

# Peptide-MHC heterodimers show that thymic positive selection requires a more restricted set of self-peptides than negative selection

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T cell selection and maturation in the thymus depends on the interactions between T cell receptors (TCRs) and different self-peptide-major histocompatibility complex (pMHC) molecules. We show that the affinity of the OT-I TCR for its endogenous positively selecting ligands, Catnb-H-2K<sup>b</sup> and Cappa1-H-2K<sup>b</sup>, is significantly lower than for previously reported positively selecting altered peptide ligands. To understand how these extremely weak endogenous ligands produce signals in maturing thymocytes, we generated soluble monomeric and dimeric peptide-H-2K<sup>b</sup> ligands. Soluble monomeric ovalbumin (OVA)-K<sup>b</sup> molecules elicited no detectable signaling in OT-I thymocytes, whereas heterodimers of OVA-K<sup>b</sup> paired with positively selecting or nonselecting endogenous peptides, but not an engineered null peptide, induced deletion. In contrast, dimer-induced positive selection was much more sensitive to the identity of the partner peptide. Catnb-K<sup>b</sup>-Catnb-K<sup>b</sup> homodimers, but not heterodimers of Catnb-K<sup>b</sup> paired with a nonselecting peptide-K<sup>b</sup>, induced positive selection, even though both ligands bind the OT-I TCR with detectable affinity. Thus, both positive and negative selection can be driven by dimeric but not monomeric ligands. In addition, positive selection has much more stringent requirements for the partner self-pMHC.

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Abbreviations used: APL, altered peptide ligand; DP, double positive; FPLC, fast protein liquid chromatography; FTOC, fetal thymic organ culture; pMHC, peptide-MHC; SP, single positive; SPR, surface plasmon resonance.

Self-peptides bound to MHCs in the thymus select a self-tolerant and self-MHC-restricted T cell repertoire. This is accomplished by immature CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) thymocytes interrogating these self-peptide-MHCs (pMHCs) with their TCRs. Strong TCR ligation induces some portion of thymocytes to apoptose in a process known as negative selection, thus preventing those clones from emigrating to the periphery where they might mount a response against those same self-ligands (von Boehmer and Kisielow, 1990). Below a certain threshold, a signal of intermediate strength can instead induce maturation, allowing positive selection of DP thymocytes into either the CD8<sup>+</sup> or CD4<sup>+</sup> single-positive (SP) T cell lineage (Huang et al., 2004). At an even lower threshold where a thymocyte's TCR binds too weakly or not at all, it eventually dies by neglect.

These thresholds are best understood, in a quantitative sense, for the class I-restricted OT-I TCR. CD8<sup>+</sup> SP T cells bearing this TCR recognize a chicken OVA<sub>257-264</sub> peptide,

SIINFEKL, which is bound to H-2K<sup>b</sup> (K<sup>b</sup>; Carbone et al., 1988; Carbone and Bevan, 1989; Fremont et al., 1995). In vitro (Hogquist et al., 1994) or in vivo (Blanas et al., 1996), OVA peptide induces clonal deletion of OT-I TCR transgenic DP thymocytes. When measured by surface plasmon resonance (SPR), this negatively selecting interaction has a dissociation equilibrium constant (K<sub>D</sub>) of ~6 μM at 25°C (Alam et al., 1996, 1999). Further studies have determined the threshold between negative and positive selection in this system (Daniels et al., 2006) in terms of the apparent K<sub>D</sub> of a tetramerized OVA-K<sup>b</sup> for the OT-I TCR and have shown that this threshold is similar across three different TCR transgenic systems (T1, S14, and OT-I; Naeher et al., 2007). With respect to positively selecting ligands in the

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OT-I system, the monomeric  $K_D$ s of two altered peptide ligands (APLs) that mediate positive selection have been measured using SPR (Alam et al., 1996, 1999). In these studies, it was demonstrated that positively selecting APLs, E1 and R4, have affinities that are 3.5–8.8 $\times$  weaker than that of OVA- $K^b$  (Alam et al., 1996, 1999). However, these APLs are not found in mice and so they could not be the natural positively selecting peptides for the OT-I TCR, and when the E1 peptide was expressed in OT-I TCR transgenic mice, it resulted in the selection of T cells that had a significantly altered reactivity toward antigen relative to normal OT-I TCR transgenic mice (Stefanski et al., 2001). Thus, it is not completely clear how the affinities of E1 and R4 relate to thymic events in vivo. More recently, 27 naturally occurring H-2K $^b$ -bound peptides were identified by mass spectroscopy analysis (Santori et al., 2002). Two of these,  $\beta$ -catenin<sub>329–336</sub> (Catnb) and F-actin capping protein A<sub>92–99</sub> (Cappa1; Hogquist et al., 1997), although they are null ligands for mature OT-I T cells, were shown to mediate positive selection, but their affinities for the OT-I TCR have not been experimentally determined. This may be a result of their weaker binding to OT-I, which likely approaches the limit of what can be measured currently by available techniques such as SPR. Similarly, whether nonselecting pMHCs bind to OT-I TCR at all is not known because their affinities for TCR are expected to be even lower than positively selecting peptides and thus are also not measurable by current techniques.

In addition to their critical role during thymic selection, several studies have shown that self-pMHCs can contribute to signaling in response to foreign agonist pMHC during peripheral T cell activation. In the case of class II-restricted T cells, experiments using soluble dimeric pMHCs showed that certain endogenous pMHCs could synergize with an agonist pMHC in T cell activation (Krogsgaard et al., 2005). These studies fit well with studies from Wülfing et al. (2002) and Sumen et al. (2004) showing that APLs that did not induce activation on their own could, at a high concentration in the synapse, contribute to CD4 $^+$  T cell activation. In contrast, another study showed that in the context of artificial or cell surfaces, endogenous pMHC did not enhance TCR signaling in response to very low numbers of agonist pMHCs (Ma et al., 2008). However, the latter study could not rule out transient dimerization events of agonist or phenotypic differences in the T cells, which could cause reduced responsiveness to self-ligands without loss of sensitivity to foreign stimuli (Hogquist et al., 2003).

The contribution of self-pMHCs to agonist stimulation has also been demonstrated in the class I-restricted OT-I system using APCs derived from RMA cells that lacked the transporter associated with antigen processing (TAP; RMA-S). Again though, the results were mixed. One study concluded that there is an enhancement of agonist signaling in the presence of a large amount of any endogenous peptide- $K^b$  on the surface of RMA-S cells that is independent of endogenous pMHCs' affinity for the TCR (Yachi et al., 2005, 2007). Comparing RMA and RMA-S cells, another study concluded

that self-peptides have a negligible effect on the response of OT-I CD8 $^+$  T cells to OVA- $K^b$  (Spörri and Reis e Sousa, 2002). One complication inherent to both studies is the fact that RMA-S cells still retain a small number of their own endogenous pMHC complexes on their cell surface (Ljunggren et al., 1990). Furthermore, these studies were performed in suspension cultures where biological outcomes, such as positive selection, cannot be observed because of the lack of some critical feature of thymic architecture. Therefore, by undertaking studies using purified pMHCs that only present a single peptide of choice, we hoped to clarify how self-peptides might work with agonist ligands to stimulate thymocytes.

In this study, we determined the affinity of interaction between the OT-I TCR and its two known naturally occurring positively selecting ligands, Catnb- $K^b$  and Cappa1- $K^b$  (Santori et al., 2002), and found that these have significantly lower affinities for the OT-I TCR than the E1 and R4 APLs. To investigate how these and other self-peptides contribute to thymic selection, we developed a whole thymus culture system where positive and negative selection can be induced using soluble pMHC dimers without complications caused by unknown endogenous self-pMHCs on cell surfaces. Using this system, we showed that TCR dimerization by homo- or heterodimeric pMHCs is absolutely required for either type of selection. We further showed that a strong agonist pMHC can partner with most self-pMHCs to mediate negative selection, but TCR binding to the weaker pMHC is still required, as peptide variants that prohibit TCR interactions do not induce any calcium signaling or selection. In contrast, a strongly positively selecting pMHC must pair with another positively selecting pMHC to induce positive selection.

## RESULTS

### The affinity threshold for naturally occurring positively selecting ligands

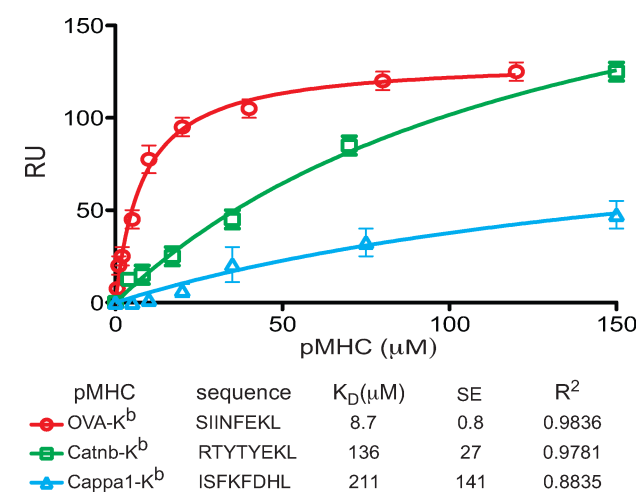
It is generally accepted that during T cell development, stronger pMHC ligands induce negative selection, whereas weaker self-pMHC ligands promote positive selection. Although multiple endogenous positively selecting peptides have been identified for the OT-I TCR, thus far dissociation constants ( $K_D$ s) have only been experimentally determined for artificial positively selecting ligands (Alam et al., 1996, 1999). These are APLs based on exogenous agonist peptide, and because they do not occur naturally in the thymus, it is not completely clear how their affinities relate to physiological T cell selection events.

We therefore took advantage of the two known endogenous self-peptides (Catnb and Cappa1) that promote positive selection of the OT-I TCR (Santori et al., 2002). Using SPR, we measured the equilibrium  $K_D$ s of OT-I binding to Catnb- $K^b$  and Cappa1- $K^b$ . Somewhat surprisingly, and in contrast to two positively selecting APLs in the OT-I system (E1 and R4), Catnb- $K^b$  and Cappa1- $K^b$  bound too weakly to accurately determine a  $K_D$  using the same conditions as used to measure the  $K_D$ s of E1 and R4 at 25°C (Alam et al., 1996, 1999). To overcome this limitation, we lowered the temperature to

10°C to slow down the dissociation phase and were able to detect specific binding by Catnb-K<sup>b</sup> ( $K_D$  136  $\mu$ M) and Cappa1-K<sup>b</sup> ( $K_D$  211  $\mu$ M; Fig. 1). We also measured the affinity of OVA-K<sup>b</sup> for OT-I ( $K_D$  8.7  $\mu$ M) at 10°C to compare the  $K_D$ s under the same conditions. In this way, we could show that Catnb-K<sup>b</sup> has an affinity that is 15.6 $\times$  lower than that of OVA-K<sup>b</sup>, and Cappa1-K<sup>b</sup>'s affinity is 24.3 $\times$  lower than that of OVA-K<sup>b</sup>. Previously, Alam et al. (1996, 1999) found that the weakest positively selecting peptide ligand (R4) has an affinity that is 8.8 $\times$  weaker than that of OVA-K<sup>b</sup>. Thus, these naturally occurring positively selecting self-peptide-K<sup>b</sup> complexes have significantly lower affinities for the OT-I TCR. At the same time, these two pMHCs have affinities that are higher than that of a previously estimated highest affinity non-positively selecting APL (K4; Alam et al., 1996). Thus, our data support the current threshold model of positive and negative selection; however, the range of affinities that lead to positive selection is somewhat broader and lower than previously appreciated.

### Dimeric TCR ligation is required for signaling and negative selection

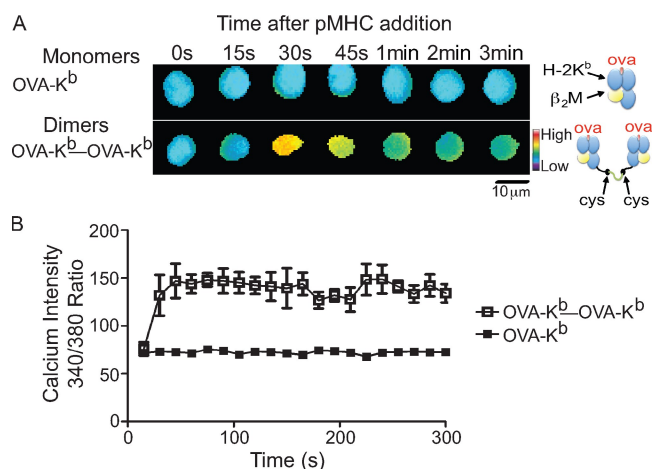
We then set out to determine how TCR engagement with these low-affinity ligands might translate into cell fate decisions. Previous studies have shown that in mature T cells, agonist pMHC monomers alone are insufficient to trigger signaling events and that dimerization of TCR is required (Cochran et al., 2001; Krogsgaard et al., 2005; Stone and Stern, 2006). Because the kinetics of ligand interaction are a critical parameter for TCR activation, the primary effect of dimerization could be to increase the operational affinity and



**Figure 1. Affinity of naturally occurring positively selecting peptide-K<sup>b</sup> ligands for the OT-I TCR.** Equilibrium binding of soluble monomeric OVA-K<sup>b</sup>, Catnb-K<sup>b</sup>, and Cappa1-K<sup>b</sup> to OT-I TCR measured by Biacore 3000 SPR at 10°C at various concentrations of peptide-K<sup>b</sup>. Error bars show SE from three to five separate runs. The table indicates the name, the sequence of the peptide, the affinity ( $K_D$ ) in micromolar, the SE ( $\pm$  SE), and the goodness of fit ( $R^2$ ). The results are representative of at least three independent experiments. RU, relative units.

thereby allow signaling. It remained possible that DP thymocytes, which are known to be more sensitive to pMHC ligands (Davey et al., 1998; Lucas et al., 1999; Li et al., 2007), might be able to detect and respond to monomeric pMHC. To test this hypothesis, we mixed soluble pMHC monomers with OT-I K<sup>b</sup>-/-D<sup>b</sup>-/- thymocytes loaded with the calcium indicator dye fura-2 and imaged them using time-lapse three-dimensional (3D) fluorescence microscopy. We found that even at a protein concentration of 1 mg/ml (17  $\mu$ M; which based on prior results should lead to  $\sim$ 50% occupancy of the thymocytes' TCRs [Naeher et al., 2007]), the high-affinity agonist OVA-K<sup>b</sup> monomer did not stimulate OT-I K<sup>b</sup>-/-D<sup>b</sup>-/- thymocytes to any measureable degree (Fig. 2). Thus, even strong agonist pMHC monomers at high concentrations could not stimulate thymocytes.

Next, we tested whether dimers of agonist peptide-K<sup>b</sup>s or of various self-pMHCs could lead to calcium signaling in DP thymocytes. K<sup>b</sup> dimers were constructed by including a free cysteine at the C terminus of the H-2K<sup>b</sup> molecule and linking them using an 8-aa peptide containing two maleimide residues (as originally described by Cochran et al., 2000; Fig. S1). We first compared calcium flux induced by fast protein liquid chromatography (FPLC)-purified homodimers of agonist OVA-K<sup>b</sup>-OVA-K<sup>b</sup> with that induced by OVA-K<sup>b</sup> monomers in OT-I K<sup>b</sup>-/-D<sup>b</sup>-/- thymocytes (Fig. 2). At 100  $\mu$ g/ml (0.87  $\mu$ M), OVA-K<sup>b</sup>-OVA-K<sup>b</sup> dimers induced a sustained 1.5-fold increase in fura 340/380 ratio in thymocytes



**Figure 2. Calcium signaling in thymocytes in response to high-affinity pMHC.** Soluble homodimers of agonist pMHCs can stimulate calcium signaling in thymocytes, whereas monomers of agonist pMHC cannot. (A) OT-I TCR transgenic K<sup>b</sup>-/-D<sup>b</sup>-/- thymocytes were loaded with fura-2, a ratiometric calcium-sensitive fluorescent dye (Tsien, 1989), and mixed with soluble OVA-K<sup>b</sup> monomers (1 mg/ml or 17  $\mu$ M) or OVA-K<sup>b</sup>-OVA-K<sup>b</sup> homodimers (100  $\mu$ g/ml or 0.87  $\mu$ M). Representative Ca<sup>2+</sup> ratio images obtained with fura-2 (340/380 nm) are shown in false color scale as indicated in the bottom right corner. (B) Fura-2 ratios (340/380 nm) were measured every 15 s for thymocytes in randomly chosen microscope fields, and their mean Ca<sup>2+</sup> ratio was plotted as a function of time (fura ratio mean  $\pm$  SEM;  $n$  = 7). The results are representative of at least three independent experiments.

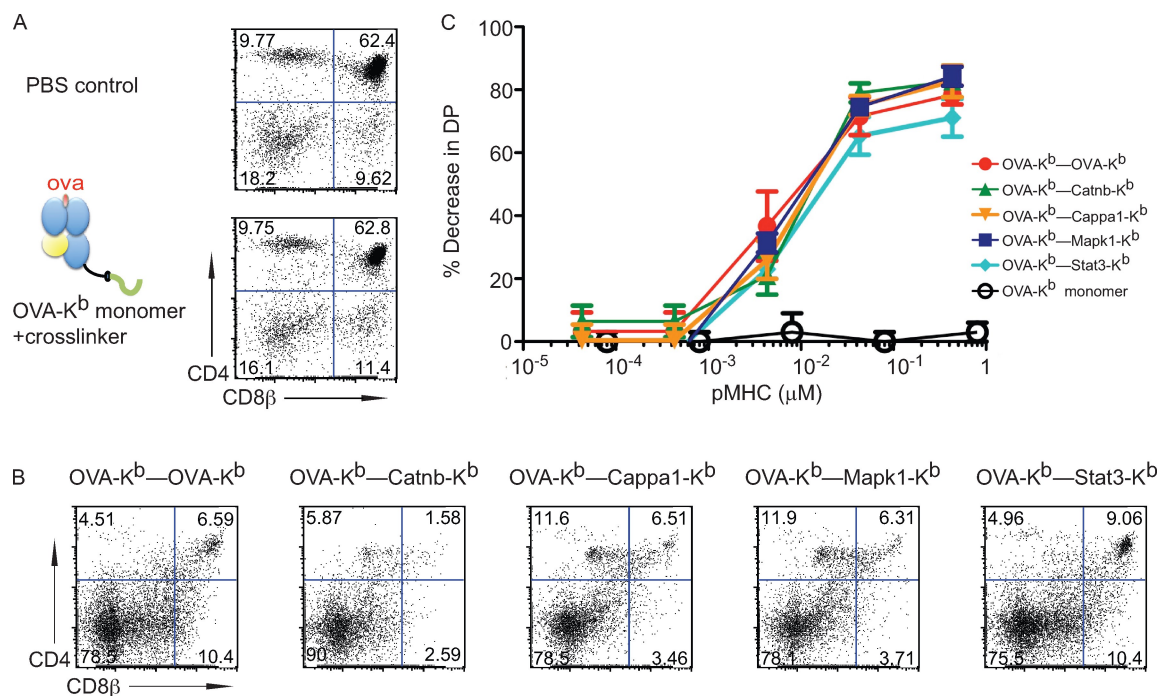
when compared with the completely nonstimulatory OVA-K<sup>b</sup> monomer (Fig. 2). Because we observed by microscopy that individual thymocytes were not in contact with each other, we could rule out any role for cell–cell contact in this dimer-mediated stimulation. Thus, OVA-K<sup>b</sup> dimers can directly stimulate calcium signaling in thymocytes.

We then set out to confirm whether these early signaling events eventually lead to phenotypic changes in a more physiological setting, which in this case was during thymic selection. Previous results have shown that adding exogenous agonist OVA peptide to OT-I fetal thymic organ cultures (FTOCs) induces negative selection of the OT-I DP thymocytes (Hogquist et al., 1993), and we examined whether addition of OVA-K<sup>b</sup>–OVA-K<sup>b</sup> dimers could induce negative selection of DPs in OT-I K<sup>b</sup>–D<sup>b</sup>–FTOCs, where the only MHC class I molecules present would be the soluble monomers or dimers that we added. Normally, a majority (50–70%) of thymocytes in OT-I K<sup>b</sup>–D<sup>b</sup>–thymi remain arrested at the DP stage because they lack endogenous positively selecting K<sup>b</sup> molecules (Fig. 3 A). When we added as little as 0.5  $\mu\text{g/ml}$  (0.0043  $\mu\text{M}$ ) of OVA-K<sup>b</sup>–OVA-K<sup>b</sup> dimers, there was a dramatic 2–4-fold decrease in the DP

population (Fig. 3 C), indicating significant negative selection. We also tested the effect of monomeric OVA-K<sup>b</sup> that included the dimaleimide linker (with one free maleimide). Even at 50  $\mu\text{g/ml}$  (0.87  $\mu\text{M}$ ), the monomer had no effect on the DP population in terms of numbers (Fig. 3 A) or up-regulation of the activation marker CD69 (not depicted). This ruled out the possibility that the dimers exerted their effects via covalent attachment to cell surfaces. Our experiments confirmed that DP thymocytes require the dimeric engagement of TCRs for negative selection (Fig. 3 B) and that monomer is insufficient even in whole thymi with normal 3D cellular interactions.

### A role for low affinity self-peptides in negative selection

Using soluble heterodimers of agonist and self-pMHCs, we have previously shown that, for an MHC class II-restricted response, only certain endogenous pMHCs can assist agonist pMHC in T cell activation (Krogsgaard et al., 2005). At the same time, using antigen-presenting cells, Yachi et al. (2007) have shown in the class I-restricted OT-I system that the enhancement of agonist signaling by endogenous peptide-K<sup>b</sup> is not endogenous peptide-specific and possibly relies more



**Figure 3. Negative selection in FTOCs in response to dimeric pMHC.** Agonist monomers cannot elicit negative selection in thymocytes, but agonist homodimers and agonist-self-peptide heterodimers induce negative selection in FTOC at a similar concentration. (A) Thymic lobes were excised from OT-I K<sup>b</sup>–D<sup>b</sup>–embryos at gestational day 16. One lobe from each pair was cultured with PBS, whereas the other was cultured with monomers of OVA-K<sup>b</sup> (with the cross-linker attached) at 50  $\mu\text{g/ml}$  (0.43  $\mu\text{M}$ ). Lobes were harvested after 4 d of culture, stained with anti-CD4 and anti-CD8 $\beta$ , and analyzed by flow cytometry. Representative FACS plots are shown from three or more experiments per condition. (B) Thymic lobes were excised from OT-I K<sup>b</sup>–D<sup>b</sup>–embryos at gestational day 16 and co-cultured with homodimers of OVA-K<sup>b</sup>–OVA-K<sup>b</sup>, or with heterodimers OVA-K<sup>b</sup>–Catnb-K<sup>b</sup>, OVA-K<sup>b</sup>–Cappa1-K<sup>b</sup>, OVA-K<sup>b</sup>–Mapk1-K<sup>b</sup>, and OVA-K<sup>b</sup>–Stat3-K<sup>b</sup> at 5  $\mu\text{g/ml}$  (0.043  $\mu\text{M}$ ). Lobes were harvested after 4 d of culture, stained with anti-CD4 and anti-CD8 $\beta$  antibodies, and evaluated by flow cytometry. Representative FACS plots are shown from three or more experiments per condition. (C) Quantification of the data at different concentrations. The percent decrease in the number of CD4<sup>+</sup>CD8<sup>+</sup> DP thymocytes was determined by dividing the difference in the percent of CD4<sup>+</sup>CD8<sup>+</sup> DP population of the experimental lobe by the percentage of CD4<sup>+</sup>CD8<sup>+</sup> DPs in the control lobe and multiplying by 100. Data are presented as mean  $\pm$  SEM of three or more independent experiments.



on the interaction of MHC class I with CD8 than with TCR. To test this with respect to thymic negative selection, we paired OVA- $K^b$  with two positively selecting peptide- $K^b$ s (Catnb and Cappa1) or two nonselecting peptide- $K^b$ s (Mapk1 and Stat3). We added heterodimers of OVA- $K^b$ -Catnb- $K^b$ , OVA- $K^b$ -Cappa1- $K^b$ , OVA- $K^b$ -Mapk1- $K^b$ , OVA- $K^b$ -Stat3- $K^b$ , or OVA- $K^b$ -OVA- $K^b$  (as a positive control) to OT-I  $K^b$ -/-  $D^b$ -/- FTOC at various concentrations. Catnb, Cappa1, Mapk1, and Stat3 are all nonstimulatory peptides for mature OT-I T cells. Catnb and Cappa1 induce positive selection in thymocytes but Mapk1 and Stat3 do not (Santori et al., 2002). As shown in Fig. 3 (B and C), all of these heterodimers lead to negative selection of OT-I  $K^b$ -/-  $D^b$ -/- thymocytes at similar concentrations as OVA- $K^b$ -OVA- $K^b$  dimers (at 0.5–50  $\mu$ g/ml or 0.0043–0.43  $\mu$ M), as indicated by the dramatic decrease in the DP population. They are all similarly potent because we titrated this effect down to 0.05  $\mu$ g/ml (0.00043  $\mu$ M), the OVA- $K^b$ -OVA- $K^b$  homodimers and all the OVA-self-heterodimers lost their ability to induce negative selection (Fig. 3 C). Our data demonstrate that OVA can synergize with any of these self-peptides to induce negative selection. Thus, our data are consistent with what has been previously observed for mature OT-I T cells interacting with APCs; most self-peptide- $K^b$ s can synergize with agonist- $K^b$ s to produce a strong signal.

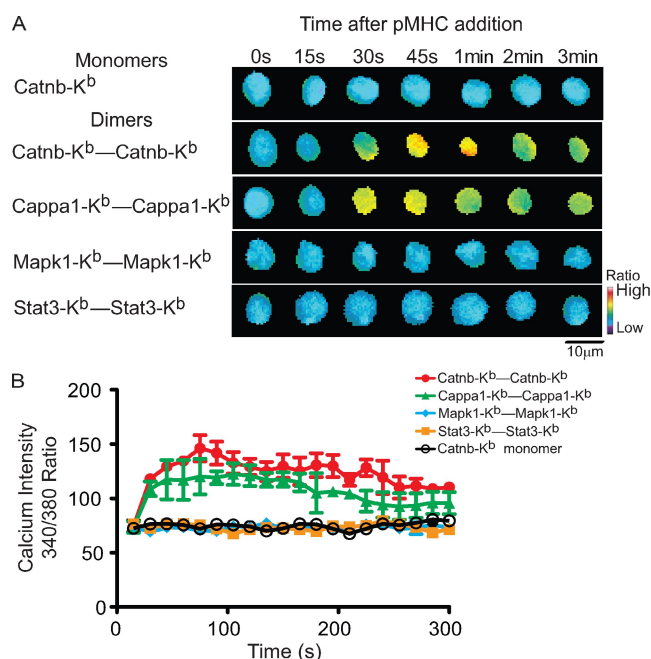
### The synergistic effect of paired pMHC is peptide sequence-specific during positive selection

In the OT-I TCR system, Catnb and Cappa1 have been previously identified as naturally occurring positively selecting peptides that bind to H-2 $K^b$  (Santori et al., 2002). In terms of their strength, Catnb can induce positive selection at lower concentrations than Cappa1. In agreement with this, we have shown that Catnb- $K^b$  has a higher affinity for OT-I than Cappa1- $K^b$  (Fig. 1). We used these two peptides to examine the difference between monomeric and dimeric pMHC ligand requirements for calcium signaling and positive selection in FTOCs. For calcium signaling, we used live cell microscopy to determine whether dimers of various naturally occurring  $K^b$ -bound peptides (Catnb- $K^b$ -Catnb- $K^b$ , Cappa1- $K^b$ -Cappa1- $K^b$ , Mapk1- $K^b$ -Mapk1- $K^b$ , or Stat3- $K^b$ -Stat3- $K^b$ ) could stimulate thymocytes. We observed that only dimers of Catnb- $K^b$ -Catnb- $K^b$  and Cappa1- $K^b$ -Cappa1- $K^b$  induced calcium elevation in OT-I thymocytes at a protein concentration of 1 mg/ml (8.7  $\mu$ M; Fig. 4). In contrast, none of the monomeric pMHC could stimulate OT-I DPs at the same concentration (Fig. 4 and not depicted) and neither could Mapk1- $K^b$  or Stat3- $K^b$  homodimers (Fig. 4).

We then examined whether these early signaling events correlated with the ability to induce positive selection in FTOC. As shown in Fig. 5 A, monomeric Catnb- $K^b$  did not elicit any increase in the CD8<sup>+</sup> SP thymocyte population when compared with the PBS-treated control lobes (and neither did monomeric OVA- $K^b$  [Fig. 3]). In contrast, Catnb- $K^b$ -Catnb- $K^b$  homodimer efficiently mediated positive selection of immature CD8<sup>+</sup> SP T cells at or above protein concentrations of

150  $\mu$ g/ml (1.3  $\mu$ M; Fig. 5 A and Fig. S2). Even at the highest concentration of 900  $\mu$ g/ml (7.8  $\mu$ M), we did not observe negative selection. As for the Cappa1- $K^b$ -Cappa1- $K^b$  homodimers, we observed an increase in the CD8 SP population in FTOCs only with very high concentrations (1.2 mg/ml or 10  $\mu$ M) of protein (Fig. 5 B and Fig. S2). We were unable to induce positive selection at this concentration with Mapk1- $K^b$ -Mapk1- $K^b$  and Stat3- $K^b$ -Stat3- $K^b$  homodimers (Fig. 5 B and Fig. S2).

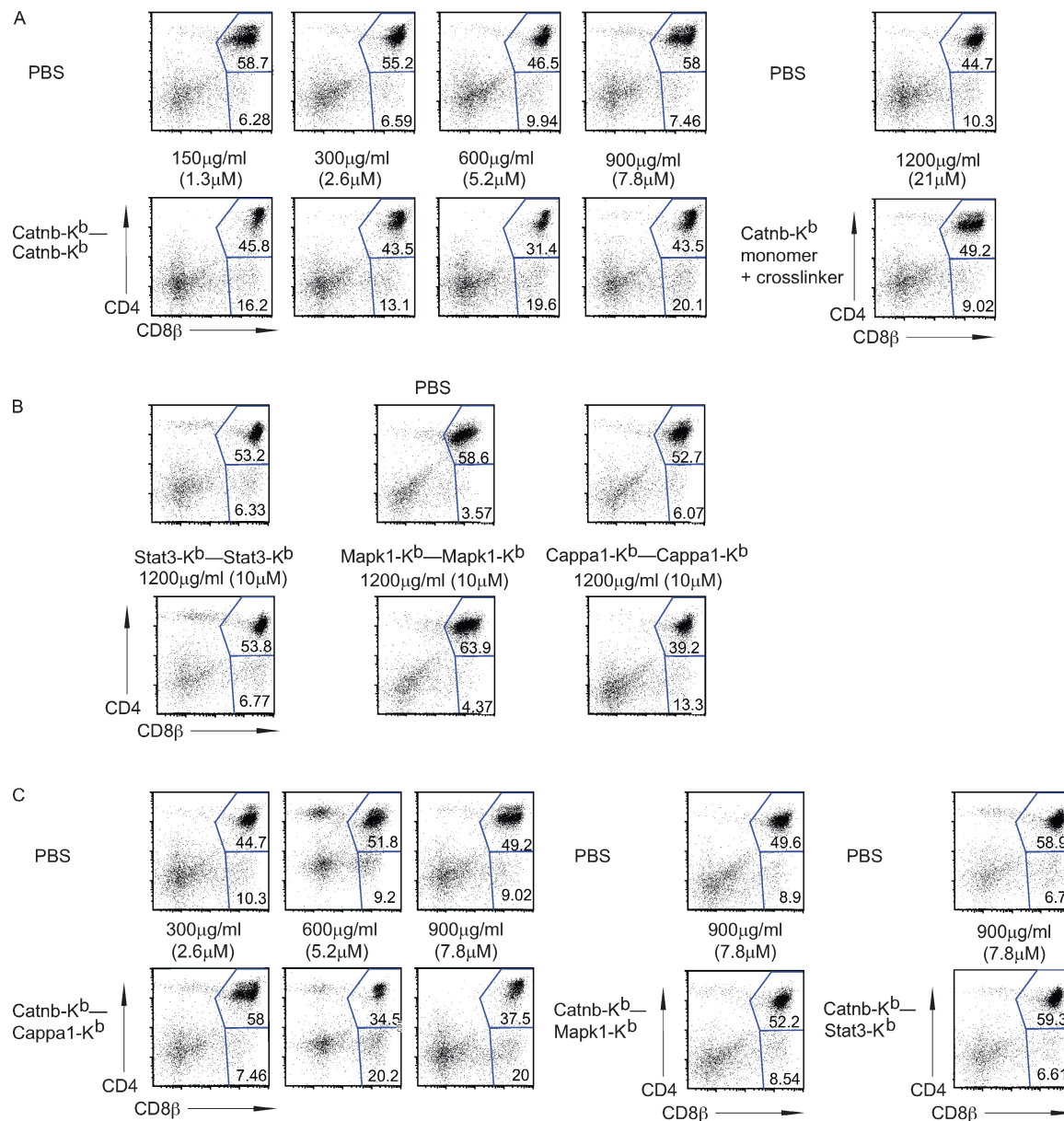
We have already shown, consistent with prior studies using mature T cells and exogenous peptides loaded onto APCs, that all of the tested self-peptides paired with OVA peptide could induce signaling and negative selection in OT-I FTOCs (Fig. 3 B). Because positive selection involves a weaker pMHC-TCR interaction than negative selection, we wondered if these self-peptides would be able to synergize with Catnb to induce positive selection as they do with OVA to induce negative selection. To test this, we added Catnb- $K^b$ -Cappa1- $K^b$ , Catnb- $K^b$ -Mapk1- $K^b$ , and Catnb- $K^b$ -Stat3- $K^b$  heterodimers to FTOCs at various concentrations.



**Figure 4. Calcium signaling in thymocytes in response to low-affinity dimeric pMHC.** Soluble homodimers of Catnb- $K^b$  and Cappa1- $K^b$  can stimulate calcium signaling in thymocytes, whereas Catnb- $K^b$  monomers and Mapk1- $K^b$  homodimers and Stat3- $K^b$  homodimers cannot. (A) OT-I TCR transgenic  $K^b$ -/-  $D^b$ -/- thymocytes were loaded with fura-2 and mixed with soluble Catnb- $K^b$  monomers at 1 mg/ml (17  $\mu$ M) or with Catnb- $K^b$ -Catnb- $K^b$ , Cappa1- $K^b$ -Cappa1- $K^b$ , Mapk1- $K^b$ -Mapk1- $K^b$ , or Stat3- $K^b$ -Stat3- $K^b$  homodimers at 1 mg/ml (8.7  $\mu$ M). Representative Ca<sup>2+</sup> ratio images were obtained with fura-2 (340/380 nm) and are shown using a false color scale as indicated in the bottom right corner. (B) Fura-2 ratios (340/380 nm) were measured every 15 s for thymocytes in randomly chosen microscopic fields and the mean Ca<sup>2+</sup> ratio was plotted as a function of time (fura ratio mean  $\pm$  SEM;  $n = 7$ ). The results are representative of at least three independent experiments.

In FTOCs cultured with Catnb-K<sup>b</sup>-Mapk1-K<sup>b</sup> and Catnb-K<sup>b</sup>-Stat3-K<sup>b</sup>, there were no significant differences between the CD4/CD8 profiles when compared with controls (Fig. 5 C) at the highest tested protein concentration. However, we observed a dramatic increase in the CD8<sup>+</sup> SP population with Catnb-K<sup>b</sup>-Cappa1-K<sup>b</sup> heterodimers at 600  $\mu$ g/ml (5.2  $\mu$ M) and 900  $\mu$ g/ml (7.8  $\mu$ M) but not at 300  $\mu$ g/ml (2.6  $\mu$ M; Fig. 5 C

and Fig. S2). This is a lower dose than is required to observe selection in response to Cappa1-K<sup>b</sup>-Cappa1-K<sup>b</sup> homodimers (1,200  $\mu$ g/ml; 10  $\mu$ M). Similarly, we found that only Catnb-K<sup>b</sup>-Cappa1-K<sup>b</sup> heterodimers are able to induce robust calcium signaling when compared with Catnb-K<sup>b</sup>-Mapk1-K<sup>b</sup> and Catnb-K<sup>b</sup>-Stat3-K<sup>b</sup> heterodimers at 1 mg/ml (8.7  $\mu$ M; Fig. S3). Therefore, in positive selection we could observe a synergistic



**Figure 5. Positive selection in FTOCs in response to low-affinity dimeric pMHC.** Soluble dimers of particular combinations of self-peptide-K<sup>b</sup> molecules induce positive selection in OT-I K<sup>b</sup>-/-D<sup>b</sup>-/- FTOC. Thymic lobes were excised from OT-I K<sup>b</sup>-/-D<sup>b</sup>-/- embryos at gestational day 16 and co-cultured with monomers and dimers of endogenous pMHCs at the indicated concentrations. Lobes were harvested after 4 d of culture, stained with anti-CD4 and anti-CD8 $\beta$  antibodies, and evaluated by flow cytometry. (A) These representative FACS plots compare lobes treated with monomeric Catnb-K<sup>b</sup> (with the cross-linker attached) to lobes treated with dimeric Catnb-K<sup>b</sup>-Catnb-K<sup>b</sup> at the indicated protein concentrations. (B) Representative flow cytometry plots comparing lobes treated with a high concentration (1,200  $\mu$ g/ml or 10  $\mu$ M) of homodimers (Stat3-K<sup>b</sup>-Stat3-K<sup>b</sup>, Mapk1-K<sup>b</sup>-Mapk1-K<sup>b</sup>, or Cappa1-K<sup>b</sup>-Cappa1-K<sup>b</sup>). (C) Representative flow cytometry plots comparing lobes treated with heterodimers (Catnb-K<sup>b</sup>-Cappa1-K<sup>b</sup>, Catnb-K<sup>b</sup>-Mapk1-K<sup>b</sup>, or Catnb-K<sup>b</sup>-Stat3-K<sup>b</sup>) at the indicated protein concentrations. The results are representative of at least three independent experiments.

effect of Cappa1-K<sup>b</sup> contributing to Catnb-K<sup>b</sup> stimulation. Moreover, our studies using lower affinity positively selecting ligands reveal something quite different to what we and others have observed for negatively selecting ligands. In this case, the synergistic effect is in fact peptide specific, as only the stronger Cappa1-K<sup>b</sup> self-ligand could partner with Catnb-K<sup>b</sup> to produce an appreciable T cell response, whereas the weaker Mapk1-K<sup>b</sup> and Stat3-K<sup>b</sup> could not.

### Nonnegligible affinity of null self-peptide-K<sup>b</sup> for OT-I

One possibility that might reconcile these results is that the ability of self-peptides to synergize with a stronger ligand is self-peptide-specific in both positive and negative selection. In this case, because of OVA-K<sup>b</sup>'s high affinity for the OT-I TCR, the affinity of the partner self-peptides involved in negative selection might span a much larger affinity range and still allow productive TCR signaling. In this view, we would predict that the various known self-peptide-K<sup>b</sup>s do in fact have some appreciable affinity for the OT-I TCR, even if alone they are null ligands in terms of stimulatory capability. These affinities must be very low, as Mapk1-K<sup>b</sup> and Stat3-K<sup>b</sup> (ligands that could partner with OVA-K<sup>b</sup> to induce negative selection) showed undetectable binding in SPR, which generally sets a limit in the range of 300  $\mu$ M for molecules of this size and solubility. Nonetheless, we have shown here that a  $K_D$  as low as 211  $\mu$ M is sufficient for Cappa1 to drive positive selection of OT-I T cells. Self-pMHC with affinities only twofold lower for OT-I would not be measureable yet might still make some peptide-specific contribution to OT-I T cell triggering.

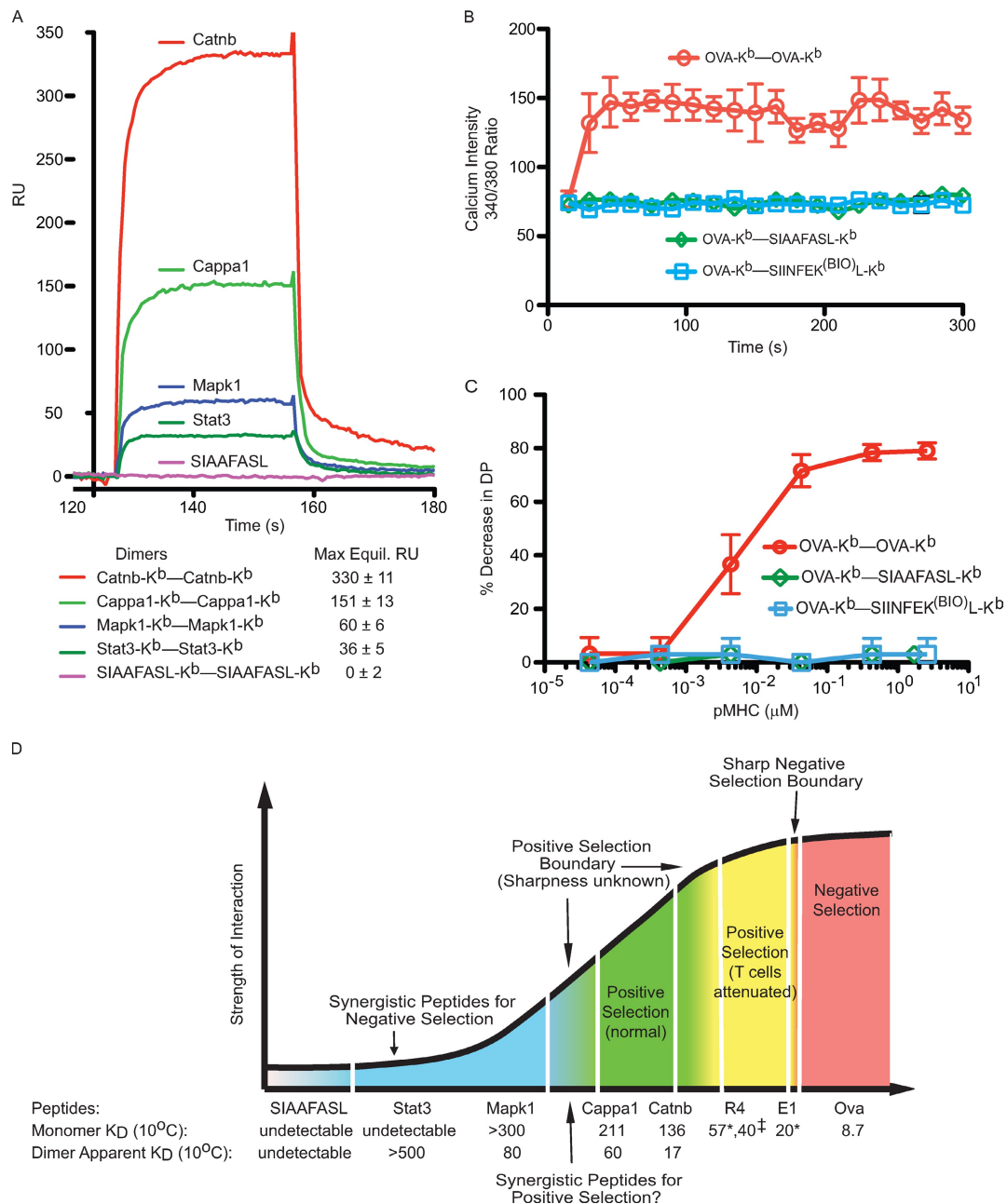
To explore this possibility, we measured the affinities of dimeric self-peptide-K<sup>b</sup>s for the OT-I TCR using SPR. Dimerization makes accurate calculation of a  $K_D$  impossible because of cooperativity effects. But using soluble dimers in SPR does allow us to determine whether or not particular self-peptide-K<sup>b</sup> can bind to the OT-I TCR at all. We saturated the surface of streptavidin sensor chips with biotinylated OT-I TCR and used different combinations of foreign and self-pMHC dimers as analytes. In these measurements, we also used the peptide SIAAFASL, an APL of OVA (SIINFELK) which we designed to minimize contacts with the OT-I TCR based on structural and T cell reactivity data (Jameson and Bevan, 1992; Matsumura et al., 1992; Saito et al., 1993; Hogquist et al., 1994; Fremont et al., 1995) and which binds to H-2K<sup>b</sup> comparably with the other peptides tested (Fig. S4). We measured the binding of 5  $\mu$ M of Catnb-K<sup>b</sup>-Catnb-K<sup>b</sup>, Cappa1-K<sup>b</sup>-Cappa1-K<sup>b</sup>, Mapk1-K<sup>b</sup>-Mapk1-K<sup>b</sup>, Stat3-K<sup>b</sup>-Stat3-K<sup>b</sup>, and SIAAFASL-K<sup>b</sup>-SIAAFASL-K<sup>b</sup> dimers on the same OT-I ligand surface, and their relative affinities are given in Fig. 6 A. As expected from SPR measurements of self-K<sup>b</sup> monomers (Fig. 1), Catnb-K<sup>b</sup> and Cappa1-K<sup>b</sup> dimers showed the highest level of binding (respectively) to OT-I TCR. Our engineered null SIAAFASL-K<sup>b</sup> complex showed no detectable binding even as a dimer. But most importantly, the other self-ligands, Mapk1-K<sup>b</sup> and Stat3-K<sup>b</sup>, which had no biological activity in our positive selection assay, nonetheless displayed weak but significant affinity for the OT-I TCR.

### Engineered null ligands abolish the synergistic effect of paired pMHC

Considering the peptide specificity requirement for both partners of the dimeric pMHC during positive selection, our data suggest that the affinity of the partner pMHC interaction is important for its synergistic contribution. However, the partner self-pMHC's affinity requirement has not been demonstrated for agonist signaling such as the signaling leading to negative selection. To reconcile the difference between positive and negative selection and to determine this self-peptide specificity requirement, we paired the lowest affinity SIAAFASL peptide-K<sup>b</sup> with OVA-K<sup>b</sup> in the context of a soluble heterodimer. This heterodimer was mixed with OT-I thymocytes loaded with fura-2 ratiometric dye to measure the intracellular increase in calcium levels by microscopy. At 100  $\mu$ g/ml (0.87  $\mu$ M), OVA-K<sup>b</sup>-SIAAFASL-K<sup>b</sup> heterodimers could not stimulate OT-I thymocytes, unlike homodimers of OVA-K<sup>b</sup>-OVA-K<sup>b</sup> (Fig. 6 B) or heterodimers of OVA-K<sup>b</sup>-Stat3-K<sup>b</sup> (Fig. 3). Similarly, FTOC co-cultured with OVA-K<sup>b</sup>-SIAAFASL-K<sup>b</sup> heterodimers at 200  $\mu$ g/ml or 1.74  $\mu$ M (which is 400-fold higher than the concentration of OVA-K<sup>b</sup>-OVA-K<sup>b</sup> homodimers which is required to cause negative selection) showed no signs of negative selection (Fig. 6 C). It is not clear how SIAAFASL disrupts TCR binding in terms of its structure. It was designed to minimize TCR contacts, but from a structural model (unpublished data) we cannot rule out the possibility that it also alters K<sup>b</sup> docking and/or the surrounding K<sup>b</sup> structure, which might also influence CD8 binding. So in addition, we used SIINFELK<sup>(BIO)</sup>L, where the only modification is to a TCR-contacting residue (a biotin group added on to K) which does not impinge on the MHC structure (Fremont et al., 1995; Turner et al., 1997). Likewise, SIINFELK<sup>(BIO)</sup>L-K<sup>b</sup> homodimers showed no detectable binding on SPR when compared with Cappa1-K<sup>b</sup> homodimers under the same conditions (Fig. S5). And similarly, this APL could not induce Ca<sup>2+</sup> when paired with OVA-K<sup>b</sup> (as an OVA-K<sup>b</sup>-SIINFELK<sup>(BIO)</sup>L-K<sup>b</sup> heterodimer; Fig. 6 B) nor promote negative selection at high protein concentrations (Fig. 6 C). Thus, even a strong OVA-K<sup>b</sup> ligand must pair with a partner that is able to bind to the TCR, although this partner only needs to bind TCR very weakly.

### DISCUSSION

In this paper, we introduce and demonstrate the utility of an *in vitro* system in which we can induce either positive or negative thymic selection with soluble pMHC dimers. We use this to show an absolute requirement for dimeric pMHC in both types of thymic selection, which strongly indicates that bringing together two distinct TCR-CD3 complexes rather than just engaging individual TCRs is essential for thymocyte signaling. This parallels work on T cell activation where most (Boniface et al., 1998; Cochran et al., 2001; Krosgaard et al., 2005; Stone and Stern, 2006) although not all (Delon et al., 1998; Ma et al., 2008) studies show that soluble pMHC dimers, and not monomers, activate mature T cells. In contrast to a prior study, which showed positive



**Figure 6. TCR affinity determines the thymic selection outcome, even for very weak ligands.** (A) Sensorgram of the interaction between immobilized OT-I TCR at high density with 5 μM Catnb-K<sup>b</sup>—Catnb-K<sup>b</sup>, Cappa1-K<sup>b</sup>—Cappa1-K<sup>b</sup>, Mapk1-K<sup>b</sup>—Mapk1-K<sup>b</sup>, Stat3-K<sup>b</sup>—Stat3-K<sup>b</sup>, and SIAAFASL-K<sup>b</sup>—SIAAFASL-K<sup>b</sup> homodimeric pMHC. (B) Mean Ca<sup>2+</sup> ratio of thymocytes mixed with 100 μg/ml (0.87 μM) of OVA-K<sup>b</sup>—OVA-K<sup>b</sup>, OVA-K<sup>b</sup>—SIAAFASL-K<sup>b</sup>, or OVA-K<sup>b</sup>—SIINFEK<sup>(BIO)</sup>L-K<sup>b</sup>. (C) Thymic lobes were excised from OT-I K<sup>b</sup>—/—D<sup>b</sup>—/— embryos at gestational day 16 and co-cultured with homodimers of OVA-K<sup>b</sup>—OVA-K<sup>b</sup> or with heterodimers OVA-K<sup>b</sup>—SIAAFASL-K<sup>b</sup> and OVA-K<sup>b</sup>—SIINFEK<sup>(BIO)</sup>L-K<sup>b</sup> at various concentrations. Lobes were harvested after 4 d of culture, stained with anti-CD4 and anti-CD8β antibodies, and evaluated by flow cytometry. The percent decrease in the number of CD4<sup>+</sup>CD8<sup>+</sup> DP thymocytes was determined by dividing the difference in the percentage of CD4<sup>+</sup>CD8<sup>+</sup> DP population of the experimental lobe by the percent of CD4<sup>+</sup>CD8<sup>+</sup> DPs in the control lobe and multiplying by 100. Data are presented as mean ± SEM of three or more independent experiments. (D) A graph summarizing the relationship between pMHC-TCR affinity and thymic selection outcomes. Steady-state K<sub>D</sub>s measured at 10°C of monomeric and dimeric peptide-K<sup>b</sup> binding to OT-I TCR are indicated in the bottom of the graph. \* and † represent monomeric K<sub>D</sub> measurements at 25°C that were obtained previously by Alam et al. (1996) and Alam et al. (1999), respectively.



selection in response to soluble OVA-K<sup>b</sup> molecules (Mintern et al., 2004), our data using OVA-K<sup>b</sup> dimers agree more with studies (Hogquist et al., 1994, 1995; Jameson et al., 1995; Daniels et al., 2006) that show that strong agonist ligands do not mediate positive selection of OT-I CD8<sup>+</sup> T cells.

We also find that the requisite peptide components of dimeric pMHC vary significantly between negative and positive selection. Although negative selection can be mediated by dimerization of a strong negatively selecting ligand partnered with various nonselecting self-pMHC ligands, positive selection requires that both of the dimer's peptides be positively selecting ligands. This observation may help to explain the extreme sensitivity of DP thymocytes toward negatively selecting ligands. That is, even if a rare high-affinity ligand is present in minute amounts, it can pair with any of many lower affinity ligands on an APC to induce negative selection. This feature could help to explain how thymocytes are able to detect and respond to very small numbers of cognate agonist peptides (Kurts et al., 1996; Peterson et al., 1999; Ebert et al., 2008), which is thought to help enforce the efficiency of thymic self-tolerance.

Our results also have implications for the stringency of positive selection. A previous study showed that only 4–8% of the preselection T cell repertoire is restricted to self-MHC (Zerrahn et al., 1997), and yet primary T cell responses in the periphery depend on the frequency of their naive precursors (Moon et al., 2007). These results underscore the critical role for positive selection in producing an optimally self-compatible repertoire. At the same time, positively selecting peptides in the thymus account for <10% of self-peptides examined in several systems (Baldwin et al., 1999; Santori et al., 2002; Ebert et al., 2009; Lo et al., 2009), and we know that positive selection is limited by the availability of these particular self-pMHCs (Hogquist et al., 1993; Merckenschlager, 1996). Our results show that positive selection requires dimerization of an already limited set of positively selecting ligands, and this mode of binding seems likely to inhibit the maturation of clones that only recognize very rare self-pMHCs. That is, if  $p$  is the probability of encountering a single positively selecting self-pMHC, then the probability of encountering two positively selecting ligands in proximity of each other would be proportional to  $p^2$ , which diminishes rapidly as  $p$  becomes small. Thus, requiring that thymocytes engage two pMHCs simultaneously would help to select TCRs that are compatible with the most abundant self-pMHCs. By the same token, this mode of binding could help to explain how positive selection encourages polyspecificity of TCRs for self-pMHC (Huseby et al., 2005). Negative selection would inhibit polyspecificity to some degree (Huseby et al., 2005), but the ability to recognize multiple distinct ligands might be useful for responding to more diverse antigens (Wucherpfennig et al., 2007).

Our experimental system successfully restricts thymocyte interactions to exogenously supplied soluble self-pMHC complexes only. This is an advantage over previous studies using antigen-presenting cells (Spörri and Reis e Sousa, 2002; Yachi et al., 2007), which involve many more components and the physiology of a second cell type, which is a possible

explanation for these studies' contradictory data. Using our system, we find that there is a TCR specificity requirement for the partnering endogenous ligand in both positive and negative selection. Our results agree more with the study of Yachi et al. (2007) in that endogenous pMHC could enhance thymocyte activation in the presence of a high-affinity ligand. However, this enhancement is peptide specific, as shown by the inability of SIAAFASL-K<sup>b</sup> and SIINFEK<sup>(BI0)</sup>L-K<sup>b</sup> to partner with OVA-K<sup>b</sup> to provoke a response. We think it likely that the seven self-peptides tested in prior studies (Yachi et al., 2007) might have some nonnegligible affinity for the OT-I TCR that could allow them to assist OVA peptide in T cell activation. We have shown by SPR that at least two of these peptides, Mapk1 and Stat3, have measurable affinity in dimeric form when compared with an engineered null peptide. Whether any truly null self-p-K<sup>b</sup> exists endogenously is unknown, but, nonetheless, our data show that the ability of self-p-K<sup>b</sup> to enable the response to OVA-K<sup>b</sup> relies on the TCR's low affinity for those self-peptide-K<sup>b</sup>s. The larger number of self-p-K<sup>b</sup>s that are able to contribute to agonist signaling (compared with the class II systems [Krogsgaard et al., 2005; Ebert et al., 2009; Lo et al., 2009]) likely reflects the ability of CD8–class I binding to compensate for weaker TCR–pMHC binding (Yachi et al., 2007).

We have measured OT-I's affinity for Catnb-K<sup>b</sup> and Cappa1-K<sup>b</sup> as monomers, but many more self-p-K<sup>b</sup>s bind too weakly to accurately measure, even though we have shown that their affinity in a dimeric form is detectable and relevant for TCR signaling. One drawback of measuring multivalent modes of binding is that the binding enhancement is a result of both the simultaneous spanning of two binding sites and of an increased local concentration of available ligand. This cooperativity factor is different for each pMHC complex, thus one cannot accurately estimate the monomeric affinity of the TCR using this method. Despite this, several studies have used measurements of multimeric affinities to examine the threshold separating negative and positive selection (Daniels et al., 2006; Naeher et al., 2007). In this study, we have provided affinity rankings for an agonist, several self-pMHCs, and a null pMHC ligand for the OT-I TCR in monomeric and dimeric form at 10°C. The dimeric affinities correlate with selection outcomes and also with monomeric affinities. Specifically, we showed that dimers of Mapk1-K<sup>b</sup> have a higher affinity than Stat3-K<sup>b</sup> dimers, whereas we could not detect binding of OT-I to Stat3-K<sup>b</sup> monomers and could barely detect binding of OT-I to Mapk1-K<sup>b</sup> monomers. Our ranking indicates that the affinity of monomeric SIAAFASL-K<sup>b</sup> is weaker than that of Stat3-K<sup>b</sup> because we could not detect any binding of the dimeric SIAAFASL-K<sup>b</sup>. The codon model (Feng et al., 2007; Rubtsova et al., 2009) of TCR recognition predicts that the OT-I TCR should retain some complementarity to the invariant helices of H-2K<sup>b</sup>'s peptide-binding cleft, regardless of the peptide (unless TCR binding is sterically blocked, as in the case of SIINFEK<sup>(bio)</sup>L-K<sup>b</sup>). In contrast, the SIINFEKL P3 position contacts K<sup>b</sup>'s  $\alpha$ -2 helix (Fremont et al., 1995), and replacing

I with A at this position may impinge on the MHC's conformation. So SIAAFASL may represent a truly null peptide, but we cannot rule out the possibility that it has an extremely low affinity that falls below the threshold required to support signaling as a partner in a heterodimer and below the threshold for detection as a dimer by SPR.

Therefore, based on an affinity gradient (Alam et al., 1996; Fig. 6 D), our data suggest that pMHCs with high TCR affinity ( $<8 \mu\text{M}$ , e.g., OVA- $\text{K}^b$ /OT-I) would induce negative selection when paired with ligands above another much lower threshold. This partner threshold falls somewhere between SIAAFASL- $\text{K}^b$  and Stat3- $\text{K}^b$  and we can only measure apparent affinities of dimers at  $10^\circ\text{C}$  in this range. But the threshold for the partner ligand during positive selection is measurably higher. So far, we have only found one other positively selecting ligand to be sufficient: Cappa1- $\text{K}^b$ , which binds OT-I with a monomeric binding affinity of  $211 \mu\text{M}$ , which is 24-fold weaker than OVA- $\text{K}^b$ , at  $10^\circ\text{C}$ . We predict that this partner threshold would lie somewhere between the monomer-measurable and dimer-measurable ranges,  $\sim 250\text{--}400 \mu\text{M}$ , although this lower bound estimate is necessarily not very accurate in terms of monomeric affinity. Finally, we showed that the range of naturally occurring positively selecting pMHC affinities is broader and lower than previously appreciated:  $100\text{--}250 \mu\text{M}$  in terms of monomeric affinity at  $10^\circ\text{C}$ . Our results extend the affinity model for predicting selection outcome to significantly lower affinities and suggest that TCR interactions with extremely weak self-ligands can play an important role in both positive and negative selection.

## MATERIALS AND METHODS

**Mice.** To obtain OT-I  $\text{K}^b$ - $\text{D}^b$  mice, OT-I mice (C57BL/6-Tg(Tcr $\alpha$ Tcr $\beta$ )1100Mjb/J; The Jackson Laboratory) were crossed with  $\text{K}^b$ - $\text{D}^b$  mice (C57BL/6Ji- $\text{K}^b$ <sup>tm1</sup> $\text{D}^b$ <sup>tm1</sup> N12; Taconic). All mice were bred and maintained at the Stanford University Department of Comparative Medicine Animal Facility (protocol 3540) in accordance with National Institutes of Health guidelines.

**Expression of soluble OT-I TCR molecules.** The OT-I TCR  $\alpha$  chain with an acidic zipper and a  $\beta$  chain with a basic zipper have been previously described (Fremont et al., 1995) and were expressed in the pAcGP67A vector. We added a BirA site to the C terminus of the OT-I TCR  $\alpha$  chain by PCR using the 5'-GGAAGATCTTTATTCATGCCATTCAATCTTTGAGCTTC-GAAAATATCATTAAGTCCTGAGCCAGTTCTTTTCCGGTG-3' primer to enable biotin binding for SPR analysis. The soluble OT-I TCR with this BirA site was expressed in a Baculovirus expression system and purified as described previously (Garcia et al., 1996). Site-specific biotinylation was performed as previously described (Boniface et al., 1998).

**Soluble p-H-2K<sup>b</sup> complexes.** The genes for murine class I H-2K<sup>b</sup> (including the connecting peptide region) and a Ser-Gly-Gly-Cys sequence (for cross-linking) were amplified by PCR from OT-I cDNA. For constructs to be used for refolding with peptides, the genes were fused in frame with T7 or His sequences in a modified PET28a(+) vector (EMD) for subsequent affinity purification (Krogsgaard et al., 2005). Soluble murine class I MHC proteins H-2K<sup>b</sup> and human Beta 2 Microglobulin were produced by expression as inclusion bodies in *Escherichia coli*, purified and refolded in vitro in the presence of peptide, as described for mice class II MHC proteins (Altman et al., 1993). The peptides OVA<sub>257-264</sub> (SIINFEKL), Catnb<sub>329-336</sub> (RTYTYEKL), Cappa1<sub>92-99</sub> (ISKFDHL), Mapk1<sub>19-26</sub> (VGPRYTNL), Stat3<sub>53-60</sub> (ATLVFHNL), SIAAFASL, and SIINFEK<sup>(B10)</sup>L (Bio-Synthesis Inc.) were purchased and

analyzed for purity by mass spectrometry. Peptide-based cross-linking reagents (Schafer-N) were constructed and purified essentially as described (Krogsgaard et al., 2005). The protein concentration was estimated by UV absorbance at 280nm. Immediately before use, pMHC complexes were purified by FPLC on a Superdex S200 column (GE Healthcare) to remove aggregates.

**FTOC.** Thymic lobes were excised from fetuses at gestational day 16. The lobes were placed on ISOPORE membrane filters (Millipore) on top of MF support Pads (Millipore) in 24-well plates with standard RPMI complete medium (Ebert et al., 2008). Soluble H-2K<sup>b</sup> molecules were added to the culture at the indicated concentrations. After 4 d in culture, the thymic lobes were harvested and thymocytes were extruded mechanically by pressing the tissue through a nylon mesh.

**BIACORE analysis.** All SPR experiments were performed at  $10^\circ\text{C}$  on a Biacore 3000 instrument (GE Healthcare) as previously described (Krogsgaard et al., 2003). In brief, biotinylated OT-I TCRs were captured on streptavidin-coated sensor chips (Sensor Chip SA; GE Healthcare) that were pretreated according to the manufacturer's instructions. As a control, biotinylated MCC-IE<sup>K</sup> was also captured on the same sensor chip. The specific binding of peptide- $\text{K}^b$  and OT-I was measured using the in-line reference subtraction feature of the Biacore 3000 instrument. Increasing concentrations of monomeric and dimeric peptide- $\text{K}^b$  were injected at a flow rate of  $60 \mu\text{l}/\text{min}$  over all four surfaces of the sensor chip. The monomeric TCR/pMHC steady-state affinity was calculated by plotting the mean maximal response at steady state for each concentration versus pMHC concentration and fitting the data in BIAevaluation software (version 4.1; GE Healthcare) to the equation  $\text{Response} = (R_{\text{max}} \times C)/(K_D + C)$ , which describes the simple 1:1 binding of a ligand that follows the law of mass action, where  $C$  is the concentration of pMHC.

**Antibodies and flow cytometry.** Cell surface molecules were stained with anti-H-2K<sup>b</sup> (AF6-88.5), anti-V $\alpha$ 2 TCR (B20.1), anti-CD69 (H1.2F3), anti-CD62L (MEL-14), and anti-CD4 (RM4-5) antibodies (all obtained from BD). The anti-CD8 $\beta$  antibody was purchased from eBioscience. Live cells were identified by their forward- and side-scatter profiles. Samples were run on a FACStar flow cytometer (BD) and analyzed with FlowJo software (version 8.8.6; Tree Star, Inc.).

**Microscopy.** Microscopy and data analysis were performed using a microscope (Axiovert S100TV; Carl Zeiss, Inc.) and MetaMorph (Universal Imaging Corporation) 3D time-lapse system using a  $40\times$  Fluor objective (NA 1.3) as previously described (Huppa et al., 2003). Thymocytes were isolated by removing whole thymi into cold complete RPMI and disrupting the tissue by pushing it through a nylon mesh. Before imaging, thymocytes were loaded with  $10 \text{ mM}$  fura-2(AM) (Invitrogen) in medium for 20 min at room temperature and washed three times in imaging medium (Hepes-buffered saline) plus 5% FCS,  $0.5 \text{ mM}$   $\text{CaCl}_2$ , and  $0.1 \text{ mM}$   $\text{MgCl}_2$ . For time-lapse experiments using purified protein, OT-I  $\text{K}^b$ - $\text{D}^b$  thymocytes in imaging medium were mixed with purified pMHC dimers or pMHC monomers (kept on ice) and imaged in an FCS2 chamber (Biopetech, Inc.) at  $37^\circ\text{C}$  as previously described (Krogsgaard et al., 2005). As an indicator of intracellular  $\text{Ca}^{2+}$  concentration, fura-2 fluorescence ratios ( $340/380 \text{ nm}$ ) were determined as previously described (Huppa et al., 2003).

**Online supplemental material.** Fig. S1 shows that pMHC dimers, not oligomers, are purified and used for calcium imaging and FTOC. Fig. S2 shows the percent change in CD8 SP in FTOC in response to an assortment of dimeric pMHC ligands at various concentrations. Fig. S3 shows the calcium signaling response of thymocytes mixed with low-affinity heterodimeric pMHC. Fig. S4 shows the relatively similar H-2K<sup>b</sup> binding affinity of peptides used in the study by a peptide competition assay. Fig. S5 shows the relative OT-I TCR affinity of SIINFEK<sup>(B10)</sup>L- $\text{K}^b$  homodimers in comparison to Cappa1- $\text{K}^b$  homodimers. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20092170/DC1>.

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