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A genetically selective inhibitor demonstrates a function for the kinase Zap70 in regulatory T cells independent of its catalytic activity

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To investigate the role of the kinase Zap70 in T cells, we generated mice expressing a Zap70 mutant whose catalytic activity can be selectively blocked by a small-molecule inhibitor. We found that conventional naive, effector and memory T cells were dependent on the kinase activity of Zap70 for their activation, which demonstrated a nonredundant role for Zap70 in signals induced by the T cell antigen receptor (TCR). In contrast, the catalytic activity of Zap70 was not required for activation of the GTPase Rap1 and inside-out signals that promote integrin adhesion. This Zap70 kinase–independent pathway was sufficient for the suppressive activity of regulatory T cells (T_{reg} cells), which was unperturbed by inhibition of the catalytic activity of Zap70. Our results indicate Zap70 is a likely therapeutic target.

After engagement of the T cell antigen receptor (TCR) by peptide-bound major histocompatibility complex molecules, the immunoreceptor tyrosine-based activation motifs in the CD3 and ζ-chains of the TCR complex are phosphorylated by the Src family kinase Lck. The tyrosine kinase Zap70 (A002396) is recruited to and associates with dually phosphorylated immunoreceptor tyrosine-based activation motifs, where it is phosphorylated by Lck and becomes catalytically active[1]. Subsequently, active Zap70 phosphorylates at least two substrates, the adaptor proteins Lat and SLP-76, which in turn facilitate the assembly of molecular complexes important for the activation of downstream signals[2,3].

The critical role of Zap70 in TCR signal transduction has been demonstrated by defects in thymic development that result from Zap70 deficiency. In mice, Zap70 expression is required for thymic development to progress beyond the $CD4^+CD8^+$ double-positive stage[4,5], whereas human Zap70-deficient patients suffer from a form of severe combined immunodeficiency characterized by a lack of peripheral $CD8^+$ T cells and the presence of peripheral $CD4^+$ T cells that fail to induce signals downstream of the TCR[6,7].

The use of knockout mice has proven to be a powerful approach for studying the role of many tyrosine kinases in TCR signal transduction and T cell biology. However, because of the paucity of normal mature T cells in Zap70-deficient mice and humans, it has been difficult to determine the requirements for Zap70 in the responses of primary peripheral T cells. Additionally, knockouts in mice are limited by genetic compensation and redundancy of functions. With inducible deletions, the uncertainty of the time at which loss of protein expression occurs and the amount of expression at which effective loss of function occurs diminishes their value for examining rapid effects on loss of function. Finally, such loss-of-function studies are not reversible, short of gene transfer.

Small-molecule inhibitors can be used to study the temporal requirements of a kinase's catalytic activity for TCR signaling and activation as well as potential therapies. However, specificity is a substantial limitation in the use of kinase inhibitors. A cell-permeable, highly specific inhibitor of Zap70 has not yet been reported. To address this issue, we generated and studied in the Jurkat human T cell line a Zap70 mutant that retains catalytic activity yet can be inhibited by an analog of the small-molecule kinase inhibitor PP1 (4-amino-1-*tert*-butyl-3-(*p*-methylphenyl) pyrazolo [3,4-D] pyrimidine), which does not inhibit wild-type kinases by virtue of a conserved bulky 'gatekeeper' residue across the kinome[8]. Specificity for the inhibitor in this system is encoded genetically, thus allowing specific inhibition of the engineered mutant Zap70 but not of wild-type Zap70. The analog-sensitive Zap70 protein Zap70(AS) has substitution of the gatekeeper methionine at position 414 (Met414) with alanine in the ATP-binding domain, which allows it to accommodate 3-MB-PP1, a bulkier analog of PP1 (ref. 9). The genetic control of inhibitor sensitivity also allows the use of mixtures of T cell populations with different sensitivities to Zap70 inhibitor for analysis of the role of the same kinase in different cellular contexts. Together these features make the analog-sensitive system a powerful tool for determining the role of the catalytic activity of Zap70 in T cells.

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To explore the requirements for Zap70 activity by mature mouse T cells, we generated mice expressing a transgene encoding Zap70(AS). The analog-sensitive system allowed us to investigate the circumstances in which T cell activation requires the catalytic activity of Zap70. Second, this system enabled us to study the role of Zap70 activity in activating the function of naturally occurring $CD4^+CD25^+$ regulatory T cell (T_{reg} cell). Third, the use of a catalytic inhibitor allowed us to identify the biological importance of a noncatalytic function of Zap70 in T_{reg} cells.

RESULTS

Generation of *Zap70*(AS) mice

To analyze the requirements for Zap70 catalytic activity by mature mouse T cells, we generated transgenic mice that express a bacterial artificial chromosome (BAC) transgene containing the mouse *Zap70* locus bearing a mutated sequence encoding substitution of the gatekeeper residue methionine at position 413 with alanine (M413A). Met413 in mouse Zap70 is homologous to the human Zap70 Met414 gatekeeper residue. We generated two independent founder strains with transgenic expression of Zap70 M413A and crossed them with Zap70-null mice[4] to generate $Zap70^{-/-}$ mice with transgenic expression of Zap70 M413A. We used a BAC transgene strategy rather than a knock-in strategy for two reasons. First, genetic changes in the endogenous *Zap70* locus, by knock-in or by spontaneous mutation, have resulted in lower Zap70 protein expression and T cell lymphopenia[10,11]. Second, the Zap70(AS) mutant kinase is hypomorphic, with catalytic activity approximately one third that of wild-type Zap70 (ref. 9). We reasoned that higher expression of Zap70(AS) protein might be needed to restore T cell development. Indeed, one founder strain, whose T cells had expression of Zap70 protein ~28% that of wild-type T cells (**Supplementary Fig. 1a**), had few peripheral $CD4^+$ and $CD8^+$ T cells (**Supplementary Fig. 1b**). The second founder strain had tenfold higher expression of Zap70 protein than that of wild-type mice (**Supplementary Fig. 2a**) but had frequencies and total numbers of thymocyte subsets similar to those of wild-type mice (**Supplementary Fig. 2b**,**c**). On the basis of these data, we continued our studies with this second founder line (called '*Zap70*(AS)' here).

Phenotypic analysis of the thymi from *Zap70*(AS) mice showed that expression of the analog-sensitive Zap70 mutant was sufficient to support the development of $CD4^+$ single-positive and $CD8^+$ single-positive thymocytes, overcoming the developmental blockade in $Zap70^{-/-}$ mice at the double-positive stage (**Supplementary Fig. 2b**,**c**). Further analysis of peripheral T cells showed that the percentage and total number of $CD4^+$ and $CD8^+$ T cells in the spleens of *Zap70*(AS) mice was similar to that of wild-type and $Zap70^{+/-}$ mice (**Supplementary Fig. 2b**,**d**). In the peripheral T cell population, the frequencies of T cells with a naive $CD44^{lo}CD62L^{hi}$ or effector-memory $CD44^{hi}CD62L^{lo}$ phenotype were more similar for *Zap70*(AS) and $Zap70^{+/-}$ mice than for *Zap70*(AS) and $Zap70^{+/+}$ mice (**Supplementary Fig. 2e** and data not shown). Given the phenotypic similarities of *Zap70*(AS) mice and $Zap70^{+/-}$ heterozygous mice, we used $Zap70^{+/-}$ T cells as the control cell type in our subsequent studies.

Inhibition of Zap70 catalytic activity impairs T cell activation

We examined the circumstances under which the catalytic activity of Zap70 is required during the following stages of a T cell response: initial activation, effector function and the memory response. The best known biochemical role of Zap70 involves phosphorylation of its substrates Lat and SLP-76, which nucleate a complex of signaling molecules, leading to the phosphorylation and activation of phospholipase C-γ and subsequent second messenger pathways, such as the inositol-1,3,4-trisphosphate–calcium flux pathway and the diacylglycerol–mitogen-activated protein kinase (MAPK) pathway. Stimulation of *Zap70*(AS) or control $Zap70^{+/-}$ $CD4^+$ T cells by crosslinking of cell surface–bound monoclonal antibody (mAb) to CD3ε in the presence of vehicle (dimethyl sulfoxide) resulted in a robust increase in the cytoplasmic concentration of free Ca^{2+} ($[Ca^{2+}]_i$; **Fig. 1a** and **Supplementary Fig. 3a**). However, simultaneous addition of the Zap70(AS)-specific inhibitor 3-MB-PP1 with mAb to CD3ε resulted in a dose-dependent decrease in $[Ca^{2+}]_i$. We did not observe inhibition of the increase in $[Ca^{2+}]_i$ mediated by 3-MB-PP1 after the addition of ionomycin (data not shown). In the presence of high concentrations of 3-MB-PP1 (such as 10 μM), there was a small inhibitory effect on the increase in $[Ca^{2+}]_i$ in $Zap70^{+/-}$ T cells and B cells; however, this effect did not reach the magnitude of inhibition seen in *Zap70*(AS) or $Zap70^{+/-}$ T cells in other assays. We also never reached a median inhibitory concentration for cells expressing wild-type Zap70. Thus, we conclude that 3-MB-PP1 is specific for Zap70(AS) but not for wild-type Zap70 or other wild-type kinases known to be required in signaling pathways for calcium increases (such as the kinase Syk or kinases of the Src or Tec family).

The use of a small-molecule inhibitor allows temporal control of inhibition of the catalytic activity of Zap70. Consistent with published experiments using Jurkat cells[9], the addition of 3-MB-PP1 to *Zap70*(AS) T cells at the peak of the TCR-induced calcium response resulted in a rapid decrease in $[Ca^{2+}]_i$ within 30 s (**Supplementary Fig. 3b**). Thus, continuous Zap70 catalytic activity is needed to sustain increases in $[Ca^{2+}]_i$.

Alternatively, we sought to determine if the effects of 3-MB-PP1 were reversible. To test this, we stimulated *Zap70*(AS) T cells in the presence of 3-MB-PP1, which inhibited the initial increase in $[Ca^{2+}]_i$ (**Supplementary Fig. 3a**). However, there was a rapid increase in $[Ca^{2+}]_i$ in *Zap70*(AS) T cells washed with fresh media (**Supplementary Fig. 3c**). These results show that the inhibition of the catalytic function of Zap70 in this chemical-genetic system not only is specific but also can be rapidly induced and reversed.

TCR-dependent MAPK activation is similarly dependent on the catalytic activity of Zap70. TCR stimulation in the presence of vehicle alone induced a robust increase in phosphorylated Erk in both $Zap70^{+/-}$ and *Zap70*(AS) $CD4^+$ cells (**Fig. 1b**). We found that 3-MB-PP1 was effective at inhibiting the induced Erk phosphorylation in *Zap70*(AS) $CD4^+$ T cells but not $Zap70^{+/-}$ $CD4^+$ T cells, in a dose-dependent manner (**Fig. 1b**). Furthermore, stimulation of Erk phosphorylation with the phorbol ester PMA was not impaired in the presence of 3-MB-PP1, consistent with a role for 3-MB-PP1 in specifically inhibiting the upstream kinase activity of Zap70 and not other downstream kinases in the GTPase Ras–MAPK pathway. TCR stimulation in the presence of 3-MB-PP1 for 16 h also had a substantial inhibitory effect on the Ras-MAPK-dependent upregulation of the activation marker CD69 on *Zap70*(AS) $CD4^+$ T cells but not on $Zap70^{+/-}$ $CD4^+$ T cells (**Fig. 1c**).

Immunoblot anlaysis of whole-cell lysates of peripheral $CD4^+$ T cells from $Zap70^{+/-}$ and *Zap70*(AS) mice (**Fig. 1d**) showed that phosphorylation of the tyrosine residue in the activation loop of Lck was not inhibited in the presence of 3-MB-PP1, consistent with a role for Lck upstream of Zap70 (**Fig. 1d**, top). As expected, phosphorylation of molecules downstream of Zap70, such as Lat Tyr132 and Erk, was impaired in the presence of 3-MB-PP1 (**Fig. 1d**, bottom). Notably, two tyrosine residues in Zap70 itself (Tyr319 and Tyr493) were still phosphorylated in the presence of 3-MB-PP1, consistent with published results[9], presumably because phosphorylation of these sites is mediated by Lck (**Fig. 1d**, middle).

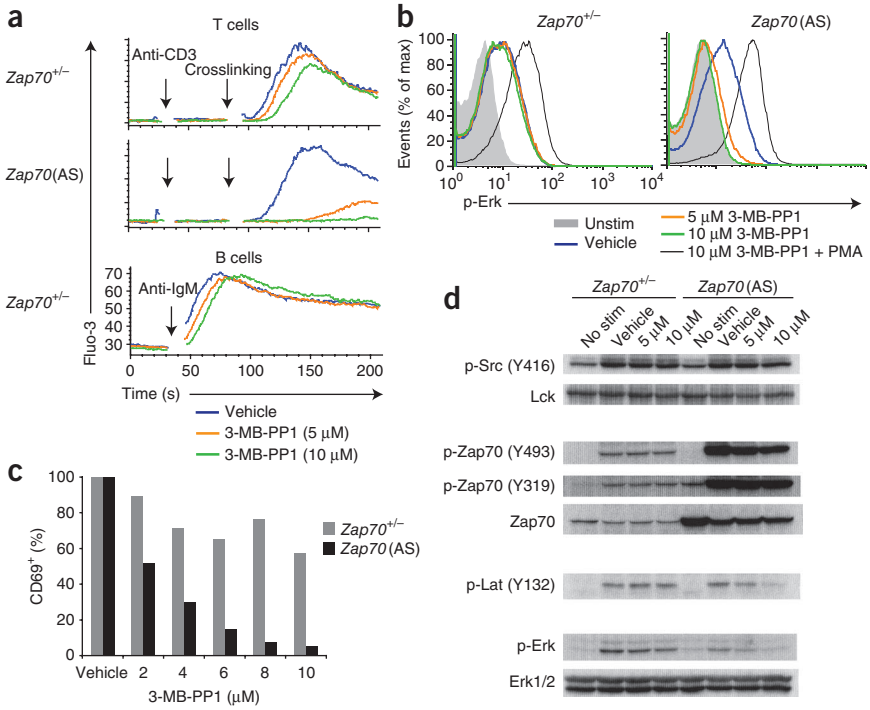

Figure 1 T cell activation is dependent on the catalytic activity of Zap70. (**a**) Flow cytometry of *Zap70*$^{+/-}$ and *Zap70*(AS) CD4$^+$ cells loaded with the fluorescent Ca^{2+} indicator Fluo-3. For T cells, arrows indicate the simultaneous addition of 3-MB-PP1 and mAb to CD3ε (left) and the addition of crosslinking antibodies (right); for B cells, arrow indicates the addition of anti-IgM plus 3-MB-PP1 (dose, key). (**b**) Flow cytometry analysis of staining for phosphorylated Erk (p-Erk) in *Zap70*$^{+/-}$ and *Zap70*(AS) CD4$^+$ T cells left unstimulated (Unstim) or stimulated with biotinylated anti-CD3ε and anti-CD4 plus streptavidin in the presence of vehicle only or 5 μM or 10 μM 3MB-PP1 alone or 10 μM 3MB-PP1 plus PMA. (**c**) Frequency of CD69$^+$ *Zap70*$^{+/-}$ and *Zap70*(AS) T cells after stimulation for 16 h with plate-bound anti-CD3ε and anti-CD28 in the presence of 3-MB-PP1 (concentration, horizontal axis); results are presented to those of vehicle-treated cells, set as 100%. (**d**) Immunoblot analysis of whole-cell lysates of purified *Zap70*$^{+/-}$ and *Zap70*(AS) CD4$^+$ T cells left unstimulated or stimulated for 2 min with anti-CD3ε and crosslinking antibodies in the presence of vehicle or 5 μM or 10 μM 3-MB-PP1; blots probed with antibodies specific for epitopes along left margin (phosphorylated (p-) residue in parentheses; Y, tyrosine). Data are representative of at least three independent experiments.

Together these results suggest that the activation of early events in mature naive T cells requires the catalytic function of Zap70.

T cell proliferation requires Zap70 catalytic activity

We hypothesized that T cell proliferation would also be highly dependent on the catalytic activity of Zap70. In response to stimulation with antibody to CD3ε (anti-CD3ε) plus anti-CD28 in the presence of vehicle alone, both *Zap70*(AS) and *Zap70*$^{+/-}$ CD4$^+$ T cells underwent a similar amount of proliferation, as measured by uptake of [^{3}H]thymidine on day 3 (**Fig. 2a**). However, the proliferation of *Zap70*(AS) T cells was roughly half in the presence of 5 μM 3-MB-PP1 and was almost completely inhibited at a dose of 10 μM. The lower sensitivity of the proliferative response (median inhibitory concentration, ~5 μM) than that of CD69 induction (median inhibitory concentration, ~2 μM) also indicated that different assays of T cell function may have different sensitivities to inhibition. For that reason, in nearly all our subsequent assays we used a high dose of 3-MB-PP1 to ensure maximum *Zap70*(AS) inhibition while minimizing off-target effects, as assessed with wild-type genetic controls.

We observed similar inhibitory results by analysis of dilution of the cytosolic dye CFSE in cultures of *Zap70*$^{+/-}$ and *Zap70*(AS) OT-II (ovalbumin-specific) TCR–transgenic CD4$^+$ T cells stimulated with an ovalbumin peptide of amino acids 323–339 in the presence of antigen-presenting cells (APCs; **Fig. 2b**). Thus, the kinase activity of Zap70 is needed to drive proliferative responses by CD4$^+$ T cells induced by anti-CD3 or antigen.

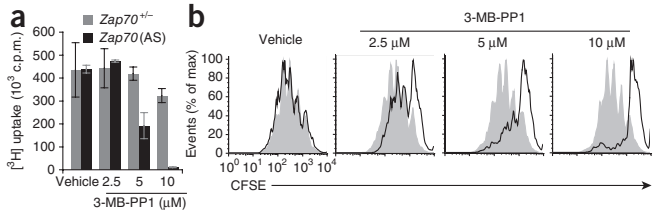

Figure 2 Proliferation of CD4$^+$ T cells requires the catalytic activity of Zap70. (**a**) [^{3}H]thymidine uptake by *Zap70*$^{+/-}$ and *Zap70*(AS) purified polyclonal CD4$^+$ T cells stimulated for 72 h with plate-bound anti-CD3ε and anti-CD28 in the presence of vehicle or 3-MB-PP1 (concentration, horizontal axis). (**b**) CFSE dilution in CFSE-loaded *Zap70*$^{+/-}$ OT-II T cells (gray filled histograms) and *Zap70*(AS) OT-II T cells (black lines) stimulated for 72 h with irradiated APCs and 1 μM ovalbumin peptide (amino acids 323–339) in the presence of 3-MB-PP1 (concentration, above plots). Data are representative of three experiments (mean and s.d. of triplicate cultures in **a**).

Effector T cell responses require Zap70 kinase activity

We next examined whether the catalytic activity of Zap70 is required at the time of a secondary TCR stimulation for the execution of effector T cell function. The Zap70 analog-sensitive system allowed us to investigate specifically whether the catalytic activity of Zap70 is required for the production of effector cytokines and cytolytic activity. To determine whether effector CD4$^+$ cells require Zap70 activity to produce effector cytokines, we cultured *Zap70*$^{+/-}$ and *Zap70*(AS) OT-II TCR–transgenic cells under conditions polarizing cells toward T helper type 1 (T_H1) or T_H2 differentiation in the absence of 3-MB-PP1. We then restimulated primed effector cells in the presence of vehicle alone or graded concentrations of 3-MB-PP1 (**Fig. 3a**). In the presence of vehicle alone, *Zap70*(AS) T_H1 and T_H2 effector cells produced interferon-γ (IFN-γ) and interleukin 4 (IL-4), respectively. However, in the presence of 10 μM 3-MB-PP1, the frequency of *Zap70*(AS) IFN-γ$^+$ T_H1 cells and IL-4$^+$ T_H2 cells was inhibited by more than 90%. Stimulation of effector cells with ionomycin and PMA, which bypass proximal TCR signaling events, was not sensitive to 3-MB-PP1 (**Fig. 3a**, right). These results suggest that previously activated CD4$^+$ T cells are dependent on the catalytic activity of Zap70 when stimulated via their TCR for the production of effector cytokines.

To address whether Zap70 activity is required for the effector function of CD8$^+$ T cells, we generated C57BL/6 (H-2^b) *Zap70*$^{+/-}$ and *Zap70*(AS) cytotoxic T lymphocytes (CTLs) in mixed-lymphocyte reactions with allogeneic stimulator cells (DBA/2; H-2^d). After

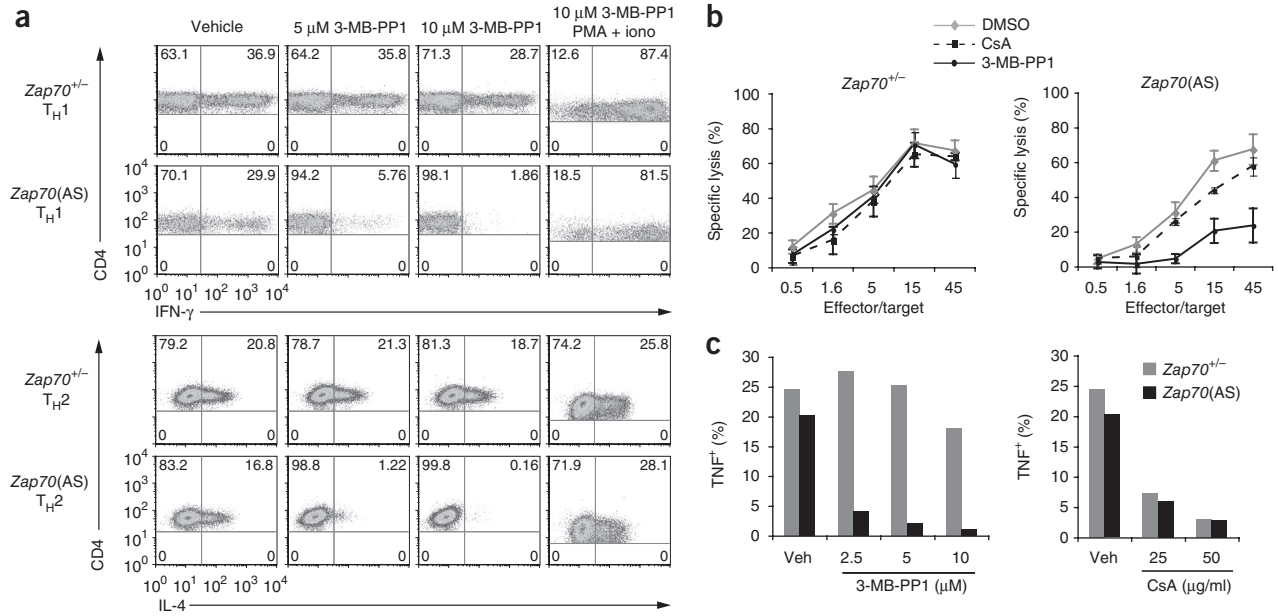

Figure 3 Execution of effector T cell functions requires the catalytic activity of Zap70. (**a**) Flow cytometry analysis of staining for IFN-γ and IL-4 in effector T_H1 and T_H2 *Zap70*$^{+/-}$ and *Zap70*(AS) OT-II cells, respectively, cultured with vehicle or with 3-MB-PP1 (concentration, above plots). Far right, separate sample of cells cultured with 3-MB-PP1 and stimulated with PMA and ionomycin (iono) as a control to bypass requirements for proximal TCR pathways. Numbers in quadrants indicate percent cells in each. (**b**) Lysis of allogeneic ^{51}Cr-labeled P815 target cells by *Zap70*$^{+/-}$ and *Zap70*(AS) CTLs in cultures containing effector and target cells (ratio, horizontal axes) supplemented with vehicle alone (dimethyl sulfoxide (DMSO)), CsA or 10 μM 3-MB-PP1. (**c**) Frequency of TNF$^+$ cells among alloreactive *Zap70*$^{+/-}$ and *Zap70*(AS) CTLs generated as in **b** and stimulated by P815 cells in the presence of 3-MB-PP1 or CsA (final concentration, horizontal axes). Data are representative of three experiments (error bars (**b**), s.d.).

5 d, we cultured the primed *Zap70*$^{+/-}$ and *Zap70*(AS) effector cells together with ^{51}Cr-labeled P815 mastocytoma target cells (H-2^d) to assess cytolytic effector function (**Fig. 3b**). In contrast to *Zap70*$^{+/-}$ control cells (**Fig. 3b**), *Zap70*(AS) CTLs were considerably impaired in their ability to lyse target cells in the presence of 10 μM 3-MB-PP1 (**Fig. 3b**). These results suggest that the cytolytic activity of primed CTLs is highly dependent on Zap70 activity. In contrast, addition of the calcineurin inhibitor cyclosporine A (CsA) failed to inhibit the CTL activity of either *Zap70*$^{+/-}$ or *Zap70*(AS) T cells.

We next sought to determine whether cytokine production by alloreactive CD8$^+$ cells is also sensitive to inhibition of Zap70 activity. Similar to *Zap70*(AS) CD4$^+$ effector cells, *Zap70*(AS) CD8$^+$ effector cells were defective in producing tumor necrosis factor (TNF) in the presence of 3-MB-PP1 (**Fig. 3c**). CsA was also effective at inhibiting the production of TNF by both *Zap70*$^{+/-}$ and *Zap70*(AS) alloreactive CD8$^+$ T cells (**Fig. 3c**). In addition, IFN-γ production by *Zap70*(AS) CD8$^+$ effector cells was also potently inhibited in the presence of 3-MB-PP1, whereas CsA inhibited IFN-γ production by both *Zap70*$^{+/-}$ and *Zap70*(AS) cells (**Supplementary Fig. 4**). Thus, in this experimental system, the TCR-dependent signals required for cytolytic activity are dependent on the kinase activity of Zap70, but independently of calcineurin activity, whereas effector cytokine production is dependent on the activity of both Zap70 and calcineurin.

Memory CD8$^+$ T cell responses require Zap70 activity

To determine whether memory T cell cytokine responses require Zap70 activity, we generated CD8$^+$ memory T cells by infecting *Zap70*$^{+/-}$ and *Zap70*(AS) mice with lymphocytic choriomeningitis

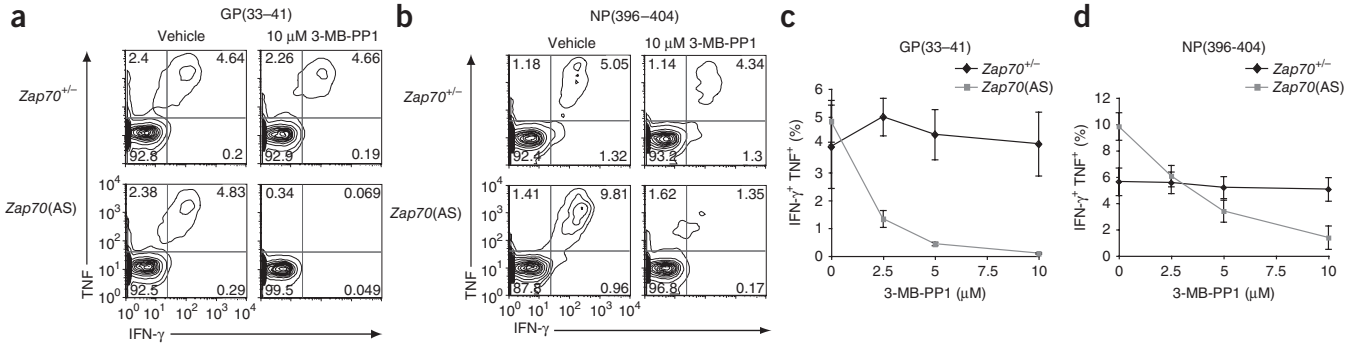

Figure 4 CD8$^+$ memory responses are dependent on the catalytic activity of Zap70. (**a**,**b**) Flow cytometry analysis of the production of IFN-γ and TNF by *Zap70*$^{+/-}$ and *Zap70*(AS) memory CD8$^+$ cells after stimulation with the LCMV peptides glycoprotein, amino acids 33–41 (GP(33–41); **a**), or nucleoprotein, amino acids 396–404 (NP(396–404); **b**), in the presence of vehicle alone or 10 μM 3-MB-PP1. Plots are gated on CD8$^+$CD44^{hi} memory cells; numbers in quadrants indicate percent cells in each. (**c**,**d**) Frequency of IFN-γ$^+$TNF$^+$ cells at various concentrations of 3-MB-PP1 (horizontal axes) among CD8$^+$CD44^{hi} memory *Zap70*$^{+/-}$ and *Zap70*(AS) cells (n = 3 mice per genotype). Data are representative of three experiments (mean and s.d. in **c**,**d**).

virus (LCMV). At >50 d after infection, we restimulated T cells *ex vivo* in the presence or absence of 3-MB-PP1. Both *Zap70*$^{+/-}$ and *Zap70*(AS) T cells produced IFN-γ and TNF after being stimulated with LCMV peptides of glycoprotein amino acids 33–41 (**Fig. 4a**) or nucleoprotein amino acids 396–404 (**Fig. 4b**) in the presence of vehicle alone. Notably, the addition of 3-MB-PP1 resulted in a dose-dependent decrease in the frequency of IFN-γ$^+$TNF$^+$ *Zap70*(AS) cells (**Fig. 4c**,**d**), which demonstrated that similar to recently activated CD8$^+$ effector cells, memory CD8$^+$ T cells are highly dependent on the catalytic activity of Zap70 for the production of effector cytokines.

Suppression by T_{reg} cells does not require Zap70 activity

Our results suggested that conventional T cells require the catalytic activity of Zap70 for the activation of naive, effector and memory cells. However, it remained unclear whether naturally occurring CD4$^+$CD25$^+$ T_{reg} cells also require Zap70 activity to exert their suppressive activity. One advantage of the Zap70(AS) system is the ability to determine the requirement for Zap70 activity in a single type of T cell in a complex culture of multiple T cell subsets. Specifically, by coculturing *Zap70*$^{+/-}$ CD4$^+$ CD25$^-$ conventional T cells (T_{conv} cells) with *Zap70*(AS) T_{reg} cells, we could address the requirements for Zap70 activity by T_{reg} cells. Published studies have suggested that T_{reg} cells require stimulation through their TCR to activate their suppressive function(s) *in vitro*[12,13]. On the basis of those reports, we hypothesized that T_{reg} cells also require the catalytic activity of Zap70 to suppress the proliferation of T_{conv} cells. Thus, we cultured T_{conv} cells and T_{reg} cells from *Zap70*$^{+/-}$ and *Zap70*(AS) mice in all combinations and at various ratios of T_{conv} cells to T_{reg} cells (**Fig. 5a**). In the presence of vehicle alone, we observed that T_{reg} cells from either *Zap70*$^{+/-}$ or *Zap70*(AS) mice suppressed the proliferation of either *Zap70*$^{+/-}$ or *Zap70*(AS) T_{conv} cells (**Fig. 5a**, left). We predicted the suppressive activity of *Zap70*(AS) T_{reg} cells would be impaired by the addition of 3-MB-PP1 to the culture, allowing uninhibited proliferation of the *Zap70*$^{+/-}$ T_{conv} population. However, *Zap70*(AS) T_{reg} cells suppressed the proliferation of *Zap70*$^{+/-}$ T_{conv} cells (**Fig. 5a**, middle and right), even in the presence of concentrations of 3-MB-PP1 that substantially inhibited the proliferation of *Zap70*(AS) T_{conv} cells (**Fig. 5b**). We obtained similar results with a CFSE-based suppression assay (**Supplementary Fig. 5**). Contrary to our initial hypothesis, these results suggest that *in vitro* T_{reg} cell–mediated suppression does not require the catalytic activity of Zap70. This led us to ask whether the activity of Zap70 is redundant in T_{reg} cells, for example, due to expression of the related kinase Syk. However, neither T_{conv} cells nor T_{reg} cells from *Zap70*$^{+/-}$ and *Zap70*(AS) mice had detectable expression of Syk protein, as assessed by immunoblot analysis (**Fig. 5c**). These results indicate that the ability of *Zap70*(AS) T_{reg} cells to suppress the proliferation of T_{conv} cells in a Zap70 kinase–independent manner is not due to redundant function provided by Syk.

Activation of Rap1 is independent of Zap70 activity

To determine how T_{reg} cells might suppress the proliferation of T_{conv} cells independently of the kinase activity of Zap70, we sought to determine whether there are TCR-induced signal-transduction pathways that are unperturbed in the presence of a Zap70 catalytic inhibitor. We first analyzed the calcium responses of *Zap70*$^{+/-}$ and *Zap70*(AS) CD4$^+$CD25$^+$ T_{reg} cell populations previously expanded *in vitro*[14]. In the absence of inhibitor, the calcium response was much lower in CD25$^+$ T_{reg} cells than in CD25$^-$ cells but was still detectable (**Fig. 6a**), consistent with published reports of diminished TCR signaling in T_{reg} cells[15,16]. The addition of 3-MB-PP1 potently inhibited the increase in $[Ca^{2+}]_i$ in *Zap70*(AS) T_{conv} cells and T_{reg} cells, which indicated that the catalytic activity of Zap70 is required even for the lower TCR-stimulated increases in $[Ca^{2+}]_i$ in T_{reg} cells. In response to TCR stimulation alone, we also observed less but still detectable phosphorylation Erk in Foxp3$^+$ T_{reg} cells relative to that in Foxp3$^-$ cells (**Fig. 6b**). Again, the amount of phosphorylated Erk was decreased to basal amounts after TCR stimulation in the presence of 3-MB-PP1 in both Foxp3$^-$ and Foxp3$^+$ *Zap70*(AS) cells. Together these results suggest that the catalytic activity of Zap70 is required for TCR-induced increases in $[Ca^{2+}]_i$ and Erk responses in T_{reg} cells but is not required for their suppressive function.

Zap70 itself was phosphorylated normally on Tyr319 and Tyr493 in the presence of a Zap70 catalytic inhibitor (**Fig. 1d**). Those results raised the possibility that Zap70 could act as a scaffolding protein in the absence of its catalytic activity via interaction between phosphorylated tyrosine residues in its interdomain B region and other Src homology 2 domain–containing proteins. One candidate that fits this model is the adaptor protein CrkII, which has been reported to bind phosphorylated Tyr315 after TCR stimulation[17]. Published work has also shown that Crk adaptor proteins associate with the guanine nucleotide–exchange factor C3G[18,19]. C3G can act as a guanine nucleotide–exchange factor for the GTPase Rap1, which potentiates the inside-out signaling pathway to the integrin LFA-1, resulting in greater affinity of LFA-1 for its

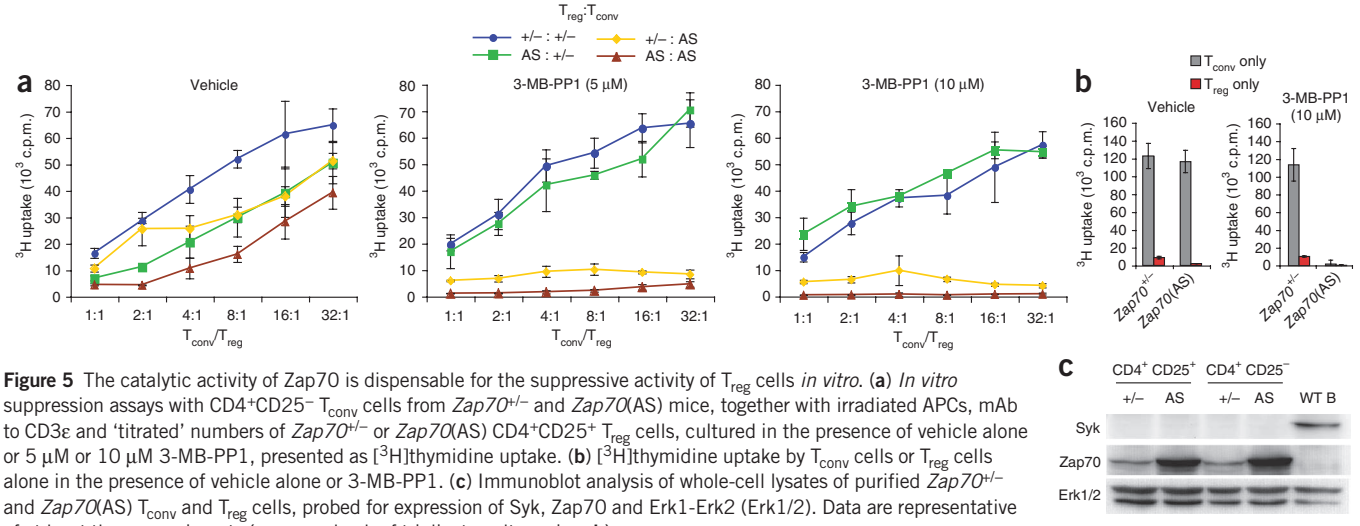

Figure 5 The catalytic activity of Zap70 is dispensable for the suppressive activity of T_{reg} cells *in vitro*. (**a**) *In vitro* suppression assays with CD4$^+$CD25$^-$ T_{conv} cells from *Zap70*$^{+/-}$ and *Zap70*(AS) mice, together with irradiated APCs, mAb to CD3ε and 'titrated' numbers of *Zap70*$^{+/-}$ or *Zap70*(AS) CD4$^+$CD25$^+$ T_{reg} cells, cultured in the presence of vehicle alone or 5 μM or 10 μM 3-MB-PP1, presented as [^{3}H]thymidine uptake. (**b**) [^{3}H]thymidine uptake by T_{conv} cells or T_{reg} cells alone in the presence of vehicle alone or 3-MB-PP1. (**c**) Immunoblot analysis of whole-cell lysates of purified *Zap70*$^{+/-}$ and *Zap70*(AS) T_{conv} and T_{reg} cells, probed for expression of Syk, Zap70 and Erk1-Erk2 (Erk1/2). Data are representative of at least three experiments (mean and s.d. of triplicate cultures in **a**,**b**).

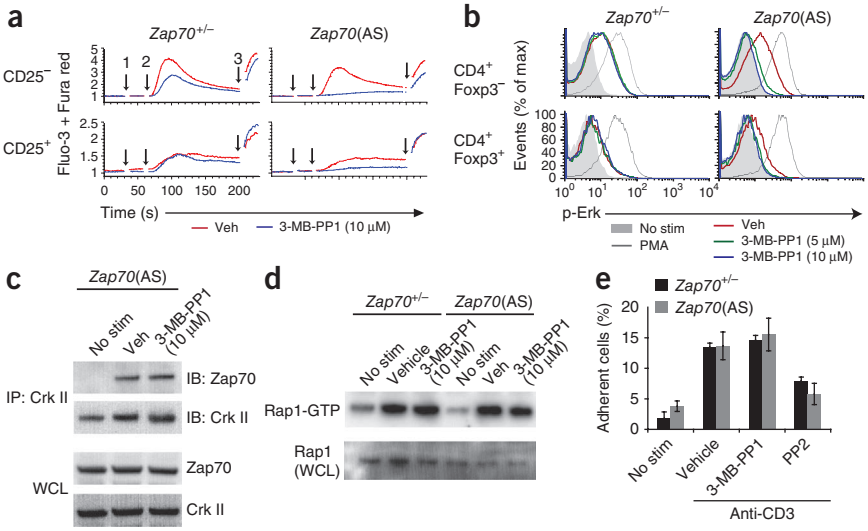

Figure 6 TCR-induced activation of Rap1 and adhesion to ICAM-1 are independent of Zap70 kinase activity. (**a**) Flow cytometry analysis of intracellular calcium in CD4+CD25– T_{conv} and CD4+CD25+ T_{reg} cells loaded with the fluorescent calcium indicators Fluo-3 and Fura red and stimulated in the presence of vehicle alone (red) or 3-MB-PP1 (blue); arrows indicate the addition of biotinylated anti-CD3ε and anti-CD4 (1), streptavidin (2) and ionomycin (3). T_{conv} cells were >99% Foxp3–; T_{reg} cells were >98% Foxp3+. (**b**) Flow cytometry analysis of phosphorylated Erk in splenocytes stimulated *ex vivo* by crosslinking mAb to CD3 and left unstimulated or stimulated in the presence of vehicle alone, 5 μM or 10 μM 3-MB-PP1 alone, or 10 μM 3-MB-PP1 plus PMA; plots are gated on Foxp3– or Foxp3+ CD4+ cells. (**c**) Immunoassay of lysates of *Zap70*(AS) thymocyte stimulated for 2 min with crosslinking mAb to CD3; samples immunoprecipitated (IP) with anti-CrkII and whole-cell lysates (WCL) were analyzed by immunoblot (IB) for Zap70 and CrkII. (**d**) Precipitation of Rap1-GTP from *Zap70*+/– and *Zap70*(AS) thymocytes left unstimulated or stimulated for 2 min with crosslinking mAb to CD3 and treated with vehicle alone or 10 μM 3-MB-PP1. Below, total Rap1 in whole-cell lysates. (**e**) Flow cytometry of *Zap70*+/– and *Zap70*(AS) CD4+ T cells stimulated for 10 min with anti-CD3ε in wells coated with recombinant ICAM-1, then washed and counted. Data are representative of three independent experiments (error bars (**e**), s.d.).

ligand ICAM-1. Thus, it is possible that even in the absence of catalytic function, phosphorylated Zap70 is able to form a complex with CrkII and C3G, leading to enhanced LFA-1-mediated cellular adhesion.

To test that model, we sought to determine whether Zap70 immunoprecipitated together with CrkII and also whether this association was dependent on Zap70's catalytic activity. Indeed, Zap70(AS) immunoprecipitated together with CrkII in a TCR-inducible manner, but this interaction was unperturbed in the presence of 3-MB-PP1 (**Fig. 6c**). To identify an additional signal in the inside-out pathway, we assayed Rap1 activation in *Zap70*+/– and *Zap70*(AS) CD4+ T cells by a Rap-1–GTP precipitation assay. After TCR stimulation in the presence of vehicle alone, Rap1 activation was induced in both *Zap70*+/– and *Zap70*(AS) T cells. In the presence of 3-MB-PP1, activation of Rap1-GTP was still induced in *Zap70*(AS) T cells (**Fig. 6d**). These results show that the catalytic activity of Zap70 is not required for TCR-induced activation of Rap1. Consistent with those results, TCR-induced adhesion to plate-bound ICAM-1 by CD4+ T cells was lower in the presence of PP2, an inhibitor of Src family kinases, but not in the presence of 3-MB-PP1 (**Fig. 6e**), which indicated that the TCR-induced inside-out signaling that regulates cellular adhesion is dependent on the activity of Src family kinases (Lck and Fyn) but not the kinase activity of Zap70.

We next sought to determine whether substitution of Tyr315 and Tyr319 of Zap70 would disrupt TCR-induced activation of Rap1. Knock-in mice bearing substitution of both of those tyrosine residues with alanine (Zap70(YYAA) mice) have been generated[10]. We found that the induction of Rap1-GTP in Zap70(YYAA) T cells was less than that in wild-type (**Fig. 7a**), which suggested that Tyr315 and Tyr319 are required for TCR-induced activation of Rap1. Similarly, Zap70(YYAA) T cells were also impaired in their TCR-induced adhesion to ICAM-1 (**Fig. 7b**). Together these results indicate a role for Zap70 Tyr315 and Tyr319 in inside-out signaling to integrin activation and cellular adhesion.

From the results reported above we conclude that a Zap70 kinase–independent scaffolding function may contribute to activation of integrin-mediated adhesion to ICAM-1. Our model proposes that *Zap70*(AS) T_{reg} cells are able to activate their suppressive function(s), even when the catalytic activity of Zap70 is inhibited, by maintaining close proximity to target T_{conv} cells through enhanced ICAM-1 adhesion. Consistent with this model, T_{reg} cells bearing the Zap70(YYAA) substitutions, which impaired Rap1 activation and ICAM-1 adhesion, also showed defective suppressive activity *in vitro*[10] (**Supplementary Fig. 6**). Also consistent with this model, stimulation of *Zap70*(AS) T_{reg} cells with IL-2 resulted in phosphorylation of the transcription factor STAT5, even when Zap70 catalytic function was inhibited (**Supplementary Fig. 7**). These data describe a previously unknown kinase-independent role for Zap70 in integrin activation and T_{reg} cell activity.

DISCUSSION

The analog-sensitive system allowed us to determine when the catalytic activity of Zap70 is required for the activation of mature, primary mouse T cells. Our results have demonstrated that many initial TCR signaling events, as well as TCR-induced proliferation, effector functions and memory responses of conventional CD4+ and CD8+ T cells, are all highly dependent on the catalytic activity of Zap70. These data are consistent with published work demonstrating failed TCR signal

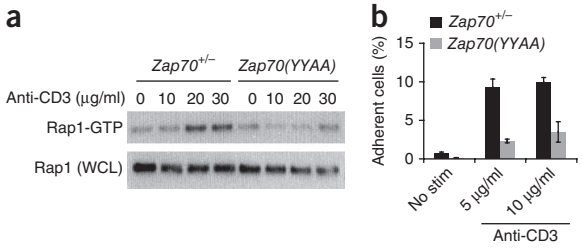

Figure 7 TCR-induced activation of Rap1 and adhesion to ICAM-1 are dependent on the adaptor function of Zap70. (**a**) Precipitation of Rap1-GTP from *Zap70*+/– and Zap70(YYAA) thymocytes stimulated for 2 min with the various doses of anti-CD3ε (above lanes) in the absence of 3-MB-PP1. (**b**) ICAM-1 adhesion by peripheral *Zap70*+/– and Zap70(YYAA) CD4+ T cells left unstimulated or stimulated (as in **Fig. 6d**) with 5 μg/ml or 10 μg/ml of anti-CD3ε. Data are representative of three independent experiments (error bars (**b**), s.d.).

transduction in Zap70-deficient Jurkat cell lines and similar impairments in peripheral $CD4^+$ T cells of Zap70-deficient patients with severe combined immunodeficiency[6].

However, we did not anticipate that the *in vitro* suppressive activity of T_{reg} cells would not require the cataytic activity of Zap70. We have shown that the TCR-induced association of Zap70 with CrkII, activation the GTPase Rap1 and adhesion to ICAM-1 occurred even in the presence of a Zap70 catalytic inhibitor. These results are in agreement with published reports showing associations among Zap70 Tyr315, CrkII, and C3G[17,20]. This model indicates a role for Zap70 as a protein scaffold that is independent of its catalytic function. Although the proposed scaffolding function of Zap70 seemed to be insufficient for the activation and effector function of conventional T cells, the same might not be true for T_{reg} cell function. We propose that *Zap70*(AS) T_{reg} cells, even in the presence of 3-MB-PP1, have TCR-induced phosphorylation of Zap70 on Tyr315, followed by the association of this phosphorylated residue with CrkII-C3G, thus facilitating the activation of Rap1, resulting in more adhesion of LFA-1 to ICAM-1 expressed on APCs. Enhanced adhesion by *Zap70*(AS) T_{reg} cells potentially enhances their close proximity to APCs and target T_{conv} cells, where they can use many mechanisms of suppression; that is, via the immunomodulatory receptor CTLA-4, competition for IL-2, suppression of the expression of costimulatory molecules on dendritic cells, or metabolic disruption of the proliferation of T_{conv} cells through the production of adenosine via the ectoenzymes CD39 and CD73 (refs. 21,22). Consistent with this model, *Zap70*(AS) T_{reg} cells did not require the catalytic function of Zap70 to respond to IL-2. Those results are compatible with computational modeling studies indicating that the distance between T_{reg} cells and T_{conv} cells is an important factor in the competition between these cells for IL-2 (ref. 23). Additionally, studies of CD18-deficient mouse T_{reg} cells, as well as of LFA-1-deficient T_{reg} cells from human patients with type 1 leukocyte adhesion deficiency, have provided evidence that LFA-1 on T_{reg} cells is important for their suppressive function[24,25]. Moreover, T_{reg} cells expressing a transgene encoding a constitutively active Rap1 mutant show enhanced suppressive activity[26].

A prediction of our model is that T_{reg} cells bearing substitutions at Zap70 residues Tyr315 and Tyr319 would have impaired Zap70 kinase–independent scaffolding function, leading to impaired adhesion and T_{reg} cell–mediated suppression. Indeed, as shown before[10] and here, T_{reg} cells from Zap70(YYAA) knock-in mice have impaired suppressive activity. Although Zap70(YYAA) T cells do have impairments in downstream signaling, such as calcium increases and Erk phosphorylation, the SKG mutation, a different hypomorphic allele of *Zap70*, results in even greater impairments in downstream TCR signaling yet has little effect on the suppressive activity of T_{reg} cells[10]. These results provide evidence of an important noncatalytic function for Zap70 in T_{reg} cell function. We conclude that at least in the context of Zap70 catalytic inhibition (that is, *Zap70*(AS) T_{reg} cells in the presence of 3-MB-PP1), Zap70 scaffolding–mediated activation of integrin adhesion is sufficient to enable the suppression of T_{reg} cells *in vitro*. It remains to be tested whether the catalytic activity of Zap70 is required for T_{reg} cell function *in vivo*.

Published reports have shown that T_{reg} cells require TCR stimulation to activate their suppressive activity[12,13]. Other data have also shown that T_{reg} cells lacking Lck expression are defective in suppression[27]. Our data are not necessarily incompatible with those studies. Instead, we conclude that *in vitro* T_{reg} cell suppression requires both TCR and Zap70 but more specifically the scaffolding function of Zap70. An implication of this is the possibility that a catalytic inhibitor of Zap70 might be used to dampen the response of pathogenic conventional T cells in settings of autoimmunity or allograft rejection while not rendering the suppressive activity of T_{reg} cells deficient.

Our results have shown that the activation of naive, effector and memory T cells required the catalytic activity of Zap70. These data indicate that it could be possible to use a catalytic inhibitor of Zap70 to both prevent a pathogenic T cell response, as in the setting of allogeneic transplantation, and also dampen previously established pathogenic T cell responses, as in the setting of autoimmunity. Furthermore, a catalytic inhibitor of Zap70 might have advantages over existing inhibitors, such as the calcineurin inhibitor CsA. Treatment with a Zap70 catalytic inhibitor was able to attenuate both cytotoxicity and effector cytokine production by CTLs, whereas treatment with CsA was effective only in impairing cytokine production.

The efficacy of a Zap70 inhibitor in the treatment of mouse models of autoimmunity remains to be tested. However, the half-life of 3-MB-PP1 *in vivo* seems too short to achieve long-term blood concentrations approaching the concentration of 10 μM required for studies at this time. Structural analogs of 3-MB-PP1 with more suitable pharmacokinetics for long-term *in vivo* use need to be characterized. Our study here has described a model system for studying the role of the catalytic activity of Zap70 in the activation of mature, primary mouse T cells. Chemical and genetic approaches may prove to be powerful techniques for defining the temporal functions, as well as the catalytic activity–dependent and catalytic activity–independent functions, of other tyrosine kinases involved in TCR signaling.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession code. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org/): A002396.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

B.B.A.-Y. did most of the experiments and wrote the paper; S.E.L. did the initial characterization of *Zap70*(AS) mice; N.K. designed the strategy for generating *Zap70*(AS) mice; D.A.C. assisted with the ICAM-1 adhesion assays and calcium-flux assays; L.-Y.H. helped with the Zap70(YYAA) T_{reg} cell–suppression assay; K.M.S. and C.Z. provided advice and synthesized 3-MB-PP1; and A.W. directed the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Generation of *Zap70*(AS) mice. *Zap70*(AS) transgenic mice were generated by the UCSF Transgenic and Targeted Mutagenesis Core Facility. BAC RP23-6M17 containing the region of chromosome 1 with the *Zap70* locus was from BACPAC Resources at the Children's Hospital Oakland Resource Institute. Mutation of the sequence encoding the mouse Zap70 methionine residue at position 413 (homologous to human Met414) to sequence encoding alanine was engineered by bacterial recombination-mediated genetic engineering. The BAC containing the *Zap70*(AS) mutation was injected into fertilized *Zap70*$^{+/-}$ embryos. Mice were genotyped with the following PCR primers: transgenic allele, 5′-TTCCTCTCTAACCCGGGAGT-3′ and 5′-TCCCGCCATCTCC GC-3′; wild-type allele, 5′-TTCCTCTCTAACCCGGGAGT-3′ and 5′-TCC CGCCATCTCCAT-3′; and knockout allele, 5′-GCACATATGCACTGTCCCT GGTCTA-3′ and 5′-TGGCTACCCGTGATATTGCTGAAGA-3′.

Mice. Mice used for these studies were 6–10 weeks of age, were housed in the specific pathogen–free facility at the University of California, San Francisco, and were treated according to protocols approved by university animal care ethics and veterinary committees in accordance with guidelines of the US National Institutes of Health. *Zap70*$^{+/-}$ and *Zap70*(AS) mice were crossed with OT-II mice. APCs were collected from TCRα-chain-deficient mice (Jackson Laboratory). DBA/2 mice were from Taconic. Knock-in mice expressing the Zap70(YYAA) substitution have been described[10].

Inhibitors. The synthesis of 3-MB-PP1 (3-methylbenzyl-pyrazolopyrimidine) has been described[8]. For CD8$^+$ effector T cell assays, CsA (Calbiochem) was added at the appropriate concentration.

Flow cytometry. Stained cells were analyzed on a FACSCalibur (Becton Dickinson) or CyAn ADP (Beckman Coulter). Anti-CD4 (GK1.5) and anti-CD8 (YTS 169.4) were from the UCSF Antibody Core Facility. Anti–IFN-γ (XMG1.2), anti-TNF (MP6-XT77), anti-IL-4 (11B11), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD69 (H1.2F3) and anti-CD62L (MEL14) were from BD Biosciences. Anti-Foxp3 (FJK-16S) was from eBioscience.

Assays of calcium flux and Erk phosphorylation. T cells were stimulated for analysis of calcium flux and phosphorylated Erk with soluble biotinylated mAb to CD3ε (10 μg/ml; 2C11; Harlan) and mAb to CD4 (2 μg/ml), followed by streptavidin crosslinking. Alternatively, cells were stimulated with anti-CD3ε and crosslinking anti–Armenian hamster (Jackson ImmunoResearch). For calcium analysis, splenocytes were loaded with Fluo-3 (2 μg/ml) and Fura Red (4 μg/ml; Invitrogen). The dump gates used mAb to B220 (RA3-6B2; BD Pharmingen), mAb to pan-NK cells (DX5; BD Pharmingen), mAb to CD11b (M1/70; BD Pharmingen), mAb to major histocompatibility complex class II (M5.114.15.2; BD Pharmingen), mAb to Gr-1 (RB6-8C5; BD Pharmingen), mAb Ter119 (BD Pharmingen) and mAb to CD8 (YTS 169.4; UCSF Antibody Core Facility).

For staining of phosphorylated Erk, cells were fixed, made permeable with ice-cold 90% methanol and stained with antibody to phosphorylated Erk (197G2; Cell Signaling). As a positive control, cells were stimulated with PMA (200 μg/ml; phorbol 12-myristate 13-acetate; Sigma).

Proliferation assays. For analysis of the uptake of [^{3}H]thymidine, T cells were stimulated for 72 h with plate-bound mAb to CD3ε (2 μg/ml) and mAb to CD28 (2 μg/ml; 37.51; BD Pharmingen). For the final 6 h of culture, 1 μCi [^{3}H]thymidine (GE Healthcare) was added. For CFSE-based proliferation assays, purified T cells were loaded with 5 μM CFSE (carboxyfluorescein diacetate succinimidyl ester; Invitrogen) and then stimulated with APCs plus 1 μM ovalbumin peptide (amino acids 323–339; New England Peptide).

Chromium-release assay. Alloreactive CD8$^+$ T cells were generated in a mixed-lymphocyte reaction of 25×10^6 *Zap70*$^{+/-}$ or *Zap70*(AS) splenocytes and 25×10^6 irradiated DBA/2 splenocytes (1,000 rads). On day 5, effector cells were 'titrated' and cultured for 4 h in triplicate with 5×10^3 ^{51}Cr-labeled P815 target cells. P815 cells were loaded with 50 μCi ^{51}Cr–sodium chromate (Perkin Elmer). Specific lysis was calculated as the percentage of lysis as follows: experimental lysis counts—spontaneous release counts (no effector T cells present) / total lysis (hypotonic lysis followed by freezing and thawing) × 100.

Staining for T_H1 and T_H2 effector cytokines. *Zap70*$^{+/-}$ and *Zap70*(AS) OT-II cells were stimulated with 1 μM ovalbumin peptide (amino acids 323–339) and irradiated APCs. T_H1-polarizing conditions included recombinant human IL-2 (10 U/ml; AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health; M. Gately, Hoffmann-La Roche), recombinant IL-12 (10 ng/ml; Peprotech) and anti-IL-4 (25 μg/ml). T_H2-polarizing conditions included recombinant human IL-2 (10 U/ml), recombinant IL-4 (50 ng/ml) and anti-IL-12 (University of California, San Francisco, Antibody Hybridoma Core Facility). Effector cells were restimulated for 6 h with plate-bound anti-CD3ε (0.5 μg/ml), with brefeldin A (10 μg/ml; Epicentre) present for the final 4 h.

LCMV infection. *Zap70*(AS) and *Zap70*$^{+/-}$ mice were infected by intraperitoneal injection of 1×10^5 plaque-forming units of LCMV (Armstrong). After >50 d, splenocytes were collected and cultured for 6 h with nucleoprotein amino acids 396–404 (0.2 μg/ml; sequence, FQPQNGQFI) or glycoprotein amino acids 33–41 (0.2 μg/ml; sequence, KAVYNFATM) in the presence of brefeldin A and 'titrated' doses of 3-MB-PP1 or vehicle. Cells were fixed and made permeable with Cytofix/Cytoperm buffers according to the manufacturer's instructions (BD Biosciences) and were stained for IFN-γ and TNF.

Immunoblot analysis and immunoprecipitation. Cells were lysed in 2× SDS sample buffer and analyzed by SDS-PAGE and immunoblot as described[9]. Blots were probed for Zap70 (2F3.2)[28], Syk (5F5)[29] and Erk1-Erk2 (sc-153; Santa Cruz). For analysis of TCR signaling, monoclonal antibody to phosphorylated Erk (197G2) and polyclonal antiserum to Src phosphorylated at Tyr416 (2101) or to Zap70 phosphorylated at Tyr319 (2701) or Tyr493 (2704) were from Cell Signaling. Antibody to Lat phosphorylated at Tyr132 (4224) was from Biosource.

For immunoprecipitation studies, anti-CD3-stimulated thymocytes were lysed in a lysis buffer of 1% (vol/vol) Nonidet P-40. Lysates were incubated for 2 h at 4 °C with protein A–Sepharose beads preincubated with polyclonal antiserum to CrkII (sc-289; Santa Cruz).

Rap1-precipitation assay. For each condition, 30×10^6 thymocytes were stimulated for 2 min with soluble anti-CD3ε (10 μg/ml) and crosslinking anti-hamster (50 μg/ml; 127-005-160; Jackson ImmunoResearch). Rap1-precipitation assays were done according to the manufacturer's instructions (Thermo Scientific).

Suppression assays. Samples were enriched for CD4$^+$CD25$^-$ T_{conv} cells and CD4$^+$CD25$^+$ T_{reg} cells with regulatory T cell enrichment kits according to the manufacturers' protocols (Miltenyi; Stem Cell Technologies). For [^{3}H]thymidine-based assays, 1×10^5 T_{conv} cells plus 1×10^5 irradiated APCs were cultured for 72 h together with T_{reg} cells in triplicate wells, with 1 μCi [^{3}H]thymidine present for the final 12 h. Also present was mAb to CD3ε at a final concentration of 1 μg/ml and 'titrated' doses of 3-MB-PP1 or vehicle (dimethyl sulfoxide).

ICAM-1-binding assay. Purified CD4$^+$ cells (1×10^6) were stimulated with soluble anti-CD3ε and crosslinking anti-hamster in 96-well plates coated with recombinant mouse ICAM-1–Fc fragment (3 μg/ml; R&D Systems). Wells were washed three times and plate-bound cells were removed with cell-dissociation buffer (Gibco). Plate-bound cells were counted by flow cytometry. The percentage of cells bound was calculated as follows: [(live CD4$^+$ cells bound to the plate)/(input cells per well)] × 100.

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