Title

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Stable interactions and sustained T cell receptor signaling characterize thymocyte-thymocyte interactions that support negative selection[a]

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Abstract

Negative selection is one of the primary mechanisms that render T cells tolerant to self. Thymic dendritic cells play an important role in negative selection, in line with their ability to induce migratory arrest and sustained T cell receptor (TCR) signals. Thymocytes themselves display self-peptide/MHC class I complexes, and while there is evidence that they can support clonal deletion, it is not clear whether they do so directly, via stable cell-cell contacts and sustained TCR signals. Here we show that murine thymocytes can support surprisingly efficient negative selection of antigen-specific thymocytes. Further, we observe that agonist dependent thymocytethymocyte interactions occurred as stable, motile conjugates led by the peptide presenting thymocyte, and in which the trailing peptide-specific thymocyte exhibited persistent elevations in intracellular calcium concentration. These data confirm that self-antigen presentation by thymocytes is an additional mechanism to ensure T cell tolerance, and further strengthen the correlation between stable cellular contacts, sustained TCR signals, and efficient negative selection.

Introduction

The importance of T cell tolerance is evidenced by the fact that its breakdown leads to devastating autoimmune diseases. Thus, there exist several overlapping mechanisms to ensure that self-reactive T cells are eliminated or held in check. Clonal deletion of auto-reactive T cells in the thymus is a major component in the establishment of central tolerance. Early studies showed that hematopoietic cells are important for efficient deletion of self-reactive thymocytes, whereas thymic stromal cells, including thymic epithelial cells, are less effective at inducing deletion (1-4). Of the hematopoietic cells in the thymus, it is clear that dendritic cells (DCs) play a particularly important role in negative selection of self-reactive thymocytes (5, 6). We have previously shown that thymic DCs bearing negative selecting peptide provide cognate thymocytes with a strong “stop signal” and sustained TCR signaling to induce cell death, whereas presentation of the same peptide by thymic stromal cells provides a weaker “stop signal” correlating with less efficient negative selection (7). Other hematopoietic cell types, including B cells and activated T cells, have also been implicated

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in negative selection, although the efficiency of negative selection and the nature of the cellular interactions involved has not been addressed (8-13).

The most abundant cells in the thymus are thymocytes. Since murine thymocytes express MHC class I, and human thymocytes express both MHC class I and II, thymocytes potentially present self-peptides to other thymocytes and participate in their selection (14-17). It is well-established that thymocyte-thymocyte interactions promote the development of certain T cell populations. For example, Natural killer T (NKT) cell development relies on CD1d ligands expressed by cortical thymocytes (18-20). In addition, expression of MHC class II molecules by human thymocytes plays a role in positive selection of other, non-conventional, PLZF-expressing $CD4^+$ T cells (21-24). While earlier studies have also suggested that thymocyte-specific antigen can induce negative selection (25-28), the mechanism of deletion and the cellular dynamics involved in this process have not yet been addressed.

To explore the contribution of antigen-presentation by thymocytes to the development of central tolerance, we determined the efficiency of negative selection when high affinity antigen presentation is restricted to thymocytes and characterized the resulting thymocyte-thymocyte interactions. Here we describe the rather efficient negative selection supported by thymocytes bearing cognate peptide and the prolonged cell-cell contacts and sustained calcium signaling that typify these interactions.

Materials and Methods

Thymocytes and thymic slices

Mice were housed in an American Association of Laboratory Animal Care approved facility at the University of California, Berkeley, under specific pathogen-free conditions, and all procedures were approved by the Animal Care and Use Committee. Thymus tissue was harvested from wild-type, C57Bl/6 mice (Jackson Labs), $\beta2m^{-/-}$ mice (Taconic, Germantown, NY), MHC class I and II deficient Ly5.1/Ly5.2 mice (MHCko, Abb-$\beta2m^{-/-}$) (Taconic), and OT1tg $Rag2^{-/-}$ $\beta2m^{-/-}$ mice at 3-6 weeks of age or from radiation chimeras at 5-12 weeks after reconstitution where $1\text{-}5\times10^6$ OT1tg $Rag2^{-/-}$ Ly5.1 bone marrow was transferred into lethally irradiated MHCko hosts. Vibratome-cut thymic slices, 400-500μm thick, were prepared as described (29), and 1×10^6 cells of each thymocyte population were added to each thymic slice as indicated. Thymic tissue was dissociated into single cell suspensions with glass tissue homogenizers.

Cell labeling and antigen loading

Cells were loaded with Indo-1LR (TEFLabs), a ratiometric calcium indicator dye, for 90 min at 37°C, washed, and incubated an additional 60 min at 37°C prior to addition to thymic slices. Thymocytes were loaded with 2μM SNARF (Life Technologies), 1μM Cell Proliferation Dye eFluor 670, or 2μM Cell Proliferation Dye eFluor 450 (eBioscience), for 10 min at 37°C and washed three times in cDMEM. Thymocyes were incubated in the presence or absence of 1nM $OVA_{257\text{-}264}$ peptide (Anaspec) for 20 min at 37°C and washed three times to remove unbound peptide prior to addition to thymic slices.

Flow cytometry

Single cell thymocyte suspensions were incubated with a fixable viability dye eFluor506 in PBS and subsequently stained with anti-mouse antibodies to: Ly5.1-PE, Ly5.2-FITC, CD4-PE-Cy7, CD8-eFluor450, CD69-PerCP, CD24-APC (eBioscience). Data were acquired on an LSRII flow cytometer (BD biosciences) and analyzed with FlowJo software (Tree Star). Statistical significance between groups was determined by performing one-way ANOVA and Tukey post-hoc tests using Prism software.

Two-photon microscopy

Thymic slices were adhered to glass coverslips with tissue glue and maintained in oxygenated, phenol-red free DMEM media at 37°C during imaging on a custom-built upright two-photon microscope with a 20x/0.95 Olympus objective and Spectra-Physics MaiTai laser tuned to 750nm. Fluorescent signals were separated with 440 and 510nm dichroic mirrors and 400/45, 480/50, and 605/75 bandpass filters. Images were acquired every 20 sec for 20 min with 3μm z-steps over an area of 172 × 143μm using custom software. Imaging volumes were recorded at a depth of > 70μm beneath the cut site. Cell coordinates and fluorescence intensity parameters were processed using Imaris software (Bitplane Scientific Software), and data converted to flow cytometry-like files using custom *DISCit* software for further analysis with FlowJo software (Tree Star) (30). For analysis of intracellular calcium levels ($[Ca^{2+}_i]$), the ratio of Ca^{2+} bound to unbound dye intensity was calculated.

Results

Thymocytes can serve as peptide presenting cells to support efficient negative selection

To determine if thymocytes presenting agonist peptide support negative selection, we examined the development and behavior of thymocytes in the presence of antigen-bearing thymocytes within thymic slices. Thymic slices maintain the three-dimensional architecture of the thymus including an intact cortex and medulla and chemokine gradients that direct added thymic subsets to their appropriate niche (31, 32). We mixed labeled pre-selection OT1 TCR transgenic $Rag2^{-/-}$ (herein called OT1tg) $CD4^+CD8^+$ (double positive, DP) thymocytes with labeled $\beta2m^{-/-}$ thymocytes (to serve as an internal control) on non-selecting ($\beta2m^{-/-}$) thymic slices and allowed them to migrate into the tissue (Fig. 1A). Subsequently, we added wild type (WT, $\beta2m^{+/+}$) thymocytes that had been pre-loaded with the agonist $OVA_{257-264}$ (OVA) peptide to serve as antigen presenting cells (APCs). WT thymocytes without added peptide served as a control. After 3 hours, substantial CD69 up-regulation was apparent on OT1tg thymocytes in the presence of OVA-loaded thymocytes, indicative of TCR signaling (Fig. 1B). However, no significant upregulation of CD69 was detected in the presence of WT thymocytes without added peptide, though they presumably express low-affinity endogenous peptides. Moreover, no CD69 upregulation was observed when thymocytes were loaded with low potency OVA peptide variant Q7, implying that recognition of peptide on thymocytes requires high affinity TCR binding (data not shown). By 24 hours, there was a significant decrease in the proportion of OT1tg cells cultured with OVA-loaded thymocytes (Fig. 1C). By comparing the number of OT1tg thymocytes remaining in the slice relative to the internal reference population, we estimate that ~80% of

OT1tg DP thymocytes had been deleted by 24 hours (Fig. 1D). No deletion was evident when $\beta 2m^{-/-}$ thymocytes were used as APCs, confirming that negative selection was not due to carry over of OVA peptide and presentation by residual MHC class I on thymic slice-resident DCs (Fig. 1D). Surprisingly, the extent of deletion induced using thymocytes as APCs is similar to previous reports using DCs as APCs with high affinity ligands (7). The purity of the overlaid thymocyte populations suggests that the observed levels of negative selection are not due to antigen-loaded DC contamination (Fig. 1E). Thus, thymocytes can serve as APCs to present negative selecting ligands to other thymocytes on non-selecting ($\beta 2m^{-/-}$) thymic slices.

Thymocytes bearing negative selecting peptide support stable interactions

We have previously shown that high affinity peptide presentation by DCs, but not cortical thymic epithelial cells (cTECs), supports stable thymocyte interactions, sustained TCR signaling, and efficient negative selection (7). To investigate the nature of cellular interactions when thymocytes serve as peptide presenting cells, we directly examined these interactions using two-photon time-lapse microscopy in the cortex of thymic slices. We labeled OVA-loaded WT thymocytes (the APC population) with a vital dye, and OT1tg thymocytes with a ratiometric calcium indicator prior to addition to thymic slices and imaged 2-4 hours after addition of OVA loaded thymocytes. In the absence of OVA peptide, both thymocyte populations migrated freely and no thymocyte-thymocyte interactions were observed (0/199 OT1tg cell tracks). However, when WT thymocytes were pre-loaded with OVA peptide, we observed ~5% of OT1tg thymocytes (13/265) forming stable, motile conjugates with OVA-bearing thymocytes. In these conjugates, the OVA-bearing thymocyte migrated in the lead, apparently dragging an OT1tg thymocyte behind (Fig. 2A and Supp. Video 1). These interactions are reminiscent of examples of mature T-B cell contacts and cytotoxic T lymphocyte-target B cell conjugates observed within intact lymph nodes (33-35), and suggest that the OT1tg thymocytes are “arrested” in tight contact with the peptide-presenting thymocytes, while the peptide-presenting thymocytes continue to migrate.

Out of the 13 examples of OT1tg thymocytes in conjugates that we observed, 9 remained in contact during the entire time that they remained in the imaging volume (7-20 minutes), whereas 4 OT1tg thymocytes encountered a peptide-loaded thymocyte and formed a conjugate during the imaging run (Fig. 2B). We did not observe any examples of conjugate dissociation. The observation that thymocytes presenting agonist peptide support sustained interactions with cognate thymocytes and result in negative selection adds to our previous data to suggest that stable cell-cell interactions support efficient negative selection (7).

Given that only 5% of OT1tg thymocytes engaged with peptide bearing thymocytes at the 2-4 hour time point, it is surprising that 80% of OT1tg thymocytes are deleted by 24 hours. However, because the OT1tg thymocytes and the peptide presenting thymocytes each make up fewer than 1% of thymocytes in this system, the low number of interactions observed during this time period may simply reflect that time is needed for the OT1tg thymocytes to “find” peptide bearing thymocytes. This is consistent with the finding that 4/13 conjugates formed, whereas 0/13 dissociated, during the time of imaging.

Encounter with thymocytes bearing negative selecting peptide leads to sustained, low-level TCR signaling

As we have previously shown that efficient negative selection is associated with monogamous interactions and sustained TCR signaling (7), we characterized TCR signaling in individual cells engaged in thymocyte-thymocyte interactions. We observed subtle, but consistent increases in $[Ca^{2+}{}_i]$ in OT1tg thymocytes engaged in conjugates as compared with non-interacting OT1tg thymocytes (Fig. 3A). The OT1tg DP cells engaged in these long-lived thymocyte-thymocyte interactions exhibited modest, but persistent elevated $[Ca^{2+}{}_i]$ relative to non-interacting OT1tg thymocytes (Fig. 3B), and the increase in cytosolic Ca^{2+} levels was observed shortly after initial engagement (Fig. 3B, track I). Together, these results suggest that negative selection is associated with stable interactions and sustained TCR signals.

Discussion

Though a central role for DCs in negative selection of thymocytes is well established, multiple other hematopoietic cell types including B cells, activated T cells, and even thymocytes themselves are also thought to play a role in the deletion of autoreactive T cells in the thymus (5, 8-13, 25-28). Despite the diversity of cells that can support negative selection in the thymus, cTECs do not support deletion of self-reactive thymocytes even when presenting high affinity antigen (1-4, 7). One striking difference between DCs and cTECs is the inability of the later to provide a strong “stop signal” to promote stable thymocyte-cTEC interactions (7). Whether stable conjugate formation is characteristic of other cellular interactions that promote negative selection was not known. Here we show that thymocytes presenting high-affinity antigen can support efficient negative selection that is associated with stable thymocyte-thymocyte interactions and sustained Ca^{2+} signaling.

Thymocytes lack many features of professional antigen presenting cells, including expression of co-stimulatory ligands that can promote negative selection (36-40). Moreover, when confronted by broadly distributed agonist peptide, cortical thymocytes preferentially arrest adjacent to DCs, implying that DCs induce a more potent migratory stop signal compared to other peptide presenting cells, including thymocytes, in the vicinity (7). In spite of this, we find that thymocytes can support prolonged peptide-specific contacts with other thymocytes presenting high affinity ligand and induce negative selection. Interestingly, thymocytes express SLAM (Signaling Lymphocyte Activation Molecule) receptors, a family of proteins that stabilize cellular contacts via homotypic interactions and play an essential role in T cell-B cell interactions and thymocyte-driven selection of innate-like T cell subsets (41-47). It is tempting to speculate that homotypic SLAM family interactions may help to stabilize thymocyte-thymocyte contacts that drive negative selection.

Our data have relevance for the role of direct versus indirect antigen presentation during negative selection. Thymic DCs are well equipped to directly present MHC class I associated peptides derived from proteins expressed by the DCs themselves. However, for thymocyte-specific proteins, it is unclear whether peptides are presented directly by thymocytes, or whether protein or peptide-MHC complexes are transferred to DCs for presentation. Although we cannot rule out the possibility that antigen-loaded thymocytes

transfer peptide-MHC complexes to thymic DCs, the stability of thymocyte-thymocyte interactions and persistent elevated Ca^{2+} levels strongly support a direct presentation route for thymocyte-driven negative selection in this system.

For MHC class II associated antigens, direct presentation by thymocytes likely does not occur in mice given that mouse thymocytes do not express detectable levels of MHC class II. Moreover, the tolerance of CD8, but not CD4, T cells to thymocyte / T cell-specific expression of an MHC class I protein argues that neither efficient transfer of antigen to DCs, nor direct presentation of class II associated antigens by thymocytes occurs in the mouse system (25-28). However, it is interesting to consider that in humans, direct presentation by thymocytes, may play a role in both MHC class I and II tolerance, given that human thymocytes express MHC class II (14-17).

Whether self-peptide presenting thymocytes share the burden of negative selection or act more as a “safety net” during negative selection remains to be determined. Our results indicate that thymocytes themselves can support efficient negative selection, and suggests yet another layer of regulation to prevent self-reactive T cells from leaving the thymus. The thymocyte-mediated negative selection we report here may be particularly relevant for tolerance to thymocyte specific proteins, such as TCR. A number of different hematopoietic cell subsets have now been identified that can support negative selection. These cells are quite diverse and the characteristics that allow these hematopoietic cells, and not cTECs, to support the stable interactions necessary to support efficient negative selection remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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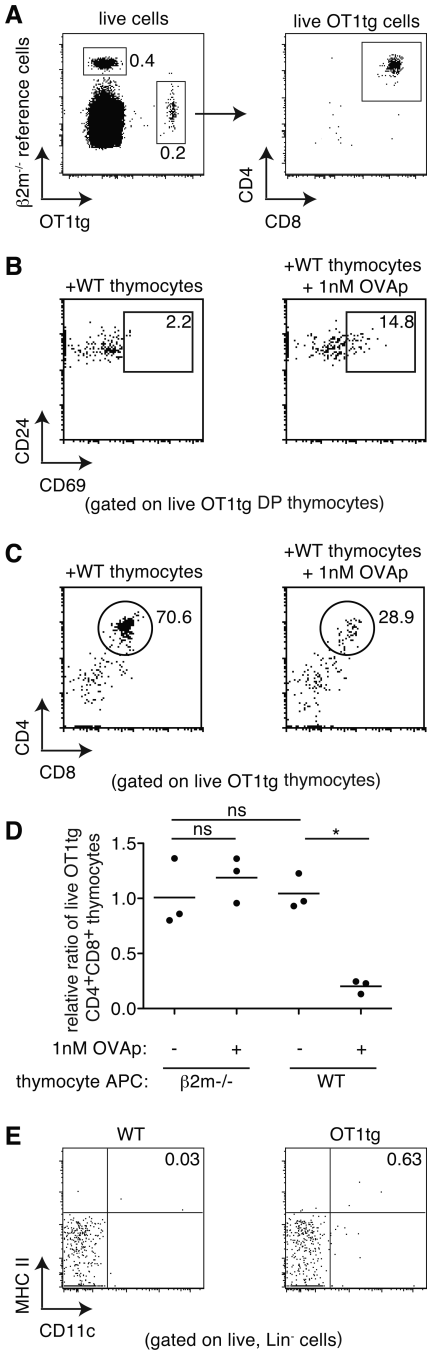

Figure 1. Thymocytes bearing agonist peptide efficiently support negative selection

Preselection cell proliferation dye eFluor670 labeled OT1tg DP thymocytes were mixed 1:1 with cell proliferation dye eFluor450 labeled, $\beta 2m^{-/-}$ thymocyte population as an internal reference, overlaid on $\beta 2m^{-/-}$ thymic slices, and allowed to migrate into the tissue for 2 hours. Thymic slices were washed, and unlabeled WT or $\beta 2m^{-/-}$ thymocytes pre-loaded with or without 1nM OVA peptide were added. After an additional 2 hours, thymic slices were washed, and thymocytes were harvested at either 3 or 24 hours after the addition of the peptide-loaded thymocyte population. (**A**) Flow cytometry gating strategy to distinguish

β2m$^{-/-}$ thymocyte internal reference cells versus OT1tg DP thymocytes from endogenous cells. (**B**) Representative flow cytometry plots of CD24 and CD69 on live, OT1tg DP thymocytes 3 hours after the addition of WT thymocytes with or without OVA peptide. (**C**) Representative flow cytometry plots of live, OT1tg DP thymocytes 24 hours after the addition of WT thymocytes with or without OVA peptide. Flow cytometry plots are representative of triplicate samples from one experiment of at least two. (**D**) Normalized ratio of live, OT1tg DP cells to internal control population 24 hours after addition of WT or β2m$^{-/-}$ thymocytes with or without OVA peptide. Each dot represents an individual thymic slice, and lines represent the mean. Data from one representative experiment of at least two. * indicates $p < 0.05$. ns, not significant (**E**) Representative flow cytometry plots of thymocyte preparations prior to addition to thymic slices. CD11c and MHC II on live, Lin^{-} (CD4, CD3, TCRβ, CD19, NK1.1) from indicated populations.

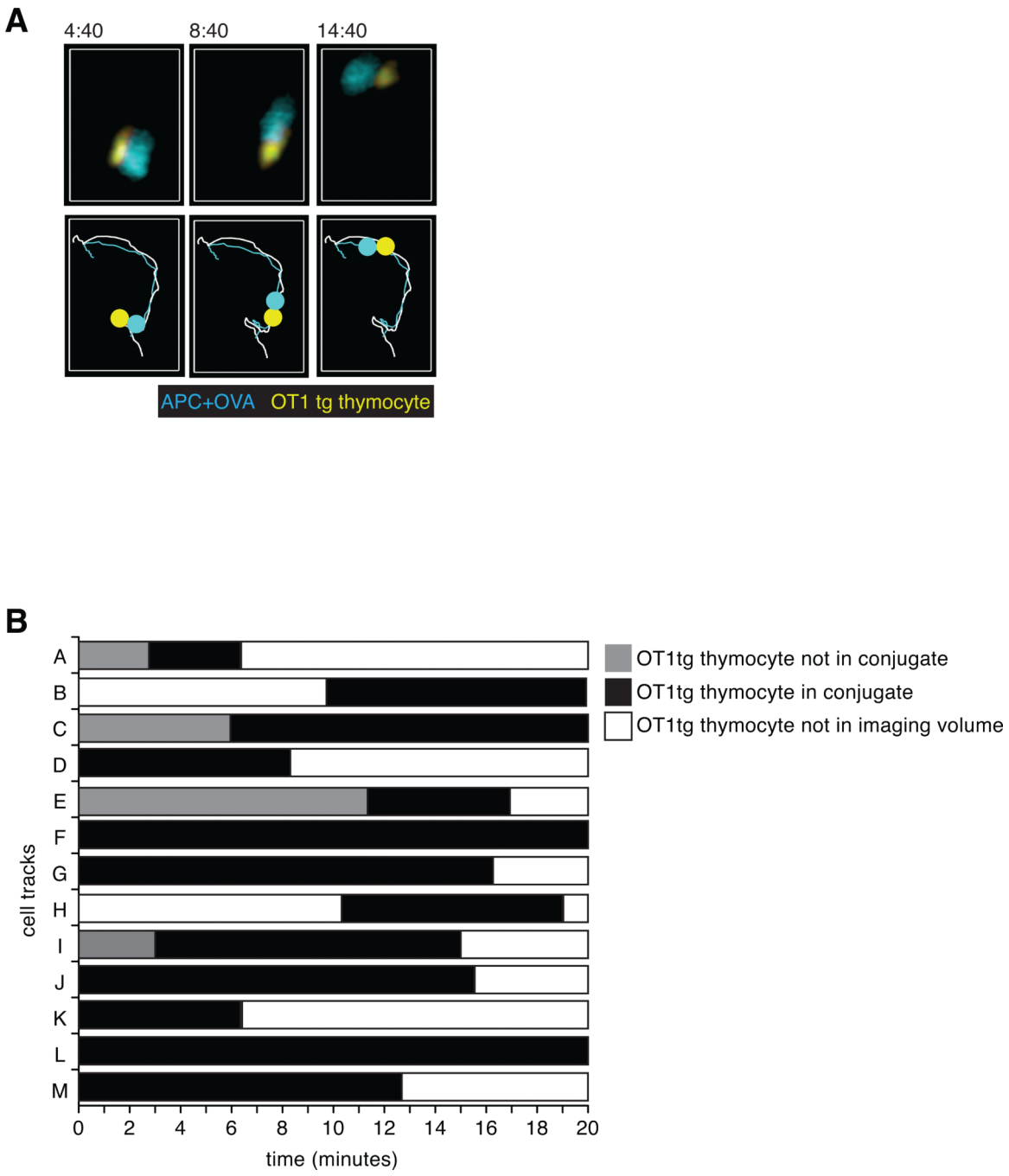

Figure 2. Thymocytes engage in prolonged, stable interactions with thymocytes presenting negative selecting peptide

(**A**) Pre-selection OT1tg DP thymocyte (yellow) were overlaid on β2m$^{-/-}$ thymic slices in the presence of WT OVA-presenting thymocytes (aqua) and imaged by two-photon microscopy. Images were recorded in the cortex as identified by the density of OT1 thymocytes and the proximity to the thymic capsule. Representative images from a time series at the times indicated are shown. Top panels, fluorescent images. Bottom panels, lines indicate cell tracks for the duration of the movie with spots overlaid to indicate the location

of each cell at the time points indicated above the top panels. (**B)** Time line indicating the duration of thymocyte thymocyte contacts of 13 interacting OT1tg thymocytes identified out of 265 OT1 cell tracks, in 3 imaging runs.

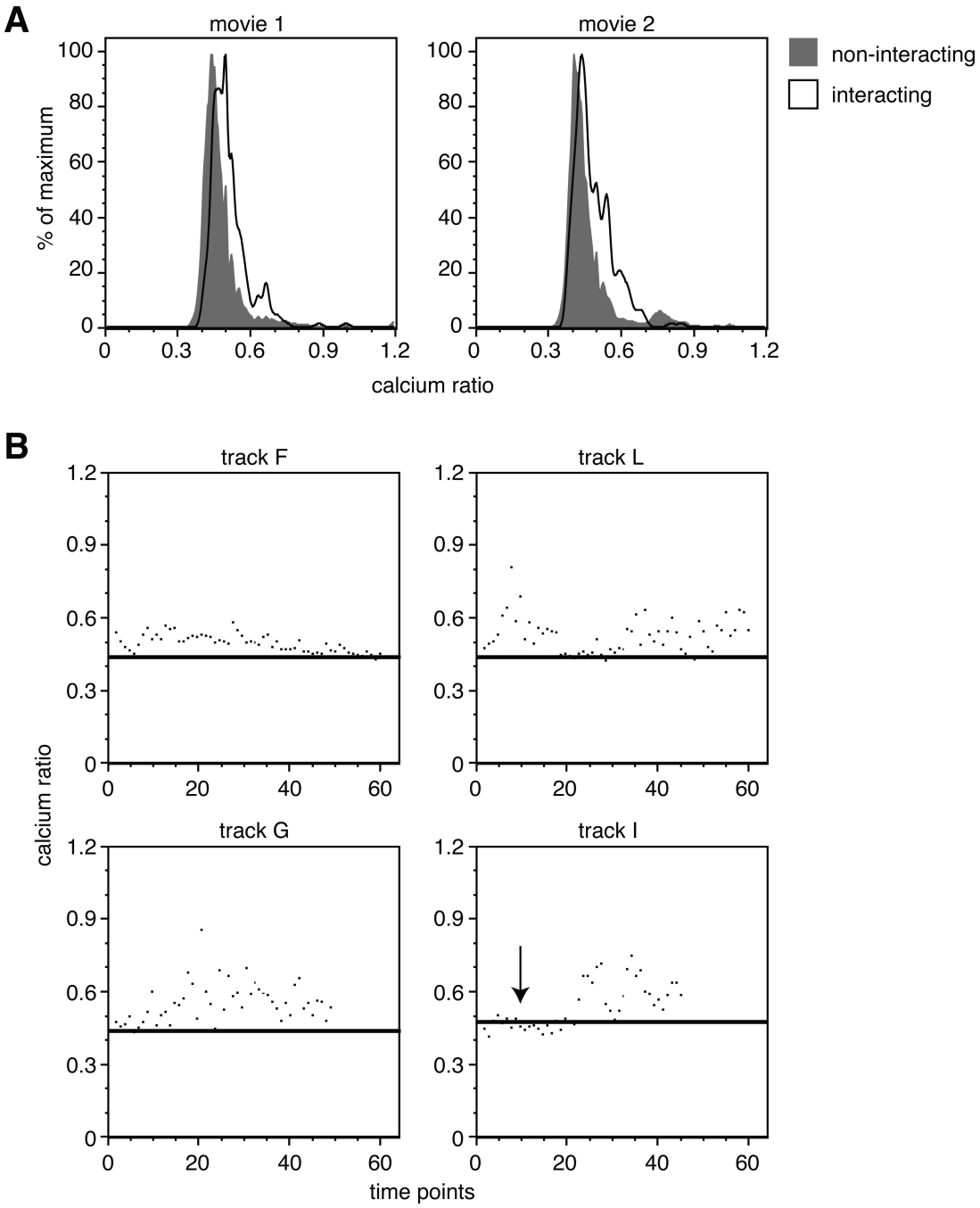

Figure 3. Thymocytes presenting negative selecting peptide support low, sustained TCR signals

(**A**) The frequency of time points plotted against the $[Ca^{2+}_i]$ ratio of Indo-1–labeled preselection OT1tg DP thymocytes incubated on $\beta 2m^{-/-}$ thymic slices in the presence of WT thymocytes alone or loaded with 1nM OVA peptide from two independent imaging runs. “Interacting” values are time points that were part of a thymocyte-thymocyte contact, and derived from ≥ 5 tracked OT1tg thymocytes. “Non-interacting” were values from all OT1tg thymocytes from the same run that were not in a conjugate, and that had a similar overall fluorescence value to the “interacting” tracks. (**B**) $[Ca^{2+}_i]$ ratios over time from

individual representative interacting OT1tg thymocyte tracks. Track labels correspond to those in Fig. 2B. Line indicates average $[Ca^{2+}{}_i]$ ratio of non-interacting cells with comparable fluorescence intensity in the unbound Indo-1 channel of the interacting cell. For track I, arrow indicates the timepoint in which the initial thymocyte-thymocyte contact occurred.