Proximal changes in signal transduction that modify CD8+ T cell responsiveness *in vivo*

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The antigen dose conditions the functional properties of CD8+ T cells generated after priming. At relatively low antigen doses, efficient memory T cells may be generated, while high antigen doses lead to tolerance. To determine the mechanisms leading to such different functional outcomes, we compared the proximal TCR signal transduction of naive cells, to that of memory or high-dose tolerant cells generated *in vivo*. *In vivo* activation led to the constitutive phosphorylation of CD3ε, recruiting Zap70, in both memory and tolerant cells. In tolerant cells, these phenomena were much more marked, the CD3ε and ζ chains no longer associated, and the Src kinases p56Lck and p59Fyn were inactive. Therefore, when the antigen load overcomes the capacities of immune control, a new mechanism intervenes to block signal transduction: the recruitment of Zap70 to CD3ε becomes excessive, leading to TCR complex destabilization, Src kinase dysfunction, and signal arrest.

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1 Introduction

A main concern in immunology is the elucidation of the mechanisms leading to adaptive tolerance or immunity. Key to this challenge is the molecular characterization of antigen receptor-mediated signal transduction that controls lymphocytes responsiveness, in both conditions of tolerance and immunity. The T cell receptor (TCR) is composed of the antigen binding αβ dimer and the signal transducing CD3 chains, assembled into γε/δε heterodimers and ζζ homodimers. These chains contain one (δ, ε, γ) to three (ζ) immunoreceptor tyrosine-based activation motifs (ITAM) that support the transmission of the activation signal into the cell. Following efficient TCR stimulation, the ITAM are doubly tyrosine-phosphorylated by the Src family kinases p56Lck and p59Fyn, resulting in the appearance of the fully phosphorylated pp23 form of the ζ chain. Fully phosphorylated ITAM recruit the Zap70 kinase, which is in turn phosphorylated and activated, thereby initiating the phosphorylation cascade as well as intracellular calcium fluxes, which ultimately leads to gene transactivation [1].

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Abbreviations: ITAM: Immunoreceptor tyrosine-based activation motif **APL:** Altered peptide ligand **Tg:** Transgenic **IP:** Immunoprecipitate **WB:** Western blot **IVK:** *In vitro* kinase assay

These proximal steps of TCR-mediated signaling were expected to be defective in tolerant T cells, since severe impairment of calcium movements characterized both *in vitro* and *in vivo* models of T cell unresponsiveness [2, 3]. The models of T cell tolerance induction *in vitro*, either by altered peptide ligands (APL) or by the absence of costimulation, or *in vivo* after superantigen stimulation, converged to the current accepted view that preferential expression of partially phosphorylated pp21 ζ chains of the TCR and the subsequent defective activation of Zap70 were responsible for T cell anergy [4–7]. This notion was contradicted by a recent report. Defective Zap70 and ζ chain phosphorylation could also be detected in functionally reactive T cells [8]. Since defective Zap70 and ζ chain phosphorylation cannot be directly correlated to the tolerant state, it is possible that tolerance implies co-existing unidentified defects in proximal TCR signaling.

Depending on the conditions of priming, antigen stimulation can either lead to tolerance or to the generation of memory. The modifications of signal transduction associated to improved responses of memory T cells to antigen stimulation yet also remain poorly understood. Comparison of proximal TCR signaling in naive and memory CD4+ T cells showed defective phosphorylation of Zap70 in memory lymphocytes [9]. Despite this, CD4+ memory cells efficiently activated the mitogen-activated protein kinases downstream of Zap70, suggesting that memory cells developed unknown mechanisms to circumvent the

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classical pathways of TCR signaling [10]. The proximal signaling of $CD8^+$ memory T cells is also likely to be modified, as antigen stimulation generated reduced amplitude of calcium peaks and the predominant generation of calcium oscillations when compared to naive cells [3]. However, the modifications of proximal signaling responsible for this behavior of memory $CD8^+$ T cells have not been studied.

We have previously described an experimental system in which the same clone of naive $CD8^+$ T cells could be stimulated by antigen *in vivo*, and divide extensively, eventually acquiring very different functional properties, those of memory or tolerant cells [3]. To generate memory and tolerant cells, naive $CD8^+$ T cells [obtained from female $Rag2^{-/-}$ mice bearing a transgenic (Tg) $\alpha\beta$-TCR specific for the male antigen] were stimulated *in vivo* by different doses of male antigen-presenting cells (APC), in the presence of CD4 T cell help, and recovered 2 months after immunization. At a low APC dose, Tg cells eliminate male APC and become very efficient memory cells. At a high APC dose, Tg cells become tolerant, *i.e.* unable to eliminate male APC, and unable to proliferate and secrete IL-2 [3]. This system is advantageous to study signal transduction, since it concerns T cells responding *in vivo* to their cognate antigen, processed by normal APC. Moreover, it allows the direct comparison of antigen-challenged T cells with the same TCR specificity in both conditions of tolerance and immunity. Finally, it involves memory and high-dose tolerant $CD8^+$ T cells, where signal transduction has not been characterized. In the present work, we show that the two regimens of *in vivo* antigen stimulation lead to different proximal modifications of TCR signaling that may explain the different functional behavior of $CD8^+$ memory and tolerant T cells.

2 Results and discussion

2.1 ζ chains expression and phosphorylation in naive, memory and tolerant $CD8^+$ T cells

We first studied ζ chains expression and phosphorylation. Antigen-experienced memory and tolerant cells contained many fewer ζ chains than naive cells (Fig. 1A). In both naive and memory cells, ζ chains immunoprecipitates (IP) contained phosphorylated pp21 ζ chains before stimulation, and fully phosphorylated pp23 forms were induced after activation (Fig. 1B). In contrast, ζ chain phosphorylation was undetectable in either resting or activated tolerant cells (Fig. 1B). Therefore, the defect of ζ chain phosphorylation we detected in these tolerant cells was even more profound than that of *in vitro* anergized cells, yet capable of partial ζ phosphorylation [4–6]. Moreover, the differential efficiency of ζ chain phosphorylation in tolerant versus memory cells could not be explained by a difference in ζ expression level, since both populations had equivalent amounts of ζ protein.

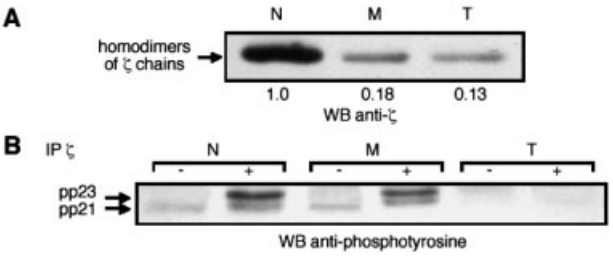

Fig. 1. ζ chain expression and phosphorylation in naive (N), memory (M), and tolerant (T) $CD8^+$ Tg T cells. (A) ζ chain quantitation in non-reduced lysates by anti-ζ WB. Cell stimulation did not induce modifications of ζ amounts in lysates. (B) ζ immunoprecipitates (IP) of resting (–) and activated (+) cells, blotted with anti-phosphotyrosine mAb. Results are representative of four experiments.

2.2 Zap70 expression, phosphorylation and association in naive, memory and tolerant $CD8^+$ T cells

We next studied Zap70 expression, phosphorylation and association, before and after TCR stimulation. We found that whole lysates of all $CD8^+$ populations contained similar amounts of Zap70 (data not shown). In Zap70 IP, resting naive cells did not contain phosphorylated Zap70. In contrast, phosphorylated Zap70 was constitutively present in resting memory cells, and even in higher amounts in resting tolerant cells (Fig. 2A). These results were surprising, as impairment of ζ chains phosphorylation is generally accompanied by a defect in Zap70 phosphorylation [4–7]. Yet, we found that tolerant T cells, that lacked ζ chains phosphorylation, contained the highest level of constitutive phosphorylation of Zap70.

Zap70 phosphorylation and association to ζ after TCR stimulation also differed between memory and tolerant $CD8^+$ populations. In both stimulated naive and memory T cells, phosphorylation of Zap70 augmented to the same high level (Fig. 2A) and Zap70 associated with fully phosphorylated ζ chains (Fig. 2B). In this respect, memory $CD8^+$ T cells differed from the $CD4^+$ memory population, unable to phosphorylate Zap70 after *in vitro* restimulation [9]. Tolerant $CD8^+$ T cells behaved differently. After activation, their medium level of constitutive phosphorylation of Zap70 did not change (Fig. 2A) and phosphorylated Zap70 did not associate to the ζ chains (Fig. 2B).

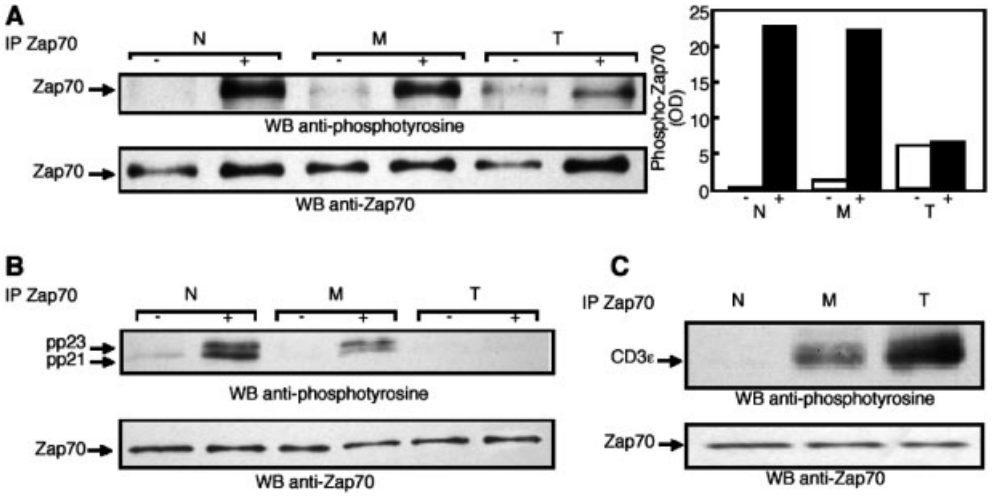

Fig. 2. Zap70 phosphorylation and association in resting and activated naive (N), memory (M), and tolerant (T) CD8+ Tg T cells. IP Zap70 of resting (–) and activated (+) cells, blotted with anti-phosphotyrosine mAb (up). Stripped blots, reprobed with anti-Zap70 mAb (down). (A) Left: Zap70 phosphorylation. Right: Zap70 phosphorylation normalized to Zap70 amounts (optical densities, OD). (B, C) Anti-phosphotyrosine WB of co-precipitated: (B) ζ chains, (C) CD3ε chains. Results are representative of four experiments.

To explore the mechanisms involved in the constitutive phosphorylation of Zap70 in memory and tolerant cells, we studied if Zap70 could be associated to CD3ε. Indeed, Zap70 phosphorylation requires its binding to phosphorylated ITAM and phospho-CD3ε was also shown to recruit Zap70 [11]. The amount of CD3ε found in total cell lysates was similar in naive, memory and tolerant cells (see below). While in naive cells, IP of Zap70 displayed no phospho-CD3ε co-precipitation, phosphorylated CD3ε was found to co-precipitate with Zap70 in memory cells, and to a much higher extent in tolerant cells (Fig. 2C). Thus, antigen-experienced cells, independently of their functional properties, displayed constitutive phosphorylation of Zap70 (Fig. 2A), bound to constitutively phosphorylated CD3ε (Fig. 2C). However, the association of Zap70 to phosphorylated CD3ε and Zap70 phosphorylation was much higher in resting tolerant, than in resting memory, T cells. Moreover, after activation, tolerant cells could not phosphorylate ζ chains and further enhance Zap70 recruitment and subsequent phosphorylation, unlike functionally competent memory cells.

2.3 Src kinase function is severely impaired and the TCR complex is destabilized in tolerant CD8+ T cells

To investigate the mechanisms responsible for the defective phosphorylation of ζ chains in tolerant cells, we analyzed the expression levels and the *in vitro* kinase activity (IVK) of p56Lck and p59Fyn tyrosine kinases, since these proteins are responsible for ITAM phosphorylation [1]. Both memory and tolerant cell lysates had doubled amounts of p56Lck and p59Fyn as compared to naive cells (Fig. 3A), excluding differential expression of Src kinases as a cause of tolerant cells inability to phosphorylate ζ chains. After TCR activation, Src kinases of memory cells could phosphorylate CD3ε and ζ chains (Fig. 3B, left). In sharp contrast, after activation of tolerant cells, p56Lck and p59Fyn activity did not increase (data not shown), and these kinases did not phosphorylate CD3ε or the ζ chains (Fig. 3B, right). These data reveal a major defect in Src kinase activity and association to the CD3 complex in tolerant cells.

T cell hybridomas expressing a mutated TCR-α chain connecting peptide motif, which impairs the association of the ζ chains with the remaining CD3 complex, were shown to lack Src kinase activity [12, 13]. We thus studied if the ζ chains could be dissociated from CD3ε in tolerant cells. The amount of CD3ε found in whole lysates was similar in naive, memory and tolerant cells (Fig. 3C). However, CD3ε associated loosely with the ζ chains in ζ IP of tolerant cells, contrary to naive and memory cells (Fig. 3D). The stability of the CD3 complex is thus severely compromised in tolerant cells.

In this report, we determine the modifications of proximal TCR signaling responsible for high-dose antigen tolerance *in vivo*. Moreover, by comparing signaling in primed (tolerant and memory) and naive cells, we can distinguish the changes induced by T cell activation shared by the antigen-experienced cells, from those resulting in the

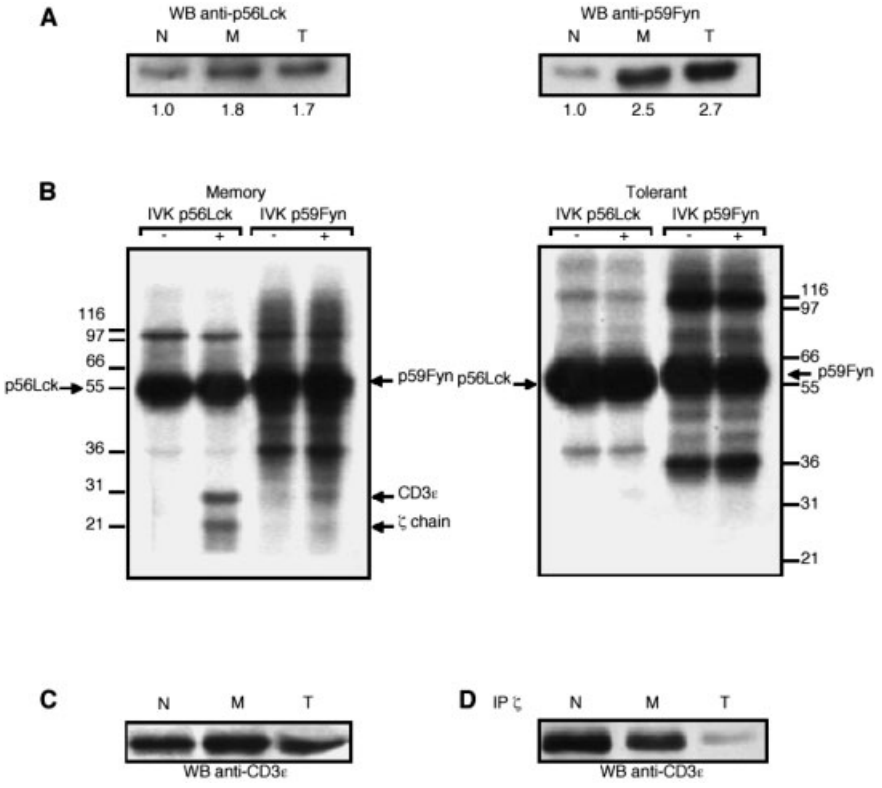

Fig. 3. (A, B) Src kinase expression and activity. Results are representative of three experiments. (A) Quantitation of Src kinase expression in lysates of naive (N), memory (M), and tolerant (T) cells by anti-p56Lck and p59Fyn WB. Samples were equalized for the same amount of protein. (B) *In vitro* Src kinase activity (IVK). p56Lck and p59Fyn IP of resting (–) and activated (+) cells were incubated in the presence of 10 μCi [γ-^{32}P]ATP. Kinase assays were normalized to equal quantities of p56Lck and p59Fyn. The gels of tolerant cells shown are overexposed, to exclude any phosphorylation/association of the CD3 complex. (C) CD3ε expression in lysates by anti-CD3ε WB. (D) Association of CD3ε to the ζ chains, in IPζ followed by anti-CD3ε WB.

arrest of signal transduction exclusively found in tolerant cells.

In this context, several characteristics are common to memory and tolerant CD8$^+$ T cells. Firstly, ζ chain expression was reduced in both cell populations, while CD3ε expression levels were unchanged. These characteristics were repeatedly reported in T cells recovered from tumor infiltrates, and were thought to contribute to the tolerant state [14]. This reduced ζ chain expression correlated with a slight decrease in TCR surface expression on both memory and tolerant CD8$^+$ T cells when compared to naive cells (data not shown), consistent with reports showing that ζ amounts are limiting for TCR surface expression [15]. However, these reduced levels of surface TCR and ζ expression did not impair ζ chain phosphorylation in memory cells. Since memory cells were capable of efficient TCR signaling, our studies correlate low ζ expression to prolonged antigen stimulation *in vivo* and exclude that low ζ expression is sufficient to induce tolerance. Secondly, both primed populations showed similar increased levels of the Src kinases, when compared to naive cells. Finally, memory and tolerant cells showed increased constitutive phosphorylation of Zap70, associated to phosphorylated CD3ε. In this respect, *in vivo* activated cells differ from *in vitro* tolerized cells, where CD3ε and Zap70 are not associated nor phosphorylated [4–6].

What are the alterations specific for CD8$^+$ tolerance induction by high antigen doses? We identified a major defect in Src kinase function. Both p56Lck and p59Fyn kinase activity was not modified after activation neither these kinases phosphorylated the CD3ε and ζ chains. This explains the lack of ζ chain phosphorylation, mainly attributed to p56Lck [16]. Since p56Lck was recently shown to be responsible for the tyrosine phosphorylation of the activating site of Zap70, impairment of p56Lck activity may also contribute to the inhibition of the up-regulation of Zap70 phosphorylation and likely catalytic activity [17, 18]. p59Fyn dysfunction in tolerant cells, on the other hand, justifies their inability to mobilize cal-

cium, since calcium fluxes are primarily regulated by p59Fyn [19]. Finally, we found a major dissociation of the ζ chains from CD3ε, suggesting a major destabilization of the signal transducing CD3 complex. This modification may explain the alterations leading to defective ζ chains phosphorylation, since the stable coupling of ζ chains with CD3ε is thought necessary for Src kinase activity [13]. In T cells from TCR-Tg mice expressing a defective TCR-α chain connecting peptide motif, the ζ chains are dissociated from the TCR complex and T cells do not respond to antigen stimulation [20], indicating that stability of the entire antigen receptor is required to control lymphocyte responsiveness. Finally, it was previously shown that *in vitro* T cell stimulation was followed by the dissociation of individual components of the TCR, may be explaining T cell refractoriness to additional TCR stimulation; it was also hypothesized that such mechanism could occur in tolerant states [21]. Our results indicate that such dissociation occurs when naive T cells are stimulated *in vivo* by high antigen doses, and thus, together with the subsequent defective function of Src kinases, may be major mechanisms of *in vivo* tolerance induction. In this way, T cell inactivation would be ensured by a double lock.

How are the specific characteristics of high-dose tolerance induced during *in vivo* stimulation? We found quantitative differences between memory and tolerant cells. Zap70 association to CD3ε and the constitutive phosphorylation of Zap70 were much higher in tolerant cells, when compared to memory cells. These results suggest that below a certain level, constitutive Zap70 phosphorylation and CD3ε association may rather facilitate signal transduction, as found in memory cells. However, during a chronic/intense stimulation, an excessive recruitment of Zap70 to CD3ε could result in the uncoupling of the ζ chains from CD3ε and subsequent extinction of Src kinase activity. In this context, changes in signal transduction during cell activation may be progressive. They may facilitate signal transduction at lower levels, while they can become deleterious when expressed at higher levels as in the case of tolerant cells.

How the dissociation of TCR components occurs, and how such uncoupling leads to signal transduction arrest, remain speculative at this moment. The forced membrane expression of a myristylated Zap70 mimicked prolonged TCR stimulation, leading to T cell anergy [18]. In our CD8+ tolerant cells, it is likely that the constitutively phosphorylated CD3ε is localized at the cell membrane [22]. Binding of Zap70 to CD3ε in these circumstances could also lead to forced membrane localization of Zap70, and consequent arrest of signal transduction.

To summarize, we find that T cells rendered tolerant by exposure to high doses of antigen *in vivo* develop so far undescribed mechanisms to block proximal TCR signaling. These results have particular relevance in the understanding of how CD8+ T cells become dysfunctional when confronted by an antigen load they cannot control, as in some infections and cancer growth [23, 24].

3 Materials and methods

3.1 Mice

Mice were C57BL/6: Ly5.2; female Rag2$^{-/-}$ expressing an MHC class I Db-restricted Tg αβ–TCR specific for the male antigen HY; Rag2$^{-/-}$ female; Ly5.1 CD3ε$^{-/-}$ male.

3.2. Naive, memory and tolerant cells

Naive CD8+ Tg cells were recovered from lymph nodes (LN) of female Rag2$^{-/-}$ mice bearing a Tg αβ-TCR specific for HY (98% purity). To immunize naive cells *in vivo*, 5×10^5 LN naive CD8+ Tg cells and the same number of purified LN CD4+ T cells (from C57BL/6 females) were injected into lethally irradiated Ly5.2 Rag2$^{-/-}$ female mice reconstituted with male bone marrow cells from Ly5.1 CD3ε$^{-/-}$ mice. The Ly5.1 allotype allowed us to track male cells elimination [3]. To generate memory cells, 5×10^5 CD3ε$^{-/-}$ male bone marrow cells were injected. To generate tolerant cells, the number of male cells injected was tenfold higher. Primed Tg cells were recovered 2 months later, from pooled LN and spleens. Memory and tolerant CD8+ Tg T cells were purified by negative selection, using monoclonal antibodies (mAb) against B cells, macrophages, CD4+ T cells and granulocytes (PharMingen), and coated Dynabeads (Dynal) (purity >95%).

3.3 Cell stimulation, lysis, immunoprecipitation, and *in vitro* kinase assays

Naive, memory and tolerant cells were activated with or without anti-CD3ε mAb (145–2C11, PharMingen) at 37°C for 4 min, lysed in 1% NP-40 lysis buffer containing inhibitors of phosphatases and proteases [17]. The Ab for immunoprecipitations were: anti-ζ (6B10.2, Santa Cruz Biotechnology), anti-Zap70 (raised against the amino acids Pro266 to Gly344 of the mouse sequence, gift of G. Criado), anti-p56Lck (3A5, Santa Cruz Biotechnology), and anti-p59Fyn (FYN3, Santa Cruz Biotechnology). Cell lysates and IP were reduced (unless specified), resolved by 7–12.5% SDS-PAGE and blotted onto nitrocellulose (Amersham Pharmacia Biotech) [17]. The Ab for Western blots (WB) were: anti-phosphotyrosine (4G10, Upstate Biotechnology); anti-ζ (6B10.2, Santa Cruz Biotechnology); anti-Zap70 (29, Transduction Laboratories); anti-CD3ε (M-20, Santa Cruz Biotechnology); anti-p56Lck (3A5, Santa Cruz Biotechnology); and anti-p59Fyn (FYN3, Santa Cruz Biotechnology). They were revealed using horseradish peroxidase (HRP)-conjugated Ab, detected with an ECL system (Amersham

Pharmacia Biotech) and quantified using a Bio-Rad camera and the Quantity one 4.0.2 software (Bio-Rad). When needed, blots were stripped (Pierce). Numbers of purified T cells were 2×10^5 for WB on cell lysates, and 2×10^6 for IP. Cell lysates were equalized for protein amounts and/or cell numbers.

Kinase activity was examined *in vitro* using p56Lck and p59 Fyn IP of resting and stimulated naive, memory and tolerant cells in the presence of 10 μCi [γ-^{32}P]ATP, resolved by 10% SDS-PAGE as previously described [17]. Kinase assays were normalized to equal quantities of p56Lck and p59Fyn. The determination of Src kinase activity requires the purification of a high number of T cells (10^7 cells/sample). Since these studies were performed with *ex vivo* isolated cells, many mice had to be pooled to obtain enough T cells for kinase assays. Due to this limitation, memory and tolerant cells could not be studied on the same day.

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