

T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation

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After homing to lymph nodes, CD8⁺ T cells are primed by dendritic cells (DCs) in three phases. During phase one, T cells undergo brief serial contacts with DCs for several hours, whereas phase two is characterized by stable T cell–DC interactions. We show here that the duration of phase one and T cell activation kinetics correlated inversely with the number of complexes of cognate peptide and major histocompatibility complex (pMHC) per DC and with the density of antigen-presenting DCs per lymph node. Very few pMHC complexes were necessary for the induction of full-fledged T cell activation and effector differentiation. However, neither T cell activation nor transition to phase two occurred below a threshold antigen dose determined in part by pMHC stability. Thus, phase one permits T cells to make integrated ‘measurements’ of antigen dose that determine subsequent T cell participation in immune responses.

The naive T cell population expresses a broad array of unique T cell antigen receptors (TCRs), each with a discrete affinity for a given complex of cognate peptide and major histocompatibility complex (pMHC). Naive T cells constantly survey and sample antigen-presenting cells (APCs) in secondary lymphoid tissues in search of rare cognate pMHC complexes¹. Due to the diversity of the TCR repertoire, only one in 1×10^5 to 1×10^6 T cells expresses a TCR with sufficient affinity for any given antigen to transmit an activating stimulus^{2,3}. Activation of naive T cells also requires costimulatory and cytokine signals^{4,5}, which are typically provided by mature dendritic cells (DCs) in secondary lymphoid tissues⁶. As they ‘hunt’ for their cognate antigen, naive T cells recirculate between the blood and lymph nodes and spend less than 1 d in any given secondary lymphoid tissue^{1,7}. When T cells encounter antigen in the proper context, they become activated and upregulate the activation marker CD69, which causes their retention in lymph nodes⁸. The trapped cells proliferate and acquire effector functions. Effector cells egress from lymph nodes and travel to peripheral tissues to seek out cells presenting cognate antigen⁹.

Although this chain of events is well established, gaps remain in understanding the rules governing these processes. For example, T cells expressing TCRs of lower affinity are known to require a larger dose of antigen to become activated than do T cells expressing high-affinity TCRs^{10,11}. The abundance of pMHC complexes per DC and of

antigen-presenting DCs per lymph node is influenced by many factors and may vary substantially within and among individuals¹². How T cells ‘measure’ antigen dose *in vivo* and how this determines whether a T cell will respond to a given antigenic constellation remain unclear.

Several groups have applied multiphoton and confocal microscopy to address related issues by analyzing T cell activity in explanted mouse lymph nodes^{13–15}. Experiments using multiphoton intravital microscopy (MP-IVM) to study CD8⁺ T cell–DC interactions in popliteal lymph nodes of anesthetized mice have provided initial evidence showing how antigen dose might be ‘measured’ by T cells¹⁶. Mice received footpad injections of fluorescence-labeled DCs pulsed with a model peptide antigen and the Toll-like receptor 4 ligand lipopolysaccharide (LPS). Fluorescence-labeled naive CD8⁺ T cells expressing a peptide-specific TCR were injected intravenously after 18 h and the injected T cells were allowed to home to lymph nodes for 2 h. At this point, further homing to lymph nodes was prevented with an antibody blocking L-selectin, which is essential for the migration of T cells from blood to lymph nodes but is dispensable for interstitial T cell migration and activation. This synchronized T cell population allows an exact measurement of the kinetics of CD8⁺ T cell priming by DCs¹⁷.

That strategy has shown that priming occurs in three distinct phases. The initial phase lasts about 8 h and is characterized by short-term interactions between cognate antigen–pulsed DCs and

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Received 15 August 2007; accepted 18 December 2007; published online 20 January 2008; doi:10.1038/ni1559

T cells that are initially indistinguishable from the random collisions of T cells with antigen-free DCs. Phase one is immunologically productive, as T cells upregulate activation markers. At the onset of phase two (6–8 h after T cell transfer), T cell–DC interactions become very prolonged. This phase lasts about 12 h and is accompanied by further upregulation of activation markers and initiation of the production of interleukin 2 and interferon- γ (IFN- γ). Finally, phase three, which begins about 24 h after the entry of T cells into the lymph node, is characterized by a return to short T cell–DC interactions and induction of T cell proliferation.

Transient interactions during phase one were somewhat unexpected. Earlier *in vitro* studies had suggested that T cells stop immediately after antigen encounter¹⁸, although serial, short-lasting T cell interactions with antigen-presenting DCs have been reported to occur in collagen gels¹⁹. Although subsequent imaging studies have confirmed and refined the ‘phased’ model of T cell priming^{20,21}, some MP-IVM experiments have failed to detect prolonged phase one–type T cell activity²². In these experiments, mice were injected with a protein antigen fused to an antibody specific for the DC marker DEC-205, which targeted the antigen to DEC-205⁺ DCs²³; antigen thus reached almost all members of the dense network of lymph node–resident DCs²⁴. The amount of DEC-205-targeted antigen and the density of antigen-presenting DCs encountered by T cells is probably much larger than that in a setting in which a small number of antigen-laden DCs enter the lymph node through afferent lymph. We speculate that this difference in antigen dose could have been responsible for the differences in T cell activity. To address this issue, here we used an altered peptide ligand in the P14 TCR-transgenic system to study three aspects of antigen-induced signals on T cell activation: peptide affinity for MHC; pMHC concentration per APC; and APC frequency in lymph nodes.

The overall dose of antigen in a lymph node determined the duration of phase one, which suggested the existence of integrative mechanisms that establish a threshold for activation based on the antigen dose encountered by a T cell. T cells rapidly formed tight stable contacts with DCs presenting large numbers of cognate pMHC complexes. However, T cells did not engage in immediate tight contacts with DCs with low antigen densities; instead, they accumulated signals through hours of signal integration during serial encounters with many DCs until sufficient antigenic stimulation enabled the

transition from phase one to phase two. T cells encountering a subthreshold antigen dose could not form prolonged contacts and continued to migrate until they egressed from the lymph node without achieving activation. Thus, serial encounters during phase one allow recirculating T cells to ‘measure’ the overall amount of antigen in lymph nodes and set a threshold for subsequent T cell responses.

RESULTS

Altered peptide ligands with distinct MHC affinities

MP-IVM experiments defining the three-phase priming paradigm¹⁶ have used the P14 TCR-transgenic mouse system, in which CD8⁺ T cells recognize a peptide from lymphocytic choriomeningitis virus (LCMV) glycoprotein (gp33–41) in H-2D^b (ref. 25). The antigenic peptide used before¹⁶ had the native amino acid sequence KAVYN-FATC, with a carboxy-terminal cysteine (C-peptide). As the carboxy-terminal sulfhydryl group in C-peptide is amenable to oxidation and the formation of peptide dimers, it is difficult to control the peptide concentration when pulsing APCs. Therefore, many investigators use an altered peptide ligand, KAVYNFATM, which terminates in a methionine residue (M-peptide).

To explore the suitability of M-peptide for quantitative studies, we compared the activity of DCs pulsed with M-peptide and C-peptide *in vivo*. We incubated purified splenic DCs with 10 μ M of either peptide, labeled them with fluorescent dyes and injected them along with LPS into the right footpads of C57BL/6 mice. After 18 h, we intravenously injected fluorescent antigen-specific P14 T cells and differentially labeled non-antigen-specific OT-I T cells. We blocked further T cell homing to lymph nodes 2 h later by intravenous injection of antibody to L-selectin (anti-L-selectin) and recorded T cell–DC interactions by MP-IVM in the popliteal lymph node¹⁶. In keeping with published findings¹⁶, C-peptide-pulsed DCs supported transient contacts with P14 T cells during the first 6–8 h after T cell transfer (Fig. 1a,b and Supplementary Video 1 online). In contrast, the transition from phase one–like to phase two–like interactions occurred more quickly (within 2–4 h) with M-peptide-pulsed DCs (Supplementary Video 2 online). This difference between C-peptide-induced and M-peptide-induced T cell activity was also reflected in the P14 T cell motility coefficients, which decreased more rapidly with M-peptide (Fig. 1c). Neither peptide induced prolonged DC interactions with OT-I T cells.

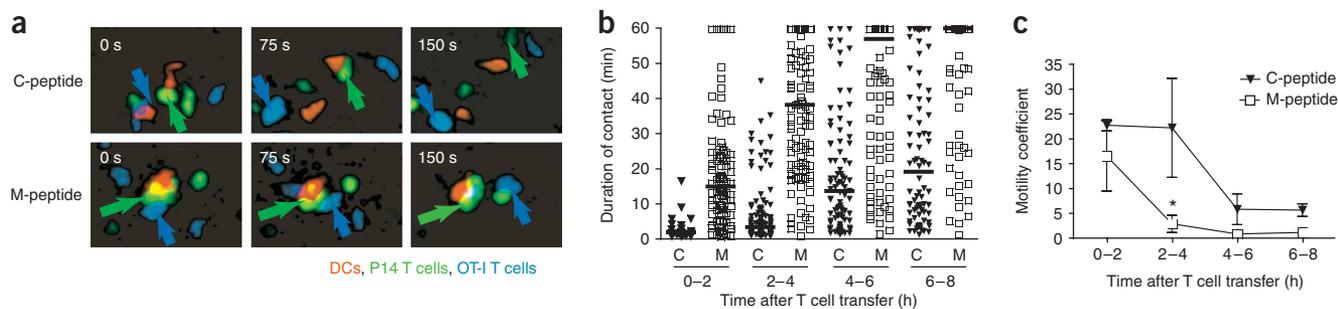


Figure 1 Interactions of P14 T cells with DCs pulsed with C-peptide or M-peptide *in vivo*. (a) MP-IVM of P14 T cells (green) and OT-I control T cells (blue) interacting with DCs (red) pulsed with 10 μ M C-peptide (top) or 10 μ M M-peptide (bottom). Still images were obtained from individual time-lapse movies at 2.5 h after T cell injection; images were chosen in 75-second intervals from movies with cycle times of 15 s. Green and blue arrows indicate P14 T cells and control T cells, respectively, interacting with DCs; $n = 3$ –4 mice for each condition. Original magnification, $\times 60$. (b) Duration of P14 T cell contacts with DCs pulsed with 10 μ M M-peptide (M) or C-peptide (C). Data are from 60-minute recordings obtained during various time intervals after T cell injection. Data are pooled from two to four mice per time point; small horizontal lines indicate the median. Contact durations are significantly longer with M-peptide than with C-peptide for all time intervals ($P < 0.0001$; Mann-Whitney U-test). (c) Motility coefficients for P14 T cells interacting with DCs pulsed with 10 μ M C-peptide or M-peptide. *, $P < 0.05$ (Mann-Whitney U-test); $n = 2$ –4 mice per time point (error bars, mean \pm s.e.m). Data are representative of two to three (a) or three to five (b,c) experiments.

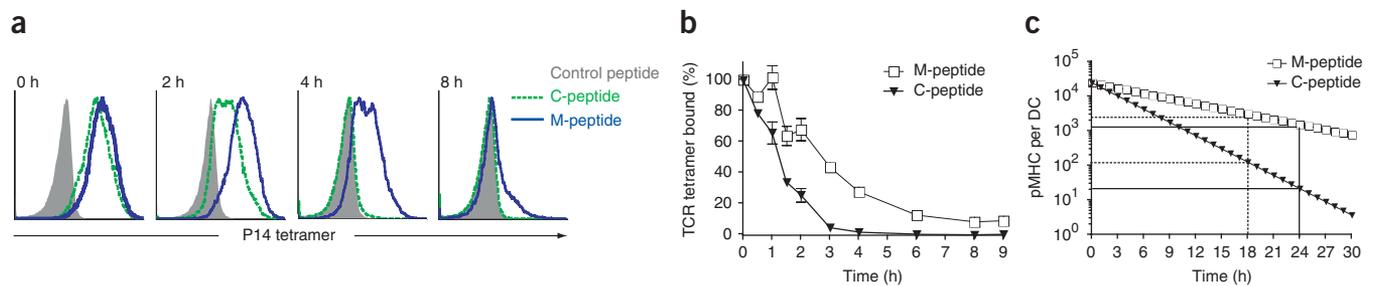


Figure 2 Number of cognate pMHC complexes on DCs. **(a)** Flow cytometry of the binding of P14 TCR tetramers to 5T33 cells (APCs) pulsed for 3 h at 37 °C with 10 μM C-peptide, M-peptide or control peptide (SIINFEKL) in the presence of peptide-loading enhancer; after cells were washed and incubated at 37 °C (time, above plots) to allow peptide dissociation from H-2D^b, cognate pMHC complexes were counted by staining of cells with phycoerythrin-labeled P14 TCR tetramers and annexin V. Histograms are gated on annexin V–negative cells. Data are representative of three experiments. **(b)** Staining intensity of P14 TCR tetramers bound to 5T33 cells pulsed with C-peptide or M-peptide. The MFI of delayed staining is presented relative to the MFI of cells stained immediately after peptide pulsing. Data represent the mean ± s.e.m. of results pooled from three independent experiments. **(c)** Cognate pMHC complexes remaining after pulsing, calculated on the basis of a measured starting value of 2.5×10^4 H-2D^b complexes per DC and calculated half-lives of 6.01 h and 2.36 h for M-peptide and C-peptide, respectively. Lines connecting data lines to axes indicate estimated number of cognate pMHC complexes per DC at the time of T cell injection (18 h after DC pulsing; dashed lines) and 6 h after T cell injection (24 h after DC pulsing; solid lines).

Because the single amino acid substitution that distinguishes C-peptide and M-peptide involves a contact residue with H-2D^b (ref. 26), it seemed likely that the observed peptide-dependent differences in T cell activity were due to differential peptide interactions with H-2D^b. Indeed, compared with C-peptide, M-peptide binds with about 260-fold higher affinity to H-2D^b (ref. 27) and with similar affinity to TCR²⁸. We confirmed the higher MHC affinity of M-peptide in an *in vitro* H-2D^b stabilization assay with RMA-S mutant mouse lymphoma cells (data not shown). Given the high peptide concentration (10 μM) used for DC pulsing in these experiments, it seems likely that most available MHC class I molecules on DCs were initially loaded with peptide, although this may be an overestimate. However, T cells were not exposed to DCs until 18 h after DC injection; this delay was necessary for migration of DCs to the draining lymph node. If the pMHC complexes formed by C-peptide and M-peptide had different dissociation kinetics, the amount of cognate pMHC complexes available for P14 T cell priming in lymph nodes may have differed substantially even if similar numbers of pMHC complexes were initially loaded.

To assess the half-lives of C-peptide and M-peptide in H-2D^b, we constructed²⁹ a single-chain P14 TCR that was biotinylated and in oligomers with phycoerythrin-streptavidin. We used the recombinant P14 tetramer in flow cytometry experiments to stain peptide-pulsed

cells of the multiple myeloma cell line 5T33 (**Fig. 2a**), which has high expression of H-2D^b (data not shown). We noted apparent half-lives of 2.93 h (95% confidence interval (CI), 2.43–3.73 h) and 1.14 h (95% CI, 0.99–1.34 h) for M-peptide and C-peptide, respectively, in H-2D^b (**Fig. 2b**). However, on the basis of theoretical considerations, we predicted that our measurements underestimated the true pMHC half-lives (procedural reasons for the underestimation and computational strategies to correct for the effects of the underestimation, **Supplementary Methods** online). After taking into account the relevant sources of error in a computational model, we arrived at a corrected half-life for M-peptide of 6.01 h (95% CI, 5.36–6.66 h) and for C-peptide of 2.36 h (95% CI, 2.15–2.57 h). These results are consistent with past estimates²⁸.

We used our knowledge of peptide half-lives to calculate the maximum number of cognate pMHC complexes per DC encountered by P14 T cells (**Fig. 2c** and **Table 1**). Using flow cytometry³⁰, we determined mean copy numbers of 2.5×10^4 and 1×10^5 H-2D^b molecules per cell on freshly isolated (immature) and LPS-matured DCs, respectively (**Supplementary Fig. 1** online). Assuming that all available H-2D^b complexes were loaded when immature DCs were incubated with 10 μM C-peptide or M-peptide (probably an overestimate), we calculated that by 18 h after DC pulsing, when T cells would be transferred, M-peptide-pulsed DCs retained an average of

Table 1 Relationship of peptide concentration to the estimated number of pMHC complexes

	pMHC/DC		pMHC/lymph node ($\times 10^3$)		pMHC in T cell–DC contact zone	
	18 h	24 h	18 h	24 h	18 h	24 h
C-peptide (10 μM)	127	22	38	6.6	13	2.2
M-peptide (100 pM)	30	15	9	4.5	3	1.5
M-peptide (200 pM)	60	30	18	9	6	3
M-peptide (10 μM)	3,133	1,568	940	470	313	156

Estimates for cognate pMHC numbers per DC, per lymph node and per T cell–DC contact zone *in vivo* are for a given concentration of C-peptide or M-peptide used to pulse DCs. For 18 h, the calculations assume that there are 2.5×10^4 H-2D^b molecules per DC (**Supplementary Fig. 1a**) and that the C-peptide and M-peptide half-lives are 2.36 h and 6.01 h, respectively. Predicted pMHC numbers are also provided for 24 h after DC injection, by which time phase two has begun for most conditions (except for 100 pM M-peptide). To estimate the number of pMHC complexes in the entire lymph node, we assumed that 300 antigen-bearing DCs reached the draining lymph node after footpad injection (data not shown). To estimate the number of pMHC complexes per T cell–DC contact zone, we assumed that immature DCs have a surface area of 500 μm² (ref. 44) and that the T cell–DC contact zone is about 50 μm² (ref. 45). Data for pMHC numbers after pulsing with 10 μM C-peptide and M-peptide were generated as described (**Fig. 2a,b** and **Supplementary Methods**); values for 100 pM and 200 pM M-peptide were calculated from those values on the basis of our model (H.Z., B. Jin, S.E.H., A. Perelson, U.H.v.A. and A.K.C., unpublished data). With that model, we calculated that DCs pulsed with 200 pM M-peptide began with about 478 loaded pMHC complexes at the time of DC pulsing; logically, for DCs pulsed with 100 pM M-peptide, there would be approximately half that number at each time point. Data are representative of 00 experiments.

Figure 3 Peptide dose dependence of T cell activation. **(a)** T cell proliferation after CD11c⁺ DCs pulsed with M-peptide or C-peptide (concentration, horizontal axis, and as labeled for M-peptide) or with 10 μ M control peptide (SIINFEKL) were injected into footpads of CD45.1⁺ recipient mice, then CFSE-labeled CD45.2⁺ P14 T cells were injected 18 h later; after 48 h, CFSE dilution was measured in single-cell suspensions of popliteal lymph nodes. *, $P < 0.0005$; **, $P < 0.0001$; ***, $P < 0.016$ (two-tailed Student's *t*-test).

Data (mean \pm s.d.) are pooled from three

independent experiments with two to six mice per concentration. **(b)** Cytotoxicity of P14 T cells stimulated *in vivo*. Purified DCs were pulsed with 10 μ M or 200 pM M-peptide or 10 μ M control peptide and injected into the footpads of recipient mice; 5×10^6 P14 T cells were injected intravenously 18 h later and, after an additional 48 h, two polyclonal B cell populations (one pulsed with 10 μ M M-peptide and labeled with 2 μ M CFSE; the other labeled with 0.1 μ M CFSE), mixed at a ratio of 1:1, were injected intravenously; 6 h later, the ratio of CFSE^{hi} B cells to CFSE^{lo} B cells was assessed in the popliteal lymph node. Numbers above bracketed lines indicate percent CFSE^{hi} B cells (right) or CFSE^{lo} B cells (left). Data are pooled from two independent experiments with four to five mice per group.

3,133 cognate pMHC complexes (95% CI, 2,434–3,838 complexes), whereas C-peptide-pulsed DCs retained, on average, 127 pMHC complexes (95% CI, 76–195 complexes). By 6 h later, these numbers decreased to 1,568 and 22 pMHC complexes per DC, respectively (95% CI, 1,120–2,055 complexes for M-peptide and 11–39 complexes for C-peptide; **Table 1**). With our DC transfer protocol, about 300 DCs reached the draining popliteal lymph node by 18 h (data not shown). Thus, P14 cells encountered a maximum total number of 3.8×10^4 cognate pMHC complexes in the lymph nodes of C-peptide-pulsed DC recipients. This was sufficient to induce activation of P14 cells but yielded prolonged phase one interactions. In contrast, T cells entering lymph nodes containing M-peptide-pulsed DCs encountered a maximum of 9.4×10^5 cognate pMHC complexes and transitioned rapidly to stable phase two-like interactions.

Effect of peptide dose on T cell activation

To delineate the relationship between peptide dose and T cell activation dynamics, we examined the effects of DCs pulsed with various concentrations of C-peptide and M-peptide on T cell proliferation and activation marker expression. *In vitro* experiments in which we exposed T cells to immature DCs showed that maximum proliferation

of P14 T cells exposed to M-peptide-pulsed DCs required 1.8% of the concentration required when using C-peptide (**Supplementary Fig. 2a** online); these findings paralleled the known difference between these peptides in MHC affinity²⁷. *In vivo* assays with T cells injected into mice 18 h after footpad injection of peptide-pulsed DCs identified a difference of 30,000-fold in apparent peptide antigenicity; the minimum peptide concentration at which pulsed DCs induced near-maximum T cell proliferation was 200 pM for M-peptide and 6 μ M for C-peptide (**Fig. 3a**). We found rapid upregulation of the activation marker CD69 (**Supplementary Fig. 2c**) and *in vivo* cytotoxicity (**Fig. 3b**) whether DCs were pulsed with 10 μ M or 200 pM M-peptide. Notably, a further twofold dilution of M-peptide to 100 pM rendered DCs incapable of inducing T cell proliferation or full expression of activation markers (**Fig. 3a** and **Supplementary Fig. 2b,c**). P14 T cells were completely unresponsive to DCs that had been pulsed with 10 μ M control peptide (SIINFEKL), which indicated that the peptide effects required recognition of cognate antigen.

Having determined concentrations of M-peptide at which pulsed DCs induced maximum proliferation (200 pM) or no proliferation (100 pM), we investigated the effect of those and higher doses on T cell–DC interaction dynamics *in vivo* (**Fig. 4**). DCs that had been pulsed with 100 pM M-peptide did not promote long-lasting T cell

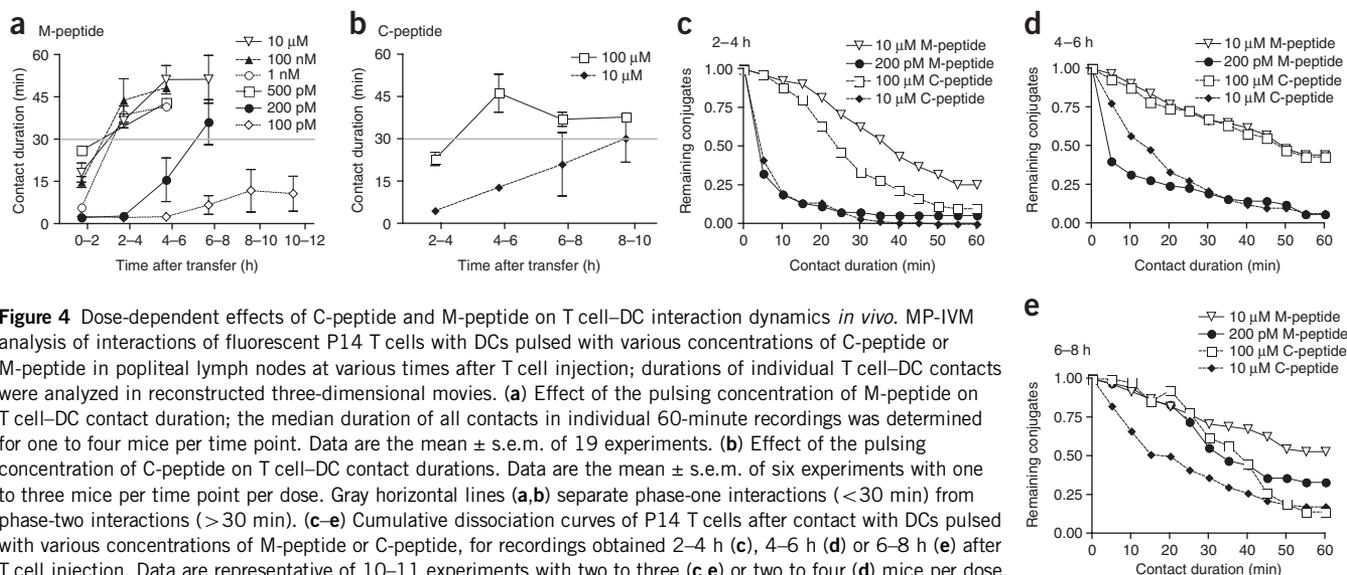


Figure 4 Dose-dependent effects of C-peptide and M-peptide on T cell–DC interaction dynamics *in vivo*. MP-IVM analysis of interactions of fluorescent P14 T cells with DCs pulsed with various concentrations of C-peptide or M-peptide in popliteal lymph nodes at various times after T cell injection; durations of individual T cell–DC contacts were analyzed in reconstructed three-dimensional movies. **(a)** Effect of the pulsing concentration of M-peptide on T cell–DC contact duration; the median duration of all contacts in individual 60-minute recordings was determined for one to four mice per time point. Data are the mean \pm s.e.m. of 19 experiments. **(b)** Effect of the pulsing concentration of C-peptide on T cell–DC contact durations. Data are the mean \pm s.e.m. of six experiments with one to three mice per time point per dose. Gray horizontal lines (**a,b**) separate phase-one interactions (< 30 min) from phase-two interactions (> 30 min). **(c–e)** Cumulative dissociation curves of P14 T cells after contact with DCs pulsed with various concentrations of M-peptide or C-peptide, for recordings obtained 2–4 h (**c**), 4–6 h (**d**) or 6–8 h (**e**) after T cell injection. Data are representative of 10–11 experiments with two to three (**c,e**) or two to four (**d**) mice per dose.

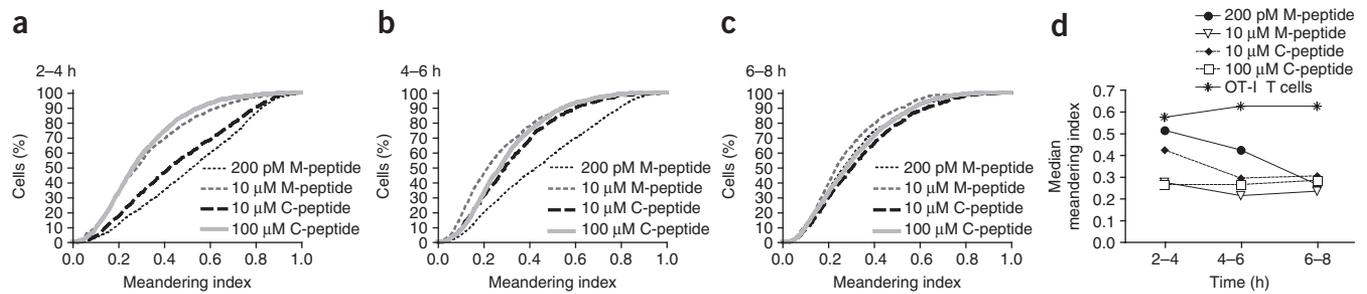


Figure 5 Effect of antigen dose on the meandering of T cells in lymph nodes. MP-IVM analysis of interactions between fluorescent P14 T cells and DCs pulsed with various concentrations of C-peptide or M-peptide, assessed in popliteal lymph nodes at various times after T cell injection; cell centroids in three dimensions were measured by semiautomated cell tracking and the 'meandering index' was calculated by division of the displacement for each cell track by the total path length for that cell track. **(a–c)** Cumulative distribution plots of the 'meandering index' of P14 T cells interacting with DCs pulsed with one of four peptide doses (key) at 2–4 h **(a)**, 4–6 h **(b)** or 6–8 h **(c)** after T cell injection. Data are representative of 00 experiments with two to three **(a,c)** or two to four **(b)** mice per dose. **(d)** Median 'meandering index' of P14 T cells interacting with DCs pulsed with one of four peptide doses (key) and of OT-I T cells (pooled interactions with DCs pulsed with 100 pM M-peptide, 200 pM M-peptide, 10 μ M M-peptide, 10 μ M C-peptide or 100 μ M C-peptide), assessed at various time intervals (horizontal axis). Data are representative of 10–12 experiments with two to four mice per dose per time point for P14 T cells and 21 experiments with eleven to fourteen mice per time point for OT-I T cells.

contacts over at least 12 h (**Supplementary Video 3** online). In contrast, DCs pulsed with peptide concentrations of at least 200 pM triggered T cell activation and phase two–like interactions, with varied kinetics. T cells underwent prolonged phase one–like interactions with DCs pulsed with 200 pM M-peptide (**Fig. 4a,c–e** and **Supplementary Videos 4** and **5** online). In contrast, M-peptide concentrations at or above 500 pM supported only a relatively short period (about 2 h) of transient interactions before the transition to phase two (**Fig. 4a,c–e**). We also noted the same inverse relationship between duration of phase one and concentration of antigenic peptide with C-peptide; as expected, DCs pulsed with 10 μ M C-peptide triggered prolonged phase one–like interactions¹⁶, whereas pulsing DCs with 100 μ M C-peptide accelerated the transition to phase two substantially (**Fig. 4b–e** and **Supplementary Video 6** online). To confirm those results, obtained by manual image analysis, we used automated cell tracking and track analysis, which showed a parallel reduction in the

'meandering' index (**Fig. 5** and **Supplementary Fig. 3** online), which indicated greater spatial confinement of T cells³¹. These rapid changes in median T cell–DC interaction times with varied doses of peptides are 'predicted' by both experimental and theoretical studies³² (H.Z., B. Jin, S.E.H., A. Perelson, U.H.v.A. and A.K.C., unpublished data).

Effect of intranodal APC density and signal integration

The observations reported above indicated that T cell responses varied in accordance with mean antigen density on individual APCs in lymph nodes. Those results are compatible with at least two different mechanisms distinguished by the presence or absence of T cell 'memory' of TCR signals perceived during previous interactions. In the memory-free scenario, each encounter of T cell with an APC has a certain probability of triggering stable binding, which is proportional to local antigen density and is independent of the cell's previous

Figure 6 Tissue concentration of cognate antigen-bearing DCs controls T cell proliferation and IFN- γ production. CFSE dilution and IFN- γ production of cells in popliteal lymph nodes after subcutaneous injection of a constant number of DCs (5×10^5) and 10 ng LPS into footpads of congenic (CD45.1⁺) recipients; the fraction of antigen-bearing DCs was varied from 0% to 100% (above plots, **a**; horizontal axes, **b,c**) by mixture of DCs pulsed with M-peptide (10 μ M or 200 pM) and DCs pulsed with 10 μ M control peptide (SIINFEKL). Each recipient received a positive control injection of 100% antigen-bearing DCs in the left footpad and 10%, 1%, 0.1% or 0% antigen-bearing DCs in the right footpad. After 18 h, CFSE-labeled P14 T cells were injected intravenously, followed 2 h later by anti-L-selectin and analysis by flow cytometry 18 h and 48 h later. **(a)** Flow cytometry of CFSE dilution versus IFN- γ production. **(b)** Percent proliferated T cells, calculated as a ratio for each mouse by division of percent proliferation in the test popliteal lymph node by that in the positive control popliteal lymph node, which received 100% DCs pulsed with M-peptide. **(c)** MFI of IFN- γ in transferred P14 T cells in the test popliteal lymph node, presented as a percentage of the MFI for transferred P14 T cells in the control popliteal lymph node, which received 100% DCs pulsed with M-peptide. Data are pooled from three independent experiments with two to five mice per condition per time point (mean \pm s.e.m., **b,c**).

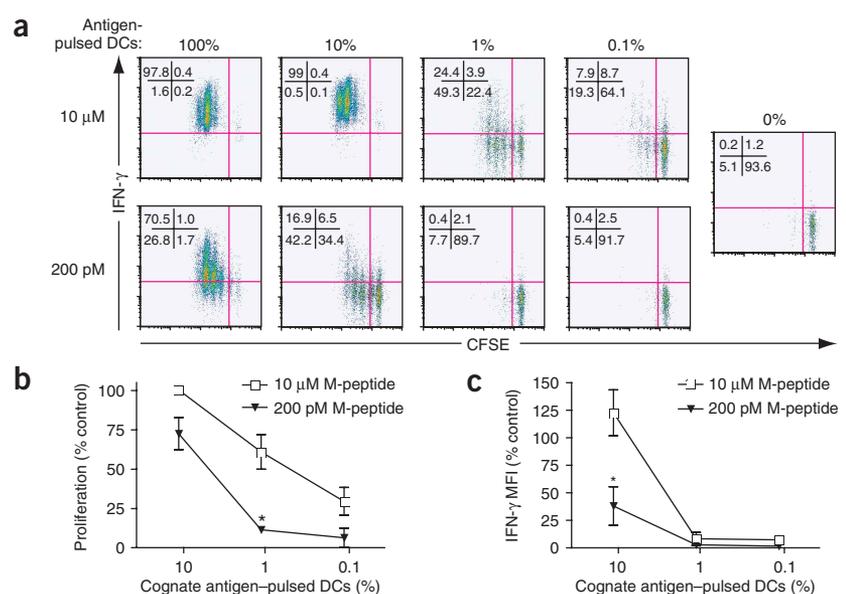
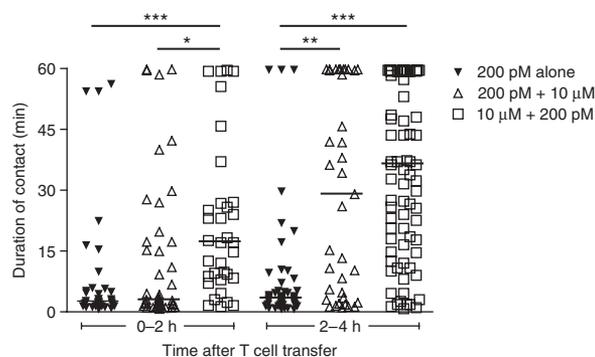


Figure 7 Early phase two for antigen-specific T cells interacting with threshold dose antigen-pulsed DCs when a second DC population pulsed with high-dose antigen is also in the lymph node. MP-IVM analysis of P14 T cell interactions with DCs in popliteal lymph nodes versus a single population or two populations of differentially peptide-pulsed DCs, assessed at various times after T cell injection. P14 T cells interacted with DCs pulsed with 200 pM M-peptide alone (200 pM alone), with DCs pulsed with 200 pM M-peptide in the presence of DCs pulsed with 10 μ M M-peptide (200 pM plus 10 μ M) or with DCs pulsed with 10 μ M M-peptide in the presence of DCs pulsed with 200 pM M-peptide (10 μ M plus 200 pM). The duration of individual T cell–DC contacts was analyzed in reconstructed three-dimensional movies; short horizontal lines indicate the median. For 0–2 h, interactions are significantly longer with DCs pulsed with 10 μ M M-peptide than with either population of DCs pulsed with 200 pM M-peptide (***, $P < 0.0001$ for 200 pM alone; *, $P = 0.0031$ for 200 pM plus 10 μ M; Mann-Whitney U-test); for 2–4 h, interactions are significantly shorter for DCs pulsed with 200 pM M-peptide than for DCs pulsed in either combination (**, $P = 0.0002$ for 200 pM plus 10 μ M; ***, $P < 0.0001$ for 10 μ M plus 200 pM; Mann-Whitney U-test). Data are pooled from four experiments with four mice per time point.



contacts. Alternatively, in the memory model, T cells and/or DCs may have the ability to ‘remember’ previous cognate interactions, thereby incrementally increasing the probability for tight binding with each successive contact.

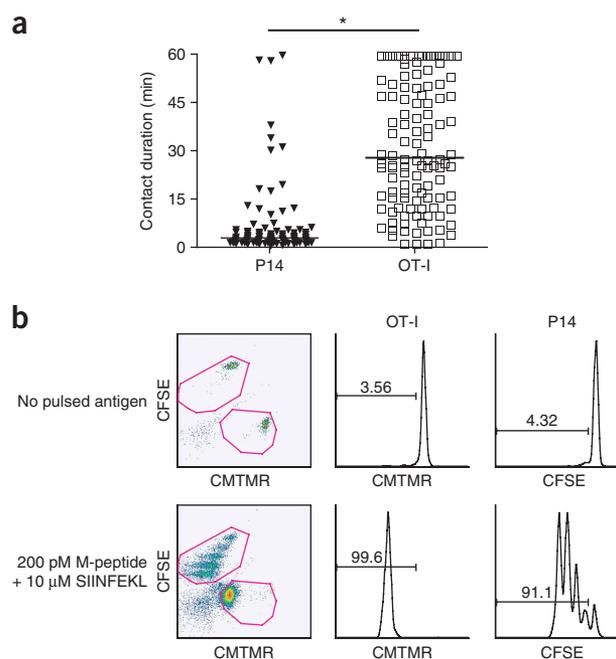
We addressed this issue in two ways. First, we predicted that if the memory model is correct, the likelihood that migrating T cells will receive the necessary dose of antigen should be greater if they can integrate information from more APCs (H.Z., B. Jin, S.E.H., A. Perelson, U.H.v.A. and A.K.C., unpublished data). Thus, T cell activation should be sensitive not only to antigen density per APC but also to APC density per lymph node. Second, we tested the idea of signal integration by antigen-specific T cells with two differentially peptide-pulsed DC populations in a single lymph node.

To test the first prediction *in vivo*, we varied antigen density and APC density in parallel. Because activated DCs in a lymph node can alter T cells even in the absence of cognate antigen^{16,33}, we injected the same total number of DCs into each recipient and adjusted the proportion of cognate antigen-pulsed DCs by mixing them at different ratios with DCs pulsed with control peptide (SIINFEKL). We loaded the cognate antigen-bearing DCs with a high dose (10 μ M) or the threshold activating dose (200 pM) of M-peptide and injected them into footpads at the appropriate ratio with control peptide-pulsed DCs and LPS. At 18 h, we gave mice an intravenous injection of P14 T cells labeled with the cytosolic dye CFSE and administered anti-L-selectin 2 h later. At 18 h or 48 h after T cell transfer, we collected popliteal lymph nodes and assessed the IFN- γ production and CFSE dilution of P14 T cells.

Figure 8 DCs can simultaneously maintain phase one- and phase two-like interactions with two different T cell populations. (a) MP-IVM analysis of T cell–DC interactions in popliteal lymph nodes for DCs pulsed with both 10 μ M SIINFEKL peptide and 200 pM M-peptide and injected into footpads; after 18 h, P14 and OT-I T cells labeled with different fluorescent dyes were injected intravenously. Duration of individual contacts was analyzed in three-dimensional time-lapse movies. *, $P < 0.0001$ (Mann-Whitney U-test). Data are representative of two experiments with results pooled from four 1-hour recordings from 1.5 h to 5 h after T cell transfer. (b) Flow cytometry of the proliferation of P14 and OT-I T cells among CD45.2⁺ cells obtained from the popliteal lymph nodes of CD45.1⁺ mice injected with DCs left unpulsed (left footpad) or pulsed with 10 μ M SIINFEKL and 200 pM M-peptide (right footpad) and, after 18 h, injected intravenously with CFSE-labeled P14 T cells and CMTMR-labeled OT-I T cells (5×10^6 of each), followed by analysis after an additional 48 h. Numbers above bracketed lines indicate percent cells that had divided at least once. Data are representative of two experiments with three mice.

Reducing the fraction of cognate antigen-bearing DCs in lymph nodes profoundly attenuated T cell proliferation and IFN- γ production to an extent inversely correlated with cognate pMHC complex density per DC. T cell activation by fewer DCs that had been pulsed with 10 μ M M-peptide was much more robust than the response to an equivalent number of DCs pulsed with 200 pM M-peptide (Fig. 6). Thus, a sizable fraction of P14 T cells responded to as little as 1% of DCs pulsed with 10 μ M M-peptide (about three cells in the popliteal lymph node), but the same number of DCs pulsed with 200 pM M-peptide failed to activate P14 T cells. At very low APC density, competition between P14 T cells may prevent some T cells from accessing DCs before cognate pMHC complexes are lost^{34,35}. Nevertheless, we conclude that the outcome of interactions between migrating T cells and DCs in lymph nodes is determined mainly by the overall antigen dose, which comprises the density of cognate pMHC-bearing DCs and the number of cognate pMHC complexes per DC.

To test the second prediction (that signal integration determines the duration of phase one), we labeled two populations of DCs with different fluorescent dyes and pulsed them with different doses of



M-peptide, one high dose (10 μM) and one threshold dose (200 pM), and then measured *in vivo* P14 T cell–DC interaction times. Although interactions with DCs pulsed with 200 pM M-peptide alone yielded phase-one durations of 6–8 h, interactions with DCs pulsed with 200 pM M-peptide in the presence of DCs pulsed with 10 μM M-peptide yielded phase-one durations of 2–4 h (Fig. 7 and **Supplementary Video 7** online). These results support the idea that signal integration occurs *in vivo*.

Influence of T cells versus DCs

Next we sought to determine whether T cells or DCs dictate T cell responsiveness during the transition from phase one to phase two. We pulsed DCs with two distinct cognate peptides for two different TCRs. We used one peptide, the ovalbumin-derived SIINFEKL, which is the cognate antigen for OT-I T cells³⁶, at a high concentration (10 μM). Simultaneously, we also loaded DCs with M-peptide at the established threshold activation dose (200 pM) or at an intermediate concentration (10 nM). We chose the latter concentration to minimize competition with SIINFEKL for binding to H-2K^b while ensuring sufficient loading of H-2D^b to induce rapid transition to phase two. We injected double-peptide-pulsed fluorescent DCs into footpads and analyzed their interactions with differentially labeled P14 and OT-I T cells by MP-IVM.

Double-peptide-pulsed DCs loaded with 200 pM M-peptide supported only phase one–like interactions with P14 T cells during the first 5 h after T cell injection (Fig. 8a). In contrast, the same DCs engaged in substantial long-lasting contacts with OT-I T cells during the same time interval (**Supplementary Video 8** online). It is not likely that this difference was due to competition between the two peptides for MHC class I binding sites. SIINFEKL binds to H-2K^b, whereas M-peptide binds to both H-2D^b and H-2K^b on C57BL/6 DCs. However, P14 T cells recognize M-peptide only in the context of H-2D^b. We also considered the possibility that P14 and OT-I T cells were competing for access to the DCs, which might have impaired P14 T cell binding^{34,35}. Although we cannot exclude the possibility that this happens at some level, it seems unlikely to be a dominant factor in this setting on the basis of two observations: first, exposure to double-pulsed DCs induced both T cell populations to proliferate, even when M-peptide was used at 200 pM (Fig. 8b); second, both P14 and OT-I T cells engaged simultaneously in early phase two–like interactions when DCs were pulsed with 10 μM SIINFEKL and 10 nM M-peptide (**Supplementary Video 9** online). Although DCs are obviously important in T cell activation and modulate the ability of T cells to become activated, our results show that the T cell ‘decides’ when to transition to phase two by integrating the antigen signal delivered by DCs.

DISCUSSION

Here we sought to decipher the rules governing the transition from a phase dominated by serial T cell–DC contacts (phase one) to a phase of stable T cell–DC interactions (phase two). We found that the duration of phase one correlated inversely with the overall antigen dose by comparing the effect of DCs pulsed with native LCMV-derived C-peptide and the altered peptide ligand M-peptide. M-peptide has a prolonged half-life in H-2D^b and is thus retained at much higher density during the voyage of DCs to lymph nodes. A dose-response analysis of the effect of peptide-pulsing concentrations on the ability of pulsed DCs to elicit phase two–like activity identified a low threshold concentration of M-peptide that supported efficient T cell activation and effector differentiation while inducing prolonged phase one–like activity. Higher doses of M-peptide yielded a rapid transition to phase two. Furthermore, activation of naive

T cells could be controlled by the amount of antigen carried by each DC and by the density of antigen-presenting DCs. However, these two variables do not influence T cell activation equally (H.Z., B. Jin, S.E.H., A. Perelson, U.H.v.A. and A.K.C., unpublished data). The dependence on DC density is linear, whereas the dependence on the number of cognate pMHC complexes per DC is nonlinear with a sharp threshold between nonactivating and activating pMHC density per DC^{32,37} (H.Z., B. Jin, S.E.H., A. Perelson, U.H.v.A. and A.K.C., unpublished data). We propose that phase one constitutes a period of signal integration allowing T cells to ‘decide’ how to respond to the overall antigen dose encountered.

What is the function of the antigen environment in T cell priming? Coordinated changes in T cell interaction dynamics with antigen-presenting DCs, consistent with our three-phase model, have been noted by other groups studying CD4⁺ and CD8⁺ T cells^{20,21,38,39}. Such studies have provided clues that multiphase interactive programs may be modified by antigen context. For example, when antigen is presented by tolerogenic DCs²⁰ or in the presence of the coinhibitory molecule CTLA-4 (ref. 38), T cells do not transition to phase two. Another study exploring anergy induction has shown that T cell activity depends on the potency of the peptide; only high-potency pMHC complexes induce calcium-dependent T cell deceleration. Thus, in certain tolerance-inducing settings, T cells maintain transient contacts without transitioning to phase two. Multiphoton microscopy has also been used to study the effect of antigen dose and APC density on P14 T cell interactions with DCs in explanted lymph nodes¹⁵. That study showed that DCs pulsed with high-dose antigen (1 μM M-peptide) form large clusters with T cells (indicative of phase two) and induce full proliferation of P14 cells, in contrast to results obtained with low-dose antigen (30 pM M-peptide). Our results have confirmed and refined those observations and provide quantitative information on how antigen dose influences the character of T cell activation.

T cell activation seems to require very few cognate DCs. Although the low frequency of DCs in our DC dilution experiments made it impractical to obtain statistically meaningful MP-IVM data, we can extrapolate from our observations with higher DC densities to propose a mechanism in which at low DC abundance, a T cell must scan a larger volume to find cognate APCs. However, as T cells migrate, they may encounter draining sinusoids through which they can egress from the lymph node and, if not activated by 20 h, 90% of T cells will have left a given lymph node⁷. Thus, although DCs pulsed with large amounts of cognate antigen probably promote tight adhesion of T cells after only a few contacts, T cells presumably need more contacts to form long-lasting conjugates with DCs bearing small numbers of cognate pMHC complexes and may leave the lymph node before this happens.

It remains unclear how many pMHC complexes are needed to induce T cell activation *in vivo*⁴⁰. *In vitro* work with effector T cells has shown that single TCR–pMHC contacts yield calcium flux, about 10 pMHC complexes are required for immune synapse formation^{41,42}, and cytotoxic T lymphocytes can lyse target cells with three cognate pMHC complexes⁴³. To estimate the number of pMHC complexes required for T cell activation, we estimated here that naive CD8⁺ T cells interacted with DCs presenting fewer than 4×10^3 cognate pMHC complexes. The true numbers are probably lower because our calculations assumed that all available H-2D^b molecules on a given peptide-pulsed DC had their endogenous self-peptide replaced. A saturating amount (such as 10 μM) of M-peptide might approach this extreme, but this seems unlikely for the threshold activating dose (200 pM). We can extrapolate from the similar effects on T cell activity

exerted by DCs pulsed with 200 pM M-peptide and those pulsed with 10 μ M C-peptide, and the fact that the TCR affinity for M-peptide and C-peptide is similar²⁸, to conclude that at 24 h after DC injection (around the time of phase-two initiation), there were approximately the same number of cognate pMHC complexes remaining on DCs initially pulsed with 200 pM M-peptide or 10 μ M C-peptide (H.Z., B. Jin, S.E.H., A. Perelson, U.H.v.A. and A.K.C., unpublished data). We can therefore calculate that DCs pulsed with 200 pM M-peptide probably start with about 480 pMHC complexes at the time of DC pulsing and retain about 60 pMHC complexes 18 h later (H.Z., B. Jin, S.E.H., A. Perelson, U.H.v.A. and A.K.C., unpublished data). Given that this is probably an overestimation of pMHC complexes, T cells can be activated by very few pMHC complexes.

With only dozens to a few thousand cognate pMHC complexes per DC, how many cognate pMHC complexes would a T cell encounter in a given interaction? The surface area of an immature DC can be estimated on the basis of its volume⁴⁴ to be about 500 μ m², and the surface area of the T cell–DC contact area is about 50 μ m² (ref. 45). Immature DCs pulsed with 10 μ M C-peptide presented about 127 cognate pMHC complexes at 18 h, whereas DCs pulsed with 10 μ M M-peptide presented about 3,133 cognate pMHC complexes. Thus, the average number of antigenic TCR ligands that may be experienced immediately after T cell transfer per contact region is about 12.7 or about 313 for DCs pulsed with 10 μ M C-peptide or M-peptide, respectively. These numbers should decrease further as the peptides dissociate from H-2D^b, and although very small, they are in agreement with *in vitro* estimates^{41,42}. Given that pMHC complexes tend to cluster in immune synapses and such synapses may include noncognate pMHC complexes, we may be underestimating the number of pMHC complexes in contact zones. On the basis of *in vitro* experiments with cognate pMHC molecules on lipid bilayers, it has been suggested that TCR microclusters initiate signaling^{46,47}. Our imaging studies cannot resolve the issue of whether there are any *in vivo* microclusters of TCRs on the T cell or pMHC complexes on the DC. However, given that transition to phase two (and subsequent T cell proliferation) is stimulated by very small numbers of cognate pMHC complexes, if microclusters do form, they most likely involve endogenous ligands.

The physiological importance of pMHC half-life for T cell immunity is unclear. Most MHC class I peptides are synthesized by APCs and are continually replenished. However, DCs can also cross-present MHC class I antigens from exogenous sources⁴⁸. If a peripheral tissue DC engulfs pathogen-derived material and carries it to a draining lymph node, the antigenic cargo is limited⁴⁹. Moreover, when DCs receive maturation signals, they substantially reduce endocytosis and focus on moving MHC molecules to the surface¹². Thus, migrant DCs that enter lymph nodes may initially present a broad repertoire of peptides, which ‘skews’ toward the longest-lived peptides over time. Prolonged phase-one interactions provide T cells with a strategy for finding optimal APCs when cognate pMHC complexes are limited or have short half-lives.

An important unresolved issue is whether and how cognate T cell–DC contacts are counted and ‘remembered’. Activation could require a single, full signal from one APC if there is no cellular ‘memory’ of previous interactions. In this case, the phase one–type interactions could reflect contacts in which T cells fail to hit the ‘sweet spot’ on DCs. We consider this unlikely for many reasons. First, even if there were only a few sites on DCs that supported tight T cell binding, the high frequency of contacts in our experiments should have allowed some T cells to rapidly transition to phase two. The delayed and coordinated timing of phase two, particularly at low antigen densities, is inconsistent with this idea. Second, we have shown that the time

necessary for antigen-specific T cells to transition to phase two while interacting with DCs pulsed with a threshold dose of antigen shrunk considerably when a second population of DCs pulsed with a high dose of antigen was also present. This supports the idea of signal integration by T cells: as T cells undergo serial interactions with DCs pulsed with both high- and low-dose peptide, they accumulate incremental activation signals until they reach a threshold that triggers stable interactions. The antigen density on the DC with which phase-two interactions are subsequently initiated seems to be irrelevant, as long as the overall signal is sufficient. Third, imaging experiments have shown that even transient encounters trigger persistent antigen-dependent calcium fluxes, regardless of whether T cells are in contact with DCs, which indicates that TCR signaling does not necessarily result in instant arrest⁵⁰. In addition, with the loss of pMHC complexes over time there should be a reduction in the frequency of suitable sites for T cell binding; however, there is an increase in tight contacts at late time points. Finally, *in silico* modeling has shown that memory of previous cognate encounters substantially improves the efficiency of T cell responses to small amounts of antigen (H.Z., B. Jin, S.E.H., A. Perelson, U.H.v.A. and A.K.C., unpublished data).

Which has the most influence on the ‘decision’ to engage in tight interactions, T cells or DCs? Our data support the idea that T cells have the main function. First, the length of phase one was modulated by antigen dose even with a fixed time for DC maturation. So, if DCs determined the onset of phase two, they would have had to determine the number and/or intensity of cognate interactions with T cells and distinguish cognate encounters. That seems unlikely, because the duration of phase one would be inversely correlated with the concentration of cognate T cells in the lymph node (although there is evidence of this type of effect on the transition from phase two to phase three³⁵). Although in published studies the number of adoptively transferred TCR-transgenic T cells have been well above physiological numbers, as cognate T cells are rare in polyclonal naive T cell populations, the rate of cognate contacts experienced by individual DCs would be low and, consequently, the hypothetical threshold signal allowing DCs to switch to phase two might not be reached before pMHC complexes dissociate or DCs perish. In addition, our finding that individual DCs simultaneously supported both phase-one and phase-two interactions with two populations of T cells of different antigen specificity also undermines the idea that DCs ‘drive’ the length of phase one. However, these data do not preclude the possibility that DCs contribute to the quality and strength of cognate interactions (perhaps by reorganizing pMHC complexes into microdomains⁵¹).

What are the consequences if the ‘decision’ to enter phase two is made by T cells? Any new antigen will be recognized by a small subset of T cells with varied TCR affinity. Given the inverse relationship between TCR affinity and the dose of antigen needed to activate a T cell^{10,11}, although high-affinity T cells may need only a few encounters with antigen-presenting DCs to commit to activation, T cells with intermediate or low affinities must somehow ‘decide’ whether to be recruited into the ongoing immune response or ignore the weak signal. Phase one provides a mechanism that allows migrating T cells to ‘measure’ the overall abundance of antigens and make an informed ‘decision’ about how to respond.

METHODS

Mice. Male C57BL/6 mice were from Taconic Farms; congenic C57BL/6 (CD45.1⁺) mice were from Jackson Laboratories and were used at 6–10 weeks of age. OT-I and P14 mice, which carry a transgenic TCR specific for ovalbumin amino acids 257–264 (SIINFEKL) in H-2K^b and LCMV gp33–41 (KAVYNEATC) in H-2D^b, respectively, were from Taconic Farms. All

experiments were in accordance with National Institutes of Health guidelines and were approved by the Committees on Animal Care and Use of both Harvard Medical School and the Immune Disease Institute.

Peptides. C-peptide (LCMV gp33–41; KAVYNFATC), M-peptide (KAVYN-FATM) and SIINFEKL (New England Peptides) were resuspended in filtered deionized H₂O. L-selectin-specific monoclonal antibody (Mel-14) was grown and purified according to standard procedures or was from BD Pharmingen.

Cells. DCs were purified by positive immunomagnetic cell sorting (about 98% CD11c⁺; Miltenyi Biotec) as described⁵² from spleens of donor C57BL/6 mice that had been implanted with a mouse melanoma cell line secreting the ligand for the receptor tyrosine kinase Flt3. CD8⁺ T cells from lymph nodes and spleens of P14 and OT-I mice were purified by negative immunomagnetic cell sorting (about 98% CD8⁺; Miltenyi Biotec).

MP-IVM. DCs were pulsed with various concentrations of C-peptide, M-peptide or SIINFEKL and were labeled for 20 min at 37 °C with 20 μM CMTMR (5-(and 6-)-(4-chloromethyl)benzoyl) amino)tetramethylrhodamine; Invitrogen) or 10 μM CMAC (7-amino-4-chloromethylcoumarin; Invitrogen). DCs (5 × 10⁵) in 20 μl Iscove's modified Dulbecco's medium (with 10% (vol/vol) FCS) containing 10 ng *Escherichia coli* LPS (Sigma) were injected into the right hind footpads of recipient mice. One population of T cells was labeled for 15 min at 37 °C with 2.5 μM CFDA (5-chloromethylfluorescein diacetate; Invitrogen) and the other population was labeled for 25 min at 37 °C with 10 μM CMAC. Cells of each subset were injected at a dose of 5 × 10⁶ cells into recipient tail veins at 18 h after DC injection. OT-I T cells, rather than polyclonal T cells, were used as control cells to prevent the presence of antigen-reactive cells in the control population; the control cells provided a means of assessing if the surgical preparation had affected baseline T cell motility. After 2 h, mice received 100 μg Mel-14 intravenously. The right popliteal lymph node was prepared microsurgically for MP-IVM on a BioRad 2100MP system as described¹⁶. For multiphoton excitation and second-harmonic generation, a MaiTai Ti:sapphire laser (Spectra-Physics) was tuned to 800 nm to balance excitation of the various fluorescent probes used. For four-dimensional analysis of cell migration, stacks of 11 square *x-y* sections with 4 μm *z* spacing were acquired every 15 s with electronic 'zooming' to 3–4× and a ×20 objective (0.95 numerical aperture) to provide image volumes 40 μm in depth and 206 μm in width with a Lasersharp 2000. Emitted light and second-harmonic signals were detected through 450/80-nm, 525/50-nm and 630/120-nm band-pass filters with non-descanned detectors for the generation of three-color images. Sequences of image stacks were transformed into volume-rendered, four-dimensional time-lapse movies with Volocity software (Improvision). Cell centroids in three dimensions were measured by semiautomated cell tracking with Volocity (Improvision) and new software written in MATLAB programming language (Mathworks) was used for further computational analysis. In total, five mice were used for experiments with 100 μM C-peptide and 10 μM C-peptide, seven mice were used for experiments with 100 pM M-peptide, eight mice were used for experiments with 200 pM M-peptide, two mice were used for experiments with 1 nM M-peptide and 100 nM M-peptide, one mouse was used for experiments with 500 pM M-peptide, and five mice were used for experiments with 10 μM M-peptide.

Quantification of H-2D^b. H-2D^b was measured on freshly prepared CD11c⁺ immature DCs at various dilutions of phycoerythrin-conjugated anti-H-2D^b (KH95) or phycoerythrin-conjugated mouse IgG2b isotype control (555058; BD Pharmingen). DCs (1 × 10⁴ to 2.5 × 10⁵ cells per well, with consistent results) were blocked for 30 min on ice with 10% (vol/vol) mouse serum and then were washed and blocked for 30 min on ice with Fc block (1 μg/100 μl) and were washed again. They were stained for 1 h on ice with antibody at various dilutions (0.2–20 μg per 100 μl), then were washed and then assayed by flow cytometry. The absolute number of H-2D^b molecules was calculated by comparison of the mean fluorescent intensity (MFI) of DCs stained with phycoerythrin-KH95 to a calibration curve derived from Quantibrite beads with known number of phycoerythrin fluorophores (BD Pharmingen)³⁰.

Quantification of pMHC half-life. Recombinant P14 TCRs in multimers were used to assess cognate pMHC on 5T33 cells⁵³. For this, 5T33 cells at a density of

3 × 10⁵ cells per well in 96-well plates were incubated for 3 h at 37 °C with 10 μM M-peptide, C-peptide or control peptide (SIINFEKL) in the presence of peptide-loading enhancer (Altor BioSciences), a PBS-based buffered solution that substantially enhances peptide loading onto various MHC class I molecules on APCs⁵⁴. Cells were washed in RPMI medium with 10% (vol/vol) FCS and were incubated for 0–9 h at 37 °C to allow peptide dissociation. The remaining pMHC complexes were assessed by being stained for 1 h at 4 °C with 1.25 μg P14 TCR tetramer in 62.5 μl PBS with 2.5 mM EDTA and 1% (wt/vol) BSA. Cells were then washed and were stained for 15 min at 25 °C with fluorescein isothiocyanate–annexin V (BD Pharmingen) for identification of apoptotic cells. Additional annexin V buffer at 4 °C was added and cells were kept on ice, then binding of TCR tetramers to annexin V–negative cells was assessed by flow cytometry. For accurate computation of peptide half-life in H-2D^b, the annexin V staining step (at 25 °C) must be taken into account. During incubation at 25 °C, TCR tetramers are lost from the APC surface. Therefore, the subsequent fluorescence 'readout' of the amount of bound TCR underestimates the number of pMHC that remain on the surface. Thus, using the data in **Figure 2b** directly without correcting for this effect would underestimate the half-life of the stability of the pMHC. The **Supplementary Methods** online show how we derived the formulas that account for this effect and explain how we used these considerations to estimate the true pMHC complex half-life for C-peptide and M-peptide.

In vivo cytotoxicity assay. CD45.1⁺ recipients received DCs pulsed with 10 μM SIINFEKL (or no DCs) in the left rear footpad and received DCs pulsed with 200 pM or 10 μM M-peptide (or DCs pulsed with 10 μM SIINFEKL) in the right footpad, followed 18 h later by intravenous injection of 5 × 10⁶ P14 T cells. B cells were incubated for 15 min at 25 °C with 0.1 μM CFSE (carboxyfluorescein diacetate succinimidyl diester; CFSE^{lo}) or 2 μM CFSE and 10 μM M-peptide (CFSE^{hi}) in RPMI medium with 10% (vol/vol) FCS. The two B cell populations were mixed at a ratio of 1:1 and 2 × 10⁷ total cells were injected intravenously. At 6 h after B cell transfer, the ratio of peptide-pulsed B cells (CFSE^{hi}) and unpulsed B cells (CFSE^{lo}) was determined by flow cytometry in single-cell suspensions of popliteal lymph nodes.

DC dilution. In each experiment, 5 × 10⁵ freshly purified CD11c⁺ DCs were injected subcutaneously with 10 ng LPS (Sigma) into each rear footpad of a CD45.1⁺ recipient mouse. All DCs injected into the left footpad were pulsed with cognate antigen (10 μM or 200 pM M-peptide, as an internal positive control). For the right footpad injection, 0–10% of the DCs injected were pulsed for 1 h at 37 °C with cognate antigen (10 μM or 200 pM M-peptide) and the rest were pulsed for 1 h at 37 °C with 10 μM control peptide (SIINFEKL). DCs pulsed with cognate antigen were stained for 20 min at 37 °C with CMTMR and were mixed with the appropriate number of unlabeled SIINFEKL-pulsed DCs to achieve mixtures of 100%, 10%, 1%, 0.1% and 0%. Labeling of the antigen-pulsed DCs with CMTMR made it possible to confirm the appropriate DC dilution in each lymph node in assays of the T cells by flow cytometry. The appropriate mixture of DCs was injected into the appropriate footpad and 5 × 10⁶ naive P14 CD8⁺ T cells (labeled for 15 min at 25 °C with 2.5 μM CFSE) were injected intravenously 18 h later. After 48 h, both popliteal lymph nodes from each mouse were collected and each lymph node was dissected into a 96-well plate with PBS containing 1% (wt/vol) BSA and 2.5 mM EDTA. Lymph node cells at a density of 1 × 10⁷ cells per ml were restimulated for 3 h at 37 °C with anti-CD3 (1 μg/ml; 145-2C11; BD) in RPMI medium containing 10% (vol/vol) FCS and secretion of IFN-γ was measured by surface capture (Miltenyi Biotec). Proliferation was measured by CFSE dilution.

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

ACKNOWLEDGMENTS

We thank T. Buschman for assistance with programming; M. van den Broek for providing cDNA constructs for the P14 TCR; and G. Cheng and J. Alton for technical and secretarial assistance, respectively. Supported by the National Institutes of Health (AI069259 and AI072252 to U.H.v.A.; HL07623 and Medical Scientist Training Program to S.E.H.; HL066987 to T.R.M.; PO1 AI071195-01 for H.Z., M.N.A. and A.K.C.).

AUTHOR CONTRIBUTIONS

S.E.H. designed the study, did and analyzed experiments, and wrote the manuscript; U.H.v.A. designed the study and wrote the manuscript; T.R.M., I.B.M., A.P., M.P.F., B.S. and T.J. did experiments; B.L. and H.C.W. provided reagents; and M.N.A., H.Z. and A.K.C. modeled the experimental data.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/natureimmunology/>.

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