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A Physiological Ligand of Positive Selection Is Recognized as a Weak Agonist[1]

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Positive selection is a process that ensures that peripheral T cells express TCR that are self-MHC restricted. This process occurs in the thymus and requires both self-MHC and self-peptides. We have recently established a TCR transgenic (TCR_{trans}^+) mouse model using the C10.4 TCR restricted to the MHC class Ib molecule, H2-M3. Having defined H2-M3 as the positively selecting MHC molecule, the severely limited number of H2-M3 binding peptides allowed us to characterize a mitochondrial NADH dehydrogenase subunit 1-derived 9-mer peptide as the physiological ligand of positive selection. Here, we demonstrate that the NADH dehydrogenase subunit 1 self-peptide is seen by mature C10.4 TCR_{trans}^+ T cells as a weak agonist and induces positive selection at a defined concentration range. We also found that the full-length cognate peptide, a strong agonist for mature C10.4 TCR_{trans}^+ T cells, initiated positive selection, albeit at significantly lower concentrations. At increased peptide concentrations, and thus increased epitope densities, either peptide only induced the development of partially functional T cells. We conclude that successful positive selection only proceeded at a defined, yet fairly narrow window of avidity. ***The Journal of Immunology,*** **2000, 165: 4209–4216.**

T cells originate in the bone marrow and seed the thymus as immature precursors. Once in the thymus, immature thymocytes rearrange the α- and β-chains of the TCR and undergo two distinct developmental processes. Positive selection ensures that T cells are self-MHC restricted and thus able to respond to foreign Ags in conjunction with self-MHC molecules (1–3). During positive selection, which is driven mainly by an interaction between T cells and thymic epithelial cells (4, 5), immature T cells see self-peptides presented by self-MHC molecules (6–9). In addition to the positive selection process, T cells are screened for autoreactivity during negative selection, in which potentially autoaggressive T cells are eliminated (10–12) or inactivated (13–15).

It has been difficult to characterize physiological ligands of positive selection. Most of the naturally occurring self-peptides that had been shown to initiate positive selection in fetal thymic organ cultures (FTOCs)[5] were used at high peptide concentrations (16–18). Therefore, most relevant data dealing with peptide recognition during positive selection were derived from studies that probed T cell development with cognate peptides or their variants. Results from different experimental systems led to the different hypotheses of positive selection. When it had been found that only antagonistic variants of cognate peptides selected immature T cells in the thymus, the antagonism theory was proposed. It predicted that physiological ligands of positive selection functioned as peripheral antagonists (19–22). Yet, other groups demonstrated that strong agonistic peptides induced positive selection at low epitope densities (23–25). Their differential avidity theory postulated that positive selection occurred as a low avidity interaction and that peripheral activation or negative selection required a high avidity interaction. This theory also explained how high levels of expression of a single MHC/peptide complex could induce the selection of T cell repertoires (26–30) and predicted that at high epitope densities positive selection of a single TCR was promiscuous (18, 31, 32). If immature thymocytes indeed exhibited promiscuity, then it could be possible that they were selected on a gemisch of peptides (22, 33). However, a recent report challenged the gemisch and promiscuity predictions. It suggested that positive selection was a specific rather than a promiscuous interaction (34).

We recently produced a TCR transgenic (TCR_{trans}^+) mouse. We used the TCR from the CTL line, C10.4, that recognized the *Listeria monocytogenes*-derived AttM peptide in conjunction with the MHC class Ib molecule H2-M3 (35). A similar TCR_{trans}^+ mouse was recently described by others (36). We chose this system because H2-M3 preferentially binds peptides that carry a formylated methionine (fM) in the N-terminal position (37). In the mouse, only 13 mitochondrial genes can give rise to fM peptides (38). This limited number of fM peptides enabled us to define a NADH dehydrogenase subunit 1 (ND1)-derived peptide as the physiological ligand of positive selection for the C10.4 TCR_{trans}^+ mouse (32).

In the present report, we used the C10.4 TCR_{trans}^+ model system to study recognition events occurring during positive selection. Using a FTOC system and CTL assays, we determined that the ND1 self-peptide, the physiological ligand of positive selection for C10.4 TCR_{trans}^+ T cells, functioned as a weak agonist. When we studied cognate AttM peptides in the FTOC system, we observed that the strongly agonistic AttM 9-mer peptide induced positive selection at significantly lowered concentrations. We

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[5] Abbreviations used in this paper: FTOC, fetal thymic organ culture; RAG, recombination activating gene; fM, formylated methionine; ND1, NADH dehydrogenase subunit 1; TCR_{trans}^+, TCR transgenic; DP, double positive; SP, single positive.

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shortened the AttM peptide and reduced its agonistic potential to the level of the ND1 self-peptide and found that its ability to positively select mimicked that of ND1. Increasing epitope densities of H2-M3/peptide complexes by increasing the concentrations of the ND1 self-peptide or either of the two cognate AttM peptides resulted in the development of partially functional C10.4 TCR_{trans}^{+} T cells. Therefore, we concluded that successful positive selection proceeded at a relatively narrow window of avidity.

Materials and Methods

Mice

C10.4 TCR_{trans}^{+} mice express Vβ8.1 and Vα4.2 TCR chains. T cells from these mice are specific for the *L. monocytogenes* AttM peptide in the context of H2-$M3^{wt}$ and have recently been described (32). C57BL/6 and TAP-$1^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Recombination activating gene (RAG)-$2^{-/-}$ mice were obtained from Fred Alt (Harvard Medical School, Boston, MA). All animal studies were approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Peptides

Crude peptides synthesized by the Molecular Research Center at National Jewish Medical and Research Center (Denver, CO) were purified using a reversed phase HPLC system (Rainin Instruments, Emeryville, CA). Amino acid sequences of the peptides are as follows: ND1 self-peptide (fMFFINILTL); AttM peptides (5 mer, 6 mer, or 9 mer) from *L. monocytogenes* recognized by C10.4 TCR_{trans}^{+} T cells (fMIVTLFYSA); LemA peptide from *L. monocytogenes* able to bind H2-M3 but not recognized by C10.4 TCR_{trans}^{+} T cells (fMIGWII; Refs. 35 and 39); and OVA-derived peptide (SIINFEKL).

Cells and cell lines

EL4 is an H-2^{b} thymoma obtained from American Type Culture Collection (Manassas, VA). The 13S2 cell line is a fibroblast transfected with a chimeric H2-$M3^{wt}/L^{d}$ molecule (40) obtained from Robert Rich (Emory University, Atlanta, GA) and John Rodgers (Washington State University, Pullman, WA). C10.4 TCR_{trans}^{+} CTLs were generated from the spleens of C10.4 TCR_{trans}^{+} mice on a RAG-$2^{-/-}$ background. C10.4 TCR_{trans}^{+} CTLs were maintained by restimulating on a weekly basis with irradiated C57BL/6 spleen cells incubated with the cognate AttM 6-mer peptide. All cells were cultured at 37°C in 7% CO_2 in IMDM (Sigma, St. Louis, MO) supplemented with 5 mM HEPES, 2 mM L-glutamine, 1 mM hydroxypyruvate, 50 mM 2-ME, nonessential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamicin (all supplements from Sigma), and 10% FCS (HyClone, Logan, UT) (complete IMDM). Media used for the T cell clones was supplemented with 4% rat Con A-stimulated culture supernatant to provide cytokines and growth factors.

Abs and flow cytometric analyses

mAbs specific for CD4 (RM4-5), CD8α (53-6.7), and CD8β (53-5.8) were purchased from PharMingen (San Diego, CA). Streptavidin Cy-Chrome and streptavidin PE for revealing biotinylated mAbs were also purchased from PharMingen. The anti-Cβ mAb, H57-597 (41); the anti-Vβ8.1-8.3 mAb, F23.1 (42); the anti-α3 L^{d} mAb, 28-14-8S (43); and the anti-CD24 mAb, J11d (44) were purified from culture supernatants using protein G-Sepharose beads (Pharmacia, Piscataway, NJ) and then biotinylated or directly conjugated to FITC as necessary. For staining, cell suspensions were made by pressing organs through either steel or nylon mesh. Single cell suspensions were then added to 96-well round-bottom plates (Becton Dickinson, Mountain View, CA) and incubated with the indicated Abs. TCR staining was performed at 37°C for 30 min. Other staining was performed at 4°C for 30 min. ells were then analyzed on a FACScan using CellQuest software (both obtained from Becton Dickinson).

FTOCs

Day 16 thymic lobes from C10.$4^{+/+}$TAP$^{-/-}$ or C10.$4^{+/+}$TAP$^{+/+}$ fetuses were cultured as previously described (23). The thymic lobes were cultured in 6-well plates (Becton Dickinson) on nitrocellulose membranes (Gelman Sciences, Ann Arbor, MI; 0.45 μM pore size) supported by gelatin sponge filters (Pharmacia and Upjohn, Kalamazoo, MI) saturated with media (18) with the indicated HPLC-purified peptides. After 6 days in culture, the resulting thymocytes were pressed through nylon mesh into a single cell suspension and stained for CD4, CD8α, CD8β, CD24, and TCR expression levels using the mAbs listed above.

Expansion of FTOC thymocytes or adult thymocytes and their use in a CTL assay

After FTOCs were cultured for 6 days, a single cell suspension was made from the resulting thymocytes. Thymocytes from adult mice (C10.$4^{+/+}$TAP$^{+/+}$ or C57BL/6) were pressed through steel mesh into a single cell suspension. These thymocytes were then added to 24-well plates (Becton Dickinson) that had been coated with 5 μg/ml of the H57-597 mAb or left untreated. Media used was complete IMDM with 4% rat concanavalin A stimulated culture supernatant. After 3 days, the cells were harvested, counted, and used as effectors at an E:T ratio of 10:1. Targets used were EL4 cells labeled with either the AttM 6-mer peptide or the LemA 6-mer peptide. After 4 h, the supernatant was harvested and ^{51}Cr release was measured on an automatic gamma counter. The percent of specific lysis was calculated as follows: ((experimental release − spontaneous release)/(maximum release − spontaneous release)) × 100. Results are expressed as the mean of triplicate determinations.

H2-M3 up-regulation assay

To measure the relative binding affinities of the peptides in Fig. 6, we used an up-regulation assay as described previously (40). The 13S2 cell line expressing the H2-$M3^{wt}/L^{d}$ chimeric molecule was plated in 48-well plates (Becton Dickinson) and 100 U/ml IFN-γ (Genzyme, Cambridge, MA) was added. After incubation overnight at 37°C, peptides were added to the cells at various concentrations and the cells were incubated overnight at 27°C in 7% CO_2. The following day, the cells were harvested and stained for expression of the chimeric H2-$M3^{wt}/L^{d}$ molecule using the 28-14-8S mAb conjugated to biotin followed by streptavidin PE.

CTL assays

C10.4 $TCR_{trans}^{+/+}$ RAG-$2^{-/-}$ spleen cells were expanded in vitro on the AttM 6-mer peptide to generate C10.4 TCR_{trans}^{+} CTLs that were used for Fig. 7. Standard CTL assays were performed as previously described (35). Briefly, targets were pulsed with ^{51}Cr (ICN Biomedical Research Products, Costa Mesa, CA) and labeled with the indicated peptide. After washing, 10^{4} targets/well were added to a 96-well round-bottom plate (Becton Dickinson). Effectors were then added at the E:T ratio of 5:1. After 4 h, the supernatant was harvested and ^{51}Cr release was measured on an automatic gamma counter. The percent of specific lysis was calculated as follows: ((experimental release − spontaneous release)/(maximum release − spontaneous release)) × 100. Results are expressed as the mean of triplicate determinations.

Results

Kinetics of positive selection of C10.4 TCR_{trans}^{+} T cells

In the C10.4 TCR_{trans}^{+} system, we had earlier defined the ND1 9-mer self-peptide as the physiological ligand of positive selection (32). In the thymus, the ND1 self-peptide was recognized as a 9-mer peptide with high specificity (45). In the periphery, C10.4 TCR_{trans}^{+} T cells see the *L. monocytogenes*-derived AttM peptide as the cognate peptide (32, 35). Interestingly, the ND1 and AttM peptides did not show any apparent sequence homologies. We had previously examined how AttM peptides of different lengths sensitized targets for killing by mature C10.4 cells. Therefore, we knew that the 9-mer version of the cognate AttM peptide acted as a strong agonist and the shortened AttM 5-mer peptide acted as a weak agonist (35). We also chose the 9-mer version of the cognate AttM peptide to be consistent with the length of the ND1 self-peptide.

Previously, we had analyzed the function of the ND1 self-peptide to induce T cell maturation in the C10.$4^{+/+}$TAP$^{-/-}$ FTOC system (32). In these experiments, we had incubated the fetal thymus organs with several changes of peptide containing tissue culture medium for 10 days. For the present studies of peptide function, we wanted to limit the number of medium changes. Therefore, we examined the time course of ND1 induced T cell maturation in the C10.$4^{+/+}$TAP$^{-/-}$ FTOC system. The first evidence of a phenotypic shift from immature $CD4^{+}CD8^{+}$ double positive (CD4CD8 DP) toward $CD4^{-}CD8^{+}$ single positive (CD8 SP) thymocytes was detected at day 2 of FTOC. At day 4, positive selection had further advanced, reaching its full extent at day 6

before the first exchange of culture medium. Between day 4 and 10, the total numbers of thymocytes retrieved from both ND1-conditioned and control FTOCs were comparable and stable (Fig. 1, Table I). In another experiment, we had found that even a 1-day pulse with the ND1 self-peptide efficiently induced T cell maturation (data not shown). Thus, no evidence of ND1-induced cell expansion was detected. These time course experiments demonstrated that we could shorten culture periods to 6 days. Furthermore, they indicated that the ND1 self-peptide had indeed induced the natural progression of T cell development.

Positive selection induced by the ND1 self-peptide and the cognate AttM peptide

The modified C10.4$^{+/+}$TAP$^{-/-}$ FTOC system was used to compare the ability of the ND1 self-peptide and two AttM peptides to induce maturation of C10.4$^{+/+}$ thymocytes. Each of the three peptides was titrated into the FTOCs from 625 nM to either 610 pM (for the ND1 self-peptide and the AttM 5-mer peptide) or 152 pM (for the AttM 9-mer peptide). After a 6-day incubation, thymocytes were harvested from the FTOCs and analyzed for CD4, CD8, TCR, and CD24. Results were depicted at the highest concentration tested, lowest concentration tested, and the concentration that resulted in the selection of the most efficient CTLs for each of the three peptides (Fig. 2; see below). Data for the entire titration range of each peptide are summarized (Fig. 3*A*). At 625 nM, all three peptides induced strong phenotypic shifts, driving immature thymocytes to become CD8$^+$Vβ8$^+$ T cells. It had been suggested by others that positive selection also resulted in the down-regulation of CD24 (heat-stable Ag; Refs. 46 and 47). Indeed, the newly appearing CD8 SP T cells no longer expressed CD24. In control FTOCs that had been incubated with 0.1% DMSO, the majority of cells consisted of immature CD4CD8 DP cells with low levels of TCR and high levels of CD24 (Fig. 2, *J* and *K*).

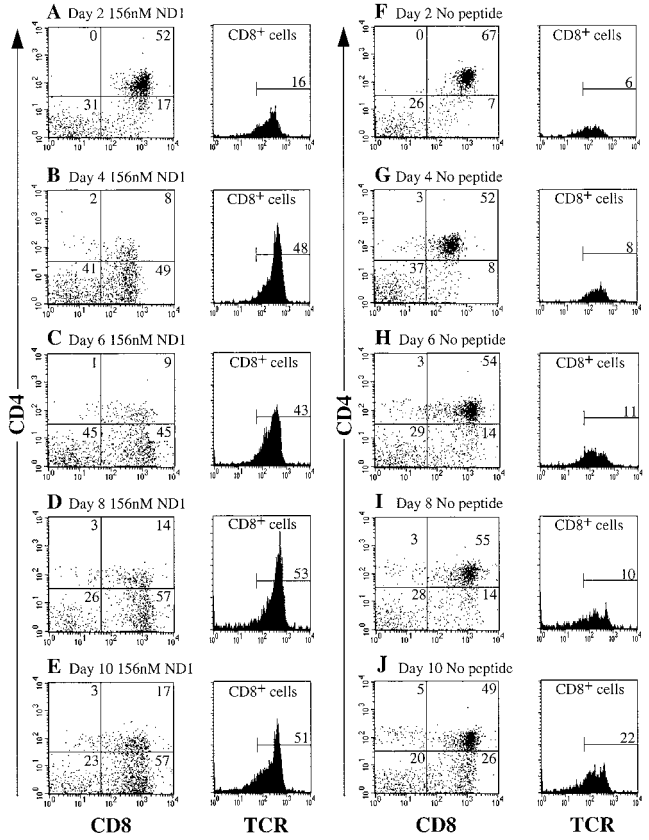

FIGURE 1. Time course of ND1-induced positive selection in C10.4$^{+/+}$TAP$^{-/-}$ FTOCs. FTOCs conditioned with the ND1 self-peptide at a concentration of 156 nM were harvested at days 2 (*A*), 4 (*B*), 6 (*C*), 8 (*D*), and 10 (*E*). Control FTOCs established in the absence of exogenous peptides were analyzed at days 2 (*F*), 4 (*G*), 6 (*H*), 8 (*I*), and 10 (*J*). For each time point, CD4 vs CD8 staining is presented as a dot plot, with the percentage of cells in each quadrant shown. The corresponding row of histograms shows TCR staining with the F23.1 mAb on the CD8 SP cells and the percentage represents the total number of CD8$^+$TCR$^+$ T cells of all the live gated events.

Table I. *Time course of thymocytes recovered from C10.4$^{+/+}$TAP$^{-/-}$FTOCs*[a]

Day of Culture	Total Numbers of Cells per Lobe ($\times 10^{-3}$)	
	ND1	Control
4	86	114
6	137	207
8	207	272
10	217	250

[a] C10.4$^{+/+}$TAP$^{-/-}$FTOCs was performed as described in *Materials and Methods*. The ND1 peptide (156 nM) or 0.1% DMSO were added at day 0, day 6, and day 8. At the time points indicated, thymus lobes were harvested and the total number of thymocytes recovered was determined.

To confirm that the observed increase in the frequency of CD8$^+$Vβ8$^+$ T cells represented a shift in phenotype, we extended our previous studies with ND1 (32) and calculated the numbers of mature-type and total thymocytes recovered from the different FTOCs. In three experimental set-ups, we never observed a significant increase in the total number of thymocytes, even though the number of CD8$^+$Vβ8$^+$ T cells increased 4- to 5-fold (Fig. 3*B*). For instance, in the case of the AttM 5-mer peptide, the total number of thymocytes ranged from 2.0 to 2.6 $\times$ 10^5 cells per lobe, whereas the number of CD8$^+$Vβ8$^+$ thymocytes increased from 0.3 $\times$ 10^5 (at the lowest peptide concentration) to 1.5 $\times$ 10^5 (at the highest peptide concentration) cells per lobe. These findings indicated that these peptides had induced a phenotypic shift toward mature-type thymocytes rather than an expansion of the low number of background CD8$^+$Vβ8$^+$ T cells seen in the control cultures. We also found that background CD8$^+$Vβ8$^+$ T cells were qualitatively different from cells positively selected by any of these three peptides (see below).

In an unmanipulated C10.4$^{+/+}$TAP$^{+/+}$ mouse, ~20% of the thymocytes are CD8$^+$Vβ8$^+$ T cells, representing the physiological situation (32). When we compared the amounts of peptides required to reproduce this level of positive selection in C10.4$^{+/+}$TAP$^{-/-}$ FTOCs, we observed that peptide concentrations of ~39 nM were required for the ND1 self-peptide and the AttM 5-mer peptide. The AttM 9-mer peptide induced these levels of positive selection at the lower concentration of ~2.4 nM. The AttM 9-mer peptide lost activity at a concentration of 152 pM, while both the ND1 self-peptide and the AttM 5-mer peptide lost activity at 2.4 nM. From these data we drew two conclusions. A strong agonist, such as the AttM 9-mer peptide, induced phenotypic shifts to CD8$^+$Vβ8$^+$ T cells at significantly lower concentrations than the physiological ligand of positive selection. The ND1 self-peptide and the weakly agonistic AttM 5-mer peptide behaved in a similar manner, even though they did not show any apparent sequence homologies. Therefore, the C10.4 TCR$_{trans}^+$ was not able to distinguish between self and foreign peptides during positive selection.

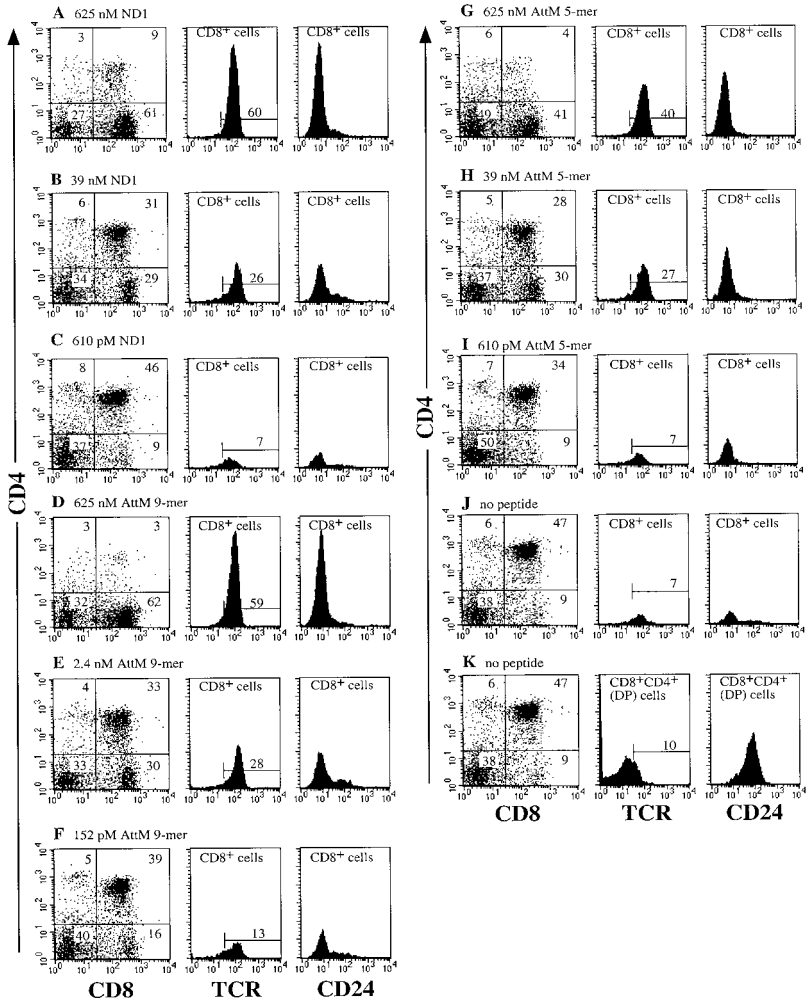

FIGURE 2. Flow cytometry data of C10.4$^{+/+}$TAP$^{-/-}$ FTOCs incubated with different concentrations of the ND1 self-peptide, the AttM 9-mer peptide, or the AttM 5-mer peptide. Different concentrations of either the ND1 self-peptide, the AttM 9-mer peptide, or the AttM 5-mer peptide were added to the media of C10.4$^{+/+}$TAP$^{-/-}$ FTOCs. After 6 days in culture, the thymocytes were stained for CD4, CD8, CD24, and TCR expression levels using the F23.1 mAb. The peptide and concentration added to the FTOC were as follows: 625 nM ND1 self-peptide (*A*), 39 nM ND1 self-peptide (*B*), 610 pM ND1 self-peptide (*C*), 625 nM AttM 9-mer peptide (*D*), 2.4 nM AttM 9-mer peptide (*E*), 152 pM AttM 9-mer peptide (*F*), 625 nM AttM 5-mer peptide (*G*), 39 nM AttM 5-mer peptide (*H*), 610 pM AttM 5-mer peptide (*I*), and no peptide (*J* and *K*). For each peptide and concentration used, CD4 vs CD8α staining is presented as a dot plot, with the percentage of cells in each quadrant shown. The first row of histograms shows TCR staining with the F23.1 mAb on the CD8α SP cells and the percentage represents the total number of CD8α^+Vβ8$^+$ T cells of all the live gated events. *K*, TCR levels are shown for the CD4CD8 DP cell population. The second row of histograms in each figure shows CD24 staining of CD8α SP cells (*A–J*) and CD24 staining of CD4CD8 DP cells (*K*).

Functionality of selected T cells was peptide-concentration dependent

Successful positive selection of class I-restricted T cells results not only in a phenotypic shift to CD8 SP cells, but also in a progression toward mature functional T cells (19, 32, 48). We had earlier found that positively selected thymocytes could be induced to proliferate by stimulation with anti-TCR mAbs in the presence of exogenous cytokines. The expanded T cells were functionally mature and able specifically to lyse targets sensitized with the cognate peptide (32). Thymocytes harvested from both a C10.4$^{+/+}$TAP$^{+/+}$ and a normal C57BL/6 mouse were expanded in the presence of the H57-597 mAb specific for the constant region of the TCR β-chain. Cultures established from C10.4$^{+/+}$TAP$^{+/+}$ thymocytes specifically lysed EL4 targets coated with a cognate AttM peptide, while they failed to lyse targets coated with an unrelated H2-M3 binding control peptide, LemA (Fig. 4*A*). Naive C10.4$^{+/+}$TAP$^{+/+}$ thymocytes or C57BL/6 thymocytes (naive or H57-597 expanded) were unable to lyse EL4 cells coated with either the AttM peptide or the LemA peptide.

We next examined the functional capabilities of thymocytes developing from the C10.4$^{+/+}$TAP$^{-/-}$ FTOCs. All cultures, even those established from control FTOCs to which peptide had not been added, underwent vigorous expansion (at least 7-fold). When the different peptides were added, expansion of thymocytes increased up to ~15-fold (data not shown). Control C10.4$^{+/+}$TAP$^{-/-}$ FTOC thymocytes which were selected in the presence of 0.1% DMSO contain qualitatively different thymocytes that give rise only to nonlytic C10.4 TCR$_{trans}^+$ T cells (Fig. 4*B*). In contrast, all three peptides induce the maturation of lytic C10.4 TCR$_{trans}^+$ T cells at specific peptide concentrations. The ND1 self-peptide and the AttM 5-mer peptide both efficiently selected CTLs at an optimal dose of 39 nM, while the AttM 9-mer peptide was able to induce the most efficient CTL activity at 2.4 nM. None of the thymocytes tested were able to lyse targets coated with an H2-M3 binding control peptide, LemA (data not shown). Although appearance of AttM specific CTLs in the in vitro expansion cultures was dependent on the presence of peptide-induced CD8$^+$C10.4 TCR$_{trans}^+$ thymocytes, not all peptide-induced T cells were fully functional. Generation of fully functional CTLs was most efficient in FTOCs that contained levels of CD8$^+$Vβ8$^+$ T cells (around 20%) seen under physiological conditions in the unmanipulated C10.4$^{+/+}$TAP$^{+/+}$ mouse (Figs. 3 and 4; Ref. 32). For the ND1 self-peptide and the weak agonist, AttM 5-mer peptide, this percentage was reached at 39 nM, while for the strong agonist AttM 9-mer peptide at the significantly reduced concentration of 2.4 nM. At lower-than-optimal peptide concentrations, the number of positively selected cells and thus the number of expanded CTLs decreased. Increasing the concentration of the relevant peptides, though resulting in an even higher frequency of CD8 SP T cells, did not boost the number of fully functional T cells. Instead, their lytic activity decreased and eventually reached background levels.

It had previously been reported that CD8 coreceptors were down-regulated when T cells were selected in the presence of strong agonistic versions of the cognate peptide (21, 48, 49). In our

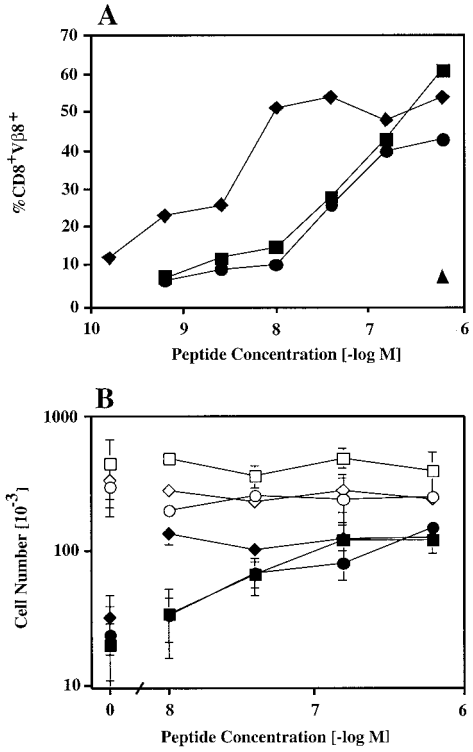

FIGURE 3. Positive selection of C10.4 TCR_{trans}^{+} T cells over the full range of peptide concentrations tested. *A*, For the ND1 self-peptide, the AttM 9-mer peptide, and the AttM 5-mer peptide, $C10.4^{+/+}TAP^{-/-}$ FTOCs were performed beginning with 625 nM and titrating down 4-fold. The *y*-axis represents the percent of $CD8^{+}V\beta8^{+}$ T cells as determined by flow cytometry for each of the peptides and concentrations used. The control culture was a $C10.4^{+/+}TAP^{-/-}$ FTOC containing 0.1% DMSO (used as peptide diluent). The peptides used are the ND1 self-peptide (■), the AttM 9-mer peptide (♦), the AttM 5-mer peptide (●), or no peptide (▲). This experiment represents averages of two independent thymic lobes and was repeated twice with similar results. *B*, For the ND1 self-peptide (squares), the AttM 9-mer peptide (diamonds), and the AttM 5-mer peptide (circles), $C10.4^{+/+}TAP^{-/-}$ FTOCs were performed beginning with 625 nM and titrating down 4-fold. After 6 days of culture, thymocytes were harvested, counted, and stained. The *y*-axis represents the total number of thymocytes harvested (open symbols) or the number of $CD8^{+}V\beta8^{+}$ T cells (filled symbols) as calculated.

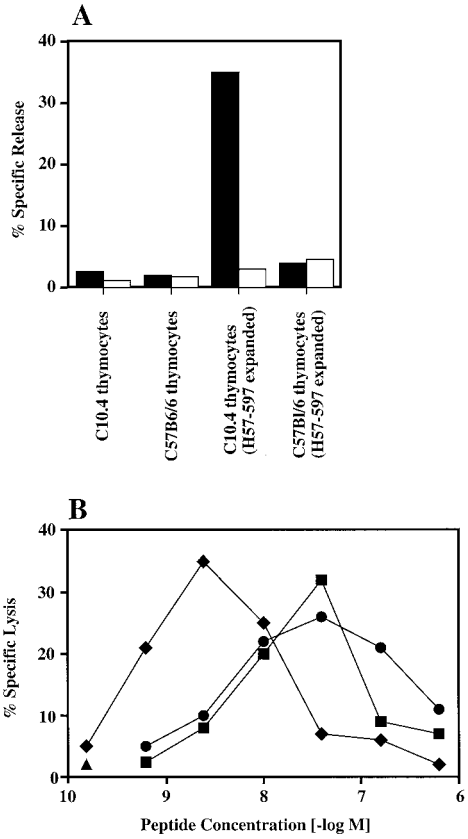

FIGURE 4. Functional characteristics of $C10.4^{+/+}TAP^{-/-}$ FTOC-derived thymocytes. *A*, Thymocytes from $C10.4^{+/+}TAP^{+/+}$ mice or C57BL/6 mice were analyzed for their cytolytic capabilities after expansion on H57-597-coated or -uncoated plates. After a 3-day expansion, the resulting cells were used in a standard CTL assay. Targets used were EL4 cells pulsed with 10 μM AttM 6-mer peptide (■) or 10 μM LemA 6-mer peptide (□). The E:T ratio was 10:1. *B*, Thymocytes resulting from $C10.4^{+/+}TAP^{-/-}$ FTOCs cultured with various concentrations of peptides were expanded for 3 days on H57-597-coated plates. After expansion, the cells were used in a standard CTL assay. Targets used were EL4 cells pulsed with 10 μM AttM 6-mer peptide or 10 μM LemA 6-mer peptide. This graph only shows killing on the AttM peptide-coated targets. Specific lysis on the targets coated with the control LemA peptide was always <5% for all of the cells tested. The E:T ratio was 10:1. The peptides used in the $C10.4^{+/+}TAP^{-/-}$ FTOCs for deriving the thymocytes are as follows: the ND1 self-peptide (■), the AttM 9-mer peptide (♦), the AttM 5-mer peptide (●), or no peptide (▲). *A* and *B*, Data is presented as the percent of specific lysis and is the mean of triplicate determinations.

system, thymocytes that matured on the cognate AttM 9-mer peptide showed similar CD8 α-chain levels as thymocytes that had matured on the physiological ligand of positive selection. In addition, there was only a small shift seen in the CD8 α-chain surface expression in the different FTOCs whether they gave rise to fully functional or nonlytic T cells (Figs. 2 and 5). Further analysis of the CD8 expression pattern after FTOC is here exemplified for the ND1 self-peptide using the three concentrations shown in Fig. 2 (Fig. 5). Similar results were obtained using the two AttM peptides at the respective peptide concentrations (data not shown). Thymocytes from FTOCs conditioned with different concentrations of the ND1 self-peptide were analyzed for both the CD8 α- and β-chains before (Fig. 5, *A–D*) and after (Fig. 5, *E–H*) in vitro expansion. Expression of CD8 α-chains was similar for thymocytes harvested from the different FTOCs with a small increase for thymocytes induced by the optimal concentration of 39 nM of the ND1 self-peptide. This relative increase became more apparent after in vitro expansion. This pattern of expression was also found for the CD8 β-chain, yet much more pronounced. Most thymocytes harvested from FTOCs cultured with 610 pM or in the absence of the ND1 self-peptide failed to express the CD8 β-chain after expansion. These thymocytes only expressed CD8 α-chain homodimers. Thymocytes induced by the highest concentration of the ND1 self-peptide expressed reduced levels of CD8 β-chains. We did not find consistent changes in the levels of TCR expression either before or after in vitro expansion. Therefore, different surface expression levels of TCR cannot explain the inability of certain thymocytes to lyse targets.

From these experiments, we concluded that thymocytes found in $C10.4^{+/+}TAP^{-/-}$ FTOCs cultured in the absence of exogenous peptides were not only quantitatively, but also qualitatively different from thymocytes that had matured in the presence of effective concentrations of the ND1 self-peptide. The reduced levels of CD8 β-chain expression on T cells selected on superoptimal peptide concentrations might not fully explain the inability of these cells to kill specific targets. Yet, they all support the notion that these T

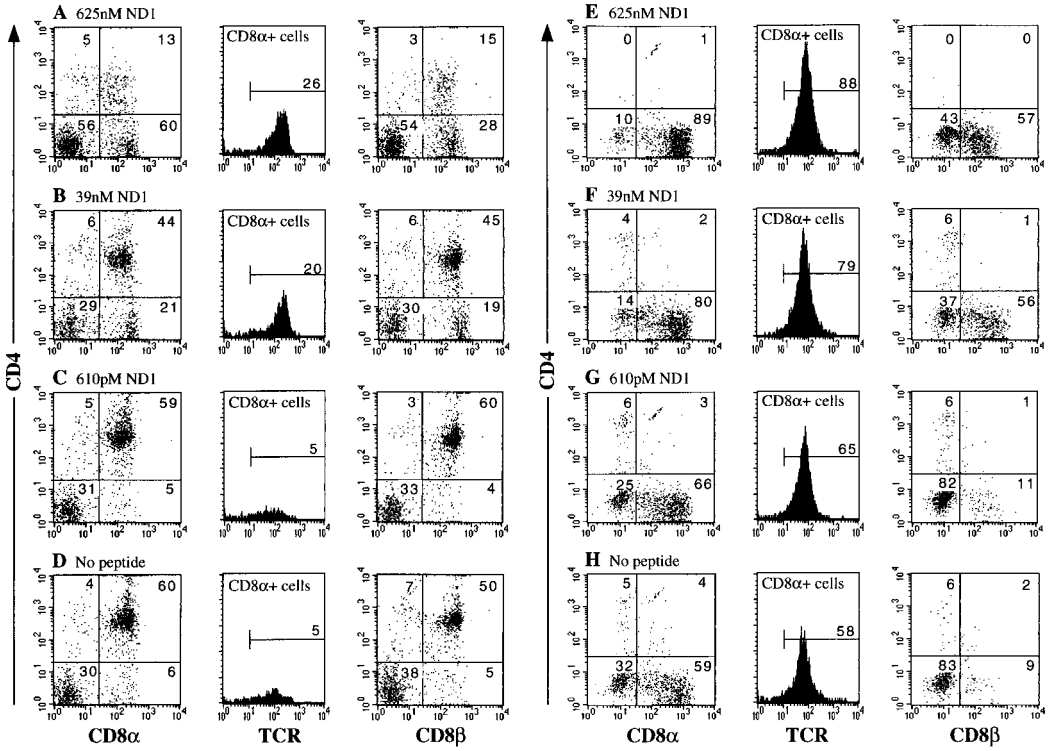

FIGURE 5. Phenotype of C10.4$^{+/+}$TAP$^{-/-}$ FTOC-derived thymocytes harvested at day 6 of organ culture and after 3 days of anti-TCR-mediated expansion. *A–D*, Thymocytes resulting from C10.4$^{+/+}$TAP$^{-/-}$ FTOCs cultured with different concentrations of the ND1 self-peptides were analyzed. *E–H*, Aliquots of these samples were expanded for 3 days on H57-597-coated plates. Before and after the anti-TCR-mediated expansion the cells were stained for CD4, CD8 α-chain, CD8 β-chain, and the TCR. Concentrations use in FTOCs were as follows: 625 nM ND1 self-peptide (*A* and *E*), 39 nM ND1 self-peptide (*B* and *F*), 610 pM ND1 self-peptide (*C* and *G*), and no peptide (*D* and *H*). Dot plots show CD4 vs CD8 α-chain or CD4 vs CD8 β-chain staining with the percentages of cells in the quadrants indicated. The histograms show staining of the CD8$^+$ T cells for TCR expression levels. Numbers within the histograms represent the percentage of CD8$^+$TCR^{high} T cells of all the live gated events.

cells had matured differently than T cells induced by optimal peptide concentrations. However, further detailed experiments will be necessary to fully characterize the phenomenon of partially functional T cells.

The ND1 self-peptide functioned as a weak agonist

Because stability of an MHC/peptide complex is a crucial component of T cell recognition, we compared the peptide binding abilities of the ND1 self-peptide and the two AttM peptides. H2-M3 preferentially binds fM peptides (50). Yet, a peptide motif that predicted binding to H2-M3 has not yet been defined. Therefore, we used an H2-M3 up-regulation assay to measure the relative binding abilities of the three peptides to H2-M3 (40). 13S2 fibroblasts that had been transfected with the chimeric H2-M3^{wt}/L^d molecule were incubated overnight with the ND1 self-peptide, the AttM 9-mer peptide or the AttM 5-mer peptide. Surface expression of the chimeric H2-M3^{wt}/L^d molecule, as a measure of MHC/peptide stability and thus affinity, was determined using the anti- L^d specific mAb, 28-14-8S (Fig. 6). A conventional non-fM peptide derived from OVA was used as a negative control. OVA was unable to up-regulate H2-M3 expression over background staining. The ND1 self-peptide bound H2-M3 slightly better than either the AttM 9-mer peptide or the AttM 5-mer peptide. Yet, all three peptides proved to be good H2-M3 binders (32, 51, 52). Therefore, the peptides' different abilities to positively select were not based on their different abilities to stabilize H2-M3.

Next, we analyzed the antigenic function of the ND1 self-peptide and compared it to that of the two cognate AttM peptides. C10.4 TCR$_{trans}^+$ CTLs were generated from C10.4$^{+/+}$RAG-2$^{-/-}$ mice. We examined the ability of the different peptides to sensitize targets for lysis by these T cells (Fig. 7). A control H2-M3 binding LemA peptide was unable to sensitize targets at any concentration tested. The AttM 9-mer peptide behaved as a strong agonist (35). Both the ND1 self-peptide and the AttM 5-mer peptide scored as weak agonists, significantly less potent than the AttM 9-mer peptide in sensitizing targets. The ND1 self-peptide and the AttM 5-mer peptide were similar in their ability to induce positive selection. Furthermore, they showed the same antigenicity. In either situation, they were both significantly less potent than the AttM 9-mer peptide.

Discussion

In a recent report, we had defined a physiological ligand of positive selection. We established that ND1 was the only H2-M3 binding self-peptide responsible for positive selection of C10.4 TCR$_{trans}^+$ T cells (32). In the present report, we demonstrated that the ND1 self-peptide induced the natural development pathway from CD4 CD8 DP to CD8 SP T cells. The ND1 self-peptide and the shortened version of the cognate AttM peptide were both able to function as weak agonists and induce positively selected C10.4 TCR$_{trans}^+$ thymocytes. Thus, in the thymus and in the periphery, the ND1 self-peptide and the AttM 5-mer peptide showed similar behavior. The AttM 9-mer peptide, a strong agonist for C10.4 TCR$_{trans}^+$ T cells, drove maturation of CD8$^+$Vβ8$^+$ T cells at lower peptide concentrations than the weakly agonistic peptides

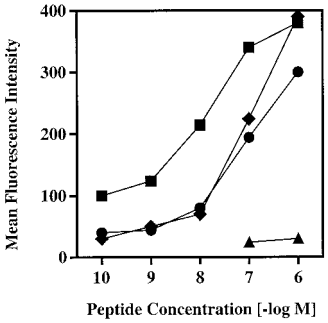

FIGURE 6. H2-M3 binding abilities of the ND1 self-peptide, the AttM 9-mer peptide, and the AttM 5-mer peptide. The 13S2 cell line was stained for expression of the H2-M3^{wt}/L^{d} chimeric molecule after stabilization of H2-M3^{wt} with the indicated peptides. Data is presented as mean fluorescence intensity (*MFI*). Peptides used are as follows: the ND1 self-peptide (■), the AttM 9-mer peptide (♦), the AttM 5-mer peptide (●), or the OVA-derived peptide SIINFEKL (▲).

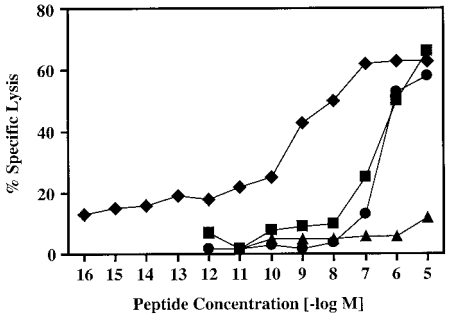

FIGURE 7. The ND1 self-peptide is a weak agonist to peripheral C10.4 TCR$_{trans}^{+}$ T cells. A C10.4 TCR$_{trans}^{+}$ T cell line derived from a C10.4$^{+/+}$ RAG-2$^{-/-}$ mouse was used in a standard CTL assay. Targets used were EL4 cells incubated with either the ND1 self-peptide (■), the AttM 9-mer peptide (♦), the AttM 5-mer peptide (●), or the LemA 6-mer peptide (▲) at the indicated concentrations. Data is presented as the percent of specific lysis and is the mean of triplicate determinations.

(Fig. 3*A*). None of these peptides induced proliferation of thymocytes (Fig. 3*B*) and because thymocytes generated in the absence of any of these peptides were qualitatively different from the peptide induced thymocytes (Fig. 5), we could exclude that any of these peptides had simply expanded the background CD8 SP thymocytes. Yet, they all bound to H2-M3 with comparable affinities (Fig. 6). Successful positive selection of functional C10.4 TCR$_{trans}^{+}$ T cells only proceeded at a narrow range of avidities. We were unable to demonstrate that the ND1 self-peptide or the AttM 5-mer peptide antagonized C10.4 TCR$_{trans}^{+}$ T cell responses (data not shown). Our data fit best with the differential avidity hypothesis of positive selection (23, 24, 53).

We demonstrated that positive selection of functional T cells depended on a certain avidity threshold. It has been shown that surface expression of H2-M3 is much lower than that of classical MHC class Ia molecules (40, 52). Therefore, it could be argued that H2-M3 induced the development of T cells with relatively high affinities. This consideration might explain why the ND1 self-peptide was a stronger agonist than variants of cognate peptides that had previously been shown to induce positive selection on the abundant MHC class Ia molecules. In these model systems, positively selecting peptide variants were either scored as very weak agonists/antagonists (19–21), or were not recognized by mature T cells (16, 17). In mice that express a single MHC/peptide complex, peripheral T cells did not react to these highly abundant epitopes, and they expressed TCRs with exceedingly low affinities to these MHC/peptide complexes (27–30, 54, 55). Therefore, it seems likely that high epitope densities select thymocytes with low TCR affinity due to deletion of thymocytes with moderate affinity.

The ND1 self-peptide is part of the ND1 protein. This protein is crucial for cellular respiration and is expressed ubiquitously (56). How is it possible that a self-peptide (ND1) that acts as a weak agonist does not activate peripheral T cells? The "differential avidity" hypothesis argued that positive selection occurred at lower triggering thresholds than peripheral activation. Another report presented data suggesting that thymocytes undergoing positive selection had a lowered threshold for signaling and thus were easier to trigger than peripheral, mature T cells (57). It was found that T cells maintained sensitivity to strong agonists throughout development, while they decreased their sensitivity to weak agonists (58). In our system, the ND1 self-peptide seems to be recognized equally well in the FTOC as compared with the CTL assay, while the strong agonist, AttM 9-mer peptide, is actually recognized slightly better in the CTL assay compared with the FTOC system (compare Figs. 3*A* and 7). However, we have established that the FTOC system is rather inefficient at presenting peptides when compared with the CTL assay (data not shown). This would indicate that the ND1 self-peptide is actually recognized at lower peptide concentrations in the thymus compared with the periphery. The AttM 9-mer peptide, which acts as a strong agonist, is recognized equally well in the thymus and in the periphery when the inefficiency of the FTOC system is factored in. Taken together, these data indicate that T cells are "refractory" to further peripheral TCR signaling induced through self-peptides that function to induce positive selection in the thymus. However, these self-peptides may be responsible for peripheral T cell maintenance (59, 60).

Selection of nonlytic C10.4 TCR$_{trans}^{+}$ T cells not only occurred on the strongly agonistic AttM 9-mer peptide, but also on weak agonists (the ND1 self-peptide and the AttM 5-mer peptide; Fig. 4*B*). These nonlytic T cells might represent a correlate to the clonally inactivated T cells seen previously that were generated in mice infected with certain mouse mammary tumor virus-encoded superantigens (13–15). Others had previously reported production of nonfunctional T cells in FTOCs systems, however only on strong agonists (20, 48). They suggested that the lack of function was due to the down-regulation of the CD8 coreceptor. In our system, down-regulation of the CD8 β-chain was seen on thymocytes induced with superoptimal peptide concentrations. Thymocytes that had not undergone positive selection failed to express the CD8 β-chain. These changes might explain why these two groups of thymocytes were only partially functional. Further studies will determine what factors determine the functionality of T cells resulting from FTOCs.

Our studies have shown that a physiological ligand of positive selection was seen as a weak agonist and that a strong agonist nevertheless induced positive selection. Assuming that the cognate AttM peptide was seen with higher affinity than the physiological ligand of positive selection, we concluded that the success of positive selection in inducing fully functional T cells depended on a fairly defined avidity range. Increasing the epitope density beyond this point resulted in the selection of partially functional T cells with the ability to proliferate, but not to mediate cytolytic functions.

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