A constant affinity threshold for T cell tolerance

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T cell tolerance depends on the T cell receptor’s affinity for peptide/major histocompatibility complex (MHC) ligands; this critical parameter determines whether a thymocyte will be included (positive selection) or excluded (negative selection) from the T cell repertoire. A quantitative analysis of ligand binding was performed using an experimental system permitting receptor–coreceptor interactions on live cells under physiological conditions. Using three transgenic mouse strains expressing distinct class I MHC–restricted T cell receptors, we determined the affinity that defines the threshold for negative selection. The affinity threshold for self–tolerance appears to be a constant for cytotoxic T lymphocytes.

Selection of mature T cells from a pool of immature CD4+CD8+ double-positive (DP) thymocytes is dependent on how their TCRs interact with self-peptide/MHC (pMHC) ligands (1–5). Thymocytes expressing TCRs that fail to recognize any self-pMHC ligand die from “neglect,” whereas weak recognition of self-pMHC complexes by the TCR and coreceptor results in the development of mature, single-positive (SP) T cells (positive selection). Strong recognition of self-pMHC leads to thymocyte death or lineage deviation, removing self-reactive cells from the T cell repertoire (negative selection). Therefore, the peripheral T cell repertoire is both self-pMHC restricted and self-tolerant.

These distinct cell fates are critically dependent on the affinity of TCR–ligand interactions (6–9). Surface plasmon resonance (BiaCore) allows the quantification of bimolecular TCR/pMHC affinities, but does not account for the contribution of the coreceptor (CD4 or CD8β) in the context of a living cell. This is an essential point because collaboration of TCR and coreceptor in pMHC binding is crucial for ligand discrimination and thymocyte selection (10–14). To circumvent these limitations, we made use of a TCR photoaffinity labeling system (15), where the antigenic pMHC complex, SYIPSAEK(ABA)I/H–2Kd, carries a photoreactive azidobenzoic acid (ABA) linked to the lysine present in the peptide. After specific binding of pMHC monomers to the appropriate T cells, photoactivation of the ABA group results in cross-linking of monomeric pMHC complexes to the TCR, allowing for quantitative analysis of pMHC monomer binding (16–18). Our results indicate that thymocytes expressing MHC class I–restricted TCRs use the same affinity threshold to initiate negative selection.

RESULTS AND DISCUSSION

Transgenic mice expressing the T1 TCR, which is a receptor specific for the SYIPSAEK(ABA)I–H–2Kd complex, were generated and backcrossed for at least 15 generations onto β2m−/− Rag−− or Rag−−–only genetic backgrounds. Peptide variants were created by replacing proline at position 4 of the agonist peptide, SYIPSAEK(ABA)I, (referred to as 4P) with leucine (4L), valine (4V), alanine (4A), serine (4S), asparagine (4N), or histidine (4H). Variant peptides bound H–2Kd with a similar affinity (within twofold), except for 4L, which bound H–2Kd with fivefold lower affinity than 4P (unpublished data).

A potency hierarchy for these SYIPSAEK(ABA)I peptide variants was determined by their ability to induce CD69 expression (Fig. 1 A) or TCR down-regulation (Fig. 1, B and C). Peptide potencies were quantified by calculating EC50 values in the CD69 assay and normalizing these values for small differences in H–2Kd binding. The pMHC monomers had a similar affinity (within twofold), except for 4L, which bound H–2Kd with fivefold lower affinity than 4P (unpublished data).

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Photoaffinity labeling in this system provides a measure of the binding strength of a monomeric pMHC ligand for a docking site formed by the TCR and CD8. Because three binding partners are involved, affinity is not the appropriate term to describe this interaction; affinity is applied to bimolecular interactions. Avidity describes the binding of a ligand to multiple partners, but is also commonly used for the binding of a multivalent receptor with a multivalent ligand, e.g., an antibody. Therefore, to best describe the binding of a monomeric ligand to a combining site composed of two distinct molecular species, the term apparent affinity may be most suitable. This term has previously been used to describe the affinity of a substrate for a complex of enzyme and coenzyme (20). This is particularly relevant to self-tolerance because the induction of negative selection is dependent on the interactions of pMHC ligand with the TCR and coreceptor (11, 13, 19, 21–23). Binding measurements were performed on live T1 thymocytes using seven different monomeric pMHC ligands (Fig. 2, A and B), and $K_d$ values were determined using nonlinear regression and Scatchard analysis (Table II and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070254/DC1). The observed apparent affinities were decreased (higher $K_d$ values) at 37°C, compared with 0°C, because of temperature-dependent differences in the contribution of CD8 coreceptors to TCR/pMHC binding (Table II and Fig. S2). Nevertheless, the apparent affinity hierarchy was the same at both temperatures.
A clear correlation between selection potential and apparent affinity was observed (Fig. 2 C). At 37°C, ligands with a $K_d > 6.1 \mu M$ induced positive selection, whereas higher affinity ligands ($K_d \leq 6.1 \mu M$) induced negative selection. These data indicated that negative selection is first attainable with a TCR–CD8/pMHC (apparent) affinity, where the $K_d < 6 \mu M$. This requirement was met by peptides 4S and 4A (Fig. 2 B and Table S2, available at http://www.jem.org/cgi/content/full/jem.20070254/DC1). These ligands were positive selectors, but could induce negative selection when used at high concentrations (Fig. 1 D). The threshold for unambiguous negative selection is likely to be slightly higher ($K_d$ between 5.4 $\mu M$ [4A] and 0.4 $\mu M$ [4V]). Examining the binding of negatively selecting pMHC monomers lacking the CD8-binding site (H-2K$^d_{\alpha 265}$) revealed that CD8 participation increases the affinity of pMHC binding to the T1 TCR by 10–15-fold (Fig. S2) (17). In the absence of the CD8 contribution, the T1 TCR/pMHC-H-2K$^d$ binomial affinity at the selection threshold had a $K_d$ of ~70 $\mu M$ (Fig. S2); this agrees with previous Biacore affinity measurements showing that weak agonist ligands exhibit $K_d$ values in this range (24). It should be noted that CD8 affinity varies for different class I MHC alleles; therefore, CD8’s contribution to apparent affinity varies for different class I MHC ligands as well.

To test whether the apparent affinity threshold for negative selection was a particular feature of the T1 TCR or represented a general feature of negatively selecting ligands, we generated transgenic mice expressing a related TCR, S14. The potencies of these ligands with S14 thymocytes and T cells were measured (unpublished data) and fell into the following hierarchy: 4A > 4P > 4S > 4L > 4N > 4V > 4H. The selecting potential of these variant peptides for the S14 TCR were also determined in FTOC (Fig. 3 A), and 4L was found to be the threshold ligand for S14 thymocytes. Photoaffinity labeling was performed with S14 thymocytes using representative ligands (Fig. 3 B) and the apparent affinity for the threshold ligand, 4L was determined (Table I). The threshold affinities for the S14 ($K_d = 6.4 \pm 1.5 \mu M$) and T1 ($K_d = 6.1 \pm 1.1 \mu M$) receptors were strikingly similar (Table I). To confirm the apparent affinity of the selection threshold using a third unrelated TCR, we used the threshold ligand for the OT-1 receptor SIITFEKL/K$^b$ (11). Because OT-1 ligands cannot be photo–cross-linked to the TCR, these threshold pMHC monomers were converted into tetramers, which were used in binding studies with their corresponding thymocytes (Fig. 3, C–E). As tetramer binding is dependent on CD8 and TCR specificity, it reflects apparent affinity. Tetramer binding of all three threshold ligands to thymocytes expressing the corresponding cognate TCR was remarkably similar (Fig. 3, C–E, and Table I), leading us to conclude that the apparent affinity threshold for negative selection of class I MHC–restricted thymocytes is a constant.

We also measured the apparent affinity on various cell populations in T1 mice (Table II). Postselection DP thymocytes had two- to threefold lower apparent affinity compared with their preselection DP counterparts, indicating that for all ligands there is a decrease of TCR/coreceptor affinity at this stage of thymocyte development. This is consistent with work
from Kao et al. (25), who demonstrated that the transition from pre- to postselection DP thymocytes is accompanied by a reduction in surface glycosylation of CD8. On the other hand, for each T1 pMHC ligand, apparent affinity remained unchanged on postselection DP thymocytes, SP thymocytes, and LN T cells (Table II). This is similar to noncognate tetramer binding, which is similar on postselection DP and SP thymocytes (25). It is interesting that SP thymocytes and LN T cells from T1 mice, lose their responsiveness to low-affinity ligands (compared with postselection DP thymocytes; Table S1). As these three cell populations have similar apparent affinities, this change in responsiveness must have another basis. In this regard, the control of phosphatase activity is important in determining T cell sensitivity to antigens (26, 27).

### Table I. Binding of threshold ligands to thymocytes from three different TCR transgenic mouse strains (T1, S14, and OT-1 TCR)

| TCR/ligand | pMHC monomers | pMHC tetramers |
|------------|---------------|---------------|
|            | DP thymocytes | SP thymocytes | SP thymocytes |
| T1/4A-K^d  | 5.4 ± 1.6 × 10^-6 | 5.5 ± 1.8 × 10^-6 | Not done |
| T1/4S-K^d  | 6.1 ± 1.1 × 10^-6 | 7.9 ± 2.0 × 10^-6 | 9.0 ± 1.5 × 10^-8 |
| S14/4L-K^d | 6.4 ± 1.5 × 10^-6 | 6.2 ± 1.7 × 10^-6 | 8.8 ± 1.6 × 10^-8 |
| OT-1OvaT4-K^b| Not done | Not done | 7.6 ± 0.7 × 10^-8 |

Mean values and SEMs of at least three independent experiments are shown.

Only negative-selecting ligands were able to fully activate naive peripheral T cells (11, 28, 29). With OT-1, T1, and S14 T cells, only high-affinity pMHC ligands (over the selection threshold) were able to induce Ca^2+ fluxes, cytotoxic responses, or proliferation (unpublished data) (11, 28). Therefore, the affinity threshold for negative selection seems to be a functional threshold, at least for the activation of naive peripheral CD8^+ T cells. Nonetheless, low-affinity ligands induced some responses from peripheral T cells, such as CD69 up-regulation (Table S1). Low-affinity, endogenous peptides play an important role as coagonists, enhancing a T cell’s sensitivity to very small numbers of agonist pMHCs (30). This might be the case during negative selection as well, where the copy number of most self-antigens expressed on the surface of thymic APCs is likely to be very low (31). Therefore, the

### Table II. K_d (M) determined for pMHC monomers bound to T1 thymocytes and lymph node T cells

| Cell type        | Ligands (pMHC monomers) | 4L-K^d | 4P-K^d | 4V-K^d | 4A-K^d | 4S-K^d | 4N-K^d | 4H-K^d |
|------------------|-------------------------|--------|--------|--------|--------|--------|--------|--------|
|                  | K_d (M)                 | K_d (M) | K_d (M) | K_d (M) | K_d (M) | K_d (M) | K_d (M) | K_d (M) |
| 0°C              |                         |        |        |        |        |        |        |        |
| Preselection DP  | 6.8 × 10^-10            | 2.9 × 10^-9 | 3.2 × 10^-4 | 2.2 × 10^-7 | 2.2 × 10^-7 | 7.1 × 10^-7 | 3.5 × 10^-6 |
| thymocyte        |                         |        |        |        |        |        |        |        |
| Postselection DP | 1.8 × 10^-9             | 6.1 × 10^-9 | 5.9 × 10^-4 | 5.0 × 10^-7 | 5.2 × 10^-7 | 2.2 × 10^-6 | 5.9 × 10^-6 |
| thymocyte        |                         |        |        |        |        |        |        |        |
| SP thymocyte     | 1.4 × 10^-9             | 5.3 × 10^-9 | 3.3 × 10^-4 | 5.6 × 10^-7 | 3.7 × 10^-7 | 2.3 × 10^-6 | 7.8 × 10^-6 |
| LN T cell        | 1.5 × 10^-9             | 6.8 × 10^-9 | 3.0 × 10^-4 | 4.5 × 10^-7 | 3.3 × 10^-7 | 1.6 × 10^-6 | 6.0 × 10^-6 |
| \Delta K_d (SP/DP) | 0.8                     | 0.9     | 0.6    | 1.1    | 0.7    | 1.0    | 1.3    |
| \Delta K_d (LN/DP)| 0.8                     | 1.1     | 0.5    | 0.9    | 0.6    | 0.7    | 1.0    |

37°C

|                  |                         |        |        |        |        |        |        |        |
| Preselection DP  | 2.8 × 10^-8             | 8.8 × 10^-8 | 2.6 × 10^-7 | 2.2 × 10^-6 | 2.9 × 10^-6 | >6 × 10^-5a |
| thymocyte        |                         |        |        |        |        |        |        |        |
| Postselection DP | 6.1 × 10^-8             | 1.7 × 10^-7 | 4.3 × 10^-7 | 5.4 × 10^-6 | 6.1 × 10^-6 | 5.5 × 10^-5a | >6 × 10^-5a |
| thymocyte        |                         |        |        |        |        |        |        |        |
| SP thymocyte     | 4.0 × 10^-8             | 1.1 × 10^-7 | 3.5 × 10^-7 | 5.5 × 10^-6 | 7.9 × 10^-6 | 5.8 × 10^-5a | >6 × 10^-5a |
| LN T cell        | 2.5 × 10^-9             | 1.2 × 10^-7 | 3.6 × 10^-7 | 4.7 × 10^-6 | 5.8 × 10^-6 | 3.1 × 10^-5a | >6 × 10^-5a |
| \Delta K_d (SP/DP)| 0.7                     | 0.6     | 0.8    | 1.0    | 1.3    | nd     | nd     |
| \Delta K_d (LN/DP)| 0.4                     | 0.7     | 0.8    | 0.9    | 0.9    | nd     | nd     |

Mean values of at least three independent experiments are shown. nd, not determined. \Delta K_d represents the fold difference in ligand affinities between T lineage cells at the indicated developmental stages.

^Extrapolated values.
presence of low-affinity endogenous ligands may increase the thymocyte’s sensitivity to the copy number of its cognate self-antigen, provided its apparent affinity is above the threshold for negative selection (11). This affinity and its corresponding “off-rate” for ligand dissociation (Table S2) (6, 8, 9, 32) are essential quantitative parameters needed to understand the basis of ligand discrimination, on which T cell specificity and self-tolerance depend.

MATERIALS AND METHODS

Antibodies. The following fluorescently labeled mAbs were purchased from BD PharMingen: α-TCR-ββ (H57-597), α-CD4 (RM4-5), α-CD8α (53–6.7), α-CD8β (53–5.8), and α-CD69 (H1.2F3). The α-CD8β (H35-17) mAb and the α-H-2Kb(a11) mAb (20–8-4S) (American Type Culture Collection) were produced by hybridomas and purified by protein G chromatography.

Mice and cells. S14 TCR (17) transgenic mice were produced as previously described for T1 TCR transgenic mice (33). Founders were backcrossed to BALB/c Rag-/- or B10.D2 Rag-/- β2m-/- mice for at least 15 generations. Similarly, C57BL/6 OT-1 Rag-/- mice were bred in our colony. Thymi or lymph nodes from these mice were harvested, and single-cell suspensions were used in the experiments described. Animal experiments were performed in accordance with the federal laws in Switzerland, and they were approved by the veterinary office of the canton of Basel.

CD69 and TCR down-regulation assays. Freshly isolated thymocytes or lymphocytes from T1 or S14 Rag-/- mice were stimulated with peptide-loaded T2-H-2Kb cells, and EC50 values were determined as previously described (11).

Fetal thymic organ culture. FTOCs were performed using thymi of fetuses (E15) from Rag-/- β2m-/- T1 or S14 TCR transgenic mice, as previously described (11). Thymocytes were harvested after 7 d, stained with α-CD4, α-CD8α, and/or α-CD8β mAbs, and analyzed by flow cytometry.

Ligand-binding measurements. Soluble peptide-MHC monomers and tetramers were produced as previously described (11, 16). Direct photoaffinity labeling experiments were performed as previously described using Cy5-labeled H-2Kb/Plasmodium berghei circumsporozoite (PbCS)252–260 SYIPSAEK(ABA)I complexes (15, 16). For two-step photoaffinity labeling experiments, thymocytes or lymph node T cells (107 cells) were incubated in DMEM complete media (+10% FCS) supplemented with indicated concentrations of unlabeled H-2Kb/PbCS252–260 SYIPSAEK(ABA)I complexes or variant peptide monomers. Samples were irradiated with UV light (312 ± 40 nm; 90 Watts) to photochemically cross-link cell-bound monomers (referred to as Step 1). Cells were washed three times at room temperature to remove monomers which were not covalently bound to TCR. The fraction of remaining free TCR was measured using a constant concentration (10−6 M) of Cy5-labeled H-2Kb/PbCS252–260 SYIPSAEK(ABA)I mAbs. Samples were subjected to a second round of UV irradiation (312 ± 40 nm; 90 Watts; Step 2), washed, and stained with α-TCR-ββ, α-CD4, and α-CD8α mAbs, followed by flow

Figure 3. T1, S14, and OT-1 TCR transgenic animals exhibit a similar affinity threshold for negative selection. (A) Thymic selection properties of peptide variants specific for S14 TCR transgenic thymocytes were determined by FTOC. Positive and negative selection were assessed as in Fig. 1 D. (B) Binding of pMHC monomers (4A-H-2Kb, 4L-H-2Kb, and 4H-H-2Kb) to thymocytes from S14 TCR transgenic mice at 37°C using the two-step photoaffinity labeling method. (C–E) Tetramer binding of threshold ligands to thymocytes from T1, S14, or OT-1 TCR transgenic mice at 37°C. (C) 4S-H-2Kb tetramers applied to T1 thymocytes. (D) 4L-H-2Kb tetramers applied to S14 thymocytes. (E) SIITFEKL-Kb tetramers applied to OT-1 thymocytes. Tetramer concentrations resulting in half-maximal TCR saturation are indicated in each panel.
cytometric analysis. In contrast to the direct photoaffinity labeling technique, Cy5 signals gained by the two-step method represented the fraction of free TCR (α free TCR) after the first round of ligand binding. Ligand concentration–dependent binding curves were fitted using Prism software performing nonlinear regression analysis (using the four-parameter equation). $K_d$ values were determined from fitted curves representing free TCR (α free TCR) or by Scatchard analysis. Tetramer binding assays were performed, as previously described (11). $r_1/2$ represents tetramer concentration to achieve 50% binding.

TCR-CD8/pMHC dissociation (off-rate) measurements. Thymocytes from T1 Rag $^{-/-}$ mice were subjected to saturating concentrations of unlabeled peptide–H-2K$^d$ complexes for defined periods of time, allowing for steady-state binding to TCR. Samples were diluted 500-fold with medium containing 40 μg/ml α-H-2K$^d$α1) mAb (20–8–4S) to prevent ligand rebinding, and UV was irradiated at defined intervals (Step 1), followed by Step 2 and flow cytometric analysis as described for the two-step photoaffinity labeling method. $r_1/2$ represents the time at which 50% of bound ligands dissociated from the TCR.

Online supplemental material. Fig. S1 shows that determination of $K_d$ values for TCR-CD8/pMHC interactions was very similar using direct photoaffinity labeling or the two-step photoaffinity labeling methods (A–C). $K_d$ values are similar when determined by nonlinear regression or Scatchard analysis (compare B and D). In the direct-binding assay, 4P-H-2K$^d$-Cy5 $1/2$ values are similar when determined by nonlinear regression or Scatchard analysis, which was not a feature of the two-step assay used for the experiments described in this report. Fig. S2 shows the contribution of CD8 to TCR/pMHC binding at 37°C (A–D) and the contribution of CD8 (E) to the temperature dependence of TCR–CD8–pMHC interactions (F). Table S1 displays the potencies of variant peptides recognized by T1 thymocytes and lymph node T cells. Table S2 displays TCR–CD8–pMHC $r_1/2$ experimentally determined with DP thymocytes from T1 TCR mice. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070254/DC1.

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