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Specificity and flexibility in thymic selection

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Abstract

During positive selection, developing thymocytes are rescued from programmed cell death by T-cell receptor (TCR)-mediated recognition of major histocompatibility complex (MHC) molecules^{1–3}. MHC-bound peptides contribute to this process^{4–8}. Recently we identified individual MHC-binding peptides which can induce positive selection of a single TCR⁹. Here we examine peptide fine specificity in positive selection. These data suggest that a direct TCR–peptide interaction occurs during this event, and strengthens the correlation between selecting peptides and TCR antagonists^{9,10}. Certain positively selecting peptides are weakly antigenic⁹. We demonstrate that thymocytes ‘educated’ on such a peptide are specifically non-responsive to it and have decreased CD8 expression levels. Similar reduction of CD8 expression on mature T cells converts a TCR agonist into a TCR antagonist. These data indicate that thymocytes may maintain self-tolerance towards a positively selecting ligand by regulating co-receptor expression.

Positive selection of T cells occurs at the CD4⁺8⁺ stage of development and results in phenotypic changes (including downregulation of CD4 or CD8) and functional maturation^{2, 3,11,12}. We previously described a TCR-transgenic mouse (OVA-tcr-1) with a receptor specific for ovalbumin residues 257–264 (OVAp) plus H-2K^b. Thymocytes bearing this receptor are positively selected in fetal thymic organ cultures (FTOCs) expressing K^b (Fig. 1a, d) (ref. 9) but maturation is impaired in FTOCs from $\beta_2M^{-/-}$ mice (Fig. 1b, e), where class I expression is drastically reduced^{7,13 15}. Selection is restored by addition of exogenous β_2 -microglobulin (β_2M) and certain variants of OVAp, exemplified by peptides E1 (Fig. 1c, f) and R4 (Fig. 1g) (ref. 9). This process shows exquisite peptide fine specificity; neither E1R4 (which combines both the E1 and R4 substitutions) nor K4 (which is chemically very similar to R4) are capable of positively selecting the OVA-tcr-1 receptor (Fig. 1h, i). Two theories have been proposed to explain peptide specificity in positive selection^{8,9,16}. The TCR may interact directly with both MHC and peptide residues (‘peptide interaction’ model), or it may only recognize MHC residues, in which case the peptide’s role is merely to avoid impeding the TCR–MHC interaction (‘peptide avoidance’ model). Our results are hard to reconcile with the latter model; thus if both E1 and R4 simply keep out of the TCR’s way, it is likely that E1R4 would do so too, and hence should positively select. Therefore, our data support a peptide interaction model.

We previously showed that peptides that induce positive selection, including E1 and R4, also act as TCR antagonists^{9,10} (Fig. 2). K4 and E1R4 did not behave as antagonists (Fig. 2), demonstrating similar fine specificity for positive selection and TCR antagonism. This suggests that the two phenomena may have a similar mechanistic basis, possibly based on TCR affinity for the peptide–MHC ligand^{9,10,17,18}. However, some peptides that are weak agonists induce positive selection at low concentrations and negative selection at higher concentrations (refs^{9, 19, 20} and K.A.H. *et al.*, manuscript in preparation). Thus, positive selection is conditioned by both qualitative (perhaps affinity) and quantitative (ligand density) aspects of the TCR interaction.

E1 not only antagonizes OVA-tcr-1 T-cell responses (Fig. 2), but is also a weak agonist, stimulating mature transgenic cytotoxic T lymphocytes (CTLs)⁹ and deleting immature

thymocytes in suspension culture (data not shown). We investigated how the presence of E1 during development alters the reactivity of mature OVA-tcr-1 thymocytes (Fig. 3a, b). Thymocytes from TCR-transgenic $\beta_2M^{-/-}$ FTOCs made a very weak response to both OVAp and E1. Addition of E1 during FTOC increased the absolute numbers of CD8⁺ T cells per lobe and thus the response to OVAp. But these cells were virtually non-responsive to E1 (Fig. 3a). This lack of E1 reactivity is all the more remarkable as the antigen-presenting cells used are $\beta_2M^{+/+}$ and so present peptides 50–100-fold better than $\beta_2M^{-/-}$ cells^{7,9}. Similarly, inclusion of E1 in OVA-tcr-1 $\beta_2M^{+/+}$ FTOC ablated the E1 response but only partially reduced proliferation to OVAp (Fig. 3b).

What is the basis of this selective non-reactivity to E1? We investigated whether thymocyte TCR and/or CD8 expression levels were affected. TCR- α -chain expression was essentially equivalent in all the populations (Fig. 3d), but CD8 expression was significantly lowered on thymocytes from FTOCs in which E1 was included (Fig. 3c). This is seen most dramatically in the $\beta_2M^{+/+}$ FTOC, but is also apparent in cells from $\beta_2M^{-/-}$ lobes in which E1 is the positively selecting ligand. These data suggest that thymocytes can regulate co-receptor expression levels to avoid overt reactivity to the positively selecting ligand. Mature cells bearing low levels of CD8 (CD8^{lo}) could arise from selective survival of CD8^{lo} precursors or by CD8 downregulation on CD8^{hi} precursors. Relative to $\beta_2M^{+/+}$ FTOC we observed a decrease in the number of TCR^{hi} CD8⁺ cells in $\beta_2M^{-/-}$ plus E1 (~20%) or $\beta_2M^{+/+}$ plus E1 (~50%) cultures (see Fig. 3 legend), which supports the former model but does not exclude the latter.

To test whether CD8 expression levels alone could explain the differential response to E1 and OVAp, we analysed mature T-cell clones from OVA-tcr-1 animals (Fig. 4a, b). TG-O.C2 is typical of most clones in that it responded to E1 as an antagonist. However, TG-I.C4 responded to E1 as a weak agonist. This response is completely blocked by an antibody to the transgenic α -chain (data not shown), suggesting that E1 reactivity is not due to expression of an endogenous TCR α -chain. Fluorescence-activated cell sorting (FACS) analysis indicated that CD8 (Fig. 4c) and TCR (data not shown) expression was higher on TG-I.C4 than TG-O.C2. To assess CD8 contribution in E1 reactivity, we tested the effects of anti-CD8 antibody. Partial CD8 blocking dramatically inhibited the TG-I.C4 response to E1 but, at this dose, did not inhibit OVAp reactivity (Fig. 4d). Interestingly, this treatment resulted in E1 now acting as a potent antagonist for TG-I.C4. Thus, the CD8 interaction can apparently determine whether a ligand is perceived as an agonist or an antagonist.

During positive selection, a T cell must interact with self MHC while avoiding negative selection on one hand and autoreactivity on the other. This could be achieved by regulation of receptor expression levels and/or signal transduction machinery. Our data suggest that flexibility in CD8 expression levels is one way for thymocytes to fine-tune the efficacy of their interaction with MHC–peptide ligands present in the thymus and achieve positive selection while maintaining self tolerance. In what may be an analogous situation, lowering CD8 expression on mature T cells converts TCR agonists into antagonists. We note that selection by agonist ligands in a different TCR transgenic model yields CD8^{lo} single positive thymocytes also, although the functional potential of these cells was not addressed^{19,20}. In keeping with our observations, CD8 overexpression apparently converts a positively selecting environment into a negatively selecting one^{21,22}. Also, CD8 and/or TCR downregulation in peripheral T cells allows survival in the face of potentially tolerizing antigens^{23,24}.

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FIG. 1.

Flow cytometric analysis of thymocytes from fetal thymic organ cultures of H-2^b OVA-tcr-1 mice. Thymi from $\beta_2M^{-/-}$ (a, d) or $\beta_2M^{+/+}$ fetuses (b, c, e-i) were cultured with the indicated peptides. Thymocytes were stained for expression of CD4, CD8 and the transgenic V- α 2 region ($V_{\alpha}2$). CD4 versus CD8 staining is shown for all live gated cells (a-c) or $V_{\alpha}2^{\text{hi}}$ cells (d-i).

METHODS. FTOC conditions were essentially as described⁹. Thymus lobes were recovered from fetuses at day 16 of gestation, and cultured in RP10 media containing human β_2M (CalBiochem) ($5 \mu\text{g ml}^{-1}$) and the indicated peptides (at $20 \mu\text{M}$), with daily replenishment for 7 days. Peptide names refer to the amino-acid substitutions in OVAp (sequence SIINFEKL). Thus, E1 has a Ser→Glu substitution at position 1. All peptides were synthesized on a Synergy peptide synthesizer (Applied Bio-systems). $\beta_2M^{+/+}$ and $^{-/-}$ lobes were distinguished by staining with Y3 (anti-K^b). Anti-CD4-PE and anti-CD8-FITC were obtained from Becton Dickinson and used directly. The anti- $V_{\alpha}2$ antibody B20 (ref. 25) was conjugated to biotin and staining

revealed with Tri-colour-streptavidin (Caltag). Analysis was done on a FACScan (Becton-Dickinson) on 20,000 live gated cells.

FIG. 2.

TCR antagonist properties of OVAp analogues used in FTOC. The CTL line TG-O is derived from an OVA-tcr-1 mouse and has been described before⁹. Lysis of EL4 target cells pre-pulsed with 2 pM OVAp was assayed in the presence or absence of the indicated peptide variants in a 4-h ⁵¹Cr-release assay. All four variant peptides bind K^b to a similar extent⁹ (data not shown). Specific lysis in the absence of variants was 37% and is shown on the abscissa.

FIG. 3.

Functional and phenotypic analysis of OVA-tcr-1 FTOCs cultured with and without peptide E1. $\beta_2M^{-/-}$ (a) and $\beta_2M^{+/-}$ (b) OVA-tcr-1 FTOCs were cultured with or without E1 at 20 μ M as indicated. Thymocytes were recovered (four lobes pooled in each group). Numbers ($\times 10^5$) of V α 2^{hi} CD4⁻ CD8⁺ cells from each group were $\beta_2M^{+/-}$, 1.50; $\beta_2M^{+/-}$ plus E1, 0.75; $\beta_2M^{-/-}$, 0.16; and $\beta_2M^{-/-}$ plus E1, 1.19. a, b, The proliferative response of FTOC thymocytes to EL4 cells, with or without E1 (20 μ M) or OVAp (10 nM) pre-coating was determined by ³H-thymidine incorporation. Data show average of triplicate cultures with standard deviation. c, d, An aliquot of the cells recovered from each FTOC was analysed by FACS for expression of V α 2, CD4 and CD8; 10,000 live thymocytes were analysed. After gating for CD4⁺ cells, CD8 (c) and V α 2 (d) expression was determined. The same symbols are used in c and d. The average ($n = 4$) mean channel fluorescence for CD8 expression on CD8 single positive cells in this experiment was $\beta_2M^{+/-}$, 154 (± 8); $\beta_2M^{+/-}$ plus E1, 74 (± 6) ($P \ll 0.001$ versus $\beta_2M^{+/-}$) and $\beta_2M^{-/-}$ plus E1, 120 (± 23) ($P < 0.05$ versus $\beta_2M^{+/-}$). In an independent experiment the values were $\beta_2M^{+/-}$, 142 (± 3); $\beta_2M^{+/-}$ plus E1, 60 (± 6) ($P \ll 0.001$ versus $\beta_2M^{+/-}$) and $\beta_2M^{-/-}$ plus E1, 115 (± 9) ($P < 0.002$ versus $\beta_2M^{+/-}$).

METHODS. As for Fig. 1 except for the proliferation assay which was as in ref. 9. Briefly, irradiated EL4 stimulator cells were pulsed with peptide for 1 h and washed. One-twelfth of

the pooled thymocytes in each group was incubated with 10^4 stimulator cells in triplicate cultures. ^3H -thymidine was added at 48 h and the culture collected 6 h later.

Fig. 4.

Functional and phenotypic analysis of OVA-tcr-1-derived CTL clones. The clones TG-O.C2 and TG-I.C4 were obtained by limiting dilution from OVA-tcr-1-derived CTL lines. Reactivity of TG-O.C2 (open symbols) and TG-I.C4 (filled symbols) toward E1 was measured on *a*, EL4 (agonist assay) or *b*, EL4 prepulsed with 6 pM OVAp (antagonist assay) in a 4-h ⁵¹Cr-release experiment. Symbols on the abscissa represent the lysis in the absence of added E1 peptide. *c*, CD8 expression levels on TG-O.C2 and TG-I.C4 (methods as in Fig. 1). *d*, Effect of anti-CD8 antibody on the specificity of TG-I.C4. Target cells were EL4 cells plus 1 μM E1 (solid bars), pre-pulsed with 6 pM OVAp (stippled bars) or pre-pulsed with 6 pM OVAp and then coated with 1 μM E1 (hatched bars). The IgG anti-CD8 antibody 53-6 was added (1/20 dilution of tissue culture supernatant) for the duration of the assay where indicated. TG-I.C4 was added at an effector:target ratio of 5:1 and lysis measured after 4 h. Inhibition of the responses by addition of anti-CD8 are: EL4-E1, 98%; EL4-OVAp, 1%; EL4-OVAp-E1, 75%.