Degree of TCR Internalization and Ca$^{2+}$ Flux Correlates with Thymocyte Selection

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Degree of TCR Internalization and Ca\(^{2+}\) Flux Correlates with Thymocyte Selection\(^1\)

Sanjeev Mariathasan,* Martin F. Bachmann,* Denis Bouchard,† Toshiaki Ohteki,* and Pamela S. Ohashi\(^2\)

Recent evidence suggests that TCR down-regulation directly reflects the number of TCRs that have engaged MHC/peptide ligand complexes. Here, we examined the influence of defined peptides on thymic selection based on their ability to induce differential TCR internalization. Our results demonstrate that there is a direct correlation: peptides that induce strong TCR down-regulation are most efficient at mediating negative selection, whereas peptides that induce suboptimal TCR internalization are more efficient at triggering positive selection. As a consequence of suboptimal TCR internalization, a proportion of TCR complexes that remain on the cell surface may be able to relay continual signals required for survival and differentiation. In addition, we show that the magnitude of Ca\(^{2+}\) influx set by these peptides reflects the hierarchy of TCR down-regulation and correlates with positive vs negative selection of transgenic thymocytes. Together, our data suggest that T cell selection is mediated by differing intensities of the same TCR-mediated signal, rather than by distinct signals.


Materials and Methods

Mice

TCR-transgenic mice (327 line) were previously generated using \(\alpha\)- and \(\beta\)-chains isolated from CTL clone P14, which recognized the LCMV-gp (peptide p33–41) presented by H-2D\(^b\) (29). This line was crossed with

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H-2βm−/− mice (30). TCR-transgenic F1 mice were subsequently backcrossed with βm−/− mice to obtain TCR βm−/− (H-2b) mice (14).

**Peptides**

The peptides p33 (KAVYNFATM), S7A (KAVYNFSTM), Y6F (KAVYNYATM), A4Y (KAVANFATM), L6F (KAVYNLATM), W4Y (KAVWNFATM), adenovirus peptide AV (SGPSNTPPEI), and LCMV nucleoprotein 118–127 (RPQASGQVYM) were synthesized by a solid-phase method using the F-moc/tBu-based protocol. Chain assembly was conducted at the Angen Institute (Thousand Oaks, CA), as previously described (14).

**Peptide binding assay**

In peptide-pulsing experiments, 10⁶ RMA-S cells, which were previously cultured overnight at 29°C in RPMI plus 10% FCS, were incubated with various concentrations of peptide at 29°C for 30 min. These cells were then transferred to a 37°C incubator for 3 h, after which the cells were washed and stained with anti-H-2D⁺ mAb from tissue culture supernatant (B22.249) (31, 32) and then FITC-conjugated rat anti-mouse Ig (Sigma, St. Louis, MO). RMA-S cells were incubated with LCMV nucleoprotein 118–127 (H-2b restricted) (33) to determine background H-2D⁺ expression.

**Induction of TCR down-regulation**

Spleen cells from TCR transgenic mice (10⁶/well) were mixed with peptide-pulsed macrophages (2 × 10⁶/well), centrifuged, and incubated at 37°C (5% CO₂) in IMDM supplemented with 10% FCS in round-bottom 96-well plates. Five hours later, cells were harvested and stained for CD8 (PE; PharMingen, San Diego, CA), and were analyzed by FCM. Median Fluorescence intensity is shown for CD8⁺ T cells.

**Proliferation assays**

Spleen cells (10⁶/well) from TCR-transgenic or TCR-transgenic recombination-activating gene-2−/− mice were incubated in triplicate in 96-well flat-bottomed plates with 10⁻⁶ M iodinated C57BL/6 (H-2b) splenocytes that were prelabeled with various concentrations of peptide for 1 h at 37°C. After 40 h of cocultivation, the cells were pulsed with 1 μCi of [³H]thymidine (Amersham, Arlington Heights, IL) for 16 h. Cells were harvested and counted on a direct β-counter (Matrix96, Canberra Packard, Canada, Mississauga, Canada).

**Fetal thymic organ cultures (FTOC)**

For positive selection assays, timed breedings were established between TCR-transgenic βm−/− and βm−/−, H-2b−/− males. To test for negative selection, TCR βm− males were bred with C57BL/6 females. On day 16 of gestation, females were sacrificed, and thymic lobes were removed from the fetuses. The thymic lobes were placed on 0.8μm polycarbonate filters (Costar, Cambridge, MA), which floated on 1 ml of IMDM, 1X Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN), 5 × 10⁻⁵ M 2-ME, penicillin, streptomycin, 2 mM glutamine, 2.5 μg human βm (Sig- ma) and designated peptides. These lobes were then cultured for 6 days at 37°C, during which time the medium and peptides were added daily. After this incubation period, the thymic lobes were teased apart and stained with mAbs at 4°C in PBS containing 2% FCS and 0.2% NaN₃. Three-color analysis was performed with rat anti-mouse PE-conjugated anti-CD4 (Cedarlane), and biotinylated anti-CD8 (Cedarlane), and peridinin-chlorophyll protein (PerCP) anti-CD3 (PharMingen). Biotinylated Abs were detected with streptavidin red 670 (Life Technologies, Gaithersburg, MD).

**Flow cytometry**

All flow cytometric analysis was performed on a FACScan instrument (Becton Dickinson, Mountain View, CA). Samples were gated for live cells based on forward and side scatter parameters (10,000 events/sample) and were analyzed using LYSIS II software (Becton Dickinson).

**FTOC proliferation assay**

 Cultured thymic lobes were teased apart and stained at 4°C in PBS containing 2% FCS with FITC-conjugated anti-CD8 (Cedarlane) and PE-conjugated anti-CD4 (Cedarlane). These cells were then sorted using a FACStar Plus (Becton Dickinson) to collect CD8⁺ thymocytes. Irradiated spleen cells from a C57BL/6J mouse were pulsed with 10⁻⁴ M peptides for 1 h at 37°C, washed, and distributed in triplicate on a flat-bottom 96-well plate at a concentration of 10⁶ cells/well. CD8⁺ thymocytes (5 × 10⁵/well) resuspended in IMDM, 10% FCS, penicillin, streptomycin, and 5 × 10⁻⁵ M 2-ME were then added to these wells. The cells were cultured at 37°C for 1 day, pulsed with 1 µCi of [³H]thymidine for 16 h, and harvested as described.

**Ca²⁺ flux**

TCR-transgenic thymocytes were loaded with indo-1 (10 μM) for 1 h at 37°C in IMDM supplemented with 2% FCS. Indo-1⁺ cells exhibiting a large forward scatter corresponding to thymocyte-APC duplexes (34) were analyzed with FACS Vantage (Becton Dickinson) and CellQuest software, using an ion laser (Innova Enterprise: Coherent, Santa Clara, CA) optimized for UV argon ions, set for 355-nm excitation at a power setting of 50 mV. For stimulation of thymocytes, peritoneal macrophages were pulsed with various peptides (p33, S7A, Y6F, A4Y, W4Y, L6F, and AV) for 1 h. Macrophages (4 × 10⁶/ml) were mixed with thymocytes (1 × 10⁶ cells/ml) at 4°C, centrifuged, and warmed to 37°C for 3 min. Cells were gently resuspended and immediately analyzed. The basal level of Ca²⁺ flux observed in thymocytes in the presence of unpulsed APCs was calibrated at 200, as an arbitrary value. The Ca²⁺ flux induced by the nonstimulatory AV peptide was superimposable on this basal Ca²⁺ flux and hence should be read as the baseline response.

**Results**

**Differential TCR down-regulation by altered peptide ligands**

Using LCMV-specific H-2Db-restricted transgenic mice we have identified peptides that were efficient at inducing either positive or negative selection (12, 14). The peptide p33 (KAVYNFATM) readily induced negative selection at high concentrations (10⁻⁶–10⁻⁷ M) and only induced detectable positive selection within a narrow concentration range (10⁻¹³–10⁻¹² M). An altered peptide ligand, A4Y (KAVANFATM), promoted efficient positive selection over a wide range of concentrations (10⁻⁴–10⁻⁹ M) in the absence of detectable clonal deletion in FTOC. To identify unique properties associated with these peptides, we tested their ability to induce TCR down-regulation on mature T cells. In this assay, macrophages were pulsed with p33, A4Y, or control H-2Db-binding adenovirus (AV) peptide and were incubated with TCR transgenic splenic cells. Five hours later, T cells were harvested and analyzed for TCR expression. The strong agonist peptide, p33, induced strong down-modulation of the TCRs. In contrast, peptide A4Y only moderately down-regulated the TCRs. The control peptide, AV, failed to alter TCR expression, similar to that in an unstimulated T cell population (Fig. 1A). This raised the possibility that positively selecting ligands stimulate the TCR but do not lead to maximal TCR triggering, while negatively selecting ligands trigger efficient TCR internalization.

To identify other variant peptides that would also induce differential TCR down-regulation, macrophages were pulsed with graded doses of peptides and incubated with TCR transgenic splenocytes for 5 h. By analyzing TCR expression on CD8⁺ T cells, peptides were divided into categories defined by TCR down-regulation profiles. Peptide variants S7A (KAVYNFSTM) and Y6F (KAVNYATM) induced strong TCR internalization similar to p33, whereas W4Y (KAVWNFATM) and L6F (KAVYN LATM) showed weak down-regulation profile approximating that of A4Y (Fig. 1, B and C). Even at the highest concentration of peptide tested (10⁻⁴ M), peptide variants A4Y, L6F, and W4Y could not reach the magnitude for TCR down-regulation that was achieved using similar concentrations of p33, S7A, or Y6F.

The kinetics of TCR down-regulation were also investigated. These studies indicate that the onset of TCR internalization begins within minutes after contact with relevant peptide/MHC complexes (25). While A4Y, L6F, and W4Y induced maximal TCR internalization levels after 3–4 h, p33, S7A, and Y6F induced TCR down-regulation with faster kinetics, reaching almost maximal TCR internalization within approximately 2 h (data not shown). Thus, the extent and kinetics of TCR down-modulation appear to be dictated by individual TCR-MHC/peptide interactions. It is
likely that this reflects the affinity of the TCR for the peptide/MHC ligands.

**TCR down-regulation correlates with the efficiency of T cell proliferation**

To evaluate the biologic significance of differential TCR internalization induced by the variant peptides, we determined the proliferative capacity of naive transgenic T cells. Proliferation assays were performed by cocultivating transgenic spleen cells with irradiated nontransgenic splenic APCs pulsed with various concentrations of peptides, and proliferation was measured. This experiment was repeated three times with similar results.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Degree of TCR down-regulation correlates with degree of T cell proliferation. Mature spleen cells from TCR transgenic mice were stimulated with H-2b spleen cells pulsed with various concentrations of peptides, and proliferation was measured. This experiment was repeated three times with similar results.

Peptides that moderately down-regulate mature TCRs mediate efficient positive selection

Since A4Y efficiently induced positive selection (14), it was possible that peptides inducing similar TCR down-modulation should...
FIGURE 4. Peptides that induce suboptimal TCR down-regulation mediate efficient positive selection of thymocytes. Three-color analysis of TCR $\beta_m^m$–/– thymic lobes cultured for 7 days with $10^{-7}$ M A4Y, L6F, W4Y, or AV (a control H-2D$^b$-restricted adenovirus peptide) were stained with Abs specific for CD4, CD8, and Vα2 or HSA. CD8$^+$ or CD4$^+$CD8$^+$ cells were gated, and the profiles of Vα2 and HSA are shown. These data are representative of five experiments. The mean CD8α expressions on thymocytes selected on AV, A4Y, L6F, and W4Y are 195, 154, 168, and 153, respectively (arbitary units).

also mediate positive selection of transgenic thymocytes. Therefore, fetal thymic lobes from TCR $\beta_m^m$–/– mice were cultured with A4Y, L6F, and W4Y in the presence of exogenous $\beta_m$ to examine their influence on positive selection. A4Y, L6F, and W4Y ($10^{-7}$ M) efficiently induced the development of CD8$^+$ T cells in the FTOC (Fig. 4). In lobes where control peptide AV was added, the percentage of CD8$^+$ T cells was 11.5 ± 1.9%. These cells expressed reduced levels of the transgenic TCR. However, upon incubation with A4Y, L6F, and W4Y the percentage of CD8$^+$ cells increased to 30.1 ± 7.9% ($p < 0.003$), 30.6 ± 8.4% ($p < 0.003$), and 29.0 ± 2.9% ($p < 0.0001$), respectively. These cells had high levels of the transgenic TCR and low expression of HSA, characteristic of mature thymocytes. As shown in Table I, the positively selecting peptides generated 3–5 times as many CD8$^+$ thymocytes as the negative control, AV peptide. Therefore, these data suggest that peptides that trigger moderate TCR internalization are efficient at positively selecting transgenic thymocytes.

We observed a modest down-regulation of CD8α expression on thymocytes maturing in the presence of A4Y, L6F, and W4Y (Fig. 4). Using the P14 transgenic system, other researchers have also shown that A4Y selects T cells with down-regulated CD8$^+$ expression (35). Given the similarity of A4Y to W4Y and L6F, we would predict that W4Y and L6F would induce a similar CD8$^+$ down-regulation. T cells being selected on moderate agonist peptides could down-modulate their coreceptors to decrease the avidity to their cognate ligands to escape negative selection.

A4Y-, W4Y-, and L6F-selected thymocytes are responsive to p33

To further examine the functional maturity of the thymocytes that were selected in the presence of the positively selecting peptides, proliferation assays were performed. Purified CD8$^+$ cells from TCR $\beta_m^m$+ thymic lobes as well as TCR $\beta_m^m$–/– thymic lobes incubated with A4Y, L6F, and W4Y were cocultured with irradiated splenocytes that were pulsed with p33, AV, or the corresponding positively selecting peptide. Although the positively selected TCR $\beta_m^m$–/– thymocytes did not respond to the selecting peptide, they mounted a proliferative response to the strong agonist peptide p33 (Fig. 5). In contrast, TCR $\beta_m^m$+ thymocytes selected by natural endogenous ligands proliferated not only in response to p33, but also in response to the positively selecting peptides. These results demonstrate that the positively selected TCR $\beta_m^m$–/– thymocytes are functionally mature, since they proliferated against p33 and are self tolerant.

Peptides that strongly down-regulate TCRs on mature T cells induce negative selection of thymocytes

The ligand p33, which induces strong TCR down-regulation, has been shown to negatively select transgenic thymocytes in FTOCs (12, 13). Based on the correlation between the activity of p33 in the TCR down-regulation assay and that in the FTOCs, it was possible that the variant peptides S7A and Y6F, which induce maximal TCR down-regulation, might also effectively delete CD8$^+$ T cells. TCR $\beta_m^m$+ thymic lobes were cultured with p33, S7A, or Y6F at $10^{-6}$ M to investigate their roles in negative selection. We consistently observed a four- to fivefold decrease in total cell recovery from cultures treated with these peptides compared with that from control cultures that were incubated with AV peptide (Table I). Notably, the residual CD8$^+$ cells that were found in these cultures expressed reduced levels of transgenic Vα2 TCRs and intermediate to high amounts of HSA, indicating their lack of maturity (Fig. 6A). In contrast, fetal thymic lobes cultured with A4Y, L6F, or W4Y did not show a decrease in total cell recovery compared with that in cultures treated with AV, suggesting that these peptides were not efficient in mediating deletion. The CD8$^+$ thymocytes were Vα2highHSAlow, demonstrating that A4Y, L6F, and W4Y did not trigger detectable negative selection of transgenic thymocytes (Fig. 6B). These data demonstrate that peptides

<table>
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<tr>
<th>Peptide</th>
<th>Total Cell Number</th>
<th>CD8$^+$ Cell Number</th>
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</thead>
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<tr>
<td>TCR, $\beta_m^m$–/– (n = 6)$^*$</td>
<td>22.4 ± 4.1 × 10⁴</td>
<td>2.5 ± 0.5 × 10⁴</td>
</tr>
<tr>
<td>AV</td>
<td>29.1 ± 4.0 × 10⁴</td>
<td>7.9 ± 1.8 × 10⁴</td>
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<tr>
<td>A4Y</td>
<td>35.7 ± 5.7 × 10⁴</td>
<td>9.7 ± 1.7 × 10⁴</td>
</tr>
<tr>
<td>L6F</td>
<td>22.3 ± 4.8 × 10⁴</td>
<td>6.3 ± 1.9 × 10⁴</td>
</tr>
<tr>
<td>TCR, $\beta_m^m$+ (n = 5)$^*$</td>
<td>49.2 ± 6.7 × 10⁴</td>
<td>17.5 ± 4.0 × 10⁴</td>
</tr>
<tr>
<td>AV</td>
<td>41.8 ± 5.2 × 10⁴</td>
<td>9.1 ± 1.0 × 10⁴</td>
</tr>
<tr>
<td>A4Y</td>
<td>38.3 ± 6.5 × 10⁴</td>
<td>11.5 ± 2.1 × 10⁴</td>
</tr>
<tr>
<td>L6F</td>
<td>42.0 ± 2.8 × 10⁴</td>
<td>7.6 ± 2.3 × 10⁴</td>
</tr>
<tr>
<td>p33</td>
<td>12.2 ± 2.8 × 10⁴</td>
<td>1.0 ± 0.1 × 10⁴</td>
</tr>
<tr>
<td>S7A</td>
<td>8.3 ± 1.2 × 10⁴</td>
<td>0.8 ± 0.3 × 10⁴</td>
</tr>
<tr>
<td>Y6F</td>
<td>11.0 ± 1.0 × 10⁴</td>
<td>1.0 ± 0.2 × 10⁴</td>
</tr>
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$^*$ $10^{-7}$ M peptides.

$^*$ $10^{-6}$ M peptides.

FIGURE 5. TCR thymocytes selected in the presence of A4Y, L6F, or W4Y are tolerant to the selecting peptide, but proliferate in response to the strong agonist p33. TCR $\beta_m^m$+ thymocytes from TCR $\beta_m^m$–/– thymic lobes cultured with A4Y (A), L6F (B), or W4Y (C; shaded bars) or TCR $\beta_m^m$+ thymic lobes cultured in medium alone (solid bars) were cocultured with peptide-coated APC. Proliferation was measured 24 h later. These results are representative of three experiments.
that induce strong TCR down-regulation also induce negative selection, whereas peptides that moderately down-regulate TCRs promote positive selection.

**Positively and negatively selecting peptides induce distinct calcium signals**

Differential calcium signaling has been implicated in the regulation of T cell effector functions in response to altered peptide ligands (26–28). Therefore, we tested positively and negatively selecting ligands for their ability to induce increases in intracellular calcium \([\text{Ca}^{2+}]_i\). To examine the changes in intracellular calcium following stimulation, thymocytes were loaded with indo-1 mixed with peptide-pulsed macrophages. \([\text{Ca}^{2+}]_i\) was determined immediately for indo-1-stained thymocytes that had formed complexes with APCs, as judged by a large forward scatter. In this assay, negatively selecting peptides p33, S7A, and Y6F induced a strong calcium flux (Fig. 7A). However, positively selecting peptides A4Y, L6F, and W4Y incubated under the same activating conditions induced a quantitatively different \([\text{Ca}^{2+}]_i\) profile. Although the initial increase in \([\text{Ca}^{2+}]_i\) was high, it declined rapidly. However, the response did not result in a convergence at a minimum. Rather, each response asymptotically approached a distinct steady state level and remained elevated for at least 1 h (data not shown). The AV peptide, which induced neither positive nor negative selection, did not induce any calcium flux over the basal level observed in thymocytes in the presence of unpulsed APCs. We also observed similar degrees of conjugate formation when incubating thymocytes with either strong or weak agonist peptide-pulsed APCs compared to the relatively low level of conjugate formation when incubating with the nonstimulatory AV-pulsed APCs. It is possible that greater differences would be observed with weaker, partial agonists and strong antagonist peptides to the P14 transgenic system, in accordance with our previously published results on mature splenic T cells (28).

**FIGURE 6.** Peptides that strongly down-regulate TCRs are most efficient at inducing negative selection of thymocytes. TCR βm

\[\text{m}^\dagger\] thymic lobes were cultured daily for 7 days with 10^{-6} M peptides. Thymocytes were then analyzed with Abs specific for CD4, CD8, and Va2 or HSA. Va2 and HSA profiles for gated CD8+ cells are shown. These data are representative of five experiments.

**FIGURE 7.** Positively and negatively selecting ligands induce different calcium signals. A, TCR transgenic thymocytes H-2D^b preloaded with indo-1 were centrifuged and incubated at 37°C with various macrophages pulsed with 10^{-5} M p33 or variant peptides. Three minutes later, free intracellular \([\text{Ca}^{2+}]_i\) was measured (by the indo-1 405/485 ratio) for the indicated time span. One representative experiment of three is shown. B, TCR transgenic thymocytes (H-2D^b) were compared with TCR transgenic mice from H-2D^d mice. Representative calcium dot plots over time are shown for thymocytes responding to the nominal peptide, p33; a strong agonist, S7A; a moderate agonist, L6F; and the nonstimulatory peptide, AV.

These experiments were performed on H-2D^b thymocytes, in which positive selection is an ongoing process. Therefore, the observed calcium flux in response to the strong and moderate agonists is in addition to the selection signals recently received by the thymocytes.
thymocytes in vivo. To measure the calcium flux in cells that have not yet undergone selection, we used thymocytes from TCR-transgenic nonselecting H-2d mice. As shown in Fig. 7B, we observed similar results with these mice. Furthermore, we observed that the calcium flux induced by these peptides tightly correlated with their TCR internalization profiles. Thus, the magnitude of calcium signaling induced by positively and negatively selecting ligands suggests that there is a quantitative role for calcium and/or calcium-dependent pathways during thymic selection.

**Discussion**

**Extent of TCR triggering correlates with positive and negative selection**

Using a defined set of peptide variants of the LCMV-gp, we have shown that these peptides induce different degrees of transgenic TCR internalization (Fig. 1C). Using mature T cells, we have previously shown that the extent of TCR down-regulation correlates with the strength of TCR-mediated signals and the induction of a variety of effector functions (21). Accordingly, peptides that strongly down-regulated TCRs (p33, S7A, and Y6F) induced a greater proliferative response than peptides that induced intermediate TCR internalization (A4Y, L6F, and W4Y; Fig. 2). We show here that all variant peptides used in this study bind to H-2Db efficiently (Fig. 3). Therefore, the relative agonist strength of the peptides in these assays (p33, S7A > Y6F > A4Y, L6F, W4Y > AV) reflected the presumed affinities of these peptides for the LCMV transgenic TCR and paralleled the TCR down-regulation profiles. By testing these peptides in FTOCs, we demonstrated that weak agonist peptides that moderately triggered the TCRs were most efficient at mediating positive selection (Fig. 4). However, these peptides were inefficient at inducing clonal deletion (Fig. 6 and Table I). Conversely, peptides that induced strong TCR-mediated signals were most efficient at triggering negative selection at similar peptide concentrations. It is worth noting that maximal TCR down-regulation seen with weak agonists never reaches the levels induced by strong, agonist peptides.

Several studies have shown that partial agonist and antagonist peptides transmit distinct TCR-mediated intracellular signals from agonist ligands (36–38). Such specialized signals induced by antagonist and agonist peptides have been implicated in triggering positive and negative selection, respectively (3). Our data demonstrate that positive or negative selection may be induced by varying intensities of the same TCR-mediated events. We have shown that the level of TCR internalization induced by each peptide is dependent on peptide concentration (Fig. 1C). This suggests that different outcomes of TCR-mediated interactions could be induced by different intensities of the same signal in a peptide-specific, concentration-dependent manner. We have observed that at high concentrations of A4Y, L6F, or W4Y some apoptosis is detected by annexin staining, demonstrating that some thymocytes are concurrently undergoing negative selection. In addition, low concentrations of Y6F (10⁻⁷ M) and S7A (10⁻¹⁰ M) have been shown to enhance maturation of CD8⁺ thymocytes (data not shown). Previous studies have also shown that the same peptide could mediate positive selection at low concentrations and clonal deletion at high concentrations (12, 13). Together, these findings are consistent with the affinity/avidity model for thymocyte selection.

**TCR dimerization, internalization, and thymic selection**

Several experiments have suggested that oligomerization of TCRs is important for TCR internalization and subsequent T cell activation (24, 25). Consistent with this idea, prolonged TCR occupancy has to occur to achieve efficient multimerization (39–41). Numerous studies have shown that low affinity APLs have a faster dissociation rate than the high affinity, agonist ligands (42–44). The decreased occupancy time associated with weak agonists would affect the degree of oligomerization and subsequent internalization, as observed by the positively selecting peptides. Thymocytes may require such weak triggering for survival, but may die if significant numbers of TCRs are engaged within a certain time frame.

Consistent with our results, parameters that modify receptor oligomerization and subsequent internalization could also affect T cell selection events. T cells interacting with target cells undergo sequential morphologic changes coincident with actin polymerization, suggesting that the cytoskeleton may play a role in mature T cell activation (45, 46). The proto-oncogene vav has been shown to function as a guanine nucleotide exchange factor for Rho-like small GTPase family members RhoA, Rac1, and Cdc42 that regulate cytoskeletal organization (47–50). Interestingly, a requirement for vav has been implicated in thymocyte selection (51, 52). Therefore, dysregulated cytoskeletal changes in the vav-deficient thymocytes may be responsible for the disturbed oligomerization and down-regulation of TCRs and the impaired positive and negative selection.

**Reactivity of mature T cells altered during positive selection**

Functional analysis of TCR transgenic CD8⁺ thymocytes maturing in the presence of defined, positively selecting ligands showed that they responded strongly to the agonist ligand p33. However, they reacted poorly to the selecting peptides. We also observed that T cells selected on positively selecting peptides, such as A4Y, W4Y, or L6F, were not only unable to proliferate in response to the corresponding selecting peptide, but also were nonresponsive to other moderate agonists in this class (e.g., thymocytes selected on A4Y did not respond to W4Y nor L6F). However, thymocytes selected on these agonist peptides showed a strong response not only to the wild-type peptide p33 but also to the other strong agonist peptides, S7A and Y6F (data not shown). Proliferation assays performed on purified CD8⁺ T cells from β₂m⁺/+ FTOCs indicate that TCR transgenic T cells developing in the presence of endogenous peptides could proliferate in response to A4Y, W4Y, and L6F and could proliferate with a stronger response to p33, S7A, and Y6F (Fig. 5 and data not shown). Therefore, maturation of thymocytes in the continued presence of A4Y, W4Y, and L6F (rather than endogenous peptides) has resulted in functional tolerance to the selecting ligand. This ability to adjust the resting threshold and alter the ligands capable of inducing a T cell response has been previously reported in this model (14, 53). These positively selected thymocytes expressed high levels of transgenic TCRs, comparable to TCR-transgenic β₂m⁻/- thymocytes that matured in the presence of endogenous peptides, arguing against the possibility that reduced TCR expression leads to unresponsiveness. However, coreceptor (10, 54), adhesion, and signaling molecules could play a role in altering the reactivity profile of thymocytes. Thus, T cells may be “tuned” during positive and negative selection by interactions with peptide/MHC complexes on thymic stromal cells, thereby limiting the spectrum of activating ligands (55, 56).

It remains controversial whether functional thymocytes may be selected in the presence of agonist ligands. Some reports have suggested that T cells selected on agonist peptides are not bonafide, functional T cells (11, 57). However, we have shown here and in previous studies that T cells selected by agonist ligands are functional, since they can fully respond to the stronger agonist peptides, but are not unresponsive to their selecting peptide or other peptides that have similar or lower reactivity. Others have
also shown that positive selection in the presence of agonist ligands leads to functional mature T cells (16, 54, 58, 59). One possible interpretation is that thymocytes are selected so that they do not respond to their positively selecting ligand (9, 12, 60). As long as the positively selecting ligand persists and continues to “contact” selected T cells, these T cells will have adjusted their basal resting threshold so that they do not respond to the selecting ligand. Only stronger stimulation will lead to activation of effector function. If the selecting peptide is not continually present to tune or alter the reactivity of the selected thymocyte, the resting threshold may be lowered so that the selecting peptide can now induce T cell activation.

Role of Ca\(^{2+}\)/calcineurin in thymocyte selection

The importance of calcium signaling pathways has been implicated in T cells undergoing thymocyte selection. Studies using calcineurin inhibitors such as FK506 and cyclosporin A have shown that Ca\(^{2+}\)/calcineurin pathways are essential for positive and negative selection (61–63). A role for elevated [Ca\(^{2+}\)]\(_{i}\), in cell death induction has also been previously reported (64, 65). However, these studies do not directly correlate differential calcium signaling with peptide-induced positive and negative selection. In this study we clearly demonstrate that peptides that efficiently trigger positive selection induce an intermediate calcium flux in immature transgenic thymocytes, whereas the peptides that efficiently trigger negative selection induce a strong calcium flux (Fig. 7). It should be noted that we performed these assays using macrophages as APCs. However, similar results were obtained using thymic epithelial cells as APCs, suggesting that these assays mimic the physiological thymic environment. Differences in the magnitude of calcium mobilization may lead to the activation of different subsets of calcium-dependent enzymes and, hence, elicit different functional results. There are precedents for this in several different systems. In B cells, the amplitude and the duration of calcium signals control different sets of transcriptional regulators that have different sensitivities to intracellular calcium (66, 67). Similarly, the chemokine RANTES can affect different functional outcomes in T cells depending on the concentration and the resulting magnitude of calcium mobilization (68). Therefore, the intensity of TCR-mediated [Ca\(^{2+}\)]\(_{i}\), flux in thymocytes may play a role in defining the outcome of T cell maturation.

Importance of continuous signaling through the TCRs for positive selection

Several studies have indicated that survival of positively selected thymocytes involves sustained interactions with the thymic microenvironment, transduced via their TCRs (6, 7, 69) It also includes an obligatory role for TCRs in cooperating with other surface molecules to drive developing thymocytes through DP to SP transition (70, 71). We have observed that strong agonists induced TCR down-regulation with faster kinetics, and TCR expression after such induction remained at minimum over a 24-h period (data not shown). Therefore, where negatively selecting peptides induce maximal TCR internalization, the thymocytes may no longer be receptive to these signals. However, thymocytes, in which positively selecting peptides do not fully internalize TCRs, can continue to perceive signals from the microenvironment, suggesting that continual TCR engagements are required to induce the full spectrum of differentiation events associated with positive selection.

The findings described in this report address the relationships among TCR internalization, intracellular calcium levels, and T cell selection events induced by altered peptide ligands. We demonstrate that peptides that induce strong TCR down-regulation accompanied by elevated Ca\(^{2+}\) levels are most efficient at mediating negative selection, whereas peptides that induce suboptimal TCR internalization and weaker Ca\(^{2+}\) elevations are more efficient at triggering positive selection. We do not propose that all selecting ligands will necessarily have a measurable ability to mediate TCR internalization. Instead, our observations with this group of peptides suggest that the quantitative differences, rather than the quality of TCR-derived signal, determine the fate of the thymocyte. This is in accordance with the affinity/avidity model for thymocyte selection. Furthermore, our data explain how positively selecting ligands, due to their inability to fully internalize TCRs, may provide a way in which thymocytes receive the constant signals necessary for differentiation and survival.

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References


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