nature immunology

CD4 enhances T cell sensitivity to antigen by coordinating Lck accumulation at the immunological synapse

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How T cells respond with extraordinary sensitivity to minute amounts of agonist peptide and major histocompatibility complex (pMHC) molecules on the surface of antigen-presenting cells bearing large numbers of endogenous pMHC molecules is not understood. Here we present evidence that CD4 affects the responsiveness of T helper cells by controlling spatial localization of the tyrosine kinase Lck in the synapse. This finding, as well as further *in silico* and *in vitro* experiments, led us to develop a molecular model in which endogenous and agonist pMHC molecules act cooperatively to amplify T cell receptor signaling. At the same time, activation due to endogenous pMHC molecules alone is inhibited. A key feature is that the binding of agonist pMHC molecules to the T cell receptor results in CD4-mediated spatial localization of Lck, which in turn enables endogenous pMHC molecules to trigger many T cell receptors. We also discuss broader implications for T cell biology, including thymic selection, diversity of the repertoire of self pMHC molecules and serial triggering.

In the context of an immune response, antigen-presenting cells (APCs) typically display both exogenous antigen–derived peptide–major histocompatibility complex (pMHC) molecules and a much larger number of endogenous pMHC molecules[1]. T cells are selected such that their T cell receptors (TCRs) bind endogenous pMHC molecules much more weakly than antigen-derived pMHC molecules (agonists)[2]. *In vitro*, even one agonist pMHC molecule can produce a signal to stop rolling and a transient increase in cytosolic calcium, and as few as ten agonist pMHC molecules can result in sustained calcium flux and the formation of a mature immunological synapse[3]. In the case of cytotoxic T cells, only three ligands are necessary for killing[4]. How this extraordinary sensitivity to antigenic pMHC molecules in a 'sea' of endogenous ones is achieved is unknown.

Many investigators have suggested that endogenous pMHC ligands are critical for the sensitivity of T cells for foreign antigen[2,3,5,6]. This possibility is supported by the fact that αβ T cells are required to interact weakly with at least some self pMHC ligands in the thymus to progress to full maturity[7], and again in the periphery some interaction seems to be required for survival[6,8]. In addition, along with agonist ligands, large quantities of endogenous pMHC molecules (about 20% of those at the interface) accumulate in the immunological synapse in a TCR-dependent way[2,3]. Thus, weak interactions are likely to occur continuously between TCRs that emerge from the thymus and some self pMHC molecules. Several models have been suggested as to how this type of interaction could augment T cell sensitivity. One of these is the 'noise' model, in which weak and numerous TCR–self pMHC interactions partially activate T cells[5,6] but stop just short of initiating synapse formation and activation. It has also been suggested that TCR–self pMHC interactions could influence the organization of TCRs on T cells in a way that maximizes their sensitivity[6]. Others have suggested that agonist pMHC and self pMHC molecules can form heterodimers that can induce dimerization (and hence, activation) of TCRs[3,5]. It was further postulated that CD4 is involved in crosslinking of pMHC heterodimers and TCRs (the 'pseudodimer' model[3]).

Here, we first address an apparent anomaly in a previous report in which blocking of CD4 was found to cause an increase in the slope of the graph of T cell responsiveness as a factor of the number of agonist peptides, albeit at a much greater number of peptides[3]. After blocking of CD4, the abrupt change in the T cell response above a threshold number of agonist pMHC molecules

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Published online 11 July 2004; doi:10.1038/ni1095

resembles a switch. The switch-like response in the presence of antibody to CD4 (anti-CD4) is somewhat unexpected, because this coreceptor is considered to facilitate rather than interfere with the T cell response to antigen[4,9–12]. We found that this response correlated with inefficient accumulation of the tyrosine kinase Lck at the synapse, suggesting that this enzyme is a key 'gatekeeper' to subsequent signaling. This finding enabled us to build on the pseudodimer model[3] to develop a molecular picture of how T cells might have such an extreme sensitivity to antigen without normally being triggered by pMHC 'noise', which could lead to autoimmunity. The key feature of this model is that spatial localization of Lck in the vicinity of TCRs that bind to both endogenous and agonist pMHC molecules allows endogenous pMHC molecules to trigger many TCRs. Thus, the response to a few agonists could be amplified by a cooperative mechanism that involves TCR triggering by the large number of endogenous pMHC molecules. We present here the results of computer simulations and biochemical experiments that support this model.

RESULTS

Anti-CD4 affects signaling by inefficient Lck recruitment

After binding of CD4 to MHC is blocked, the number of agonist pMHC complexes required for sustained calcium flux increases[3]. However, as noted earlier, the use of anti-CD4 results in a switch-like (all-or-nothing) response to agonist, whereas normal T cells show a continuous dose-response curve that is hyperbolic in shape. The information contained in this change in shape effected by anti-CD4 is best understood in terms of a simple phenomenological model of the biochemical reactions involved in signal transduction. The enzymatic reactions that result in calcium flux constitute a cascade of protein modifications (**Supplementary Fig. 1** online). Each individual protein modification involves a forward step and a backward step (typically a phosphatase-mediated dephosphorylation). A modified protein then serves to effect downstream protein modifications. Such pathways in which enzymatic protein modification schemes are concatenated have been analyzed mathematically[13,14]. Those analyses demonstrated that the activity of pathways in which enzymatic protein-modification schemes are concatenated is qualitatively the same as that of a single protein-modification cycle that involves a forward activation step and a backward deactivation step. Specifically, we have considered the pertinent protein modification scheme to be Lck-mediated phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs; **Supplementary Fig. 1** online).

If Lck and intracellular phosphatases act on their substrates according to standard Michaelis-Menten kinetics, the fraction (f) of the substrate (ITAMs) that is triggered obeys the following mean-field equation[13–15]:

$$\frac{df}{dt} = \frac{\alpha(1-f)}{K_M + (1-f)} - \frac{f}{K_M + f} \qquad (1)$$

where α is the ratio of maximum rates for the two enzymes ($k_{Lck}E_{Lck}/k_PE_P$); k_{Lck} and E_{Lck} are the rate constants for phosphorylation and Lck concentration, respectively; and k_P and E_P are the rate constants for dephosphorylation and phosphatase concentration, respectively. Thus, α is proportional to the concentration of Lck recruited to the TCR complex. K_M is the standard Michaelis-Menten constant divided by the concentration of the substrate and, for simplicity, because it does not affect the qualitative characteristics[15], its value is considered to be the same for both enzymes here.

Previously the calcium concentration was integrated over time (up to 10 min) to show how it varies with the number of agonist pMHC complexes[3]. In our scheme, this was equivalent to considering the integral over time of the quantity f in equation (1) as a function of the amount of effector molecules (agonist pMHC complexes). This integral was defined as P here, as follows:

$$P = \int_0^T f(t)dt \qquad (2)$$

where T = 10 min in the experiments (that is, T is large). Equations (1) and (2) allowed us to compute how P depends on α (proportional to the concentration of recruited Lck) but not how it depends on the number of agonists. Binding of agonist pMHC complexes to TCRs recruits Lck. Thus, it seems reasonable to assume (demonstrated by experiments below) that recruited Lck is related to the agonist pMHC amount by the following Langmuir-like relation (**Fig. 1a**):

$$\alpha = \frac{b[\text{pMHC}]}{a + [\text{pMHC}]} \qquad (3)$$

where [pMHC] is agonist pMHC concentration. The constants b and a reflect the shape of the curve relating recruited Lck to agonist pMHC concentration. Thus, the values of a and b represent the effectiveness with which Lck is recruited by the agonist pMHC complexes. Equations (1)–(3) allow computation of the analogs of the dose-response curves measured before[3]; that is, P as a function of the agonist pMHC amount.

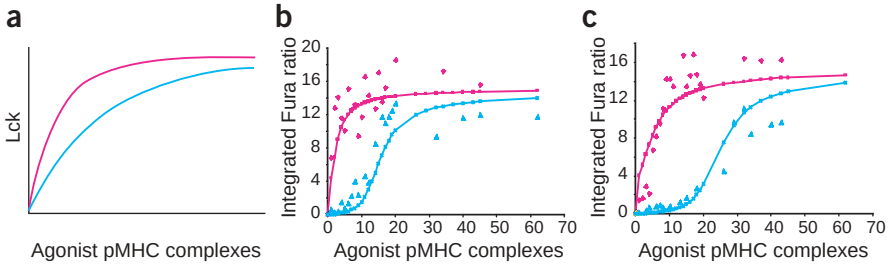

Figure 1 Blocking of CD4 affects T cell sensitivity by impairing Lck recruitment. (**a**) Postulated change in Lck recruitment efficiency (vertical axis) as a function of the number of agonist pMHC complexes (horizontal axis). (**b**,**c**) Fits of experimental data for integrated calcium flux as a function of the number of agonist pMHC complexes. Data for two different agonist pMHC complexes are fit with the 'master equation' analogs of equations (1)–(3). Data are for the MCC (88–103) peptide (**b**) and for the K5 peptide (**c**), which binds more strongly to TCRs. The presence (blue curves and points) or absence (pink curves and points) of anti-CD4 is modeled only as a change in shape of the Lck recruitment efficiency as shown in **a**. This corresponds to adding a term to equation (3) that is identical in form with values of a and b equal to 0.01 and 1, respectively. In the presence of anti-CD4, the data for K5 are best fit by a = 25 and b = 4, and the data for MCC (88–103) are best fit by a = 60 and b = 4.

Biochemical protein-modification schemes such as that described above show a switch-like response for the fraction of substrate converted (f) as a function of enzyme concentration (α) when the parameter K_M is small (that is, the reaction is limited by availability of the enzyme)[14]. This phenomenon is called 'zero order ultrasensitivity'. We solved the stochastic 'master equations'[15,16] corresponding to equations (1–3) to account for number fluctuations (equations (S1 and S2), **Supplementary Note** online) and found that when K_M was small, a switch-like response was obtained for the dose-response curve measured previously[3] over a wide range of values of a and b. Given the data obtained in the presence of anti-CD4 (ref. 3), this indicates that Lck-mediated ITAM phosphorylation initiated by binding of TCRs to pMHC complexes operates with zero-order ultrasensitivity. Thus, biochemical modification of ITAMs is limited by recruitment of Lck to the TCR complex.

This characteristic of the Lck-catalyzed ITAM phosphorylation reaction does not change when CD4 is not blocked. However, Lck should be recruited to the TCR complex far more efficiently when CD4 can bind to the MHC, because Lck is associated with the cytoplasmic tail of CD4 (refs. 11,17). This suggests that binding of CD4 to the MHC changes the relationship between available Lck and the number of agonist pMHC complexes to reflect that Lck is recruited more efficiently, particularly with small numbers of agonist pMHC complexes (**Fig. 1a**). Incorporation of such a change in the 'master equation' analogs of equations (1)–(3) resulted in dose-response curves that mirrored the effects of CD4 blocking *in vitro*, and we obtained excellent fits for data from two different experimental systems (**Fig. 1b**,**c**). For both peptides studied (the agonist peptide moth cytochrome *c*, amino acids 88–103 (MCC (88–103)) and the superagonist peptide K5), simply a change in the relationship describing Lck recruitment efficiency captured the observed effect of blocking CD4. This simple picture is accurate only if, after CD4 was blocked, the relationship between available Lck and the number of agonist pMHC complexes changed in the manner postulated in **Figure 1a**. We therefore did experiments to test this hypothesis emerging from the mathematical model.

Visualizing, quantifying Lck recruitment

Lck is not recruited efficiently in CD4-deficient T cells[18]. However, no direct evidence exists to show that Lck recruitment efficiency changes after binding of CD4 to MHC is blocked (**Fig. 1a**). Thus, we used time-lapse three-dimensional microscopy to image Lck recruitment as a function of the number of agonist pMHC complexes in the presence and absence of anti-CD4. We transferred a fusion protein of Lck and green fluorescent protein (Lck-GFP) into 5C.C7 T cell blasts through retroviral infection as described[19] and pulsed CH27 B cells to present K5 (ref. 20). Before imaging, we loaded Lck-GFP T cells with the calcium indicator Fura-2 to measure T cell activation and stained CH27 cells with streptavidin-phycoerythrin to label the surface K5 peptide[3]. The 5C.C7 T cells rapidly recognized K5 peptides presented in the I-E^k context and were activated, as shown by the morphological changes and the increase in cytosolic calcium concentration (**Fig. 2a**). We acquired a three-dimensional stack 20 μm in thickness in the phycoerythrin channel to count the peptides in the T cell–B cell interface and followed this by recording in the GFP channel to visualize Lck dynamics. Only two K5 peptides in the T cell–B cell interface were required to markedly drive Lck accumulation into the central supramolecular

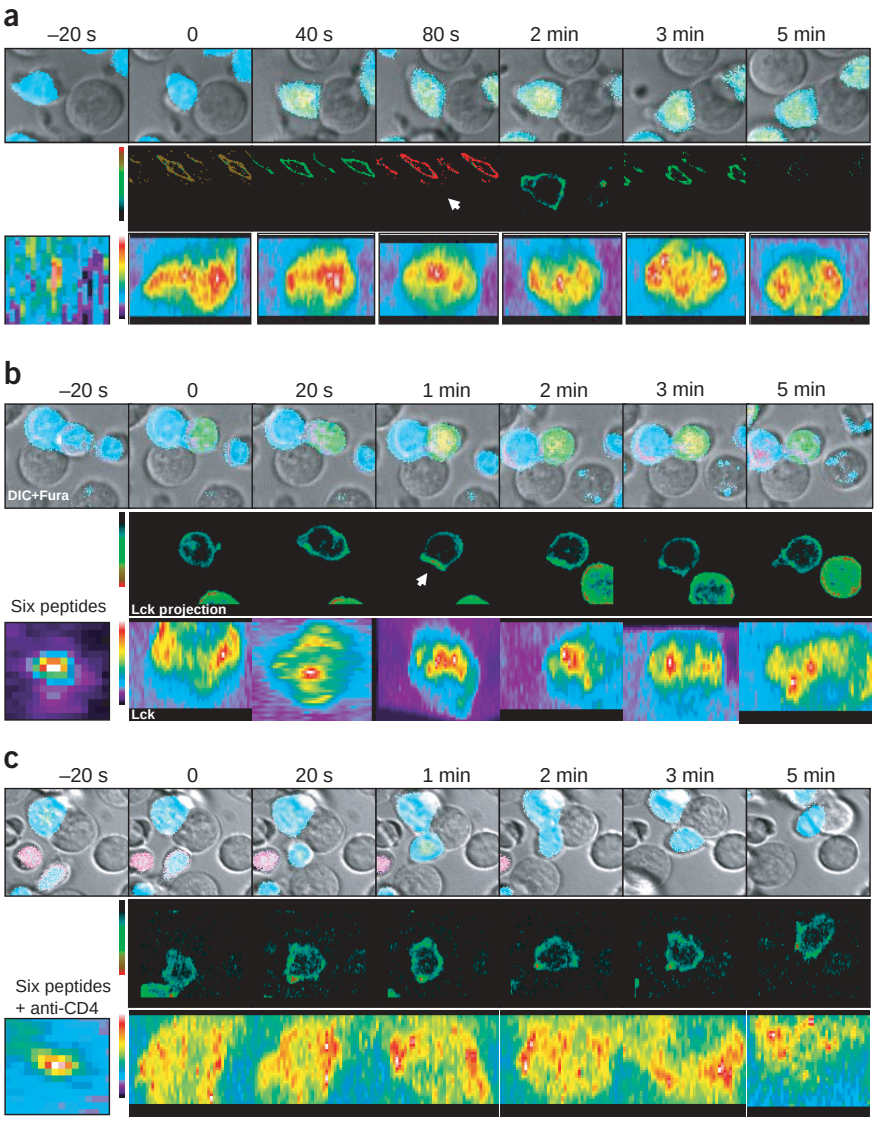

Figure 2 Effects of blocking CD4 on Lck recruitment to the synapse. Lck-GFP images were recorded at 20-second intervals for 15 min. Top rows are overlaid images that combine DIC and Fura-2 (calcium) channels; middle rows are views showing projections of the GFP (Lck) channel after three-dimensional reconstruction; bottom rows show the Lck distribution in the immune synapse viewed *en face*. Bottom left, peptide images; the peptide number was determined by measurement of the integrated intensity after three-dimensional reconstruction and comparison to the standard curve of phycoerythrin fluorescence. 0 in the time labels (above images) represents the first frame of T cell–B cell conjugate formation (**Supplementary Videos** 1–3 provide complete time courses of **a**–**c**, respectively).

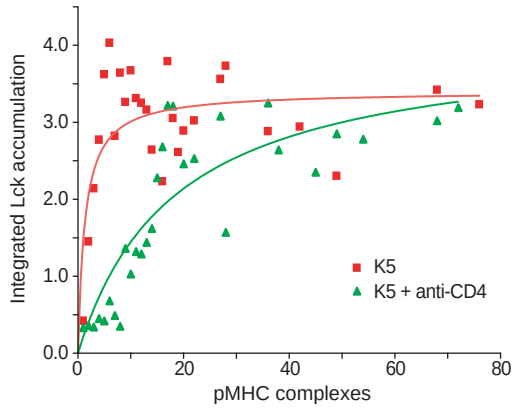

Figure 3 Lck recruitment efficiency in the absence or presence of anti-CD4 blocking. Lck accumulation ratios were determined by normalization of GFP intensity in the T cell–B cell interface to the cytosolic background. The integrated accumulation ratio was generated by combination of values acquired in the first 5 min after conjugate formation. Each data point represents an average from at least three cells. Normally these cells were from different batches that were prepared using the same procedure.

activation cluster. The peak in Lck recruitment appeared shortly after the peak in calcium mobilization. Within 3–5 min, Lck molecules were excluded from the central supramolecular activation cluster (**Fig. 2a** and **Supplementary Video 1** online). These data are consistent with previous results in which 5C.C7 T cells were stimulated by MCC[19]. Lck accumulation was slightly higher and more accelerated for K5 than for MCC (88–103) when the number of peptides was small (**Fig. 2b** and **Supplementary Video 2** online). When T cells were preincubated with a monoclonal antibody blocking CD4 function, the Lck accumulation rate was retarded and the peak ratio was greatly reduced (**Fig. 2c** and **Supplementary Video 3** online). Our data on Lck recruitment in the presence and absence of anti-CD4 (**Fig. 3**) demonstrate that the change in shape of the relationship between recruited Lck and number of agonist pMHC complexes after blocking CD4 mirrored that postulated (**Fig. 1a**).

Thus, blocking of the binding of CD4 to MHC results in impaired Lck recruitment and this in turn leads to the observed changes in sensitivity to antigen and the shape of the dose-response curve. It also provides important clues for the development of a molecular model to address the broader question of how T cells discriminate sensitively between antigen-derived and endogenous pMHC complexes.

In silico studies of a molecular model

Several experimental observations, including the fact that endogenous pMHC complexes are recruited to the immunological synapse, have prompted the hypothesis that endogenous pMHC complexes are involved in T cell activation[2,3,5,6,21]. One way that they could do so is that thymic selection could finely tune the T cell repertoire such that interactions with endogenous pMHC complexes results in a signal strength that is just below that required for activation. Then, addition of agonist pMHC complexes could push the system over this threshold. Given the very small number of agonists required for activation[3,4], this scenario would be dangerous. If the endogenous pMHC repertoire is maintained very close to the activation threshold, stochastic noise would often result in spurious T cell activation without need for agonists and thus autoimmune responses (as demonstrated below with computer simulations of this model). To prevent this, thymic selection should select a T cell repertoire with only very weak affinities for endogenous pMHC complexes[2], thereby limiting signals due to interactions with endogenous pMHC complexes to levels sufficiently below the activation threshold.

Thus, signaling due to a few agonists must be amplified by a cooperative mechanism. The response to a few agonists that can contribute to productive signaling could be enhanced greatly if an agonist bound to a TCR could help the large pool of endogenous pMHC complexes to trigger additional TCRs. Combining our findings regarding the importance of spatial localization of Lck in determining T cell sensitivity with this idea led us to study the molecular model discussed below (**Fig. 4**).

TCR–agonist pMHC binding leads to complexes that are sufficiently long lived to mediate the recruitment and binding of CD4 to the MHC (**Fig. 4a**). This results in the recruitment of Lck associated with the cytoplasmic tail of CD4 (refs. 11,17; **Fig. 4b**). Many studies suggest that CD4 could bring two pMHC complexes together[3,12,22–28], and if pMHC complexes exist as monomers on APC surfaces, we assume that CD4 can bind more than one MHC. This could be mediated either by CD4 working as a bridge that links an additional pMHC complex to a TCR–agonist pMHC complex[3] or by CD4 dimers[12,22–26]. If CD4 bound to an MHC associated with an agonist peptide recruits another MHC, the second MHC molecule is statistically most likely to be associated with an endogenous peptide. This is because an overwhelming majority of pMHC complexes on the APC surface are endogenous. Thus, the agonist and endogenous pMHC complexes, TCR, CD4 and Lck are spatially localized in a signaling complex (**Fig. 4c**). The pertinent structural data are reviewed below, but the results reported in this section do not depend on the geometric details of the signaling complex (**Fig. 4c**), which is the key to signal amplification.

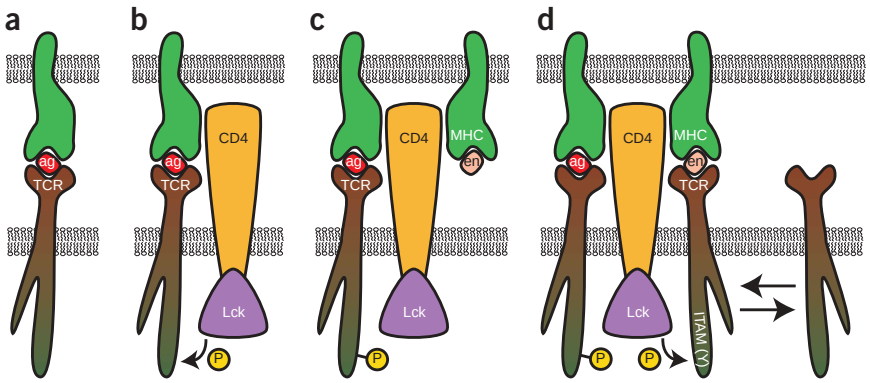

Figure 4 A sequence of molecular events that can amplify the T cell response to a few agonist pMHC complexes. (**a**) The TCR binds to an agonist (ag) pMHC complex. (**b**) CD4 (and thus Lck) is recruited and binds to the MHC. (**c**) CD4 binds to another MHC that is statistically most likely to be an endogenous pMHC complex. This spatially localizes agonist pMHC complexes, endogenous (en) pMHC complexes, CD4 and Lck. (**d**) The TCR binds to an endogenous pMHC complex and can be triggered despite the small half-life of this interaction, because Lck is 'ready and waiting'. P (in circle), phosphorylated. ITAM (Y), phosphorylation at the tyrosine of ITAM.

When a TCR binds to the endogenous pMHC complex associated with this complex, Lck is already spatially localized in the vicinity of this TCR. Thus, even though the TCR–endogenous pMHC bond is not long lived, its ITAMs can be phosphorylated by Lck (**Fig. 4d**). Thus, the large 'off rate' characterizing TCR–endogenous pMHC binding, which normally precludes efficient ITAM phosphorylation, is compensated for by the fact that Lck is 'ready and waiting' in the complex formed by binding of the TCR to an agonist pMHC complex. Thus, the presence of a few agonist pMHC complexes allows the endogenous pMHC complexes to trigger many TCRs.

We have studied the molecular mechanism proposed above using synergistic *in silico* and *in vitro* experiments. Membrane-proximal intermolecular binding and activation events are modeled in detail in the *in silico* experiments. In a recent study, downstream signaling, including the Rac, Ras and PLCγ pathways that ultimately lead to upregulation of gene transcription factors, were simulated with Monte-Carlo methods[29]. All downstream signaling requires phosphorylation of ITAMs associated with the CD3 and ζ chains, and here we sought to understand the origin of signal amplification due to a few agonists (recently measured with calcium flux as the 'readout'[3]). Thus, we used the number of triggered TCRs and the resulting calcium flux as the 'readout' of signal strength. Intermolecular complex formation, catalytic phosphorylation and phosphotransfer reactions involving TCRs, endogenous and agonist pMHC complexes, CD4, Lck, and phosphatase molecules are modeled in detail. We used the Gillespie algorithm[30] to solve the stochastic equations that describe the 741 chemical reactions involved (**Supplementary Fig. 2** online and equations S4 and S5, **Supplementary Note** online). The purpose of these computer simulations was not quantitative accuracy. Instead, they were aimed at elucidating qualitative differences between the consequences of the cooperative molecular model described above and one in which agonist and endogenous pMHC complexes act independently.

The agonist peptide used in these simulations was MCC (88–103), which has been show to have an 'off rate' (k_{off}) equal to 0.019 s^{-1} and an 'on rate' (k_{on}) equal to 2200 s^{-1} M^{-1} with TCRs derived from 2B4 cells[31]. We also did simulations with values of k_{off} and k_{on} taken from earlier measurements[32] and obtained qualitative results identical to those in **Figures 5** and **6** (**Supplementary Fig. 3** online). For the cooperative model, in agreement with existing data[3], as few as two agonists produced a transient increase in cytosolic calcium (**Fig. 5a**). However, a larger number (for example, 24) was required for sustained calcium flux (**Fig. 5b**). The minimum number of agonists required for sustained calcium flux depended on the lifetime of the TCR–endogenous pMHC bond: the larger the 'off rate' characterizing the endogenous pMHC complexes, the larger the number of agonists required for sustained calcium flux in our cooperative model (**Fig. 6a**).

As a control, we did computer simulations with a model in which endogenous and agonist pMHC complexes acted completely independently in triggering TCRs. For small values of the 'off rate' characterizing the binding of endogenous pMHC complexes to TCRs, this model also showed high sensitivity to antigen (**Fig. 6b**). However, for the conditions used in these simulations (for example, about 30,000 endogenous pMHC complexes), if the k_{off} value for TCR binding to the endogenous pMHC complexes was less than 110 s^{-1}, even without any agonist pMHC complexes, stochastic noise due to number

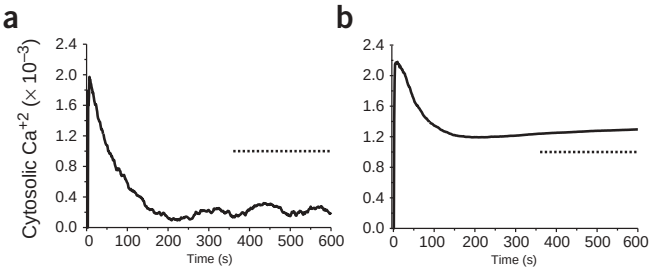

Figure 5 Computer simulations of the cooperative model. Calcium traces (Methods and **Supplementary Note** online) for 2 (**a**) and 24 (**b**) agonist pMHC complexes; the endogenous pMHC k_{off} is 170 s^{-1}. A trial is considered to sustain calcium if its calcium trace remains above the dashed line ($[Ca^{+2}] > 0.001$ for a time between 360 and 600 s).

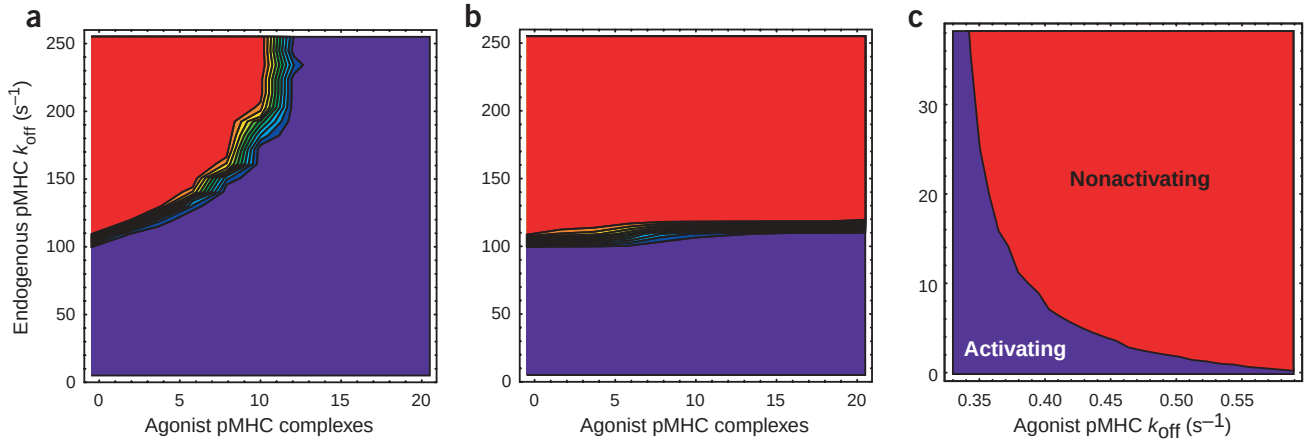

Figure 6 Fraction of trials (*in silico* experiments) with sustained calcium. (**a**) Cooperative model in which two pMHC complexes, CD4 and Lck can be spatially localized (**Fig. 4**). (**b**) Noncooperative model in which endogenous and agonist pMHC complexes act autonomously. The fraction of trials with sustained calcium in these plots ranges from 0 (red) to 1 (violet); the contour spacing is 0.1. The criterion for sustained calcium is given in **Figure 5**; each point was calculated from 20 independent trials. (**c**) Asymptotic analysis (equations (S7)–(S9), **Supplementary Note** online) showing that increasing the avidity of TCR binding to agonist pMHC complexes enables endogenous pMHC complexes with larger 'off-rate' values to participate in signaling. These results are for a specific number of agonist pMHC complexes. Increasing the number of agonists shifts the boundary between activating and nonactivating endogenous pMHC complexes to the right.

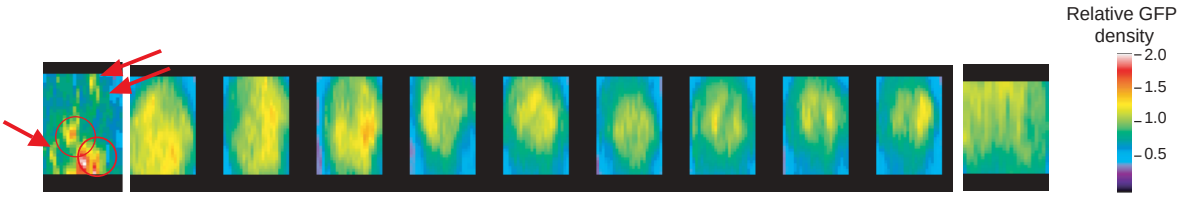

Figure 7 Endogenous pMHC complex recruitment. Biotinylated T102S peptide was labeled with streptavidin-phycoerythrin, mixed with 5C.C7 T cells and imaged. Representative data are shown for 40 T102S pMHC complexes. Left to right: reconstructed false-color fluorescence of labeled pMHC complexes in the T cell–APC interface, as viewed from the T cell 'looking at' the APC; nine time-lapse frames of the reconstructed GFP-YFP fluorescence in the T cell–APC interface (starting immediately after the pMHC complex 'snapshot'); and, for comparison, the GFP-YFP fluorescence on the APC surface reconstructed for one (noninteracting) side of the APC at the initial GFP time point. Relative GFP accumulations are calibrated by the false-color scale bar (far right). Arrows and circles on the pMHC reconstructions indicate peptide locations. Compared with similar images and data obtained for MCC (88–103) previously[3], very few endogenous pMHC complexes are recruited in to the immunological synapse by the T102S peptide.

fluctuations of the endogenous pMHC complexes resulted in sustained calcium flux (autoimmune response) in at least 1% of the *in silico* experiments. Thus, endogenous pMHC complexes with k_{off} values close to 110 s^{-1} are irrelevant, as thymic selection should eliminate T cells expressing such TCRs. For k_{off} values for endogenous pMHC complexes that are larger than 110 s^{-1}, this model, in which endogenous and agonist pMHC complexes act autonomously, was unable to effect sustained calcium flux for small numbers of agonist pMHC complexes (**Fig. 6b**).

In contrast, as in *in vitro* experiments[3], when agonist and endogenous pMHC complexes acted in concert (**Fig. 4** and **Supplementary Figs. 4** and **5** online), endogenous pMHC complexes with large k_{off} values supported sustained calcium flux when T cells were stimulated by small numbers of agonists (**Fig. 6a**). Also, in this model, spurious activation was not triggered in the absence of agonists by endogenous pMHC complexes characterized by large k_{off} values (**Fig. 6a**). This is because these endogenous pMHC complexes, by themselves, bind TCRs for a time that is not sufficiently long for CD4 recruitment and binding to MHC. Thus, signaling complexes (**Fig. 4c**) in which both pMHC molecules are endogenous form very rarely.

As the number of agonist pMHC complexes increased, endogenous pMHC complexes with larger 'off rates' participated in productive signaling (**Fig. 6a**). An asymptotic analysis (equations (S7–S9), **Supplementary Note** online) of the continuum equations that correspond to the model we have simulated showed that this characteristic was mirrored if the 'off rate' for binding of TCRs to the agonist pMHC complexes decreased (**Fig. 6c**). Although the asymptotic analysis is not quantitatively accurate, the mathematical structure of the equations shows that the following qualitative result is robust: agonists with larger k_{off} values recruit fewer endogenous pMHC complexes to participate in productive signaling. This relationship derives from the fact that as the number of agonists or their avidity for TCR increases, the average number of signaling complexes (**Fig. 4c**) increases. There is always a stochastic chance that a TCR bound to an endogenous pMHC complex even fleetingly will undergo ITAM phosphorylation when it is part of such a signaling complex. The probability of this happening decreases with the half-life of the TCR–endogenous pMHC complex. The number of TCRs that can be triggered by endogenous pMHC complexes characterized by a particular half-life is proportional to the product of this probability (p) and the number (N) of signaling complexes (**Fig. 4c**). Thus, as the number (N) of such signaling complexes increases (because of a larger number or avidity to TCRs of agonists), endogenous pMHC complexes that bind more weakly (that is, lower values of p) can trigger more TCRs and contribute to signal amplification.

Endogenous pMHC molecules that can participate in productive signaling are expected to be part of more stable complexes (**Fig. 4**) than those that do not contribute to signaling. Agonists characterized by larger 'off rates' enabled a smaller part of the pool of endogenous pMHC molecules to contribute to productive signaling (**Fig. 6c**). Hence, agonists with larger off-rates should result in a smaller number of endogenous pMHC molecules that can form stable molecules. Because pMHC molecules that form stable complexes should be recruited to the immunological synapse, agonists with larger k_{off} values should be able to recruit fewer endogenous pMHC molecules to the immunological synapse.

Imaging pMHC complexes in the synapse

To test this hypothesis emerging from the *in silico* experiments, we imaged synapse formation with the T102S pMHC complex as the agonist[33]. This pMHC complex stimulates 2B4 T cells much less robustly than does MCC (88–103) (ref. 33). We obtained images of synapse formation and recruitment of endogenous pMHC complexes with phycoethrythrin-streptavidin–labeled T102S versus GFP-labeled I-E^k as described[3]. Consistent with expectations from the *in silico* experiment results, few if any endogenous peptides were recruited to the synapse by T102S (**Fig. 7**) compared with MCC (88–103) (ref. 3). An alternative explanation for this finding is that T102S, which binds TCRs more weakly than does MCC (88–103), stimulates cytoskeletal motion less efficiently. However, this would also result in lower TCR accumulation in the synapse. Because quantitative data on TCR accumulation in the synapse for different pMHC complexes are not available, we cannot unequivocally rule out this alternative explanation for our data (**Fig. 7**).

The key element of the molecular mechanism that we have proposed is that binding of CD4 to agonist pMHC complexes localizes Lck in the vicinity of the TCR binding to the endogenous pMHC complexes so that these receptors can be triggered even though they bind for only a short time interval. Thus, blocking the binding of CD4 to the MHC should lead to a change in the response of a T cell to agonist pMHC complexes. Computer simulations of the molecular model we have proposed reproduced (data not shown) both the shift and change in shape of the dose-response curve after blocking binding of CD4 to the MHC (**Fig. 1**).

DISCUSSION

The coreceptor CD4 has long been known to enhance the T cell response to antigen[4,9–12,22]. Thus, previous data showing that blocking the binding of CD4 to the MHC results in shifting the sensitivity threshold to higher numbers of pMHC complexes was not unexpected[3]. However, those data also showed that blocking CD4 resulted

in a sharper switch-like response in the peptide number dose-response curve. The underlying reason for this apparent anomaly is harder to understand. Here, our results from a quantitative model, peptide-counting experiments and imaging of Lck recruitment suggest that both changes in the characteristics of the dose-response curve after blocking CD4 are due to impaired recruitment of Lck to the TCR complex. It could be argued that the shape of the curve changes because a more cooperative pathway involving lower Lck activity, rather than lower Lck amounts, is operative when binding of CD4 to the MHC is blocked[34]. However, our imaging experiments demonstrate that the amount of available Lck is reduced after blocking CD4 in the way suggested by the phenomenological model.

The observation that as few as ten agonist pMHC complexes can stimulate sustained calcium flux[3] could be interpreted to mean that thymic selection finely tunes the T cell repertoire such that interactions with endogenous pMHC complexes result in signaling that is very close to the activation threshold. Addition of a few agonists could then result in activation. The results of our computer simulations of such a model in which agonist and endogenous pMHC complexes act independently showed that the observed sensitivity to small amounts of antigen would be accompanied by noise-induced autoimmune responses. This simulation suggests that to show extraordinary sensitivity to antigen without a propensity for autoimmune responses, T cell signaling elicited by a few agonists needs to be amplified greatly.

This point, and our findings regarding the importance of Lck recruitment in determining T cell sensitivity to agonists, led us to propose a molecular mechanism for how T cells discriminate between 'self' and 'non-self' with great sensitivity. In this model, the presence of a few agonist pMHC complexes allows the large pool of endogenous pMHC complexes to contribute to productive TCR signaling; at the same time, endogenous pMHC complexes cannot trigger TCRs with high probability in the absence of agonists. TCR–agonist pMHC complexes are sufficiently long lived to allow CD4 binding and concomitant recruitment of the CD4-associated Lck. Spatial localization of Lck in a signaling complex allows endogenous pMHC complexes that are part of this complex to trigger multiple TCRs despite the short half-lives of endogenous TCR-pMHC complexes. This is because the enzyme required for ITAM phosphorylation (Lck) is 'ready and waiting' in this complex when TCRs bind to endogenous pMHC complexes. Our computer simulations show that spatial localization of Lck in signaling complexes involving endogenous and agonist pMHC complexes results in a large enhancement (3,500-fold) in the effective rate of Lck-TCR association. Such molecular complexes rarely form in the absence of agonists because TCR–endogenous pMHC bonds are not sufficiently long-lived to recruit CD4 and Lck, thus preventing spurious activation. We have presented results of computer simulations and imaging experiments in support of this model.

One implication of our model is that serial triggering[35] of TCRs by endogenous pMHC complexes, rather than agonist pMHC complexes, could be the critical factor in TCR signaling. For small numbers of agonists, our model predicts that the T cell response would continuously increase as the 'off rate' of TCR–agonist pMHC binding becomes smaller. This is because this would enhance the formation of signaling complexes (**Fig. 4**), which in turn would allow more endogenous pMHC complexes to participate in triggering TCRs. According to our model, the competition between serial triggering and kinetic proofreading[35–38] leading to an optimal value of half-life should be observed for the endogenous pMHC complexes rather than the agonist pMHC complexes. This may be the reason that a monotonic increase in T cell activation efficacy is often seen with increasing half-lives of stimulatory agonists[39,40].

A natural consequence of the signal amplification mechanism that we propose is that varying the number of agonists on APCs or the half-life characterizing their binding to TCRs enables different subpopulations of the endogenous pMHC repertoire to contribute to signaling. For example, if the 'off rate' characterizing TCR–agonist pMHC binding decreases, endogenous pMHC complexes with larger k_{off} values can contribute to productive signaling. This suggests that the broad diversity of endogenous pMHC complexes on APC surfaces may help T cells respond to diverse types and stages of infection.

Several proposals have been made about how endogenous pMHC complexes can participate in triggering TCRs[2,3,5,6,21]. As in the pseudodimer model[3], we assume that CD4 brings together endogenous and agonist pMHC complexes. In the model presented here, this leads to spatial localization of Lck in signaling complexes that in turn enables endogenous pMHC complexes to trigger many TCRs. The concomitant signal amplification contributes to the high sensitivity of T cells to antigen and is consistent with the close linkage we found between Lck recruitment and T cell sensitivity.

As noted earlier, the association of two pMHC complexes in signaling molecules could also be mediated by CD4 dimers. Evidence that CD4 dimerizes comes from X-ray diffraction analysis of protein crystals[24], immunoprecipitation experiments[12,26], functional studies of chimeric proteins[25] and site-directed mutagenesis[12,22]. The structural data indicate that CD4 dimerizes through a D4:D4 interface, which is compatible with binding of CD4 to the MHC at an angle of almost 90° through the D1 domain (as seen in a crystal structure[23]). Recent data also suggest that CD4 dimers are key in T cell activation[12].

The key signaling complex could also be formed if pMHC dimers form constitutively on APC surfaces[41–43]. In this case, agonist and endogenous pMHC complexes, CD4 and Lck are spatially localized after CD4 recruitment and binding initiated by binding of the TCR to an agonist pMHC complex (**Supplementary Fig. 6** online). This is because a preponderance of dimers that involve an agonist will be heterodimers of agonist and endogenous pMHC complexes, as the latter are in large excess.

Our results (data not shown) were robust for variations up to tenfold in the stability of the CD4-MHC-TCR-Lck complex. However, the stability of this complex must exceed a threshold value for the model to show high sensitivity to antigen. At present, experimental measurements for the stability of such a complex are not available. Only BIAcore measurements for the binding affinity of the CD4 ectodomain to the MHC are available, and it seems to be rather small[44]. It is well documented[45–49], however, that the quality of TCR signaling depends on the association of TCR with CD4 (suggested to be mediated by Lck), which is not reflected in the BIAcore measurements. Furthermore, because of topological constraints imposed by confinement between cell membranes, the two-dimensional affinity between cell surface receptors and ligands is higher than that measured in solution[32]. The results reported here should stimulate experiments probing the stability and detailed molecular structure of the signaling complexes described here. More generally, our proposal of a molecular mechanism that enables T cells to detect antigen with great sensitivity has broad implications for T cell biology, and it should provide a useful framework for future experimentation.

METHODS

Microscopy and image analysis. Peptide loading and labeling on CH27 B cells were done as described[3]. Anti-mouse CD16/32 (5 μg/ml; BD PharMingen) was used during the labeling process to avoid nonspecific binding of streptavidin-phycoerythrin (BD PharMingen) on Fc receptors.

T cell calcium flux, agonist-MHC and Lck-GFP dynamics were observed as described[3,19]. A Zeiss Axiovert-100TV station equipped with a CoolSNAP$_{HQ}$ charge-coupled device camera (Roper Scientific) and a high-speed piezzo Z-motor for *Z*-stack recording were used for microscopy. Fura-2-loaded T blasts from day 5 or 6 and CH27 APCs were in a humidified stage at 37 °C and 5% CO_2. The recording was controlled by Metamorph software (Universal Imaging) and differential interference contrast (DIC)-Fura time-lapse recordings were made at 30-second intervals to monitor the T cell response. Phycoerythrin 1-μm *Z*-stack 'snapshots' (for pMHC quantification) were obtained when T cells were engaged with CH27, and a time-lapse DIC-Fura-GFP Z-stack (1 μm interval for Lck observation) recording was obtained immediately after the phycoerythrin stack with 30-second intervals for 10 min. Images were analyzed with the MetaMorpher software suite. Three-dimensional image reconstruction and image rotation were done for the face-on phycoerythrin-GFP fluorescence density quantification. The number of agonist peptide presented in the synapse was quantified based on the integrated phycoerythrin density and single-molecule standard calibration[3]. The Lck accumulation ratio was generated by comparison of the averaged synaptic GFP fluorescence density to the rest of the T cell membrane. Each data point represents the average of more than three cells (number varies) from different batches of primary cell preparation. CD4 blocking experiments used methods identical to those described[3].

Computer simulations and 'master equations'. A detailed description of the computer simulations with the Gillespie stochastic algorithm and Monte-Carlo simulations is provided in the **Supplementary Note** online, which also provides the specific 'master equations' (equations (S1) and (S2)) that were solved to generate **Figure 1**.

Calculation of calcium traces from the computer simulations. Through a series of adapter proteins, ITAM phosphorylation leads to activation of PLC-γ, which hydrolyzes phosphatidylinositol-4,5-bisphosphate to produce inositol-1,4,5-triphosphate and diacylglycerol. Inositol-1,4,5-triphosphate allows the endoplasmic reticulum to empty its intracellular calcium stores. The transient increase in cytoplasmic calcium is approximaterly the same for different numbers of agonist pMHC complexes[3], which suggests that sustained calcium requires additional factors. This is consistent with the capacitive model for calcium flux[50].

The equations used to generate the traces are as follows:

$$\frac{dZ_{ER}}{dt} = -k_{ER} H\left(\sum_i P_i - P_0\right) Z_{ER} \qquad (4)$$

$$\frac{d\,[Ca^{+2}]}{dt} = k_{ER} H\left(\sum_i P_i - P_0\right) Z_{ER} - \mu[Ca^{+2}] + k_1\left(\frac{[Ca^{+2}]}{[Ca^{+2}] + K_{Ca}}\right)\left(\frac{\sum_i P_i}{\sum_i P_i + K_P}\right) \qquad (5)$$

where Z_{ER} is the concentration of calcium in the endoplasmic reticulum ($Z_{ER} = 1.0$ initially); $[Ca^{+2}]$ is the concentration of calcium in the cytoplasm; $k_{ER} = 0.02$ is the rate at which the endoplasmic reticulum empties; $\Sigma_i P_i$ is the total number of triggered TCR; $P_0 = 5.0$ is a phosphorylation threshold for opening the endoplasmic reticulum; and $\mu = 10.0$ is the rate of removal of calcium from the cytoplasm; and $k_1 = 0.02$, $K_{Ca} = 0.0006$ and $K_P = 100.0$ control for the intake of extracellular calcium. Changing these parameter values does not affect any of the qualitative results.

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

ACKNOWLEDGMENTS

We thank M. Krogsgaard for providing TCR binding data before publication. Supported by the National Institutes of Health, Howard Hughes Medical Institute, Helen Hay Whitney Foundation (Q.J.L.), QB3 Institute at UC Berkeley (S.Y.Q.), National Science Foundation (A.R.D.) and Burroughs Wellcome Fund (A.R.D.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 3 February; accepted 18 June 2004
Published online at http://www.nature.com/natureimmunology/

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