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-2Kb and Cappa1-H-2Kb, 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-2Kb ligands. Soluble monomeric ovalbumin (OVA)-Kb molecules elicited no detectable signaling in OT-I thymocytes, whereas heterodimers of OVA-Kb 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-Kb–Catnb-Kb homodimers, but not heterodimers of Catnb-Kb paired with a nonselecting peptide–Kb, 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. 

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+CD8+ 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+ or CD4+ 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+ SP T cells bearing this TCR recognize a chicken OVA257-264 peptide, SIINFEKL, which is bound to H-2Kb (Kb; 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 thymocytes. When measured by surface plasmon resonance (SPR), this negatively selecting interaction has a dissociation equilibrium constant (KD) 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 KD of a tetramerized OVA-Kb for the OT-I TCR and have shown that this threshold is similar across three different TCR transgenic systems (T1, S14, and OT-I; Naehler et al., 2007). With respect to positively selecting ligands in the
OT-I system, the monomeric K\textsubscript{b}\textsubscript{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× weaker than that of OVA-K\textsubscript{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\textsubscript{b}-bound peptides were identified by mass spectroscopy analysis (Santori et al., 2002). Two of these, β-catenin\textsubscript{29-36} (Catnb) and F-actin capping protein A\textsubscript{92-99} (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 receptor expression events. 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\textsubscript{D}) 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\textsubscript{D}s of OT-I binding to Catnb-K\textsubscript{b} and Cappa1-K\textsubscript{b} bound too weakly to accurately determine a K\textsubscript{D} using the same conditions as used to measure the K\textsubscript{D}s of E1 and R4 at 25°C (Alam et al., 1996, 1999). To overcome this limitation, we lowered the temperature to...
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 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\textsuperscript{b}−/− D\textsuperscript{b}−/− 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 µM; 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\textsuperscript{b} monomer did not stimulate OT-I K\textsuperscript{b}−/− D\textsuperscript{b}−/− thymocytes to any measurable 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\textsuperscript{b} or of various self-pMHCs could lead to calcium signaling in DP thymocytes. K\textsuperscript{b} dimers were constructed by including a free cysteine at the C terminus of the H-2K\textsuperscript{b} 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\textsuperscript{b}–OVA-K\textsuperscript{b} with that induced by OVA-K\textsuperscript{b} monomers in OT-I K\textsuperscript{b}−/− D\textsuperscript{b}−/− thymocytes (Fig. 2). At 100 µg/ml (0.87 µM), OVA-K\textsuperscript{b}–OVA-K\textsuperscript{b} 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\textsuperscript{b}−/− D\textsuperscript{b}−/− thymocytes were loaded with fura-2, a ratiometric calcium-sensitive fluorescent dye (Tsien, 1989), and mixed with soluble OVA-K\textsuperscript{b} or OVA-K\textsuperscript{b}–OVA-K\textsuperscript{b} homodimers. Representative Ca\textsuperscript{2+} 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\textsuperscript{2+} ratio was plotted as a function of time (fura ratio mean ± SEM; \(\bar{n} = 7\)). The results are representative of at least three independent experiments.
when compared with the completely nonstimulatory OVA-K\textsuperscript{b} 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\textsuperscript{b} 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\textsuperscript{b}–OVA-K\textsuperscript{b} dimers could induce negative selection of DPs in OT-I K\textsuperscript{b}–/–D\textsuperscript{b}–/– 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\textsuperscript{b}–/–D\textsuperscript{b}–/– thymi remain arrested at the DP stage because they lack endogenous positively selecting K\textsuperscript{b} molecules (Fig. 3 A). When we added as little as 0.5 µg/ml (0.0043 µM) of OVA-K\textsuperscript{b}–OVA-K\textsuperscript{b} 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\textsuperscript{b} that included the dimaleimide linker (with one free maleimide). Even at 50 µg/ml (0.87 µM), 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\textsuperscript{b} is not endogenous peptide–specific and possibly relies more...
on the interaction of MHC class I with CD8 than with TCR.

To test this with respect to thymic negative selection, we paired OVA-Kb with two positively selecting peptide-Kbs (Catnb and Cappa1) or two nonselecting peptide-Kbs (Mapk1 and Stat3). We added heterodimers of OVA-Kb–Catnb-Kb, OVA-Kb–Cappa1-Kb, OVA-Kb–Mapk1-Kb, OVA-Kb–Stat3-Kb, or OVA-Kb–OVA-Kb (as a positive control) to OT-I Kb−/−/Db−/− FTOCs 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 Kb−/−/Db−/− thymocytes at similar concentrations as OVA-Kb–OVA-Kb dimers (at 0.5–50 µg/ml or 0.0043–0.43 µM), as indicated by the dramatic decrease in the DP population. They are all similarly potent because as we titrated this effect down to 0.05 µg/ml (0.00043 µM), the OVA-Kb–OVA-Kb 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-Kbs can synergize with agonist-Kbs 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-2Kb (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-Kb has a higher affinity for OT-I than Cappa1-Kb (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 Kb-bound peptides (Catnb-Kb–Catnb-Kb, Cappa1-Kb–Cappa1-Kb, Mapk1-Kb–Mapk1-Kb, or Stat3-Kb–Stat3-Kb) could stimulate thymocytes. We observed that only dimers of Catnb-Kb–Catnb-Kb and Cappa1-Kb–Cappa1-Kb induced calcium elevation in OT-I thymocytes at a protein concentration of 1 mg/ml (8.7 µM; Fig. 4). In contrast, none of the monomeric pMHC could stimulate OT-I DP cells at the same concentration (Fig. 4 and not depicted) and neither could Mapk1-Kb or Stat3-Kb homodimers (Fig. 4).

We then examined whether these early signaling events correlated with the ability to induce positive selection in FTOCs. As shown in Fig. 5 A, monomeric Catnb-Kb did not elicit any increase in the CD8+ SP thymocyte population when compared with the PBS-treated control lobes (and neither did monomeric OVA-Kb [Fig. 3]). In contrast, Catnb-Kb–Catnb-Kb homodimer efficiently mediated positive selection of immature CD8+ SP T cells at or above protein concentrations of 150 µg/ml (1.3 µM; Fig. 5 A and Fig. S2). Even at the highest concentration of 900 µg/ml (7.8 µM), we did not observe negative selection. As for the Cappa1-Kb–Cappa1-Kb homodimers, we observed an increase in the CD8 SP population in FTOCs only with very high concentrations (1.2 mg/ml or 10 µM) of protein (Fig. 5 B and Fig. S2). We were unable to induce positive selection at this concentration with Mapk1-Kb–Mapk1-Kb and Stat3-Kb–Stat3-Kb 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-Kb–Cappa1-Kb, Catnb-Kb–Mapk1-Kb, and Catnb-Kb–Stat3-Kb heterodimers to FTOCs at various concentrations.
In FTOCs cultured with Catnb-Kb–Mapk1-Kb and Catnb-Kb–Stat3-Kb, 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+ SP population with Catnb-Kb–Cappa1-Kb heterodimers at 600 µg/ml (5.2 µM) and 900 µg/ml (7.8 µM) but not at 300 µg/ml (2.6 µM; Fig. 5 C and Fig. S2). This is a lower dose than is required to observe selection in response to Cappa1-Kb–Cappa1-Kb homodimers (1,200 µg/ml; 10 µM). Similarly, we found that only Catnb-Kb–Cappa1-Kb heterodimers are able to induce robust calcium signaling when compared with Catnb-Kb–Mapk1-Kb and Catnb-Kb–Stat3-Kb heterodimers at 1 mg/ml (8.7 µM; 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–Kb molecules induce positive selection in OT-I Kb−/− Dβ−/− FTOC. Thymic lobes were excised from OT-I Kb−/− Dβ−/− 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-CD8β antibodies, and evaluated by flow cytometry. (A) These representative FACS plots compare lobes treated with monomeric Catnb-Kb (with the cross-linker attached) to lobes treated with dimeric Catnb-Kb–Catnb-Kb at the indicated protein concentrations. (B) Representative flow cytometry plots comparing lobes treated with a high concentration (1,200 µg/ml or 10 µM) of homodimers (Stat3-Kb–Stat3-Kb, Mapk1-Kb–Mapk1-Kb, or Cappa1-Kb–Cappa1-Kb). (C) Representative flow cytometry plots comparing lobes treated with heterodimers (Catnb-Kb–Cappa1-Kb, Catnb-Kb–Mapk1-Kb, or Catnb-Kb–Stat3-Kb) at the indicated protein concentrations. The results are representative of at least three independent experiments.
effect of Cappa1-Kb contributing to Catnb-Kb 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-Kb self-ligand could partner with Catnb-Kb to produce an appreciable T cell response, whereas the weaker Mapk1-Kb and Stat3-Kb could not.

Nonnegligible affinity of null self-peptide–Kb 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-Kb’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–Kb’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-Kb and Stat3-Kb (ligands that could partner with OVA-Kb to induce negative selection) showed undetectable binding in SPR, which generally sets a limit in the range of 300 µM for molecules of this size and solubility. Nonetheless, we have shown here that a Kd as low as 211 µM 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 measurable 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–Kbs for the OT-I TCR using SPR. Dimerization makes accurate calculation of a Kd impossible because of cooperativity effects. But using soluble dimers in SPR does allow us to determine whether or not particular self-peptide–Kb 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 (SIINFEKL) 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; Hoggquist et al., 1994; Fremont et al., 1995) and which binds to H-2Kb comparably with the other peptides tested (Fig. S4). We measured the binding of 5 µM of Catnb-Kb–Catnb-Kb, Cappa1-Kb–Cappa1-Kb, Mapk1-Kb–Mapk1-Kb, Stat3-Kb–Stat3-Kb, and SIAAFASL-Kb–SIAAFASL-Kb 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-Kb monomers (Fig. 1), Catnb-Kb and Cappa1-Kb dimers showed the highest level of binding (respectively) to OT-I TCR. Our engineered null SIAAFASL-Kb complex showed no detectable binding even as a dimer. But most importantly, the other self-ligands, Mapk1-Kb and Stat3-Kb, 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–Kb with OVA-Kb 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 µg/ml (0.87 µM), OVA-Kb–SIAAFASL-Kb heterodimers could not stimulate OT-I thymocytes, unlike homodimers of OVA-Kb–OVA-Kb (Fig. 6 B) or heterodimers of OVA-Kb–Stat3-Kb (Fig. 3). Similarly, FTOC co-cultured with OVA-Kb–SIAAFASL-Kb heterodimers at 200 µg/ml or 1.74 µM (which is 400-fold higher than the concentration of OVA-Kb–OVA-Kb 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 Kb docking and/or the surrounding Kb structure, which might also influence CD8 binding. So in addition, we used SIINFEKR(BIO)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, SIINFEK(BIO)L–Kb homodimers showed no detectable binding on SPR when compared with Cappa1-Kb homodimers under the same conditions (Fig. S5). And similarly, this APL could not induce Ca2+ when paired with OVA-Kb (as an OVA-Kb–SIINFEK(BIO)L–Kb heterodimer, Fig. 6 B) nor promote negative selection at high protein concentrations (Fig. 6 C). Thus, even a strong OVA-Kb 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; Krosggaard 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 Catenb-Kb–Catenb-Kb, Cappa1-Kb–Cappa1-Kb, Mapk1-Kb–Mapk1-Kb, Stat3-Kb–Stat3-Kb, and SIAAFASL-Kb–SIAAFASL-Kb homodimeric pMHC. (B) Mean Ca²⁺ ratio of thymocytes mixed with 100 µg/ml (0.87 µM) of OVA-Kb–OVA-Kb, OVA-Kb–SIAAFASL-Kb, or OVA-Kb–SIINFEK(BIO)L-Kb. (C) Thymic lobes were excised from OT-I K⁻/⁻ Db⁻/⁻ embryos at gestational day 16 and co-cultured with homodimers of OVA-Kb–OVA-Kb or with heterodimers OVA-Kb–SIAAFASL-Kb and OVA-Kb–SIINFEK(BIO)L-Kb 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⁺ CD8⁺ DP thymocytes was determined by dividing the difference in the percentage of CD4⁺ CD8⁺ DP population of the experimental lobe by the percent of CD4⁺ CD8⁺ 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 Kᵣₛ measured at 10°C of monomeric and dimeric peptide-Kb binding to OT-I TCR are indicated in the bottom of the graph. * and ‡ represent monomeric Kᵣₛ 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-Kb molecules (Mintern et al., 2004), our data using OVA-Kb 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+ 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-tolerization.

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 (Zerahn 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; Merkenschlager, 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 (Kurts et al., 1996; Peterson et al., 1999; Ebert et al., 2008), which is thought to help enforce the efficiency of thymic self-tolerization.

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-Kb and SIINFEKL-Kb to partner with OVA-Kb 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-Kb exists is unknown, but, nonetheless, our data show that the ability of self-p-Kb to enable the response to OVA-Kb relies on the TCR’s low affinity for those self-peptide–Kbs. The larger number of self-p-Kbs 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-Kb and Cappal-Kb as monomers, but many more self-p-Kbs 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; Naehler 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-Kb have a higher affinity than Stat3-Kb dimers, whereas we could not detect binding of OT-I to Stat3-Kb monomers and could barely detect binding of OT-I to Mapk1-Kb monomers. Our ranking indicates that the affinity of monomeric SIAAFASL-Kb is weaker than that of Stat3-Kb because we could not detect any binding of the dimeric SIAAFASL-Kb. 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–2Kb’s peptide-binding cleft, regardless of the peptide (unless TCR binding is sterically blocked, as in the case of SIINFEKL-Kb). In contrast, the SIINFEKL P3 position contacts Kb’s α–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 μM, e.g., OVA-Kb/OT-I) would induce negative selection when paired with ligands above another much lower threshold. This partner threshold falls somewhere between SIAAFASL-Kb and Stat3-Kb and we can only measure apparent affinities of dimers at 10°C in this range. But the threshold for the partner ligand during positive selection is measureably higher. So far, we have only found one other positively selecting ligand to be sufficient: Cappa1-Kb, which binds OT-I with a monomeric binding affinity of 211 µM, which is 24-fold weaker than OVA-Kb, at 10°C. We predict that this partner threshold would lie somewhere between the monomer-measurable and dimer-measurable ranges, ~250–400 µM, 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: 100–250 µM, in terms of monomeric affinity at 10°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 Kb⁻/⁻D⁻/⁻ mice, OT-I mice (C57BL/6-Tg(Tcrαβerb)1100Mjb/J; The Jackson Laboratory) were crossed with Kb⁻/⁻D⁻/⁻ mice (C57BL/6J-Kb⁻/⁻D⁻/⁻ 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 α chain with an acetic zipper and a β 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 α chain by PCR using the 5'GGAAATATCATTAAGTCCCTGAGCCAGTTCCTTTTCCGGTG-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-2Kb complexes.** The genes for murine class I H-2Kb (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 sub-sequent affinity purification (Krogsgaard et al., 2005). Soluble murine class I MHC proteins H-2Kb 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 as class II MHC molecules (Altman et al., 1993). The peptides OVA257-264 (SIINFEKL), Catnb329-336 (RTYTYEKL), Cappa1α29 (ISFKFDHL), Mapk1α2.5 (VPYRTYNL), Stat3α3.6 (ATLYFHN), SIAAFASL, and SIINFER200L (Bio-Synthesis Inc.) were purchased and analyzed for purity by mass spectrometry. Peptide-based cross-linking re-agents (Schafer-N) were constructed and purified essentially as described (Krogsgaard et al., 2005). The protein concentration was estimated by UV absorbance at 280 nm. 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-2Kb 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°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-IEK was also captured on the same sensor chip. The specific binding of peptide-Kb and OT-I was measured using the in-line reference subtraction feature of the Biacore 3000 instrument. Increasing concentrations of monomeric and dimeric peptide-Kb were injected at a flow rate of 60 µl/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 Response = (Rmax × C)/(Kd + 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-2Kb (AF6-88.5), anti-Vu2 TCR (B20.1), anti-CD69 (H.2F3), anti-CD62L (MEL-14), and anti-CD4 (RM4-5) antibodies (all obtained from BD). The anti-CD8β 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 S100; TV; Carl Zeiss, Inc.) and MetaMorph (Universal Imaging Corporation); 3D time-lapse experiments using a 40× Fluor objective (NA 1.3) were performed essentially as described (Huppa et al., 2003). Thymocytes were isolated by removing whole thymus into cold complete RPMI and disrupting the tissue by pushing it through a nylon mesh. Before imaging, thymocytes were loaded with 10 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 mM CaCl2, and 0.1 mM MgCl2. For time-lapse experiments using purified protein, OT-I Kb⁻/⁻D⁻/⁻ thymocytes in imaging medium were mixed with purified pMHC dimers or pMHC monomers (kept on ice) and imaged in an FCS2 chamber (Bioptechs, Inc.) at 37°C as previously described (Krogsgaard et al., 2005). As an indicator of intracellular Ca²⁺ concentration, fura-2 fluorescence ratios (340/380 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-2Kb binding affinity of peptides used in the study by a peptide competition assay. Fig. S5 shows the relative OT-I TCR affinity of SIINFER200L-Kb homodimers in comparison to Cappa1-Kb homodimers. Online supplemental material is available at http://www.jem.rupress.org/cgi/content/full/jem.20092170/DC1.
We thank K.A. Hogquist and L. McNeil for invaluable help in the early stages of this effort. We also thank Q.-I. Li, F. Tyran, E. Newell, and N. Gascoigne for helpful discussions.

J. Juang is supported by a Medical Scientist Training Program Grant to Stanford University. P.L.R. Bert was supported by a Howard Hughes Medical Institute predoctoral fellowship. M. Krogsgaard is supported as a Pew Research Scholar by the Pew Trust. This work is supported by a grant from the US National Institutes of Health (R01 AI022511) and the Howard Hughes Medical Institute (to M.M. Davis).

The authors have no conflicting financial interests.

Submitted: 8 October 2009
Accepted: 8 April 2010

REFERENCES


Figure S1. Dimers, not oligomers, are purified and used for calcium imaging and FTOC. FPLC purification of dimeric pMHC ligands used in this study. Chromatogram shows a single peak of peptide-K\(^b\) dimer (in red) versus molecular weight standards (in blue). A representative of OVA-K\(^b\) dimer is shown here.
Figure S2. Percent change in CD8 SP in FTOCs in response to an assortment of dimeric pMHC ligands at various concentrations. Positive selection requires that both dimeric peptides be strongly positively selecting ligands. Thymic lobes were excised from OT-1 K\(^{b}\)-D\(^{b}\)-I\(^{d}\) embryos at gestational day 16. One lobe from each pair was cultured with PBS and the other was cultured with one of various homo- and heterodimers 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. The percent change in CD8\(^{+}\) SP thymocytes was determined by dividing the difference in the percentage of CD8\(^{+}\) SP population between experimental and control lobe by the percentage of CD8\(^{+}\) SP in the control lobe and multiplying by 100. Data are presented as mean ± SEM of three or more independent experiments.
Figure S3. Calcium signaling in thymocytes in response to low-affinity heterodimeric pMHC. Soluble heterodimers of Catnb-Kb–Cappa1-Kb can stimulate calcium signaling in thymocytes, whereas heterodimers of Catnb-Kb–Mapk1-Kb and Catnb-Kb–Stat3-Kb cannot. (A) OT-I TCR transgenic H-2Kb−/−Dkb−/− thymocytes were loaded with fura-2 and mixed with soluble Catnb-Kb monomers or with Catnb-Kb–Cappa1-Kb, Cappa1-Kb–Mapk1-Kb, or Catnb-Kb–Stat3-Kb heterodimers at 1 mg/ml (8.7 µM). Representative Ca²⁺ 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 microscope fields, and the mean Ca²⁺ ratio was plotted as a function of time (fura ratio mean ± SEM; n = 7). The results are representative of at least three independent experiments.
Figure S4. Peptide competition assay shows relatively similar H-2Kβ binding affinity of peptides used in the study. SIINFEKL-BIO-Kβ proteins were first attached to the surface of 96-well plates, and then unlabeled OVA, Catnb, Cappa1, Mapk1, Stat3, and SIAAFASL peptides at various concentrations were added and allowed to compete with the labeled SIINFEKL for binding to H-2Kβ. Detection of SIINFEKL-BIO-Kβ was performed by EUSA using europium streptavidin. The percent response represents the amount of residual SIINFEKL-BIO-Kβ divided by the amount of initial SIINFEKL-BIO-Kβ multiplied by 100. LogIC50 for each peptide is presented in the table on the right. The results are representative of at least three independent experiments.
Figure S5. Relative TCR affinity of very weak pMHC homodimers. Sensorgram of the interaction between immobilized OT-I TCR at high density on a CM5 sensor chip with 5 μM Cappa1-K\textsuperscript{\alpha}–Cappa1-K\textsuperscript{\alpha} and SIINFEK\textsuperscript{BIO}L-K\textsuperscript{\beta}–SIINFEK\textsuperscript{BIO}L-K\textsuperscript{\beta} homodimeric pMHC ligands. The results are representative of at least three independent experiments.