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Real-Time Analysis of Calcium Signals during the Early Phase of T Cell Activation Using a Genetically Encoded Calcium Biosensor

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Proper T cell activation is promoted by sustained calcium signaling downstream of the TCR. However, the dynamics of calcium flux after stimulation with an APC in vivo remain to be fully understood. Previous studies focusing on T cell motility suggested that the activation of naive T cells in the lymph node occurs in distinct phases. In phase I, T cells make multiple transient contacts with dendritic cells before entering a phase II, where they exist in stable clusters with dendritic cells. It has been suggested that T cells signal during transient contacts of phase I, but this has never been shown directly. Because time-dependent loss of calcium dyes from cells hampers long-term imaging of cells in vivo after antigenic stimulation, we generated a knock-in mouse expressing a modified form of the Cameleon fluorescence resonance energy transfer reporter for intracellular calcium and examined calcium flux both in vitro and in situ. In vitro, we observed transient, oscillatory, and sustained calcium flux after contact with APC, but these behaviors were not affected by the type of APC or Ag quantity, but were, however, moderately dependent on Ag quality. In vivo, we found that during phase I, T cells exhibit weak calcium fluxes and detectable changes in cell motility. This demonstrates that naive T cells signal during phase I and support the hypothesis that accumulated calcium signals are required to signal the beginning of phase II. *The Journal of Immunology*, 2016, 196: 1471–1479.

A hallmark of the adaptive immune response is T cell activation by APCs. T cells are activated when their TCR binds to cognate peptide: MHC complexes on the surface of APCs. The ligation of the TCR initiates a cascade of intracellular signaling events including calcium mobilization, MAPK activity, and activation of the NF- κ B pathway. These pathways converge to promote the autocrine secretion of IL-2, which promotes T cell proliferation and differentiation into effector cells, a critical arm of the adaptive immune response. Thus, understanding the regulation of events downstream of the TCR and their functional consequences is of vital importance.

One of the earliest detectable signaling events is a rise in intracellular calcium that occurs within seconds of TCR engagement. This rise of calcium occurs initially from the depletion of calcium stores in the endoplasmic reticulum, which then stimulates store-operated

calcium entry from the extracellular space. Increased intracellular calcium activates the phosphatase calcineurin, resulting in dephosphorylation of NFAT and its translocation to the nucleus. However, changes in intracellular calcium can also occur after engagement of other receptors on the surface of the T cell (1). The quality and duration of calcium signals significantly impact proliferation and cytokine production. Notably, artificially induced calcium oscillations increase NFAT activation and IL-2 production, especially if the overall calcium elevation is low (2). Studies of T cells activated in vitro suggest that oscillations, as well as overall intracellular calcium concentrations, may control cytokine production in effector T cells (3).

Intravital two-photon microscopy has revealed that events concerning T cell activation in vivo may be more complex (4–6). In vitro, T cells rapidly form stable complexes with APCs and immediately begin exhibiting changes in calcium levels. In vivo, T cells make multiple transient contacts with the APC before arresting their mobility to make long stable contacts (6–8). Von Andrian and colleagues have labeled the transient stage as phase I and the stable stage as phase II (6).

Initially, there was controversy about the existence of phase I because it was seen in some models and not in others. Current evidence indicates that the quantity of Ag is inversely correlated to the length of phase I (9). One potential explanation is that multiple T cell interactions with the APC are required to generate a productive interaction especially when Ag abundance is low. Alternatively, von Andrian and colleagues (9) proposed that T cells accumulate signals during multiple transient contacts and then make long, stable contacts once they have reached a certain threshold of signal.

Whether T cells are actively signaling during phase I interactions is not clear. This would require a sensor that would allow signaling to be detected in vivo by two-photon imaging. Parker and colleagues (10) imaged calcium flux using dye-labeled CD4⁺ T cells to examine the dynamics of early signaling events in the lymph

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Abbreviations used in this article: BMDC, bone marrow–derived DC; BMDM, bone marrow–derived macrophage; DC, dendritic cell; GECl, genetically encoded calcium indicator; LLO, listeriolysin; mCameleon, modified form of Cameleon; OVAp, OVA peptides 257–264.

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node. In their study, they were mainly focused on phase II interactions and used an Ag dose that exhibited a short phase I (~50 min). Their study clearly shows that the initiation of stable interactions during phase II is associated with calcium spikes. Although this study did not specifically focus on phase I interactions, it reported cells fluxing calcium after disengagement from the APC (10).

In this study, we sought to focus specifically on whether signaling occurs during phase I. We reasoned that if transient contacts between naive T cells and dendritic cells (DCs) were generating signals, induced signaling events should be detectable. In contrast, if productive interactions were of low probability and stochastic, no statistically significant signaling would be evident during phase I interactions. Our strategy entailed monitoring calcium flux as a surrogate for evidence of TCR engagement *in vivo*. Other studies have used calcium-sensitive dyes (10, 11); however, imaging using these methods is limited by time, because these dyes tend to leak out or be actively exported out of the cells. The use of genetically encoded calcium indicators (GECI) circumvents this issue.

Although widely used in other fields like neuroscience, only a few studies have used GECIs in T cells. One group introduced a fluorescence resonance energy transfer (FRET)-based GECI into activated TCR transgenic cells *in vitro* and then imaged calcium responses to Ag after transfer into mice (12, 13). They could detect calcium fluxes and oscillations after Ag administration *in vivo*. Another group studied calcium flux of T follicular helper cells in the germinal center response using GCaMP3 (14). They found that the magnitude of calcium signaling induced by the T follicular helper cell–B cell interactions in the lymph node was related to Ag quantity. Notably, these previous studies using GECIs analyzed preactivated or differentiated T cells.

Cameleon is a FRET-based sensor that takes advantage of the calcium dependence of calmodulin binding to the M13 peptide (15). In this study, we generated inducible knock-in mice using a modified form of Cameleon (mCameleon) in which the acceptor fluorophore, YFP, was replaced by a brighter variant, Ypet (16). We used these knock-in mice to monitor calcium flux in naive T cells. We were able to observe distinct calcium patterns *in vitro* including transient, sustained, and oscillatory, as has been previously reported for effector cells. We then showed by peptide titration that the biosensor was sensitive to low concentrations of peptide. After administration of Ag-loaded DCs, we measured calcium fluxes during phase I interactions. We found that calcium fluxes were low but increased in the presence of Ag-loaded DCs. Importantly, these fluxes occurred when T cells were not in direct contact with the Ag-loaded DCs. This supports the idea that transient interactions of naive T cells with DCs induce weak signals that are accumulated over time to initiate phase II.

Materials and Methods

Mice

All mice were housed under specific pathogen-free conditions in the Washington University animal facilities with the approval of the Washington University Animal Studies Committee. OT-1 Rag1^{−/−} mice were provided by Dr. H. Virgin (Washington University, St. Louis, MO). 5CC7, LLO118, and LLO56 TCR-transgenic mice (17) were provided by Dr. P. Allen (Washington University). B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J used for purification of CD11c⁺ cells were originally obtained from The Jackson Laboratory.

Generation of mCameleon reporter mice

The cDNA coding for mCameleon (16) was inserted into the pBS31 targeting vector cells under the control of the CMV minimal promoter containing tetracycline-responsive operator binding sequences (18). The vector, together with the pCAGGS-FLPe-puro vector, was used to transfect the KH2 embryonic stem cell line (harboring the *Rosa26*^{M2-rtTA} allele), as previously described (18). After electroporation, embryonic stem cells were selected with hygromycin, and genomic DNA from individual clones was subject to SpeI digestion and Southern blot using the *Col1* 3′ probe. Laser-assisted

injection of selected ES cell clones into eight-cell embryos was performed to generate chimeric mice, which were bred for germline transmission of the targeted *tetO-mCameleon* allele and the *Rosa26*^{M2-rtTA} allele.

To induce mCameleon expression in mice, we administered doxycycline (2 g/l; Sigma) in drinking water supplemented with 10 g/l sucrose starting 2 wk after birth unless otherwise stated.

Retroviral transduction of naive T cells

For retroviral transduction of activated T cells, viral supernatants were prepared by transfection of Plat-E packaging cells with 30 μg pMX-mCameleon plasmid using Lipofectamine 2000 (Invitrogen), and viral supernatant was collected 48 and 72 h after transfection. 5CC7 T cells were purified from spleens by negative selection using Dynabeads Untouched Mouse CD4 Cells Kit (Invitrogen), and purity was checked by flow cytometry. T cells were cultured in IMDM supplemented with 10% FBS (Hyclone) in the presence of B10.Br irradiated splenic cells and 5 μM peptide specific for TCR-transgenic T cells. After 24 and 48 h of stimulation, retroviral supernatant was added to the T cell cultures and spun for 45 min at 1800 rpm at 25°C in the presence of Lipofectamine 2000 (Invitrogen) and 125 U/ml IL-2. Five days after activation, transduced activated cells were used for *in vitro* imaging experiments.

Generation of bone marrow–derived macrophages and DCs

Femurs and tibias from four to eight-week-old C57BL/6J and B10.Br mice were manually flushed to harvest bone marrow cells, and RBCs were lysed in ACK lysis buffer. Cells were cultured in complete DMEM containing 20% of L929 cell-conditioned medium (containing M-CSF) for 8 d to obtain bone marrow–derived macrophages (BMDMs). Alternatively, to generate bone marrow–derived DCs (BMDCs), we cultured bone marrow cells in medium containing murine GM-CSF (1000 U/ml) for 8 d. DC and macrophage yield was determined by flow cytometry.

Confocal microscopy and FRET analysis

In vitro–generated BMDMs or BMDCs were stimulated with IFN-γ (250 U/ml) and loaded with 10 μM of the following peptides (unless otherwise stated): wild-type and mutated OVA peptides 257–264 (OVAp), listeriolysin peptides 190–205, and moth cytochrome *c* peptides 88–103; all the peptides were gifts from P. Allen (Washington University). The cells were allowed to adhere overnight to eight-well coverglass chambers (Lab-Tek). Before imaging, wells were washed in Ringers imaging solution (150 mM NaCl, 10 mM glucose, 5 mM HEPES, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂). For naive T cells, T cells were purified from TCR-transgenic *tetO-mCameleon*; *Rosa26*^{M2-rtTA} mice treated with doxycycline by negative selection using Dynabeads Untouched Mouse CD4 Cells Kit (Invitrogen). Cells expressing high levels of Cameleon were sorted with a FACSARIA II sorter (BD Biosciences). For activated T cells, after negative selection, the cells were stimulated for 5 d *in vitro* with irradiated splenocytes (2000 rad) in the presence of 10 μM specific peptide and 1 μM doxycycline, before flow cytometry cell sorting of mCameleon^{high} cells. Cameleon-expressing T cells were added in Ringer's imaging solution right before imaging started. Time-lapse movies were acquired every 15 s by using an Olympus FV1000 confocal microscope in a humidified temperature-controlled chamber (37°C). The cells were excited at 440 nm, and differential interference contrast images, as well as the donor and acceptor emission, were detected simultaneously using a 510-nm beamsplitter and two photomultipliers with optical filters: 465–495 nm (CFP) and 535–565 nm (Ypet). Images were analyzed using Imaris Bitplane Software and MetaMorph (Molecular Devices, Sunnyvale, CA). The Ypet/CFP ratio is displayed on a pseudocolor scale, with calculations done on randomly selected cells. To calculate the proportion of cells that were displaying calcium flux, we divided the number of cells that fluxed calcium by the total number of mCameleon-positive cells that were observed in the imaged fields over the course of the experiments.

Proliferation assay

T cells purified from spleen and lymph nodes of doxycycline-treated *tetO-mCameleon*; *Rosa26*^{M2-rtTA} mice were loaded with 5 μM Cell Trace Violet (Molecular Probes) according to manufacturer's instructions. T cells were stimulated with anti-mouse CD3 (2C11, 5 μg/ml) and anti-mouse CD28 Abs (37.51, 2 μg/ml) in the presence of irradiated CD45.1⁺ splenocytes (2000 rad). After 48 and 72 h, T cells were stained and proliferation was assessed by flow cytometry.

In vivo migration assay

T cells purified from spleen and lymph nodes of C57BL6/J (Ly5.2) were loaded with 5 μM CMTPIX (Molecular Probes) according to manufacturer's instructions and mixed at a 1:1 ratio with T cells purified from

doxycycline-treated *tetO-mCameleon*; *Rosa26*^{M2-rtTA} mice. The cells were injected i.v. into congenic CD45.1⁺ mice. Twenty-four hours after transfer, spleen and lymph nodes of recipient mice were harvested and made into single-cell suspension, and donor T cell migration was assessed by flow cytometry by gating on CD45.2⁺CD3e⁺ cells.

Flow cytometry

Single-cell suspensions were generated from the indicated organs followed by lysis of RBCs with ACK solution. After Fc receptor blocking with anti-CD16/32 (2.4G2), cells were stained with the following Abs obtained from BD Biosciences or Biolegend: anti-mouse CD8-allophycocyanin-Cy7 (53-6.7), anti-mouse CD8-PECy7 (53.6-7), anti-mouse CD4-PECy7 (RM4-5), anti-mouse CD3-allophycocyanin (145.2C11), anti-mouse CD3-Pacific blue (17A2), anti-mouse CD19-PE (6D5); anti-mouse CD45.1-PECy7 (A20); anti-mouse CD45.2-allophycocyanin (104); anti-mouse CD11c-allophycocyanin (N418), anti-mouse CD11b-BV421 (M1/70). FACS analyses were performed on a FACSCalibur or a FACSCanto II (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

DC isolation and immunization

DCs were isolated from spleens of CAG-mRFP1 mice using EasySep Mouse CD11c Positive Selection Kit with spleen dissociation medium (Stemcell Technologies) according to manufacturer's instructions. Purity was assessed by flow cytometry (>90%). Isolated DCs were loaded with 10 μ M OVAp for 2 h at 37°C. A total of 1×10^7 DCs were injected with 50 ng LPS into the footpad of recipient mice.

Two-photon laser scanning microscopy

Fifteen hours after transfer of OVAp-loaded DCs, wild-type mice were injected with Cameleon-expressing OT1⁺ T cells purified from spleen and lymph nodes. Two to 4 h later, excised popliteal or inguinal lymph nodes were placed in a flow chamber and maintained at 37°C by perfusion with RPMI 1640 bubbled with a mixture of 95% O₂ and 5% CO₂. Time-lapse imaging was performed with a custom-built two-photon microscope, fitted with two Chameleon Ti:sapphire lasers (Coherent) and an Olympus XLUM-PlanFl 20 $\times$ objective (water immersed; numerical aperture, 0.95), and controlled and acquired with ImageWarp (A&B software). For imaging of Cameleon, the excitation wavelength was 850 nm; signals from the second harmonic, CFP, YPet, and CMTMR, were separated by dichroic mirrors (458, 510, and 560 nm). To create time-lapse sequences, we typically scanned volumes of tissue of $250 \times 225 \times 50 \mu\text{m}$ (X,Y,Z; 2.5- μm Z steps) at ~30-s intervals for up to 60 min. Multidimensional rendering and manual cell tracking were done with Imaris (Bitplane), and statistical analysis was performed with GraphPad Prism.

Results

mCameleon is a sensitive calcium biosensor in T cells

To measure calcium flux in T cells, we used an mCameleon, a genetically encoded FRET-based calcium biosensor. The mCameleon construct that we used is composed of CFP (the FRET donor) and YPet (the FRET acceptor) that are linked by calcium-binding calmodulin and calmodulin-binding peptide M13 (16). In the absence of calcium, the M13 peptide has low affinity for apo-calmodulin, and the excitation of CFP at a 440-nm wavelength does not lead to energy transfer to YPet. The binding of calcium to calmodulin increases its affinity toward the M13 peptide, promoting close approximation of CFP and YPet, resulting in FRET after CFP excitation.

A robust calcium sensor should be able to report physiologically relevant changes in intracellular free calcium concentrations. To assess the viability of mCameleon as a T cell calcium sensor, we determined the sensitivity of mCameleon through in vitro calibration experiments. The plasma membrane was permeabilized and incubated in a range of calcium concentrations. We found that the mCameleon could reliably detect calcium concentrations from 50 nM to 2 μ M (data not shown), well within the range of calcium concentrations that occur in the T cell: 100 nM to 1 μ M (1). Next, we tested mCameleon sensitivity in T cells contacting APCs, a key event in T cell priming and activation. We retrovirally transduced T cells from 5CC7 TCR-transgenic mice with mCameleon and imaged them 5 d later upon restimulation with APCs. We observed that when

T cells made contacts with APCs, there was a decrease in CFP emission and an increase in YPet emission when CFP was excited, resulting in a change in the FRET ratio (Fig. 1A, 1B).

Interestingly, the YPet/CFP emission ratio often remained elevated for several minutes after the initial increase (Fig. 1B). This result could reflect a sustained increase in calcium flux, or more trivially, lack of reversible FRET of the mCameleon reporter. The addition of EDTA to chelate calcium while T cells exhibited elevated YPet/CFP emission ratio immediately decreased FRET (Fig. 1C), indicating sustained intracellular calcium. Strikingly, long imaging sessions revealed that elevated YPet/CFP emission ratio could be detected up to 5 h after the initial increase (Fig. 1D). Because calcium dyes leak out of the cell over a period of minutes to hours, the use of mCameleon allowed us to assess calcium signaling at late time points in vitro for the first time, to our knowledge.

mCameleon expression in vivo in doxycycline-inducible mCameleon mice does not affect T cell development, proliferation, or homing

Understanding the molecular events required for T cell priming requires reporters to be introduced into naive T cells. Retroviral transduction, a common method for genetic manipulation of T cells, requires their activation and proliferation. Consequently, this protocol precludes analysis of calcium flux with GECIs in naive T cells. To circumvent this issue, we generated doxycycline-inducible mCameleon knock-in mice. We used KH2 embryonic stem cells, which carry a *Rosa26*^{M2-rtTA} allele and a modified *Col1A1* allele that promotes Flp-assisted recombination (18). We targeted a construct containing a tetracycline-responsive element upstream of the mCameleon cDNA to the *Col1A1* locus (referred to as the *tetO-mCameleon*).

Doxycycline administration of 2-wk-old *tetO-mCameleon*; *Rosa26*^{M2-rtTA} mice induced expression of mCameleon in the spleen and the thymus (Fig. 2A, 2B), as well as in lymph nodes, liver, kidney, pancreatic acini, and kidney tubules (data not shown). Expression was high in all thymocyte subsets (Fig. 2C), whereas only ~20–30% of peripheral T cells expressed a high level of mCameleon (Fig. 2D). Similarly, high levels of expression were achieved in only a fraction of B cells and myeloid cells. This partial expression is likely due to epigenetic silencing of the collagen I locus that occurs in mature T and B lymphocytes (20). mCameleon expression did not affect T cell development, as T cell numbers were normal in the thymus and the spleen (data not shown). mCameleon-expressing cells also demonstrated normal proliferation in vitro after anti-CD3 and anti-CD28 stimulation (Fig. 2E), and normal homing to spleen and lymph nodes after transfer in vivo (Fig. 2F). Altogether, these data demonstrate that mCameleon can be expressed in naive T cells and that its expression does not substantially alter their biology.

Calcium patterns are heterogeneous during the activation of naive T cells

To validate the mCameleon construct, we measured calcium after stimulating naive T cells in vitro. Diverse calcium signaling patterns have been described previously in preactivated and polarized T cells (3, 19); however, data regarding naive T cells are lacking. Therefore, we imaged naive mCameleon⁺ T cells isolated from TCR-transgenic mice while they interacted with Ag-loaded BMDMs in vitro. We observed three different calcium patterns after the initial calcium increase: 1) a calcium spike followed by a return to baseline in a few minutes (transient), 2) a calcium flux that remains elevated for a long period (sustained), or 3) a calcium flux that oscillates for a sustained period (oscillating) (Fig. 3A). In the sustained pattern, we found that intracellular calcium levels often stayed elevated for

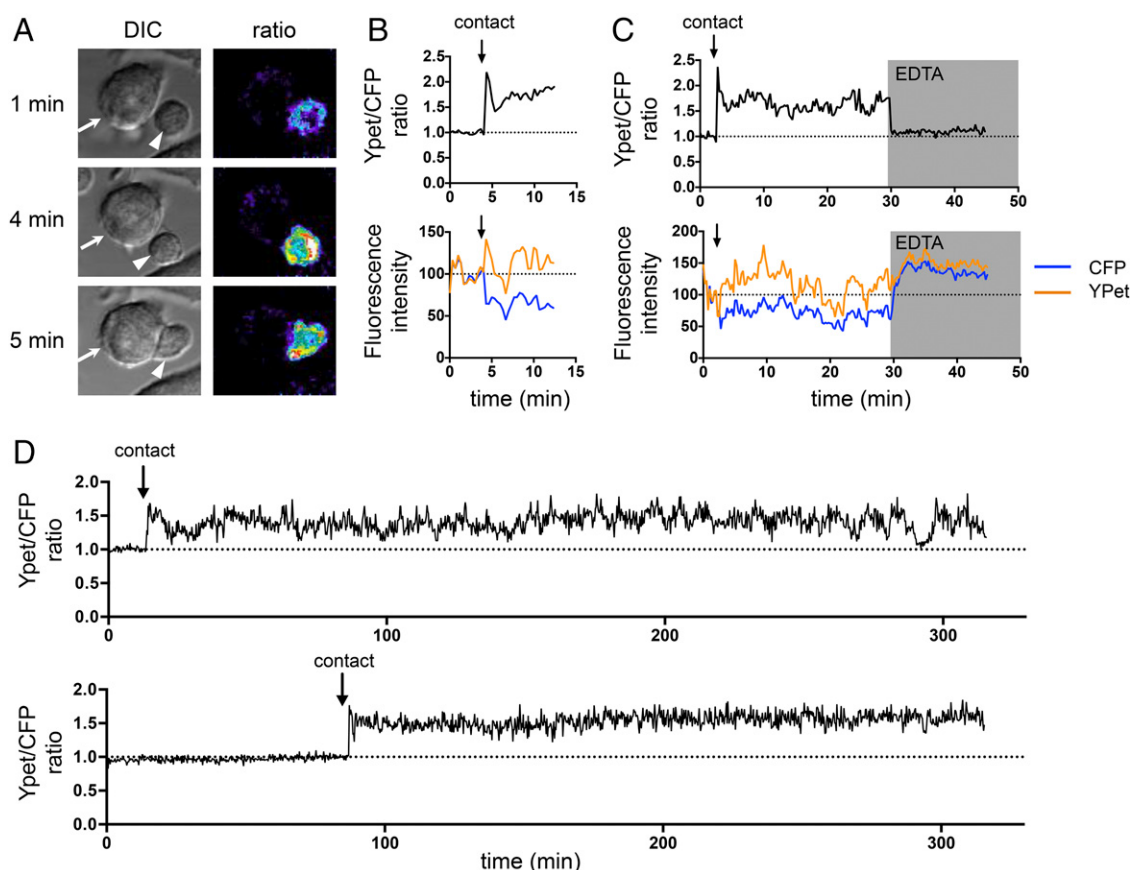


FIGURE 1. Calcium signaling in mCameleon-transduced T cells in vitro. 5CC7 TCR transgenic T cells were activated in vitro and transduced with mCameleon. After 5 d, they were added to moth cytochrome *c* peptide 88–103 (MCCp)-loaded BMDMs and imaged by confocal. **(A)** Differential interference contrast and YPet/CFP emission fluorescence ratio of one T cell (arrowheads) interacting with one BMDM (arrows) at different time points after the initial contact (original magnification $\times 400$). The ratio was color coded from purple for low ratio to red for high ratio. **(B)** YPet/CFP emission fluorescence ratio and YPet (orange line) and CFP (blue line) fluorescence intensity of the T cell shown in **(A)**. Arrows indicate the initial contact. **(C)** Thirty minutes after the addition of T cells to BMDMs, EDTA was added to the culture. **(D)** YPet/CFP emission fluorescence ratio of two representative T cells over 6 h after the addition of T cells to BMDMs.

several hours (Fig. 1D). Interestingly, T cells expressing the same TCR could display all three patterns of calcium flux. To determine whether the calcium signaling patterns could be influenced by the TCR, we bred *tetO-mCameleon*; *Rosa26*^{M2-rtTA} to four different TCR transgenics. We found that the distribution of calcium signaling patterns was specific to the TCR with different TCRs favoring different patterns (Fig. 3B). For example, T cells expressing the OT-1 and 5CC7 TCRs displayed mainly sustained calcium patterns, whereas LLO118 and LLO56 TCR-transgenic T cell stimulation displayed all three patterns equivalently. We also found that the T cells exhibiting the sustained pattern had the highest peak ratio compared with transient and oscillatory; however, there were no differences in T cell motility at subsequent time points after APC contact (Fig. 3C–E).

Calcium patterns are independent of Ag quantity but moderately dependent on Ag quality

Sustained and oscillating calcium patterns have been suggested to influence the efficiency of downstream TCR signaling events (21). Furthermore, it has been demonstrated that stable contacts (synapses) with APCs promote stronger TCR signals in vivo, and that migratory contacts (kinapses) elicit either weak or strong TCR signals (22). Therefore, we tested whether the calcium patterns were related to TCR signal strength and/or the type of contact (synapse or kinapse).

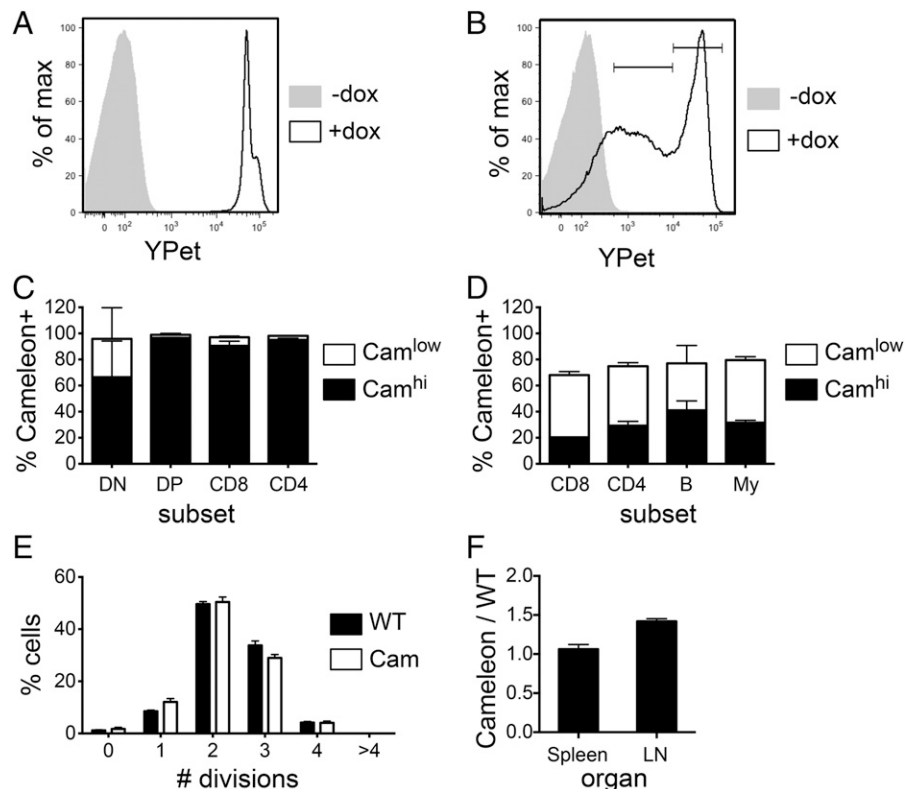
To test whether sustained calcium patterns were associated with stronger TCR signals, we used three different strategies. To ex-

amine the role of Ag dose, we varied the peptide load on APCs. Lower peptide dose diminished the number of T cells displaying calcium flux (Fig. 4A); however, among the cells that responded, the proportion of cells with sustained, oscillating, or transient calcium patterns did not change (Fig. 4B, 4C). This result suggests that the Ag quantity affects the percentage of cells that will respond, but not the magnitude of their response.

Next, we focused on the issue of Ag quality. We used the OT-1 system, which has well-established altered-peptide ligands: N4, Q4, and V4, single-residue variants of the SIINFEKL OVA_{257–264} peptide. N4 has the strongest affinity and induces the strongest TCR signals, whereas V4 has the weakest affinity and elicits only weak TCR signals. Furthermore, N4 is associated with stable contacts, whereas V4 associates with migratory contacts and Q4 can induce both types of contacts (22). Therefore, if sustained patterns were associated with stronger TCR signaling, we predicted that fewer cells would exhibit sustained signaling when OT-1 T cells recognized V4-loaded APCs. We found that the proportion of cells with various calcium patterns was somewhat different among the three peptides, with slightly but significantly fewer cells displaying sustained calcium patterns with the Q4 and V4 peptides compared with the N4 peptide (Fig. 4D). Further analysis revealed a modest increase in the peak of calcium flux with N4 peptide compared with Q4 and V4 peptide (Fig. 4E), suggesting formation of a more stable contact.

The extent of T cell activation depends not only on peptide–MHC interactions but also on the nature of the APC. In vivo, DCs

FIGURE 2. mCameleon expression in transgenic mice does not affect T cell development, proliferation, or homing. (**A** and **B**) mCameleon expression was assessed by flow cytometry in the thymus (**A**) and spleen (**B**) of *tetO-mCameleon*; *Rosa26^{M2-rtTA}* mice treated (black line) or not (gray histograms) with doxycycline (dox) for 3 wk. (**C** and **D**) Expression of mCameleon in thymocyte (**C**) and splenocyte (**D**) subsets (My = CD11b⁺). Black bars and white bars represent high- and low-expressing cells, respectively, as gated in (**B**). (**E**) Proliferation of mCameleon-expressing (white bars) or wild-type (black bars) T cells stimulated in vitro with anti-CD3 + anti-CD28 Abs for 48 h. (**F**) Equal numbers of CMTPX-loaded wild-type T cells and mCameleon-expressing T cells were cotransferred i.v. to congenic hosts. Twenty-four hours later, the numbers of transferred mCameleon and wild-type T cells recovered in the spleen and peripheral lymph nodes was assessed by flow cytometry.



play a pivotal role in the stimulation of naive T cells, whereas other APCs such as macrophages and B cells play a secondary role (23). We hypothesized that DCs might induce more stable calcium patterns than macrophages. However, incubation of LLO118 T cells displayed similar calcium patterns whether they were stimulated with BMDCs or BMDMs (Fig. 4F, 4G). Altogether, Ag quantity or the nature of the APC did not affect calcium patterns that were

induced during activation of naive T cells, whereas Ag quality had a modest impact.

Mature DCs promote T cell calcium flux

Naive T cells interact with DCs in lymphoid organs in a transient fashion for a period that is related to the amount of Ag present on the surface of the DC. At the end of this transient phase (phase I),

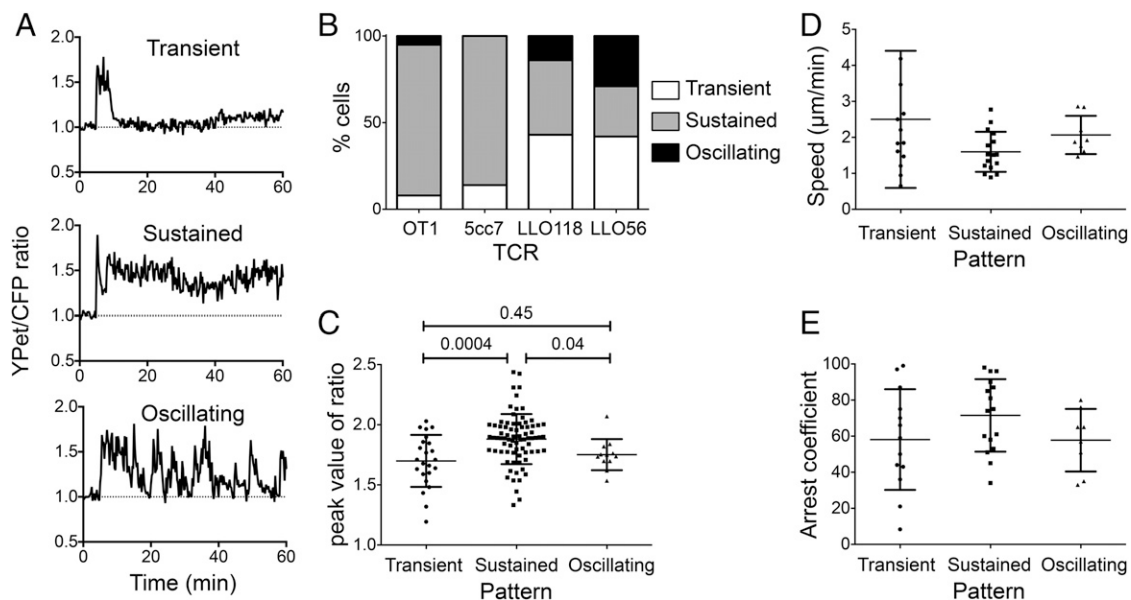
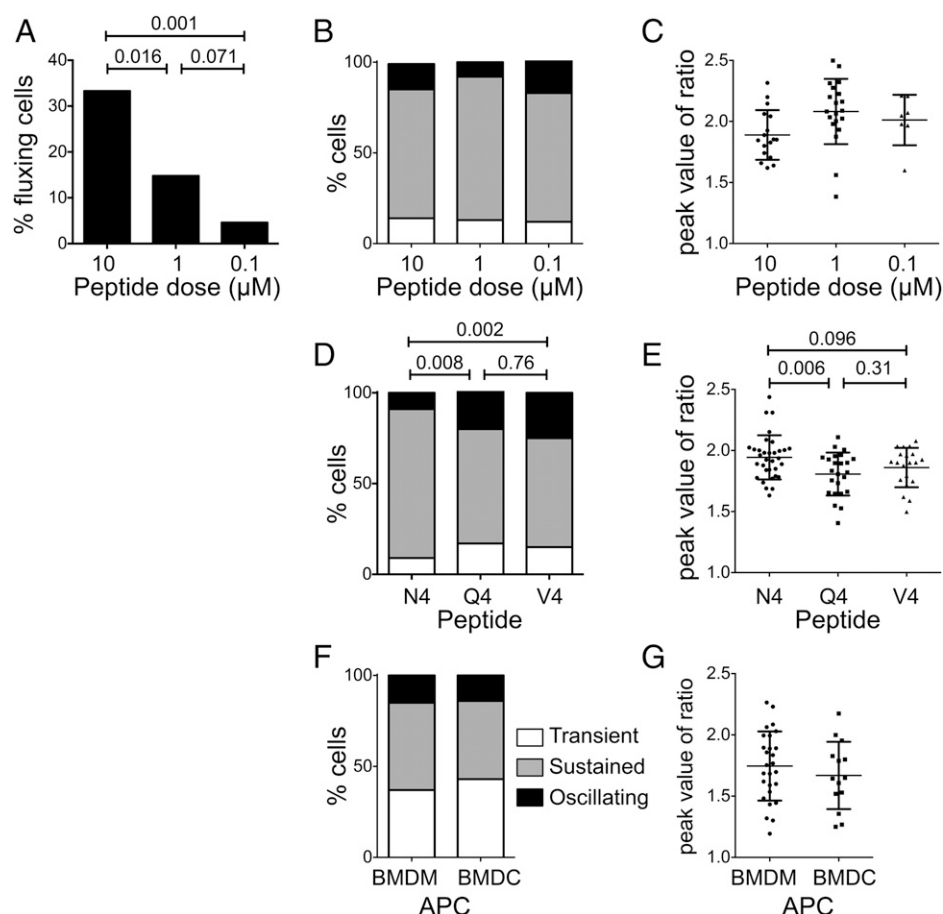


FIGURE 3. Heterogeneity of calcium signaling pattern in vitro. mCameleon-expressing TCR-transgenic T cells were purified from spleen and lymph nodes, added to BMDMs that had been loaded with peptide (10 μ M), and imaged by confocal microscopy. (**A**) YPet/CFP fluorescence emission ratio of T cells displaying transient (*top*), sustained (*middle*), or oscillating (*bottom*) calcium patterns after the initial calcium concentration increase. (**B**) Proportion of T cells displaying transient (white), sustained (gray), or oscillating (black) calcium patterns in different TCR-transgenic populations. (**C**) Intensity of the initial calcium concentration increase, expressed as the YPet/CFP fluorescence emission ratio at the peak of the initial calcium concentration increase divided by the YPet/CFP fluorescence emission ratio before contact. The *p* values of Student *t* test are indicated. (**D** and **E**) Average velocity and arrest coefficient of T cells from 5 to 30 min after the initial calcium increase (not significantly different; Student *t* test, *p* > 0.05). (**C–E**) Data from all TCR-transgenic T cells have been pooled.

FIGURE 4. Calcium patterns are independent of the dose and of APCs, but moderately dependent on quality of Ag. mCameleon-expressing OT-I (**A–E**) or LLO118 (**F and G**) TCR-transgenic T cells were added to peptide-loaded BMDMs or BMDCs, and imaged by confocal microscopy. Proportion of total T cells displaying calcium flux (A), T cells displaying transient (white), sustained (gray), or oscillating (black) calcium patterns (B, D, and F), and intensity of the initial calcium concentration increase, expressed as in Fig. 3C (C, E, and G), from pooled data from two to three experiments. (A–C) BMDMs were loaded with 10, 1, or 0.1 μ M OVA-peptide (SIINFEKL). (D and E) BMDMs were loaded with 10 μ M SIINFEKL (N4), SIIQFEKL (Q4), or SIIVFEKL (V4) peptides. (F and G) BMDMs or BMDCs loaded with 10 μ M listeriolysin-peptide were used as APCs. The p values of χ^2 test (A, B, D, and F) or Student t test (C, E, and G) are indicated if $p < 0.05$.



naive T cells make stable contacts with the DC that last for a period of hours (phase II). An important unresolved issue is whether T cells are signaling during phase I or whether the increased length of phase I when Ag is low is due to a requirement for multiple contacts before an antigenic peptide is recognized by the T cell.

To this end, mCameleon⁺ OT-I T cells were transferred into C57BL/6J hosts in the absence of Ag. Explanted lymph nodes were imaged 2–4 h later by two-photon microscopy. To measure changes in intracellular calcium with Ag recognition, we added

SIINFEKL peptide to the perfusion medium. This induced rapid T cell motility arrest and a sustained increase in the YPet/CFP emission ratio that was detectable with doses as low as 100 nM (Fig. 5). These results validated the sensitivity of the mCameleon knock-in T cells for calcium imaging studies in vivo.

To characterize calcium fluxes in naive T cells during phase I interactions with DCs, we purified CD11c⁺ cells from CAG-mRFP1 mice, loaded them with SIINFEKL peptide, and injected them s.c. into the footpad of WT mice. Fifteen hours later, mCameleon⁺ OT-I

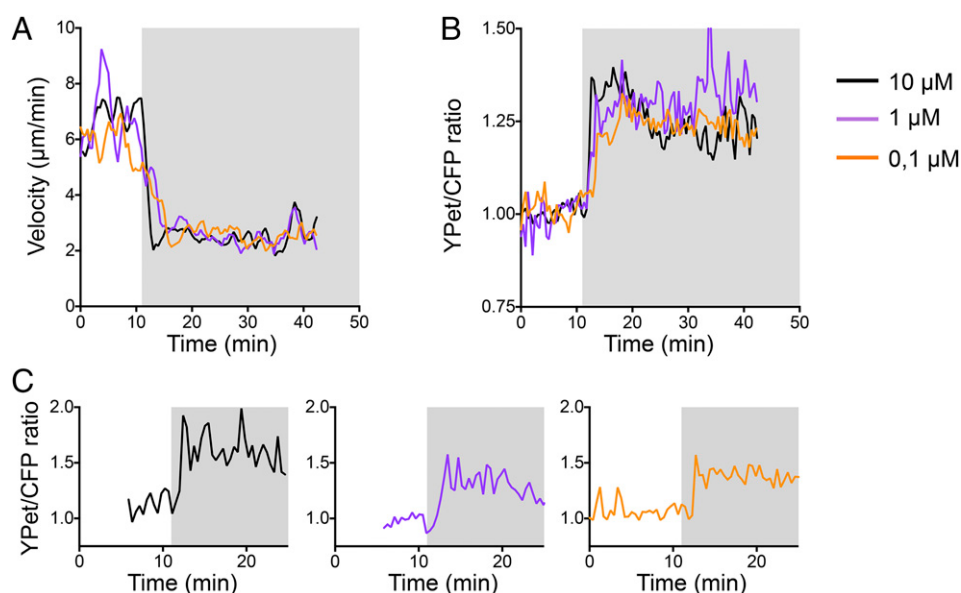


FIGURE 5. Sensitivity of mCameleon in vivo. Lymph nodes of mice that received a transfer of mCameleon-expressing OT-I TCR-transgenic CD8⁺ T cells were explanted and imaged by 2P. After 11 min of imaging, 10 μ M (black), 1 μ M (purple), or 100 nM (orange) of OVA-peptide was added to the lymph nodes. Presence of OVA-peptide is indicated by gray shading. (**A and B**) Average velocity (A) and YPet/CFP fluorescence emission ratio (B) of 15–30 cells/group. (**C**) Ratio of single cells.

cells were transferred i.v. Two to four hours later, draining and nondraining lymph nodes were harvested and imaged by two-photon microscopy. At this time point, few, if any, of the T cells had arrested their movement, and most made only short contacts with DCs (average length of contact: 5.7 min), confirming that with this peptide dose and at this time point, the T cells were in the phase I period (Fig. 6A, 6B).

To determine whether there was an effect on T cells when Ag-bearing DCs were present, we measured both cell motility and calcium. Motility measurements showed that the velocity of the T cells when peptide-loaded DCs were present was slower than the velocity of T cells in the absence of transferred DCs (Fig. 6E). Imaging of multiple single cells, however, did not demonstrate any cells with a clearly detectable change in FRET ratio when T cells contacted DCs (Fig. 6C, 6D), and this is consistent with previous studies using calcium dye-labeled T cells. Averaging multiple cells, however, demonstrated that intracellular calcium concentrations were clearly elevated in T cells when Ag-loaded DCs were present, compared with T cells in nondraining lymph nodes

(Fig. 6F). Specifically, a clear and significant proportion of T cells displayed an elevated intracellular calcium concentration in draining lymph nodes (Fig. 6G). Comparing mCameleon⁺ OT-1 T cells between the draining and the nondraining lymph node also showed that the calcium levels had a much broader distribution as compared with the nondraining lymph node where the calcium signal was relatively homogeneous (Fig. 6H). Importantly, increased calcium concentration was not dependent on ongoing T cell–DC contacts, because T cells undergoing contact with DCs did not display elevated FRET or decreased velocity compared with T cells that were not in contact with DCs (Fig. 6E), and because cells with elevated calcium did not correlate with decreased velocity (Fig. 6I, 6J). These results suggest that during the phase of transient interactions with DCs, T cells flux calcium, but at levels that are much lower than occur when stable contacts are formed.

Discussion

Although it is clear that TCR ligation results in calcium flux, the exact nature of this phenomenon in vivo remains elusive. What is

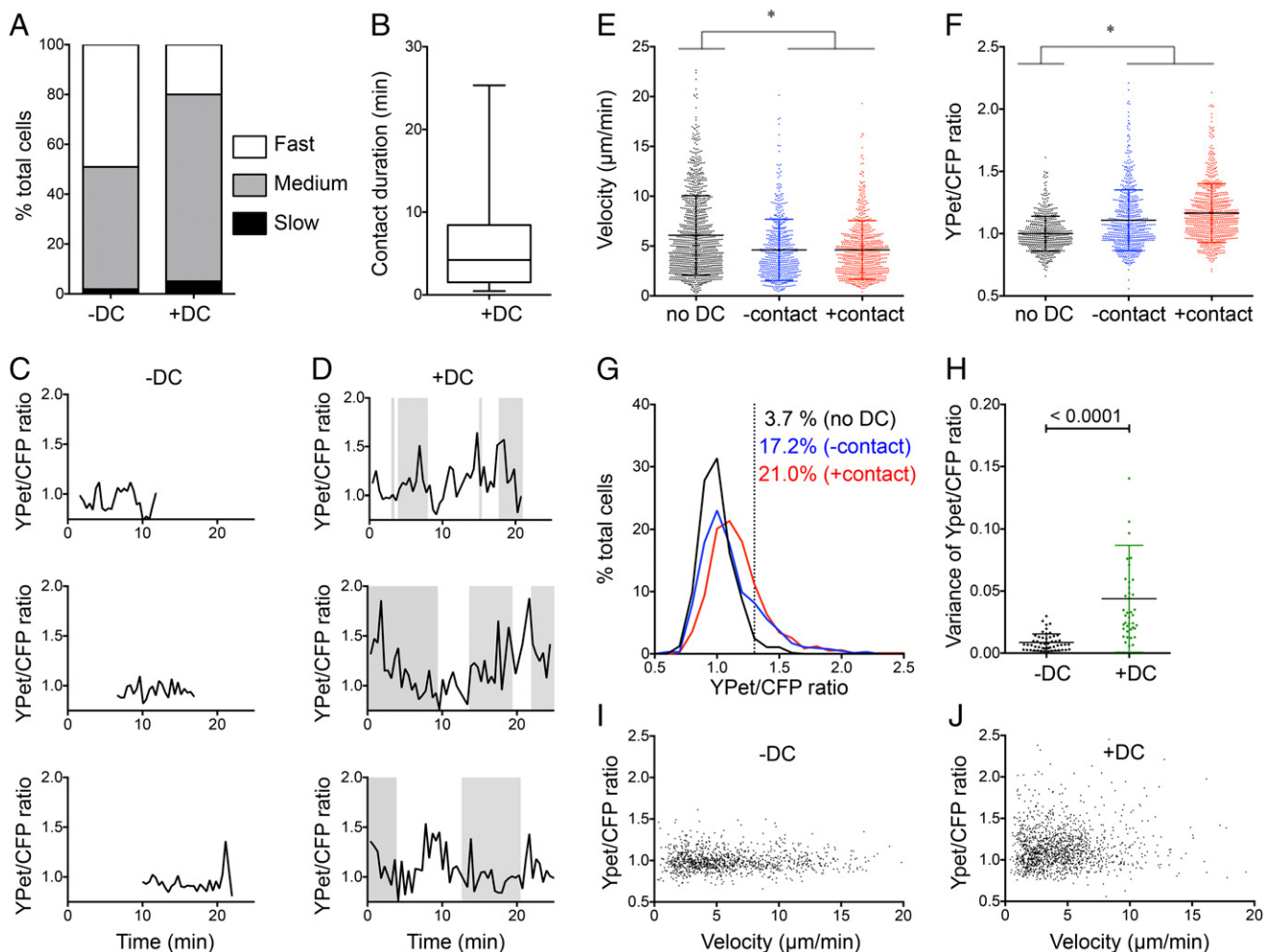


FIGURE 6. Ag-induced calcium signals in vivo. mCameleon-expressing OT-1 TCR-transgenic CD8 T cells were transferred to mice 15 h after the injection of Ova-peptide-loaded DCs in the right footpad. Two to 4 h later, popliteal lymph nodes were explanted and imaged by 2P. **(A)** Proportion of cells exhibiting slow (<2.5 $\mu\text{m}/\text{min}$), intermediate (2.5–6 $\mu\text{m}/\text{min}$), or fast (6 $\mu\text{m}/\text{min}$) average speed in the presence (right popliteal lymph node) or absence (contralateral lymph node) of Ag-loaded DCs. **(B)** Duration of T cell/DC contacts. **(C)** and **(D)** YPet/CFP fluorescence emission ratio over time of single cells in lymph nodes without **(C)** or with **(D)** DCs. Gray regions indicate contact between T cells and DCs. **(E)** and **(F)** Instantaneous velocity **(E)** and YPet/CFP fluorescence emission ratio **(F)** of T cells in contact (red) or not (blue) with DCs in the right popliteal lymph node, and in the absence of DCs in the contralateral lymph node (black). Each dot represents one cell at one time point. Asterisks are shown when $p < 0.0001$ (ANOVA with post hoc Tukey test). **(G)** Frequency distribution of ratio [color code as in **(A)**]. Percentages indicate the proportion of T cells with a ratio >1.3 . **(H)** Variance of YPet/CFP ratio of T cells in lymph nodes with or without DCs. Each dot represents one cell. The p value of Student t test is indicated. **(I)** and **(J)** YPet/CFP ratio over instantaneous speed in the absence **(I)** or presence **(J)** of DCs (Pearson's correlation of linear regression, $r^2 = 0.004$).

clear, from a variety of different experimental methods, ranging from studying T cells in suspension to in vivo imaging, is that the nature of calcium flux is context dependent. Thus, to understand the nature of T cell activation and the role of calcium signaling, it is important to perform experiments in the appropriate physiologic context.

Intravital imaging of T cells loaded with calcium dyes allows for measurement of calcium flux, optimally for 1–2 h before leakage becomes an issue. This precludes imaging calcium responses that occur later during the adaptive immune response. The use of GECIs that are stably integrated into the genome allows potentially for long-term calcium imaging of T cells. Previous studies used retroviral transduction of activated T cells to express GECIs, precluding its use for naive T cells (12, 13). We solved this problem by generating a knock-in mouse expressing the mCameleon FRET reporter under the control of a tetracycline-responsive promoter.

We found that the expression of the mCameleon reporter did not substantially alter the biology of T cells and was a sensitive indicator of calcium flux at both early and late time points. We showed that we could measure calcium fluxes in vitro continuously for up to 5 h. It had been previously reported that T cells exhibit different patterns of calcium flux, and it had been suggested that these different patterns might be associated with distinct outcomes for T cell activation. Using a variety of different TCRs, we found that the proportion of cells exhibiting a given pattern was dependent on the specific TCR. This bulk behavior of T cells from a specific TCR transgenic was not affected by the type of APC or the quantity of Ag, and was moderately dependent on the quality of the Ag.

Our main goal was to examine the nature of TCR signaling by naive T cells in lymphoid organs. In vitro, T cells begin signaling within seconds of making contact with peptide-loaded APCs. However, in vivo, the nature of TCR signaling appears to be more complex. The biology of naive T cells when they enter a lymph node is to first make multiple transient interactions with DCs before making stable contacts. This first transient period, called phase I, was initially controversial, because some laboratories did not see phase I interactions. Von Andrian and coworkers demonstrated that the length of the phase I period is related to Ag dose (9); using high Ag doses results in a short phase I period that is followed by T cells forming stable, long-lived interactions during the second or phase II period. Because T cells appear to synchronously enter phase II, von Andrian proposed that during the phase I period, T cells were accumulating signals from transient interactions with antigenic peptide and after a threshold level of signaling was generated, T cells stop moving and phase II begins (9). An alternative explanation is that when Ag density is low, T cells required multiple contacts with the DC before a productive interaction with cognate peptide–MHC complex occurs. We hypothesized that if our mCameleon sensor was sensitive enough to detect signaling during phase I, we could resolve this issue.

We first established that we could detect calcium influx in mCameleon⁺ OT-1 T cells using two-photon microscopy in lymph node explants by perfusing cognate peptide into the flow chamber. We observed immediate motility arrest and increased FRET after addition of as little as 100 nM peptide, indicating that the calcium sensor and T cells are highly sensitive to low levels of antigenic peptide. Surprisingly, the magnitude of calcium fluxes induced by APC contact was much less after transfer of BMDCs. The analysis of individual cells did not reveal an obvious increase in calcium during the course of a 30- to 45-min imaging period. However, increased calcium was detectable in T cells in the presence of Ag-loaded DCs compared with their absence. This result is consistent with results from Parker and coworkers, who found that the calcium signal was much weaker when T cells were stimulated by transferred DCs versus when T cells were stimulated by immunization (10).

One possible explanation is that immunization creates a generalized inflammatory environment in the lymph node, and these additional signals can synergize with the TCR to enhance calcium influx.

Parker and coworkers used calcium-sensitive dyes to examine T cell signaling in explanted lymph nodes (10). Most of their studies used immunization with adjuvant and CFSE added to Ag to stimulate and label migratory DCs. Their conditions were relatively strong as they found that naive T cells were forming clusters and fluxing calcium shortly after they began imaging, ~50 min after T cell transfer. This suggests that they were imaging in phase II. Similarly, when they transferred exogenous Ag-bearing BMDCs for imaging, they saw that most T cells had formed clusters soon after they began imaging.

We established conditions allowing us to image naive T cells during the phase I period. During our entire imaging period, T cells were transiently interacting with our labeled, transferred DCs with few, if any, T cells forming stable contacts or clusters. In addition, velocity measurements showed that T cells transiently interacting with Ag-bearing DCs were moving with similar velocities to Ag-specific T cells that were not close to any labeled DC. This implies that, although the transient interactions stimulate a small but significant increase in calcium, this level of calcium was not sufficient to have an effect on cell motility.

Our data are thus the first, to our knowledge, to focus on the transient interactions between naive T cells and DCs during the phase I period. Our data show that during this period, there is a small but discernable average increase in intracellular calcium, but no change in the velocity of the cells. Because this low signal was seen only with Ag-bearing APCs, it suggests that these motile cells have previously had at least one encounter with Ag. The low signal can be explained by the possibility that most of the interactions are nonproductive, and thus, no high spikes are seen. The low average signal is due to a previous productive encounter. Importantly, the small but detectable calcium increase in the population of cells suggests that some recognition of Ag had already occurred and that phase II interactions require at least two or more productive interactions consistent with the model of signal accumulation proposed by von Andrian (9).

This suggests that phase I interactions are a mechanism to allow naive T cells to assess the density of Ag present on the APC. The requirement for two or more productive interactions within a specific period introduces a thresholding phenomenon to activation in vivo. Unlike in vitro, where a single antigenic peptide is sufficient to activate a naive T cell, this high sensitivity in vivo might lead to poor discrimination between antigenic and nonantigenic peptides, especially if both cognate and noncognate peptides can contribute to T cell activation.

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Disclosures

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