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Quantitative and Temporal Requirements Revealed for Zap-70 Catalytic Activity During T Cell Development

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Abstract

The catalytic activity of Zap-70 is crucial for T cell 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 Zap-70 catalytic activity in a model of synchronized thymic selection, we showed that $CD4^+CD8^+$ thymocytes integrate multiple, transient, Zap-70-dependent signals over more than 36 h to reach a cumulative threshold for positive selection, whereas one hour of signaling was sufficient for negative selection. Titration of Zap-70 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, revealing heterogeneity, even among $CD4^+CD8^+$ thymocytes expressing identical TCRs undergoing positive selection.

Introduction

The Syk family tyrosine kinases, Zap-70 and Syk, are activated upon TCR engagement and promote downstream signal transduction essential for T cell development[1–3]. Expression of Zap-70 and Syk varies throughout T cell development, with Syk expressed at high amounts during β selection whereas Zap-70 is the dominant kinase in DP cells[4]. In mice, Zap-70 has

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Author Contributions

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

a nonredundant role in positive selection; deficiency causes a complete block at the DP stage and expression of hypomorphic *Zap70* alleles impairs positive selection[5–9].

Different experimental models have manipulated Zap-70 expression as a means of limiting TCR signals during positive selection or to synchronize positive selection[10,11]. While genetic systems are useful for inducible or developmental stage-specific expression, it is difficult to titrate or temporally halt Zap-70 expression with precision. We reasoned that a cell permeable, reversible pharmacologic inhibitor would enable titration and temporal control of Zap-70 activity to study the requirements for TCR signaling magnitude and duration for thymic selection. Such control over TCR-derived Zap-70-dependent signal strength was not previously possible. To inhibit Zap-70 activity, we developed a chemical-genetic approach in which bulky analogs of the kinase inhibitor PP1 selectively inhibit an "analog-sensitive" mutant of Zap-70 (referred to as *Zap-70(AS)*), but not wild-type Zap-70[12]. Activation of primary mouse T cells expressing *Zap70(AS)* was sensitive to 3-MB-PP1 in a rapid, reversible, and dose-dependent manner[13].

Here, we use catalytic inhibition of Zap-70 as a method to manipulate the strength of TCR signaling during T cell development. Our studies focus on the timing and dose of Zap-70 inhibition. These data provide unanticipated insights regarding the thresholds for the duration and magnitude of Zap-70 activity required for positive and negative selection.

Results

Zap-70 and Syk-specific inhibition

We first confirmed the specificity of inhibitors of Zap-70(AS) and Syk. Consistent with previous studies on mature T cells[13], treatment of thymocytes with the *Zap70(AS)*-specific inhibitor, 3-MB-PP1, impaired CD3 crosslinkinginduced increases in cytosolic free Ca^{2+} (hereafter referred to as $[Ca^{2+}]_i$) and Erk phosphorylation in a dose-dependent manner in *Zap70(AS)*, but not control *Zap70*$^{+/-}$ thymocytes that express the wild-type kinase (Supplementary Fig. 1a,b). Further, we simultaneously stimulated splenic T cells (expressing Zap-70(AS)) and B cells (expressing Syk) and detected antigen receptor-induced increases in $[Ca^{2+}]_i$. Indeed, 3-MB-PP1 treatment impaired increases in $[Ca^{2+}]_i$ induced upon CD3 crosslinking in $CD4^+$ T cells, but not IgM crosslinking in B cells, suggesting that 3-MB-PP1 specifically inhibits Zap-70(AS) but not Syk (Supplementary Fig. 1c). Conversely, treatment with BAY61–3606[14] impaired IgM but not CD3-induced $[Ca^{2+}]_i$ increases, demonstrating the specificity of BAY61–3606 for Syk and not Zap-70(AS).

Differential importance of Zap-70 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 Zap-70. Furthermore, catalytic inhibitors enable the interrogation of non-catalytic functions of Zap-70 to T cell development. Therefore, we revisited the relative functions of Syk and Zap-70 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 Zap-70, robustly impaired expression

of CD27, a marker associated with the DN3b post-selection population (Fig. 1a[15]. Syk inhibition also profoundly inhibited the transition from DN3 to DN4 cells and total thymocyte numbers after 4 days of culture (Fig. 1b,c). Following 4 days of 3-MB-PP1 treatment in FTOC, there was a ~2-fold impairment in the proportion of $CD25^{-}CD44^{-}$ DN (DN4) cells in 3-MB-PP1- versus DMSO-(vehicle control) treated FTOCs (Fig. 1b). Total FTOC cell numbers were 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).

Zap-70 activity is required for positive selection

To determine the effect of titrating Zap-70 activity on positive selection, we performed FTOC of e15.5 *Zap70(AS)* thymic lobes for 5 days with graded concentrations of 3-MB-PP1. Analysis of total thymocytes showed little apparent inhibitory effect of 3-MB-PP1 on the frequency of $CD4^{+}$SP and $CD8^{+}$SP cells. However, gating on $TCR\beta^{hi}$ 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 $TCR\beta^{hi}$ $CD5^{hi}$ 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 signaling[16], and coincided with a block in the generation of $CD4^{+}$SP and $CD8^{+}$SP cells with a mature $TCR\beta^{hi}$ $CD24^{lo}$ phenotype. Titration of 3-MB-PP1 resulted in dose-dependent reductions in the percentages of $TCR\beta^{hi}$ $CD5^{hi}$ DP cells, as well as mature $TCR\beta^{hi}$ $CD24^{lo}$ $CD4^{+}$SP and $CD8^{+}$SP cells, suggesting that the capacity of DP cells to complete positive selection is proportional to the magnitude of Zap-70 dependent signals (Supplementary Fig. 2b,c). Furthermore, analysis of only the $TCR\beta^{hi}$ cells revealed that titration of 3-MB-PP1 inhibited the development of mature $CD4^{+}$SP and $CD8^{+}$SP to a comparable extent, suggesting that Zap-70 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 Zap-70(AS) catalytic activity, independently of the rate of Zap-70 protein turnover. Previous work demonstrated that addition of 3-MB-PP1 to activated T cells could decrease $[Ca^{2+}]_i$ to baseline levels within one minute of addition[13]. We next asked how acute inhibition of Zap-70 activity, after normal T cell development has been initiated, affects positive selection. To address this question, we performed FTOC of e15.5 *Zap70(AS)* thymic lobes for 4 days in the absence of inhibitor, followed by continuous treatment with a high concentration of 3-MB-PP1 or DMSO alone, for 48 hours (Fig. 2b). After 4 days without inhibitor, there was a population of $TCR\beta^{hi}$ $CD5^{hi}$ DP cells, but few mature single positive cells. On day 6, following a pulse with vehicle alone, there was a 3-fold increase in the percentage of DP cells with a $TCR\beta^{hi}$ $CD5^{hi}$ phenotype, and a marked accumulation of $CD24^{lo}$ $CD4^{+}$SP and $CD8^{+}$SP cells. In contrast, FTOC pulsed with 3-MB-PP1 from days 4–6 had significantly fewer $TCR\beta^{hi}$ $CD5^{hi}$ DP cells, and decreased percentages of mature $CD24^{lo}$ SP cells, suggesting that abrupt inhibition of Zap-70 catalytic activity interrupts positive selection (Supplementary Fig. 2d).

Positive selection in thymic slices requires Zap-70

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 TCR transgenic thymocytes from a non-selecting background (pre-selection thymocytes) are added to thymic tissue slices containing endogenous positive selecting ligands, allowing for a relatively synchronous wave of positive selection over a period of 2–3 days of culture[17,18]. To generate pre-selection thymocytes expressing Zap-70(AS), we reconstituted irradiated β_2-microglobulindeficient (*B2m*$^{-/-}$) recipients with *Zap70*$^{+/-}$ OT-I or *Zap70(AS)* OT-I TCR transgenic bone marrow. The pre-selection DP cells from the reconstituted mice were then introduced onto thymic slices from congenic CD45.1^{+} *B2m*$^{-/-}$ or WT mice and incubated for up to 72 h. As previously reported[18], mature CD8^{+}SP thymocytes appeared between 24–72 h of culture, whereas no detectable CD8^{+}SP cells arose on non-selecting (*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 Zap-70 inhibition (Fig. 3b).

We showed previously that OT-I thymocytes in thymic slices undergo transient signaling events characterized by transient elevations in $[Ca^{2+}]_i$ and migratory pauses[18]. To examine the impact of Zap-70 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 Movie 1). In the absence of inhibitor, thymocytes in WT thymic slices displayed brief (1–10 minutes) elevations in $[Ca^{2+}]_i$ coinciding with migratory pauses (Fig. 3c,d). Plots of calcium ratios for individual time points show that these events correspond to occasional elevations in $[Ca^{2+}]_i$ with the majority (>70%) of time points remaining at background levels (Fig. 3e). Within 10 minutes of inhibitor addition, we observed a sharp decrease in the frequency of elevations in $[Ca^{2+}]_i$, corresponding to the decreased number of time points displaying elevated $[Ca^{2+}]_i$ (Fig. 3d,e). This confirms that addition of inhibitor to thymic slices effectively and rapidly abrogates the TCR signaling events associated with positive selection. To confirm the reversibility of Zap-70 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 media without inhibitor just prior to imaging. Calcium signaling events were detectable 7 minutes after inhibitor removal, and by 2 h, the frequency of cells signaling was comparable to non-inhibited cells (Supplementary Fig. 3a,b).

To more precisely define the temporal requirements for Zap-70 signaling during positive selection, we added 3-MB-PP1 at different times after initiating the culture (Fig. 4a).

Supplementary Video 1. Two-photon imaging of pre-selection *Zap70(AS)* OT-I DP cell migration and intracellular calcium concentration in thymic slices. Left panels, OT-I thymocytes in non-selecting (*B2m*$^{-/-}$) slices in the absence of inhibitor. Middle panels, OT-I thymocytes under positive selecting (WT slice) conditions with DMSO addition after 10 minutes of a 40-minute movie. Right panels, positive selecting (WT slice) conditions with the addition of 3-MB-PP1 after 10 minutes of a 40-minute movie. Top panels, *z*-projection of fluorescence emission of Indo-1 dye in unbound (green) and calcium bound (red) forms. Bottom panels, intracellular calcium ratio of calcium bound/unbound Indo-1 dye represented as a heat map. Frames were collected every 20 seconds for 40 minutes, 2–4 hours after cells were added to the slice. Imaging data are representative of three movies from two independent experiments.

Appreciable accumulation of $CD8^+$SP cells was only detected under conditions where inhibitor was added after at least 36 h of culture, in good agreement with the timing of the appearance of $CD8^+$SP cells (Fig. 3a) and consistent with inducible Zap-70 expression studies[11]. This indicates that Zap-70 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 Zap-70-dependent signals. To determine whether a transient interruption of Zap-70 activity would affect the completion of positive selection, we inhibited Zap-70 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 Zap-70 inhibition (Fig. 4b). The inhibitor pulses were performed in consecutive 12-h blocks covering 0 to 72 h. The feasibility of such an experiment is possible because the effects of 3-MB-PP1 on TCR signaling are rapidly reversible (Supplementary Fig. 3a,b)[13]. Addition of 3-MB-PP1 between 0–12 h or 60–72 h had moderate inhibitory effects on the appearance of $CD8^+$SP cells, correlating with the two conditions that had the longest intervals (60 hours) of uninterrupted Zap-70 activity. In contrast, 12-h periods of Zap-70 catalytic inhibition, especially between 12 and 48 h all resulted in markedly impaired generation of $CD8^+$SP cells, despite an identical aggregate of 60 h of uninhibited Zap-70 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-Zap-70 signaling requirement, consisting of a minimum of 36 h of continuous Zap-70 activity.

In addition to the temporal requirement for Zap-70-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, with concomitant shifts in CD5 expression (Fig. 4d,e). These data reveal that even modest inhibition of TCR signal strength significantly impairs positive selection. Moreover, DP cells with the same TCR behave heterogeneously in response to varying the strength of TCR/Zap-70 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 level[19]. By combining both the Zap-70(AS) and the Nur77-GFP systems we simultaneously titrated Zap-70 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 demonstrate this capacity, we stimulated OT-I pre-selection *Zap70(AS)*-Nur77-GFP DP thymocytes with graded concentrations of anti-CD3 or with a single concentration of anti-CD3 plus graded 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, consistent with other studies showing that mature T cells make a digital response to limited TCR triggering[20]. In contrast, the GFP mean fluorescence intensity of the responding cells was dependent on the concentration of 3-MB-PPI. These data demonstrate that, while varying TCR triggering at the membrane leads to a digital "off/on" response in thymocytes, titration of Zap-70 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 is at "saturating" levels, we analyzed DP thymocytes from WT and Bim-deficient Nur77-GFP mice. Comparison of pre-selection (TCRβ^{lo} CD69^{lo}) and post-selection (TCRβ^{hi} CD69^{hi}) DP cells within each genotype revealed elevated GFP fluorescence in the post-selection population (Supplemetary Fig. 4b). Further comparison of WT and *Bim*$^{-/-}$ post-selection DP cells showed skewing toward a higher GFP fluorescence intensity in the absence of Bim, consistent with stronger TCR signals experienced by cells rescued from negative selection[21] (Supplementary Fig. 4c). These results indicate that GFP fluorescence associated with positive selection is within the dynamic range of the Nur77-GFP reporter and does faithfully report on TCR signal strength.

To determine the effects of titration of Zap-70 catalytic activity on TCR signal integration during positive selection, we added various concentrations of the 3-MB-PPI to OT-I *Zap70(AS)*-Nur77-GFP DP cells under positive selection conditions. Interestingly, while 3-MB-PP1 titration led to a dose-dependent reduction in CD8^{+}SP frequency, it had little effect on GFP fluorescence intensity among the CD8^{+}SP populations generated (Fig. 5a,b). Given evidence that 3-MB-PPI titration leads to an analog reduction in TCR signaling in individual thymocytes (Supplementary Fig. 4a), these data imply that dampening the magnitude of TCR-Zap-70 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 further explore the impact of Zap-70 activity titration on positive selection, we cultured polyclonal e15.5 *Zap70(AS)*-Nur77-GFP fetal thymic lobes in the constant presence of different 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 TCRβ^{hi}CD69^{+} DP cells (Fig. 5c, top row). Comparison of the CD69^{-} and CD69^{+} DP populations in FTOC treated with DMSO revealed elevated GFP fluorescence only in the CD69^{+} cells (Fig. 5c, bottom row). Further analysis of the TCRβ^{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, DP cells were re-examined based on TCRβ^{-}CD5^{lo} (DP1), TCRβ^{int}CD5^{hi} (DP2), and TCRβ^{hi}CD5^{hi} (DP3)

subsets, transitional stages through which DP cells pass during positive selection[11]. Consistent with the effects of Zap-70 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 Zap-70 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 cumulative TCR signaling that is similar to cells that undergo positive selection in the absence of Zap-70 inhibition.

Requirements for Zap-70 during negative selection

The temporal TCR signaling requirements for negative selection, were studied by inducing a synchronous wave of negative selection by introducing pre-selection *Zap70(AS)* or *Zap70*$^{+/-}$ OT-I DP cells onto WT thymic slices with cognate OVA (ovalbumin 257–264) peptide. To quantitatively assess negative selection, we co-introduced F5 thymocytes 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 an alternative PP1 analog (HXJ42), which has greater selectivity and potency for Zap-70(AS) over wild-type Zap-70, as assessed by Erk and Lat phosphorylation, as well as by proliferative responses of mature CD4+ cells (Supplementary Fig. 5b,c,d). Consistent with its more potent inhibitory effect on Zap-70(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, suggesting an efficient block in TCR signals that drive negative selection (Fig. 6b).

We assessed the tme-dependency of deletion in this system, by examining the OT-I to F5 ratio over time (Fig. 6c). A relative decrease in OT-I abundance was detectable between 6 and 9 h, consistent with the kinetics of negative selection in previous studies[22]. 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 μM) to WT 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 one hour after the OVA peptide (Fig. 6e). Addition of HXJ42 from 3–9 h after 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 Zap-70 dependent signaling is sufficient for

deletion, but prolonged signaling (up to 9 h) increases 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 Zap-70 dependent signal threshold for negative selection, we incubated *Zap70(AS)* OT-I DP cells in WT slices with OVA and graded concentrations of HXJ42. Although high concentrations of HXJ42 (1.0, 2.0 μM) blocked negative selection and low concentrations (0.3, 0.6 μM) did not prevent negative selection, intermediate concentrations of HXJ42 weakly inhibited negative selection in a dose-dependent manner (Fig. 6f). This dosedependency was also reflected by CD69 expression on the remaining viable DP cells after 24 hours, 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-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. Since Zap-70 catalytic function is required for nearly all TCR signal propagation, its activity should be reflective of strength of TCR signaling. Here we use a genetically selective pharmacologic inhibitor of Zap-70(AS) as a means of controlling TCR signal transduction quantitatively and temporally in DP thymocytes within living, three-dimensional thymic tissue. We show that while thymocytes can commit to negative selection after only one hour of TCR signaling, completion of positive selection requires a minimum duration 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 die. On the other hand, encounter with positive selecting ligands was associated with brief, weak, and discontinuous $[Ca^{2+}]_i$ increases interspersed with periods of migration[18]. These observations, together with evidence presented here that positive selection requires many hours of uninterrupted Zap-70 activity. Thymocytes must accumulate sufficient TCR signaling to overcome a fixed threshold, suggesting that DP cells can “remember” and

integrate recent TCR signaling events over time to 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. There is evidence that genes that are differentially regulated during the DP substages of development could reflect such ordered or sequential transcriptional changes[27].

Our results provide new insights regarding the quantitative requirements for TCR signal strength between positive versus negative selection. In particular, while 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 Zap-70-dependent TCR signal induced by negative selecting ligands is in substantial excess over that required to induce thymocyte death. In contrast, modest Zap-70 inhibition leads to a profound block in positive selection. This quantitative difference in the requirement for Zap-70 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 Zap-70 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 Zap-70 activity led to a graded inhibition of both positive and negative selection. This suggests heterogeneity amongst 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 Zap-70 inhibition.

The graded inhibition of positive or negative selection observed upon quantitative reduction of Zap-70 catalytic activity is in contrast to the sharp threshold for positive versus negative selection observed upon varying affinities of altered peptide ligands-MHC for TCR[28]. In that study, the altered affinities change kinetic parameters of TCR: peptide-MHC engagement. Our experimental system focuses on inhibition of Zap-70 catalytic activity, which is proximal to the TCR, but is nevertheless activated downstream of TCR engagement. We speculate that the amount by which TCR signaling is perturbed may be a crucial factor in determining the quality of the output of downstream signals.

Our results with Syk and Zap-70(AS) catalytic inhibitors largely recapitulate the developmental impairments observed with *Syk* and *Zap70* gene knockout T cells prior to the DP stage, with Syk catalytic activity predominating at the β-selection checkpoint, and Zap-70 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 Zap-70 and Syk during T cell development require catalytic activity and argues against any prominent role for a non-catalytic function of either kinase or for compensatory

changes. These observations raise the question of why a switch from Syk to Zap-70 is necessary. One important difference between the two kinases is that Syk, unlike Zap-70, has the capacity to phosphorylate ITAMs even in the absence of Src kinase activity[29,30]. Moreover, Zap-70 activation is more dependent on Src kinases than is Syk since Zap-70 is more subject to autoinhibitory control[31,32]. We propose that the Syk to Zap-70 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 αβ T cells[33]. Thus, Syk expression could facilitate pre-TCR signal transduction in the absence of coreceptor-mediated Lck recruitment in DN cells. Conversely, expression of Zap-70, as occurs in DP cells, would render these cells highly dependent upon 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 further insights into complex events not possible during asynchronous events that occur at a population level.

Online Methods

Mice

Mice used in these studies were housed in the specific pathogen-free facility at the University of California, San Francisco, and were treated according to protocols approved by the Institutional Animal Care and Use Committee in accordance with NIH guidelines. *Zap70*$^{+/-}$ were generated by interbreeding wild-type C57BL/6 mice with Zap-70 deficient B6.129×1-*Zap70*^{tm1Weis} mice. B6.Cg-Tg(Zap70*M413A)2Weis (referred to as *Zap70(AS)*) mice were described previously[13]. *Zap70(AS)* mice crossed to TCRα-deficient B6.129S2-*Tcra*^{tm1Mom}/J, OT-I TCR transgenic C57BL/6-Tg(TcraTcrb)1100Mjb/J strains (Jackson) or to Nur-77-GFP transgenic mice described previously[19]. Bim-deficient B6.129S1-*Bcl2l11*$^{tm1.1Ast}$/J (Jackson) were also crossed to Nur77-GFP mice. Bone marrow chimeras were generated by transferring bone marrow into irradiated β2mdeficient, B6.129P2-*B2*^{mtm1Unc}/J mice (Jackson).

Reagents

Zap-70(AS) Inhibitor compounds 3-MB-PP1 and HXJ42 have been described previously[12,34]. Syk inhibitor BAY61–3606 was purchased from Sigma. For positive selection experiments on thymic slices, 2.5 μM 3-MB-PP1 was used and for negative selection experiments on thymic slices, 5 μM 3MBPP1, 1 μM HXJ42, or 1 μM BAY61–3606 inhibitor were used unless otherwise indicated.

FTOC

Timed breedings were performed and fetal thymic lobes were harvested from embryonic day 15.5 (e15.5) mice. Lobes were cultured on 0.4 μm pore cell culture inserts (Costar) in 6-well culture dishes atop 1 ml complete DMEM with 10% fetal bovine serum containing DMSO alone, 3-MB-PP1, or BAY61–3606. Media 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 (1200 rad) recipient *B2m*$^{-/-}$ mice. Pre-selection thymocytes from chimeras were harvested at least five weeks after bone marrow transplantation.

Thymic slices

Thymic slices were prepared essentially as previously described[35]. 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 μm for flow cytometry experiments or 500μm for two-photon microscopy experiments using a Vibratome 100 Plus Sectioning System and feather blades (Leica Microsystems). Thymic slices were maintained on 0.4μm cell culture inserts (BD Biosciences) atop 1 ml complete DMEM media with 10% fetal bovine serum containing DMSO alone or inhibitors in a 37°C incubator. For positive selection experiments, 3×10^6 pre-selection OT-I *Zap70*$^{+/-}$ or Zap-70(AS) thymocytes in 10 μL were overlaid on thymic slices. For negative selection experiments, 1×10^6 of each pre-selection F5 *Rag1*$^{-/-}$*B2m*$^{-/-}$GFP$^+$ and OT-I thymocytes labeled with 1 μM Cell Proliferation Dye eFluor 670 (eBioscience) in 10 μL were overlaid on thymic slices. Cells were allowed to migrate into the tissue for two hours before excess cells were removed by indirect pipetting of media to wash the slices. For negative selection experiments, thymocytes were allowed to migrate into the tissue for two hours, washed, and the media beneath the transwell was subsequently changed to media containing 0.1 nM OVA (257–264) peptide (Anaspec) with inhibitors. Media was exchanged daily or as indicated. For two-photon microscopy experiments, thymocytes were pre-labeled with 2 μM Indo-1 LR (Teflabs) for ninety minutes at 37°C and subsequently allowed to recover for an additional sixty minutes before addition to thymic slices. Thymic slices were affixed to a coverslip with tissue glue (3M Vetbond) prior to 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; TCRβ-APC (clone H57–597), CD24-Pacific Blue (clone M1/69), and TCRγδ-PerCPCy5.5 (clone GL3) from BioLegend; CD25-PE (clone PC61.5) and CD8α-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-PerCPCy5.5 (clone H1.2F3), CD5-PE (clone 53-7.3), CD4-PE-Cy7 (clone RM4–5), CD8α-eFluor450 (clone 53-6.7) from eBioscience. For negative selection experiments, cells were pre-stained with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Molecular Probes) for thirty minutes in PBS. Data were acquired on an LSRII or LSR Fortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star).

T and B cell cross-linking calcium experiments

Splenocytes from *Zap70*$^{+/-}$ and Zap-70(AS) mice were loaded with the calcium indicator dye Indo-1 (Invitrogen). Labeled cells were analyzed with a LSR Fortessa cytometer with a UV 355 nm laser (BD Biosciences). Baseline $[Ca^{2+}]_i$ measurements were acquired for thirty seconds, followed by addition of soluble anti-CD3ε (clone 2C11) and inhibitor for thirty seconds, then followed by addition of polyclonal goat anti-Armenian hamster antibodies for CD3 crosslinking and polyclonal anti-IgM $F(ab')_2$ antibodies (both from Jackson Immunoresearch) for BCR crosslinking. 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, up-right two-photon microscope with a 20X/0.95 objective (Olympus). The Ti:Sapphire MaiTai laser (Spectra-Physics) was tuned to 720 nm and fluorescence collected using 440 nm and 510 nm dichroic mirrors with 400/45 and 480/50 bandpass filters. 173 × 142μm images were collected every twenty seconds for ten to forty minutes at 3 μm *z*-intervals starting beneath the tissue cut site using custom software. For addition of DMSO or inhibitor while imaging, the perfusing media was switched after ten minutes of imaging to media containing 10 μM 3-MB-PP1 or DMSO.

Immunoblot analysis

SDS PAGE and western blot analyses of phosphorylated Tyrosine 132 of Lat and phosphorylated Threonine 202/Tyrosine204 of Erk in *Zap70*$^{+/-}$ and Zap-70(AS) thymocytes were performed as previously described[13]. Briefly, thymocytes were stimulated with soluble anti-CD3ε (clone 145-2C11) and crosslinking goat anti-Armenian hamster IgG antibodies (catalog# 127-005–099) from Jackson Immunoresearch for two minutes and lysed in SDS lysis buffer. Whole cell lysates were analyzed by western blot with primary antibodies against phospho-Lat Y132 (catalog# 44224) from Life Technologies, phospho-Erk T202/Y204 (clone 197G2) from Cell Signaling, or Actin (catalog #A2066) from Sigma, and goat anti-rabbit IgG-HRP (catalog# 4050-05) secondary antibodies from Southern Biotech.

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. The data was processed using custom written MATLAB scripts (Mathworks), Image J, and Excel programs (Supplemental ___). To determine corrected calcium values to determine signaling cells, 0.675 (the historical average value of OT-I cells under non-selecting conditions) was subtracted from raw fluorescence values, and cells were considered to be signaling when corrected calcium values were ≥ 0.2 for at least 1 time point of a cell track. Imaging data was converted to flow cytometry-like files using custom *DISCit* software for further analysis with FlowJo software (Tree Star)[36].

No statistical tests were used to pre-determine 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. A *P*-value of less than 0.05 was considered statistically significant. Statistical analysis was performed on sample groups with similar variance. Limited variance was observed within sample groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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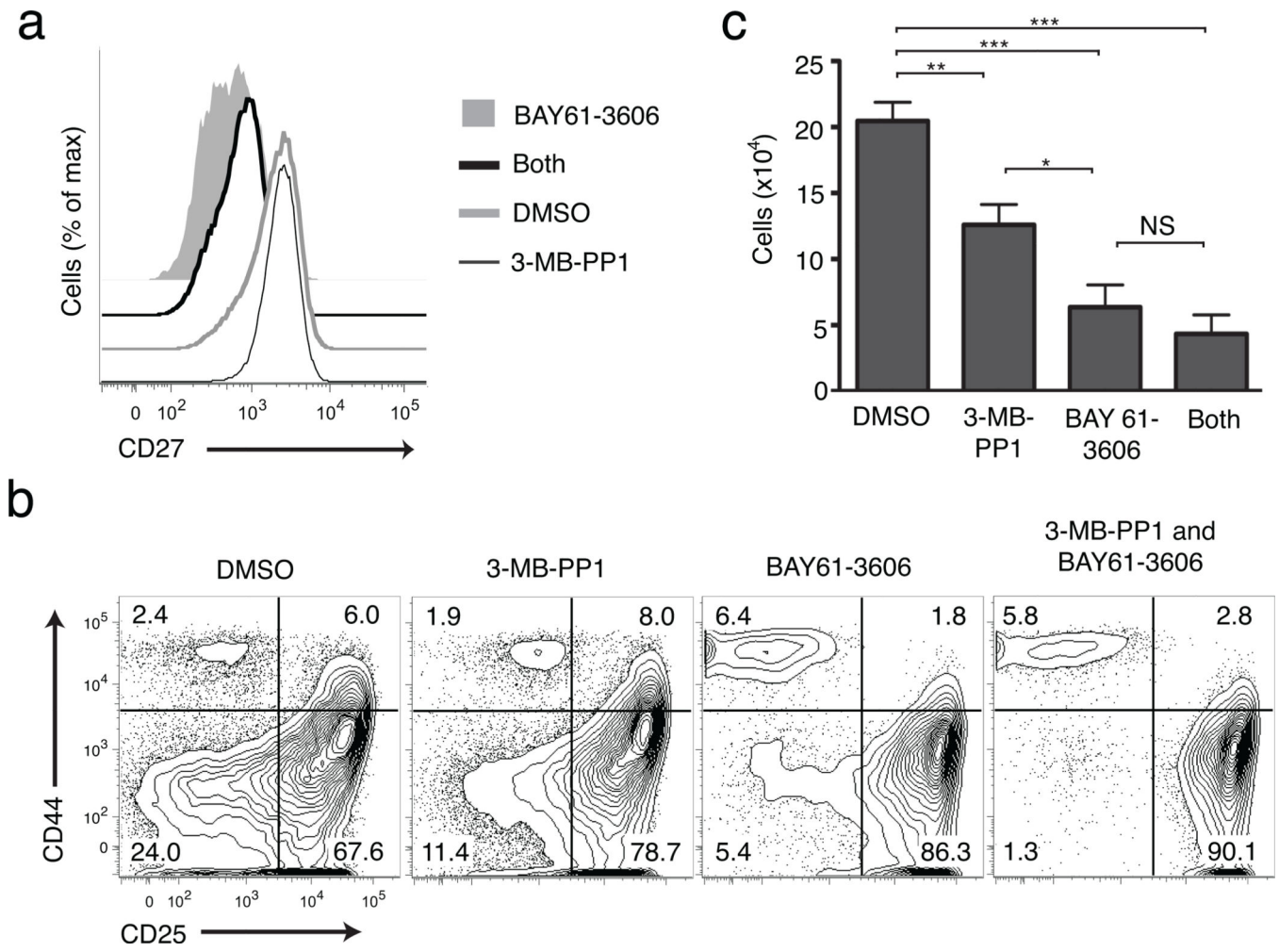

Figure 1. Greater dependence on catalytic activity of Syk versus Zap-70 for β selection

(a) FTOC of e15.5 *Zap70(AS)* thymic lobes was performed for 4 days with vehicle alone (DMSO), 5 μM 3-MB-PP1, 1 μM BAY61-3606, or both inhibitors. Overlayed histograms show CD27 expression on gated $CD25^+$ $CD44^-$ DN3 cells from fetal thymic lobes cultured with the indicated inhibitors. **(b)** Flow cytometry plots are gated on total $CD4^-CD8^-$ DN and TCR γδ negative cells. The numbers indicate the percentage of cells within each quadrant. **(c)** Total cell numbers for a single fetal thymic lobe cultured under the indicated inhibitor conditions on day 3. Bar graphs display the mean total cell numbers (± s.e.m.) from three independent experiments. Data in panels **(a,b)** are from one representative experiment out of 3 independent experiments. **P* <0.05, ***P* <0.005, ****P* <0.0005, NS not significant (Student's *t*-test).

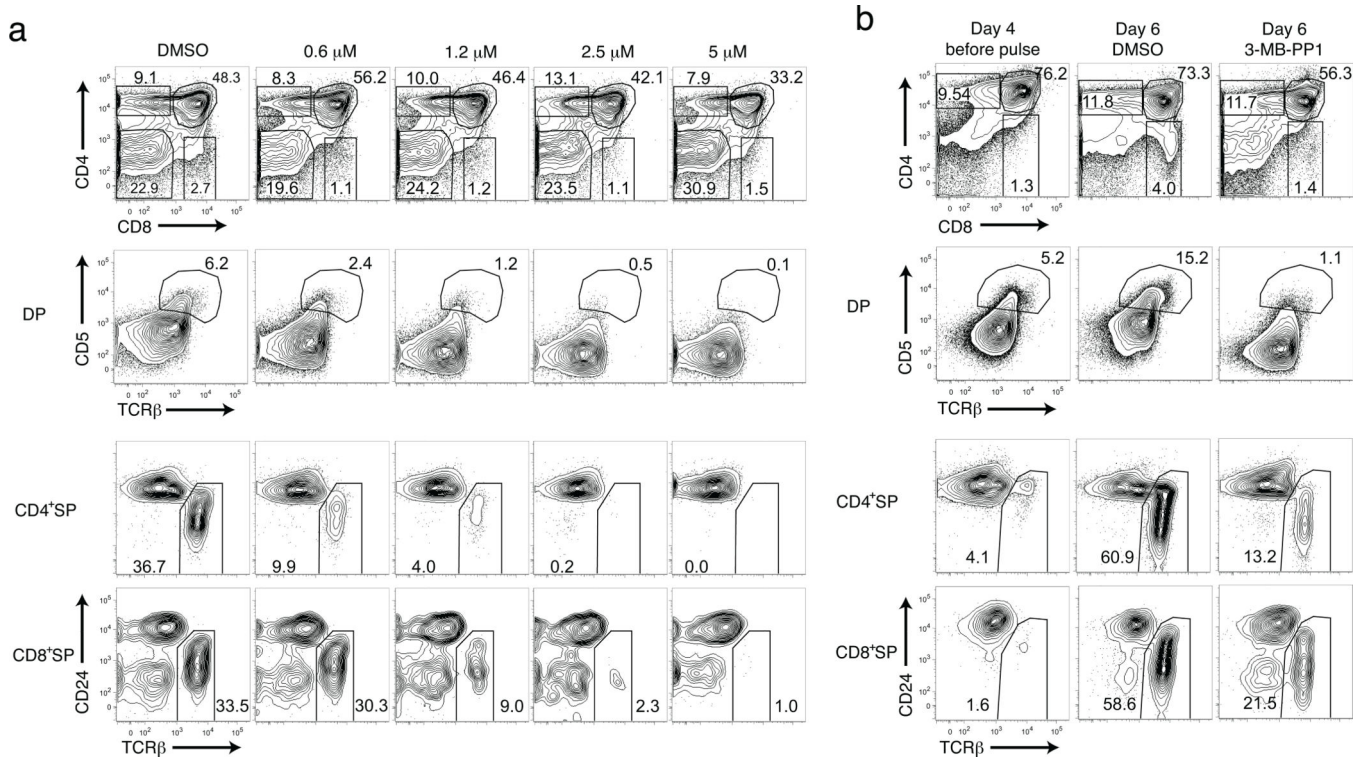

Figure 2. Positive selection requires continuous Zap-70 catalytic activity

(a) *Zap70(AS)* FTOC samples after 5 days of culture in the presence of the indicated concentrations of 3-MB-PP1. Flow cytometry plots are gated on total viable cells (top), DP cells (second row), $CD4^+$SP cells (third row), and $CD8^+$SP cells (bottom row). The numbers indicate the percentage of cells within each gate. **(b)** Fetal thymic lobes were cultured in the absence of inhibitor for 4 days (left column, "before pulse"). On day 4, lobes were cultured with DMSO only (middle column, "Day 6 DMSO") or 5 μM 3-MB-PP1 (right column, "Day 6 3-MB-PP1") for an additional 48 hand analyzed. Data in (**a**) and (**b**) are representative of 2 and 3 independent experiments, respectively.

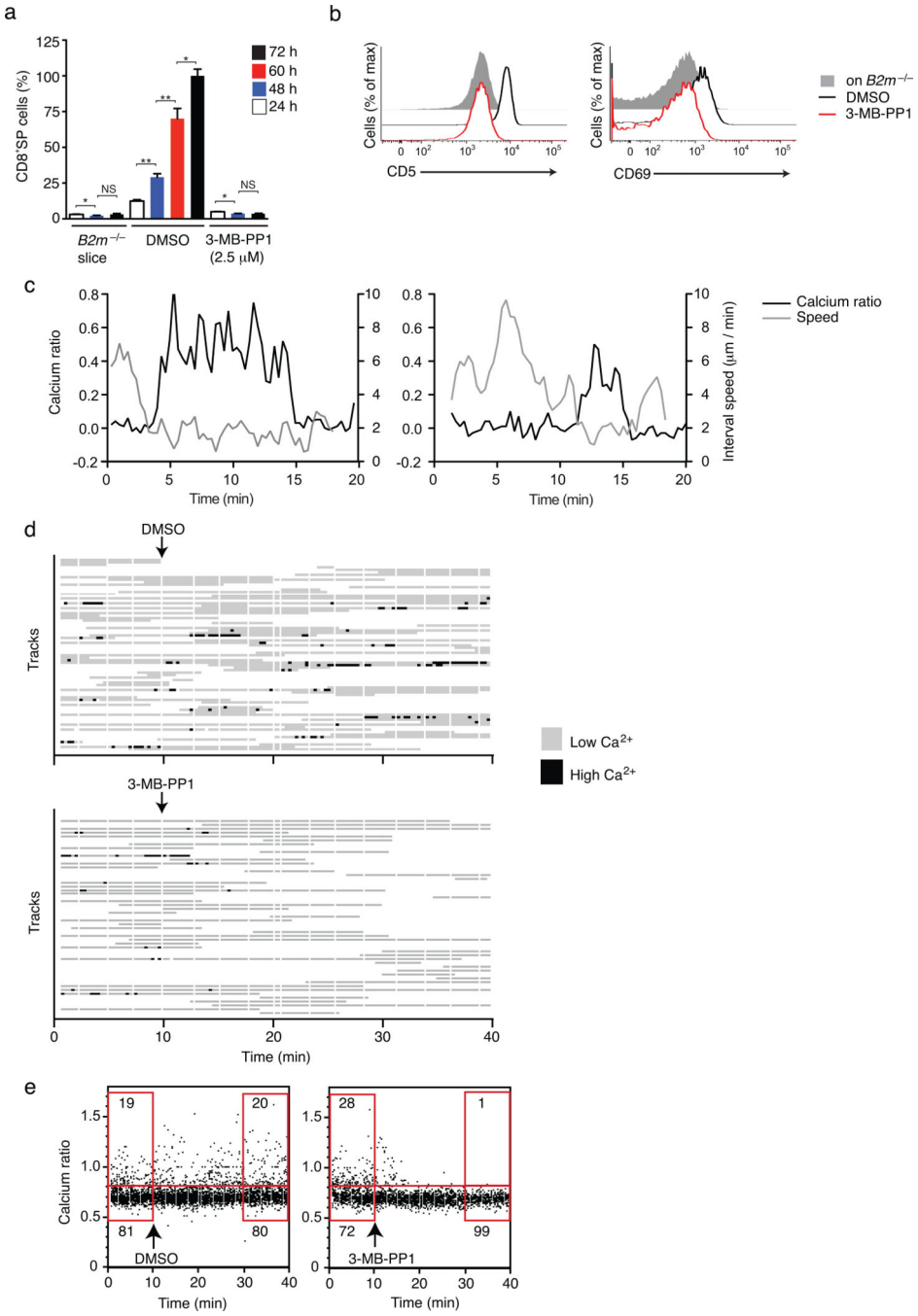

Figure 3. Positive selection in thymic slices requires Zap-70 catalytic activity

Pre-selection DP cells were generated by transferring bone marrow from Zap-70(AS) OT-I donor mice into irradiated *B2m*$^{-/-}$ recipients. **(a)** Pre-selection CD45.2^{+}*Zap70(AS)* OT-I DP cells were introduced onto CD45.1^{+}*B2m*$^{-/-}$ or WT thymic slices in the presence of DMSO alone or 3-MB-PP1 and analyzed at 24, 48, 60, or 72 hs for the presence of CD8^{+}SP cells. The percentages of CD8^{+}SP cells were determined among the CD45.2^{+} cells, and normalized relative to the average of the 72hr DMSO samples. The graph displays the average percentage of CD8^{+}SP (± s.e.m.) from technical triplicate samples. Data are from

one representative experiment out of three independent experiments. **(b)** Histograms show the expression of CD5 (top) and CD69 (bottom) on DP cells from panel (**a**) after 24 h of culture. **(c)** Graphs show the corrected calcium ratio and interval speed of two (each panel) representative individual *Zap70(AS)* OT-I cells detected by two-photon imaging in WT thymic slices. **(d)** Each horizontal line represents an individual *Zap70(AS)* OT-I cell track within a WT thymic slice. The arrows represent the time point at which DMSO or 10 μM 3-MB-PP1 was added to the thymic slices. The black segments represent the time points during which elevated $[Ca^{2+}]_i$ was detected. **(e)** Imaging data was converted to dot plot form to display the calcium ratio versus time. Each dot represents a single time point for each cell. The horizontal red line delineates signaling from non-signaling cells. The numbers represent the percentage of events with low or high $[Ca^{2+}]_i$ levels within each gate. Imaging data are representative of three movies from two independent experiments. **P* <0.05, ***P* <0.005, NS not significant (Student's *t*-test).

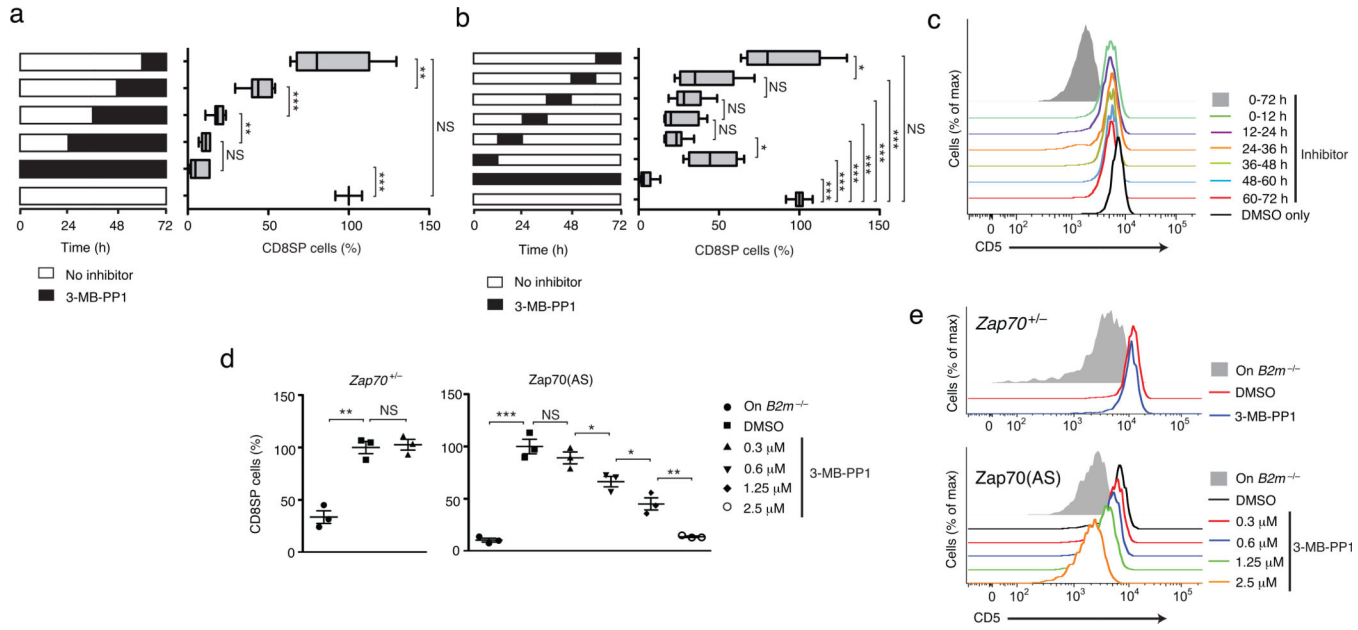

Figure 4. Temporal and dose-dependent requirements for Zap-70 catalytic activity during positive selection

(a,b) Pre-selection Zap-70(AS) OT-I DP cells were cultured on WT thymic slices for a total of 72 h. Schematics on the left indicate the time intervals during which 2.5 μM 3-MB-PP1 was added (closed bars), or cultured without inhibitor (open bars). Graphs display the mean percentage (± s.e.m.) of $CD8^+$SP cells from technical triplicate samples normalized relative to the DMSO treated control sample. Data in panel **(a)** are from one representative experiment out of three independent experiments. Panel (**b**) displays data compiled from 5 independent experiments. **(c)** CD5 expression on DP cells from panel (**b**), after 72 h of culture on thymic slices. Data are from one representative experiment out of three independent experiments. **(d)** Pre-selection *Zap70*$^{+/-}$ or Zap-70(AS) OT-I DP cells were introduced onto *B2m*$^{-/-}$ or WT thymic slices in the constant presence of the indicated concentrations of 3-MB-PP1 or DMSO alone for 72 h. Graph shows technical triplicate samples for each condition, and the horizontal line represents the mean and error bars show (± s.e.m.). **(e)** CD5 expression on DP cells after 48 h of culture. Data shown in (d) and (e) are from one representative experiment out of three independent experiments. **P* <0.05, ***P* <0.005, ****P* <0.0005, NS not significant (Student's *t*-test).

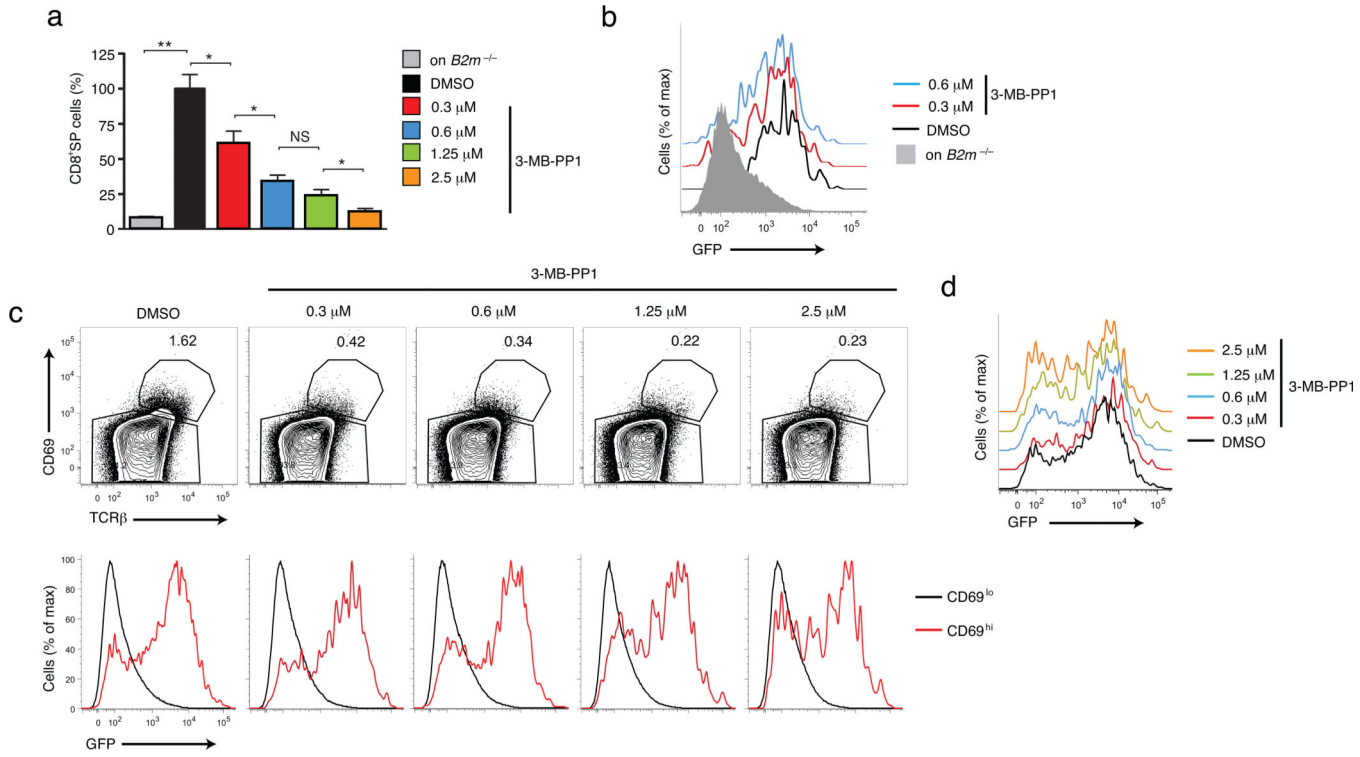

Figure 5. Invariant Zap-70 dependent signal threshold for positive selection

(a) Pre-selection OT-I Zap-70(AS)-Nur77-GFP DP cells were cultured on WT thymic slices for 72 hours in the presence of the indicated concentrations of 3-MB-PP1. Graph displays the average percentage of $CD8^+SP$ (± s.e.m.) from technical triplicate samples detected at 72 hours, normalized to the average of the DMSO only control samples.**(b)** Histograms show GFP expression by the $CD8^+SP$ populations from indicated inhibitor treatment conditions. Closed gray histogram shows the GFP expression on DP cells cultured on non-selecting *B2m*$^{-/-}$ thymic slices. **(c)** Zap-70(AS)-Nur77-GFP thymic lobes were cultured for 7 days in the presence of the indicated concentrations of 3-MB-PP1. Flow cytometry plots are gated on DP cells and the numbers indicate the percentage of cells within the $TCR\beta^{hi}$ $CD69^+$ population (top). Histograms show GFP expression of $TCR\beta^-$ $CD69^-$ DP cells (black) versus $TCR\beta^{hi}$ $CD69^+$ cells (red) (bottom). **(d)** GFP expression of the $TCR\beta^{hi}$ $CD69^+$ cells from Zap-70(AS)-Nur77-GFP FTOC. Data from (**a,b**) are representative of 2 independent experiments. Data from (**c,d**) are representative of 3 independent experiments. **P* <0.05, ***P* <0.0005, NS not significant (Student's *t*-test).

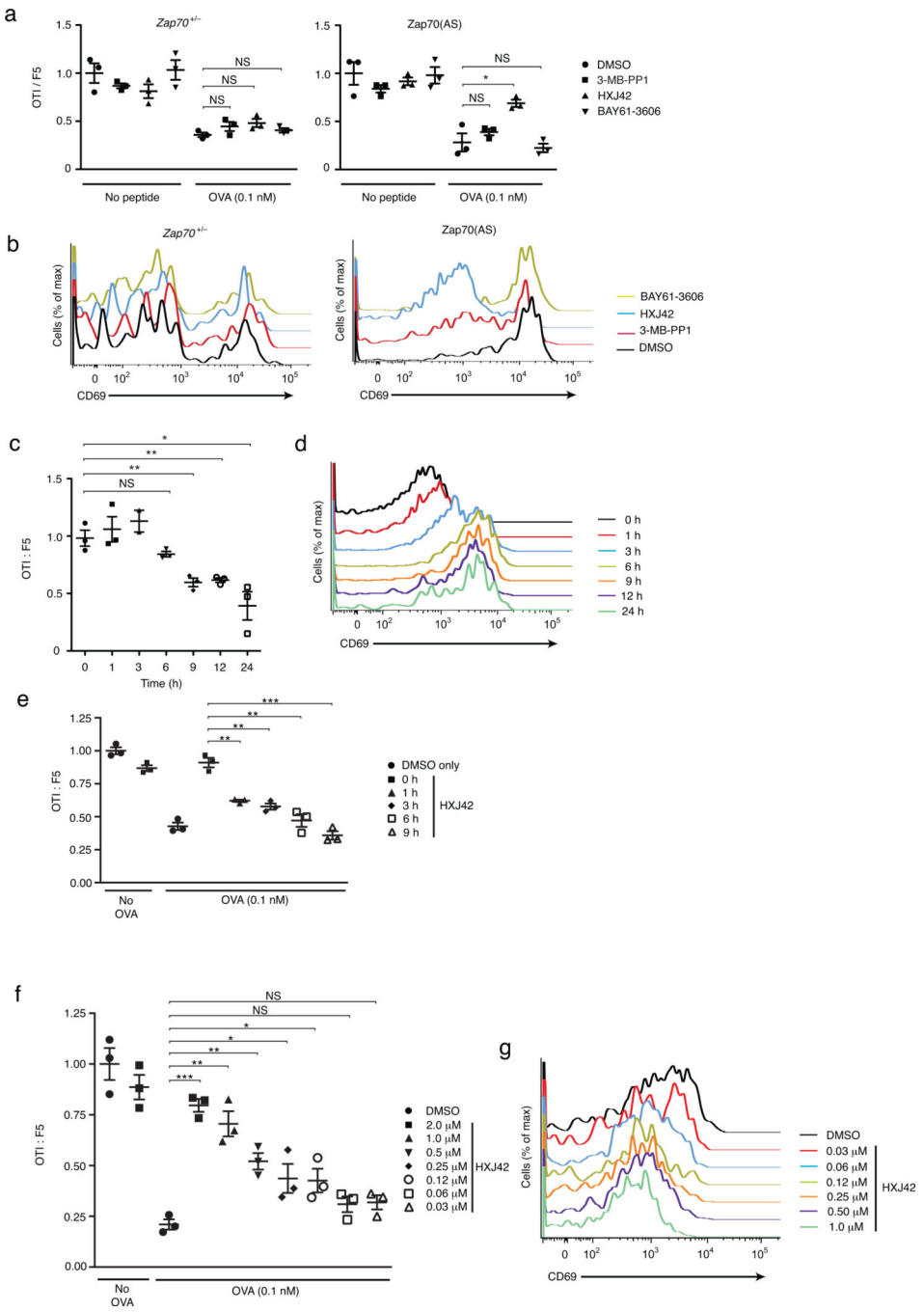

Figure 6. Negative selection requires Zap-70 catalytic activity in a dose and time-dependent manner

Pre-selection CD45.2^{+}*Zap70*$^{+/-}$ or *Zap70(AS)* OT-I DP cells were co-transferred with dye-labeled, CD45.2^{+} F5 TCR DP cells at a 1:1 ratio, onto CD45.1^{+} WT thymic slices. Slices were then cultured with or without 0.1 nM OVA peptide to induce negative selection. All graphs in this figure display the technical triplicate samples for each condition, with the horizontal line representing the mean OT-I: F5 ratio (± s.e.m.), normalized to the average ratio from the control samples, specified below. **(a)** After 24 h of culture with constant

exposure to the indicated inhibitors, slices were analyzed for the ratio of viable OT-I cells to F5 cells. Ratios were normalized to the control sample (DMSO alone, 0 nM OVA). Inhibitor concentrations: 3-MB-PP1 (5 μM), HXJ42 (1 μM), BAY61-3606 (1 μM). **(b)** Histograms show CD69 expression on viable DP cells from slices cultured with the indicated inhibitors. **(c)** Zap-70(AS) OT-I DP thymocytes were cultured on *B2m*$^{-/-}$ or WT thymic slices with OVA and analyzed at the indicated time points. Ratios were normalized to the 0 hr samples. **(d)** Histogram shows CD69 expression on viable DP cells at each indicated time point. **(e)** HXJ42 (1 μM) was added to thymic slices at the indicated times after co-introduction of pre-selection OT-I thymocytes and OVA peptide. Ratios were normalized to the average of the (DMSO, 0 nM OVA) control samples. Horizontal bars represent the average of technical triplicate samples (± s.e.m.). **(f)** Histogram shows CD69 expression on viable DP cells at 24 h. **(g)** Zap-70(AS) OT-I DP thymocytes were cultured on WT thymic slices for 24 h with OVA in the presence of the indicated concentrations of HXJ42. Horizontal bars represent the average of technical triplicate samples (± s.e.m.). Ratios were normalized to the average of the (DMSO, 0 nM OVA) control samples. All panels show data from one representative experiment out of 3 independent experiments. **P* <0.05, ***P* <0.005, ****P* <0.0005, NS not significant (Student's *t*-test).