymic slices and in the presence of the indicated concentrations of 3-MB-PP1 (mean \pm s.e.m.; $n = 3$ samples). (b) GFP expression by the CD8⁺SP populations from a. Gray histogram shows GFP expression on DP cells cultured on nonselecting *B2m*^{-/-} thymic slices. (c) Flow cytometry analysis of *Zap70(AS) nur77-GFP* fetal thymic lobes cultured for 7 d in the presence of the indicated concentrations of 3-MB-PP1. Plots were gated on DP cells, and numbers indicate the percentage of cells in the TCR β^{hi} CD69⁺ population. Histograms show GFP expression of TCR β -CD69⁻ DP cells (CD69⁻) versus TCR β^{hi} CD69⁺ cells (CD69⁺). (d) GFP expression of the TCR β^{hi} CD69⁺ cells from c. Data are representative of two independent experiments (a,b) and three independent experiments (c,d). * $P < 0.05$; ** $P < 0.0005$; NS, not significant (unpaired two-tailed Student's t -test).

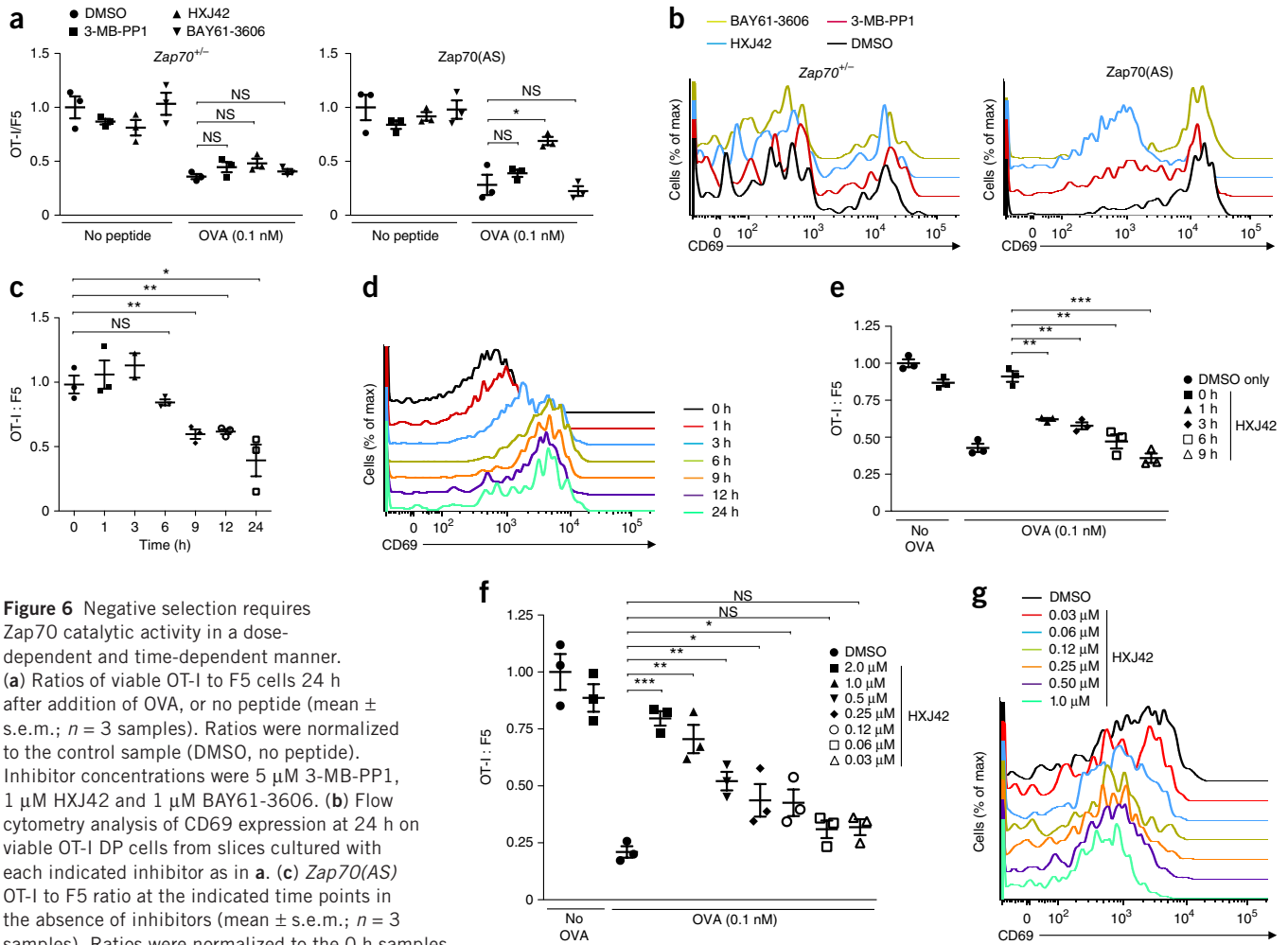


Figure 6 Negative selection requires Zap70 catalytic activity in a dose-dependent and time-dependent manner. (a) Ratios of viable OT-I to F5 cells 24 h after addition of OVA, or no peptide (mean \pm s.e.m.; $n = 3$ samples). Ratios were normalized to the control sample (DMSO, no peptide). Inhibitor concentrations were 5 μ M 3-MB-PP1, 1 μ M HXJ42 and 1 μ M BAY61-3606. (b) Flow cytometry analysis of CD69 expression at 24 h on viable OT-I DP cells from slices cultured with each indicated inhibitor as in a. (c) Zap70(AS) OT-I to F5 ratio at the indicated time points in the absence of inhibitors (mean \pm s.e.m.; $n = 3$ samples). Ratios were normalized to the 0 h samples. (d) Flow cytometry analysis of CD69 expression on viable OT-I DP cells at each indicated time point. (e) OT-I to F5 ratio 24 h after addition of OVA or no peptide (mean \pm s.e.m.; $n = 3$ samples). HXJ42 (1 μ M) was added to thymic slices at the indicated times. Ratios were normalized to the control sample (DMSO, no OVA). (f) OT-I to F5 ratio 24 h after addition of OVA or no peptide to thymic slices in the presence of each indicated concentration of HXJ42 (mean \pm s.e.m.; $n = 3$ samples). Ratios were normalized to the control sample (DMSO, no OVA). (g) Flow cytometry analysis of CD69 expression on viable OT-I DP cells at 24 h for each indicated concentration of HXJ42. Data are representative of three independent experiments. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; NS, not significant (unpaired two-tailed Student's t -test).

cumulative TCR signaling that is similar to cells that undergo positive selection in the absence of Zap70 inhibition.

Requirements for Zap70 during negative selection

To study the temporal TCR signaling requirements for negative selection, we induced a synchronous wave of negative selection by introducing preselection Zap70(AS) or Zap70^{+/-} OT-I DP cells onto wild-type thymic slices with cognate OVA peptide (ovalbumin amino acids 257–264). To quantitatively assess negative selection, we co-introduced thymocytes expressing F5 TCR transgene (specific for the influenza nucleoprotein peptide residues 366–374 in the context of H-2D^b), which are positively selected in this system. We then quantified negative selection as a decrease in the ratio of viable OT-I DP cells to F5 cells. Within 24 h after addition of OVA peptide to the slices in the absence of any inhibitors, there was a significant decrease in the normalized ratio of OT-I to F5 cells from 1 to ~0.25 (Fig. 6a and Supplementary Fig. 5a). The surviving viable OT-I cells expressed elevated levels of CD69 (Fig. 6b).

We predicted that addition of 3-MB-PP1 to this experimental system would enable the rescue of Zap70(AS) DP cells from negative

selection. However, contrary to our prediction, addition of a high concentration of 3-MB-PP1 did not substantially increase the ratio of viable OT-I DP cells to F5 cells relative to addition of DMSO alone or a Syk inhibitor (Fig. 6a). We hypothesized that the potency of 3-MB-PP1 was not sufficient to dampen downstream TCR signals enough to rescue deletion. Therefore, we used a different PP1 analog (HXJ42), which has greater selectivity and potency for Zap70(AS) over wild-type Zap70, as assessed by Erk and Lat phosphorylation as well as by proliferative responses of mature CD4⁺ cells (Supplementary Fig. 5b–d). Consistent with the more potent inhibitory effect of HXJ42 on Zap70(AS) kinase-dependent signals, addition of HXJ42 more effectively rescued Zap70(AS) OT-I cells from negative selection. Additionally, the majority of remaining viable Zap70(AS) OT-I cells in HXJ42-treated thymic slices did not highly express CD69, which suggested an efficient block in TCR signals that drive negative selection (Fig. 6b).

We assessed the time dependency of deletion in this system by examining the OT-I to F5 ratio over time (Fig. 6c). In the absence of inhibitors, we detected a relative decrease in OT-I abundance between 6 h and 9 h after addition of the ovalbumin peptide, consistent with

the kinetics of negative selection in previous studies²². Additionally, upregulation of CD69 expression was detectable by 3 h, with the majority of remaining viable cells staining positive for CD69 by 6 h (Fig. 6d). Addition of a high concentration of HXJ42 (1 μ M) to wild-type thymic slices with *Zap70(AS)* OT-I cells concurrently with OVA peptide resulted in a nearly complete block in deletion. However, a substantial proportion of OT-I DP cells was deleted if addition of HXJ42 was delayed by 1 h after addition of the OVA peptide (Fig. 6e). Addition of HXJ42 from 3 h to 9 h after addition of the OVA peptide resulted in increased negative selection and approached deletion comparable to treatment with vehicle alone. These data imply that a short duration of Zap70-dependent signaling was sufficient for deletion but that prolonged signaling (up to 9 h) increased the efficiency of negative selection. The shorter period of TCR signaling required for negative selection when compared to positive selection is consistent with the more intense and prolonged $[Ca^{2+}]_i$ increase observed during negative selection as compared to positive selection (Supplementary Fig. 5e)¹⁸.

To determine whether there is a Zap70 dependent signal threshold for negative selection, we incubated *Zap70(AS)* OT-I DP cells in wild-type slices with OVA and graded concentrations of HXJ42. Although high concentrations of HXJ42 (1.0 μ M, 2.0 μ M) blocked negative selection and low concentrations (0.3 μ M, 0.6 μ M) did not prevent negative selection, intermediate concentrations of HXJ42 weakly inhibited negative selection in a dose-dependent manner (Fig. 6f). This dose dependency was also reflected by CD69 expression on the remaining viable DP cells after 24 h, such that increasing the inhibitor concentration resulted in decreased CD69 expression (Fig. 6g). These studies suggest that even with a fixed TCR repertoire and a single agonist peptide, heterogeneity in responsiveness to titration of TCR signaling intensity is observed at the population level.

DISCUSSION

How TCR recognition of self peptide-major histocompatibility complex (MHC) can lead to either positive or negative selection is a question that has long attracted the attention of immunologists, with most explanations focusing on differences in TCR signal strength. A resolution to this question has proved elusive, in part due to the inability to precisely determine the quantitative and temporal requirements for TCR signaling for positive and negative selection of thymocytes. As Zap70 catalytic function is required for nearly all TCR signal propagation, its activity should be reflective of strength of TCR signaling. Here we used a genetically selective pharmacologic inhibitor of Zap70(AS) as a means of controlling TCR signal transduction quantitatively and temporally in DP thymocytes in living, three-dimensional thymic tissue. We found that although thymocytes can commit to negative selection after only 1 h of TCR signaling, completion of positive selection requires a minimum of 36 h of TCR signaling, in line with previous studies^{10,21–24}. Our data indicate that models to explain positive versus negative selection based on TCR signal strength alone are insufficient, and that the temporal pattern and cumulative TCR signaling may be as important as the quantity of TCR signal.

The distinct temporal and quantitative requirement for TCR signaling between positive and negative selection reported here fits well with the dynamics of individual TCR-dependent calcium signaling events reported previously^{18,22}. Encounter with negative selecting ligands led to prolonged migratory arrest and sustained elevations in $[Ca^{2+}]_i$, a pattern of TCR signaling that would allow for rapid accumulation of TCR signaling intermediates and thus account for the relatively brief period of TCR signaling required for a thymocyte to commit to death. On the other hand, encounter with positive

selecting ligands was associated with brief, weak and discontinuous $[Ca^{2+}]_i$ increases interspersed with periods of migration¹⁸. These observations, together with evidence presented here that positive selection requires many hours of uninterrupted Zap70 activity and that thymocytes must accumulate sufficient TCR signaling to overcome a fixed threshold, suggest that DP cells can ‘remember’ and integrate recent TCR signaling events to eventually reach a signal threshold required for positive selection. The mechanisms responsible for the integration of TCR signaling leading to positive selection remain unclear but may involve the accumulation of relatively stable signaling intermediates^{25,26}. Given the prolonged time required for this cumulative signaling integration, distinct developmental stages driven by an ordered cascade of transcriptional events might also be involved. This is consistent with progressive changes in thymocyte speed and calcium signaling dynamics during positive selection and with differential gene expression during substages of DP cell development^{27,28}.

Our results provide insights regarding the quantitative requirements for TCR signal strength between positive versus negative selection. In particular, although positive selection can be completely blocked using the moderately potent inhibitor 3-MB-PP1, inhibition of negative selection requires the more potent inhibitor HXJ42. This difference implies that the level of Zap70-dependent TCR signal induced by negative selecting ligands is in substantial excess over that required to induce thymocyte death. In contrast, modest Zap70 inhibition leads to a profound block in positive selection. This quantitative difference in the requirement for Zap70 activity also fits with the temporal pattern of TCR signaling. During negative selection, thymocytes arrest and can continue to accumulate TCR signals until they achieve a sufficient amount of TCR signaling for negative selection. In contrast, modest inhibition of Zap70 during positive selection may disrupt the TCR ‘signal memory’ required for positive selection by reducing the intensity, frequency or duration of transient signaling events.

In spite of the distinct thresholds involved, titration of Zap70 activity led to a graded inhibition of both positive and negative selection. This suggests heterogeneity among DP thymocytes, even if they express a fixed TCR. This heterogeneity may reflect the stochastic nature of thymocyte encounters with selecting ligands. Alternatively, stochastic variations in the expression of TCR signaling proteins may render certain thymocytes more or less resistant to inhibition of Zap70.

The graded inhibition of positive or negative selection observed upon quantitative reduction of Zap70 catalytic activity is in contrast to the sharp threshold for positive versus negative selection observed upon varying affinities of altered peptide ligands–MHC for TCR²⁹. In that study, the altered affinities change kinetic parameters of TCR binding to peptide–MHC. Our experimental system focuses on inhibition of Zap70 catalytic activity, which is proximal to the TCR but is nevertheless activated downstream of TCR engagement. We speculate that a perturbation of TCR binding versus intracellular signal transduction may be a crucial factor in determining the quality of the output of downstream signals.

Our results with Syk and Zap70(AS) catalytic inhibitors largely recapitulate the developmental impairments observed with *Syk* and *Zap70* gene knockout T cells before the DP stage, with Syk catalytic activity predominating at the β -selection checkpoint, and Zap70 activity being more important for the generation or maintenance of DN4 and DP cells. The similarity of phenotypes between knockout and inhibitor studies suggests that the functions of Zap70 and Syk during T cell development require catalytic activity and argues against

any prominent role for a noncatalytic function of either kinase or for compensatory changes. These observations raise the question of why a switch from Syk to Zap70 is necessary. One important difference between the two kinases is that Syk, unlike Zap70, has the capacity to phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) even in the absence of Src kinase activity^{30,31}. Moreover, activation of Zap70 is more dependent on Src kinases than is the activation of Syk because Zap70 is more subject to autoinhibitory control^{32,33}. We propose that the Syk to Zap70 transition in thymic development is consistent with a model whereby coreceptor-mediated recruitment of the Src kinase Lck to the TCR complex becomes a critical factor in enforcing MHC restriction on developing $\alpha\beta$ T cells³⁴. Thus, Syk expression could facilitate pre-TCR signal transduction in the absence of coreceptor-mediated Lck recruitment in CD4⁻CD8⁻ cells. Conversely, expression of Zap70, as occurs in DP cells, would render these cells highly dependent on coreceptor binding to MHC and Lck recruitment for phosphorylation of TCR associated ITAMs to propagate downstream signals.

The temporal and quantitative control and the rapid reversibility of inhibitor effects, as exemplified in these studies, may allow for opportunities to titrate or synchronize developmental events and transitions allowing for additional insights into complex events not possible during asynchronous events that occur at a population level.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

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AUTHOR CONTRIBUTIONS

B.B.A.-Y., H.J.M., E.A.R. and A.W. designed the experiments. B.B.A.-Y., H.J.M., J.O.R. and D.A.C. performed the experiments. J.Z. and K.M.S. provided advice and reagents. B.B.A.-Y., H.J.M., E.A.R. and A.W. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Mice used in these studies were housed in the specific pathogen-free facilities at the University of California, San Francisco, and the University of California, Berkeley, and were treated according to protocols approved by the Institutional Animal Care and Use Committee in accordance with US National Institutes of Health guidelines. *Zap70*^{+/-} mice were generated by interbreeding wild-type C57BL/6 mice with *Zap70*-deficient B6.129X1-*Zap70*^{tm1Weis} mice. B6.Cg-Tg(*Zap70*^{*M413A})2Weis (referred to as *Zap70*(AS)) mice have been described previously¹³. *Zap70*(AS) mice were crossed to TCR α -deficient B6.129S2-*Tcrat*^{tm1Mom/J}, OT-I TCR transgenic C57BL/6-Tg(*Tcrat*Tcrb)1100Mjb/J strains (Jackson) or to *Nur-77*-GFP transgenic mice described previously¹⁹. Bim-deficient B6.129S1-*Bcl2l1*^{tm1.1Ast/J} (Jackson) were also crossed to *nur77*-GFP mice. F5 TCR transgenic mice C57BL/6-Tg(*CD2-TcrAF5, CD2-TcrbF5*)1Kio (ref. 35) were crossed to *Rag1*^{-/-}B6.129S7-*Rag1*^{tm1Mom} (Taconic) and ubiquitin-GFP transgenic C57BL/6-Tg(UBC-GFP)30Scha/J mice (Jackson). Bone marrow chimeras were generated by transferring bone marrow into irradiated β 2m-deficient, B6.129P2-*B2mtm1Unc/J* mice (Jackson). Male and female mice aged between 6 and 12 weeks were used in this study.

Reagents. *Zap70*(AS) inhibitor compounds 3-methyl-benzylpyrazolopyrimidine (3-MB-PP1) and HXJ42 have been described previously^{12,36}. Syk inhibitor BAY61-3606 was purchased from Sigma. For positive-selection experiments on thymic slices, 2.5 μ M 3-MB-PP1 was used, and for negative-selection experiments on thymic slices, 5 μ M 3MBPP1, 1 μ M HXJ42 or 1 μ M BAY61-3606 inhibitor was used unless otherwise indicated.

Fetal thymic organ culture. Timed breedings were performed and fetal thymic lobes were harvested from e15.5 mice. Lobes were cultured on 0.4 μ m pore cell culture inserts (Costar) in 6-well culture dishes atop 1 ml complete DMEM with 10% FBS containing DMSO alone, 3-MB-PP1 or BAY61-3606. Medium was exchanged daily or as indicated until lobes were dissociated into single-cell suspensions for staining with antibodies for flow cytometric analysis.

Bone marrow chimeras. At least 1×10^6 bone marrow cells were transferred to lethally irradiated (1,200 rad) recipient *B2m*^{-/-} mice. Preselection thymocytes from chimeras were collected at least 5 weeks after bone marrow transplantation.

Thymic slices. Thymic slices were prepared essentially as previously described³⁷. Individual thymic lobes from CD45.1 or *B2m*^{-/-} CD45.1 congenic mice were embedded in 4% GTG-NuSieve Agarose (Lonza) in HBSS and cut to a thickness of 400 μ m for flow cytometry experiments or 500 μ m for two-photon microscopy experiments using a Vibratome 100 Plus Sectioning System and feather blades (Leica Microsystems). Thymic slices were maintained on 0.4 μ m cell culture inserts (BD Biosciences) atop 1 ml complete DMEM with 10% FBS containing DMSO alone or with inhibitors in a 37 °C incubator. For positive-selection experiments, 3×10^6 preselection OT-I *Zap70*^{+/-} or *Zap70*(AS) thymocytes in 10 μ l were overlaid on thymic slices. For negative-selection experiments, 1×10^6 of each preselection F5 *Rag1*^{-/-}*B2m*^{-/-}GFP⁺ and OT-I thymocytes labeled with 1 μ M Cell Proliferation Dye eFluor 670 (eBioscience) in 10 μ l were overlaid on thymic slices. Cells were allowed to migrate into the tissue for 2 h before excess cells were removed by indirect pipetting of medium to wash the slices. For negative-selection experiments, thymocytes were allowed to migrate into the tissue for 2 h, tissue was washed and the medium under the transwell was then changed to medium containing 0.1 nM OVA peptide (ovalbumin residues 257–264) (Anaspec) with inhibitors. Media were exchanged daily or as indicated. For two-photon microscopy experiments, thymocytes were prelabeled with 2 μ M Indo-1 LR (Teflabs) for 90 min at 37 °C and subsequently allowed to recover for an additional 60 min before addition to thymic slices. Thymic slices were affixed to a coverslip with tissue glue (3M Vetbond) before imaging.

Flow cytometry. FTOCs or thymic slices were dissociated into single cell suspensions, filtered and stained with antibodies for cell surface markers. The following antibodies were used for FTOC samples: CD5-FITC (clone 53-7.3)

and CD44-PE-Cy7 (clone IM7) from BD Biosciences; TCR β -APC (clone H57-597), CD24-Pacific Blue (clone M1/69) and TCR $\gamma\delta$ -PerCPy5.5 (clone GL3) from BioLegend; CD25-PE (clone PC61.5) and CD8 α -APC-eFluor780 (clone 53-6.7) from eBioscience, and CD4-Qdot605 (clone RM4.5) from Life Technologies. The following antibodies were used for thymic slice samples: CD45.1-FITC (clone A20), CD69-PerCPy5.5 (clone H1.2F3), CD5-PE (clone 53-7.3), CD4-PE-Cy7 (clone RM4-5) and CD8 α -eFluor450 (clone 53-6.7) from eBioscience. For negative-selection experiments, cells were prestained with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Molecular Probes) for 30 min in PBS. Data were acquired on an LSRII or LSR Fortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star).

T cell and B cell cross-linking calcium experiments. Splenocytes from *Zap70*^{+/-} and *Zap70*(AS) mice were loaded with the calcium indicator dye Indo-1 (Invitrogen). Labeled cells were analyzed with an LSR Fortessa cytometer with a UV 355 nm laser (BD Biosciences). Baseline [Ca²⁺]_i measurements were acquired for 30 s, followed by addition of soluble anti-CD3 ϵ (clone 2C11) and inhibitor for 30 s, then followed by addition of polyclonal goat anti-Armenian hamster antibodies for CD3 cross-linking and polyclonal anti-IgM F(ab')₂ antibodies (both from Jackson ImmunoResearch) for BCR cross-linking. The mean ratio of Indo-(violet) to Indo-(blue) over time was calculated using FlowJo software (Tree Star).

Two-photon microscopy. Thymic slices were continually perfused with 37 °C, oxygenated, Phenol Red-free DMEM (Gibco) during image acquisition. Images were acquired in the cortex, as determined by proximity to the capsule, using a custom-built, upright two-photon microscope with a 20 \times , 0.95 numerical aperture objective (Olympus). The Ti:Sapphire MaiTai laser (Spectra-Physics) was tuned to 720 nm, and fluorescence was collected using 440 nm and 510 nm dichroic mirrors with 400/45 and 480/50 bandpass filters. 173 μ m \times 142 μ m images were collected every 20 s for 10–40 min at 3- μ m z intervals starting under the tissue cut site using custom software. For addition of DMSO or inhibitor while imaging, the perfusion medium was switched after 10 min of imaging to medium containing 10 μ M 3-MB-PP1 or DMSO.

Immunoblot analysis. SDS-PAGE and western blot analyses of phosphorylated Tyr132 of Lat and phosphorylated Thr202 and Tyr204 of Erk in *Zap70*^{+/-} and *Zap70*(AS) thymocytes were performed as previously described¹³. Briefly, thymocytes were stimulated with soluble anti-CD3 ϵ (clone 145-2C11) and cross-linking goat anti-Armenian hamster IgG antibodies (127-005-099) from Jackson ImmunoResearch for 2 min and lysed in SDS lysis buffer. Whole-cell lysates were analyzed by western blot with primary antibodies against phospho-Lat Y132 (44224) from Life Technologies, phospho-Erk T202 and Y204 (clone 197G2) from Cell Signaling or actin (A2066) from Sigma, and goat anti-rabbit IgG-HRP (4050-05) secondary antibodies from SouthernBiotech.

Data and statistical analysis. Image analysis was performed using Imaris (Bitplane) software to determine *x*, *y* and *z* coordinates as well as mean fluorescence intensities for calcium-bound and calcium-free Indo-1 LR dye of individual cells over time. Data were processed using custom-written Matlab (MathWorks) scripts, ImageJ and Excel (Microsoft) programs (**Supplementary Data 1**). To determine corrected calcium values to determine signaling cells, 0.675 (the historical average value of OT-I cells under nonselecting conditions) was subtracted from raw fluorescence values, and cells were considered to be signaling when corrected calcium values were ≥ 0.2 for at least one time point of a cell track. Imaging data were converted to flow cytometry-like files using custom DISCIT software for further analysis with FlowJo software (Tree Star)³⁸.

No statistical tests were used to predetermine experimental sample size. Sample sizes were determined empirically for sufficient statistical power. We did not exclude samples from analysis, and our analyses did not include randomized samples or blinding. Prism software (GraphPad software) was used for statistical analysis. Statistical significance between groups was determined by unpaired two-tailed Student's *t*-test. *P* < 0.05 was considered statistically significant. Statistical analysis was performed on sample groups with similar variance. Limited variance was observed within sample groups.

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Erratum: Quantitative and temporal requirements revealed for Zap70 catalytic activity during T cell development

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In the version of this article initially published online, the second sentence of the legend to Figure 6e was incorrect. It should read “HXJ42 (1 μ M) was added...”. The error has been corrected in the HTML version of this article.