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Delineation of Signals Required for Thymocyte Positive Selection

Fabio R. Santori* and Stanislav Vukmanovic 2*†

Peptide/MHC complexes capable of inducing positive selection in mouse fetal thymic organ cultures fail to do so in suspension culture. Furthermore, this type of culture does not promote initial stages of differentiation, such as coreceptor down-modulation, unless peptides used for stimulation have (at least) weak agonist activity. We show in this study that signals provided in suspension culture by nonagonist peptide/MHC complexes on the surface of macrophages, even though apparently silent, are sufficient to promote complete phenotypic differentiation when CD4+ CD8+ thymocytes are subsequently placed in a proper anatomical setting. Furthermore, the synergistic actions of suboptimal concentrations of phorbol esters and nonagonist peptide/MHC complexes can make the initial stages of positive selection visible, without converting maturation into negative selection. Thus, the correlation between efficiency of positive selection and the degree of coreceptor down-modulation on CD4+ CD8+ thymocytes is not linear. Furthermore, these results suggest that the unique role of thymic stromal cells in positive selection is related not to presentation of self-peptide/MHC complexes, but most likely to another ligand.


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cell differentiation in the thymus requires TCR recognition of the self-peptide/MHC complexes. As a result of this interaction, known as positive selection, CD4+ CD8+ thymocytes receive survival and differentiation signals (1). Ultimately, CD4+ CD8+ thymocytes differentiate into mature CD4+ CD8+ or CD4+ CD8+ T cells depending on whether MHC class II or class I molecules, respectively, were engaged. In addition to CD4+ CD8+, CD4+ CD8−, and CD4− CD8+ cells, a number of intermediate transitional stages of maturation can be found in the thymus in vivo. These additional stages are CD4low CD8low, CD4+ CD8low, or CD4low CD8+ and are a result of the complex transition of CD4+ CD8+ into the CD4− CD8+ or CD4− CD8+ cells after engagement with self-peptide/MHC complexes. Although the precise precursor-product relationship between the transitional stages is not entirely clear (2, 3), there is a general agreement that down-modulation of both coreceptors is a transient phase of thymocyte maturation regardless of whether MHC class I or class II molecule is the inducing stimulus.

Differentiation of CD4+ CD8+ into CD4+ CD8− or CD4− CD8+ cells is not the only possible outcome of TCR engagement by self-peptide/MHC complexes in the thymus. Thymocytes expressing TCRs with potentially harmful reactivity to self-peptide/MHC complexes are eliminated through a process called negative selection (4–6). The intermediate stages, if any, between interaction of CD4+ CD8+ thymocytes with peptide/MHC complexes that induce negative selection have been extensively studied in vitro. Peptide/MHC complexes that promote negative selection in vivo induce down-modulation of CD4 and CD8 in vitro (7), mimicking the effect of positive selection in vivo. Hence, “coreceptor dulling” has been promoted as a surrogate assay of negative selection (7). However, although apoptosis can be clearly detected within the population of thymocytes stimulated in vitro with ligands that promote negative selection (8–10), the numbers of thymocytes with down-modulated CD4/CD8 exceeds the number of apoptotic cells (10–12). This finding suggests that a proportion of cells undergoing coreceptor dulling induced by negatively selecting ligands in vitro may have paradoxically initiated differentiation into one of the mature thymocyte subpopulations. This conclusion is consistent with the results showing enhanced positive selection of thymocytes stimulated in vitro with negatively selecting ligands and then assembled in MHC-disparate thymus reaggregation cultures (12).

Despite the fact that these experiments suggest that coreceptor dulling is a marker of thymocyte activation, rather than cell death, attempts to recreate in vitro coreceptor down-modulation after activation of CD4+ CD8+ thymocytes with positively selecting ligands were initially unsuccessful. This was most likely because positively selecting ligands deliver weaker signal than negatively selecting ones (13). However, culture of CD8 and TCR, double-transgenic, β2-microglobulin (β2m−/−) (or TAP1−/−) thymocytes with positively selecting peptides successfully induced coreceptor down-modulation in vitro (14). The purpose of using a coreceptor transgenic strain was to increase the strength of signal induced by peptide, whereas the purpose of β2m−/− or TAP1−/− deficiency was to prevent any selection events in vivo. This assay was used to discover endogenous self-peptides involved in positive selection of the OT-I TCR (14, 15). Subsequently, coreceptor down-modulation without the help of the CD8 transgene was used to detect an endogenous peptide involved in positive selection of another TCR (16). However, coreceptor dulling caused by this peptide was barely detectable. Given that this peptide exhibited weak agonist activity, it could be anticipated that peptides with weaker stimulatory potential, but still capable of efficient induction of positive selection, would be missed using the screening without artificial signaling enhancement. It is clear that coreceptor dulling is a very useful assay for easy detection of ligands that could be potentially
involved in positive or negative selection. More importantly, a more thorough characterization of the assay will lead to a better understanding of the early stages of positive and/or negative selection. We developed a novel coreceptor dulling assay relevant for positive selection, and we used this assay to characterize early events during positive selection.

**Materials and Methods**

**Mice and peptides**

TAP1−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 as well as B10.D2 H-Y TCR transgenic, RAG-2 −/− mice were purchased from Taconic Farms (Germantown, NY). Custom-synthesized peptides were purchased from Research Genetics (Huntsville, AL).

**Coreceptor down modulation dulling assay**

Fetal thymus lobes (days 14.5/15.5) from C57BL/6 TAP1−/− mice were injected i.p. with 3 ml of thioglycolate solution. After 3–4 days mice were killed, and the activated macrophages were collected with cold PBS. The cells were washed, resuspended in PM medium (17) supplemented with 20% FCS and plated in flat-bottom, 96-well plates at a density of 2 × 10^5 cells/well. Macrophages were incubated for at least 1 h at 37°C, then they received 4 × 10^5 thymocytes/well derived from either male or female HYB10.D2 RAG−/− mice. The cultures were pulsed with the desired concentration of peptide and incubated overnight at 37°C. The next day thymocytes were collected and stained for FACS analysis with anti-mouse CD4-PE, CD8-CyChrome and CD24-FITC mAbs (BD Pharmingen, San Diego, CA). Dulling was quantified based on the shift from the gate of untreated DP thymocytes from those of peptide-treated DP thymocytes. Peptide-treated thymocytes were also collected and tested for apoptosis using an FITC-based TUNEL assay kit (Roche, Indianapolis, IN).

**Fetal thymus organ cultures (FTOC)**

The FTOCs were performed using gestation day 16 fetuses derived from time-mated pregnancies of H-Y TCR transgenic mice on the TAP1−/− background (17). Fetal thymus lobes were cultivated on sponge-supported filters (Millipore, Bedford, MA) in medium supplemented with peptide at 300 μM (unless otherwise stated). Cultures were arranged so that one lobe was treated as experimental, whereas the other lobe from the same fetus was treated as a control. As a negative control we used peptides that bind H-2D^d well, but do not induce positive selection of H-Y thymocytes. After 10 days, lobes were dissociated, and cells and fetal thymuses were screened with anti-mouse V8/8-FITC (BD Pharmingen) to ascertain expression of H-Y TCR and anti-H-2K^d-PE (BD Pharmingen) to ascertain for TAP1−/− status. Fetal lobes were further analyzed by triple FACS staining with anti-mouse CD4-PE, CD8-CyChrome and CD24-FITC Abs (BD Pharmingen). Remaining cells were used in the proliferation assay as described above.

**Reaggregation thymus organ cultures (RTOC)**

Fetal thymus lobes (days 14.5/15.5) from C57BL/6 TAP1−/− mice were placed on nitrocellulose filters suspended on gelfoam sponges embedded with PM10 medium containing 1.35 mM deoxyguanosine for 5 days. Lobes were then collected, and deoxyguanosine and FCS were carefully washed out with serum-free medium. The lobes where then homogenized by trypsinization until a cell suspension was formed. All cell clumps, debris, and DNA were removed, and the remaining cells were counted, spun down, and resuspended in fresh PM10 medium. These cells were used as a source of thymic stromal cells. To generate RTOCs, 2 × 10^5 stromal cells were mixed with 1 × 10^5 HY thymocytes derived from HY mouse on the B10.D2 RAG2−/− background that were previously incubated in suspension cultures with TAP1−/− microphages, peptide, and/or PM as described above for the dulling assay. The mixed cells were spun, and aggregates were reconstituted over nitrocellulose filters suspended in gelfoam sponge embedded in fresh PM10 medium. After 72 h, the reaggregates were homogenized, and cells were stained for FACS analysis with anti-mouse CD4-PE, CD8-CyChrome and CD24-FITC Abs (BD Pharmingen).

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1. Abbreviations used in this paper: FTOC, fetal thymus organ culture; β-m, β2-microglobulin; RTOC, reaggregation thymus organ culture.

**Results**

**Synergistic action of self-peptide and suboptimal doses of phorbol ester stimulates coreceptor down-modulation**

We asked whether using suboptimal concentrations of pharmacological stimulators of pathways known to be involved in positive selection, such as the Ras/MAPK pathway (18, 19), could replace the requirement for the use of coreceptor transgene. Phorbol esters activate the Ras/MAPK pathway through protein kinase C activation (20) and induce coreceptor down-modulation in immature thymocytes (21). We therefore tested whether presence of phorbol ester (PMA) could help induce coreceptor down-modulation by a peptide that induces positive selection. As shown in Fig. 1, PMA at a concentration of 1.0 ng/ml, but not 0.5 ng/ml, induced coreceptor down-modulation in H-Y TCR transgenic, RAG-2−/− thymocytes on a nonselecting background (H-2^d). Similarly, culture of the same thymocytes with a self-peptide, Ube1x509−517, capable of inducing positive selection (17) failed to induce coreceptor down-modulation (Fig. 1). However, Ube1x509−517 and 0.5 ng/ml PMA synergized to induce significant coreceptor down-modulation. We call this synergistic effect assisted coreceptor dulling to distinguish it from the dulling induced by peptides that promote negative selection and require no signaling enhancement (either pharmacological or genetic). An example of such unassisted dulling is given in Fig. 1C using Smcy738–746 peptide. Time-course experiments have indicated that assisted dulling could be observed as early as 2 h after initiation of cultures (data not shown). Furthermore, the ability to induce assisted dulling was not restricted to peptide Ube1x509−517. For example, some variants of Smcy738–746 that were able to induce positive selection in FTOC also induced assisted dulling (Fig. 2). Thus, stimulation of CD4^+CD8^− thymocytes with suboptimal PMA concentrations acts synergistically with signals initiated by peptides that induce positive selection to induce coreceptor down-modulation.

**Assisted dulling correlates better with positive selection than with antagonist activity**

The abilities of peptides to promote positive selection and to induce TCR antagonist activity in peripheral T cells overlap in many cases, but these two activities are clearly distinguishable. Thus, it is important to determine whether suboptimal PMA stimulation enhances weak signals in general or specifically those that induce positive selection. We have recently characterized ligands capable of stimulating antagonist activity, positive selection, or both activities in the H-Y TCR transgenic model (17). We therefore asked whether suboptimal PMA concentrations would amplify signals generated by peptides that have TCR antagonist activity, but do not promote positive selection. Peptide ARX44−52 (VSNLNRQFL) is identical with Ube1x509−517 (KSNLNRQFL) except at position 1, where lysine in the latter is substituted by valine in the former peptide. This difference produces distinct biological activities of the two peptides: Ube1x509−517 antagonizes Ag-induced CD8^-cell proliferation and promotes thymocyte positive selection, whereas ARX44−52 induces antagonist activity, but not positive selection (17). ARX44−52 was unable to induce CD4^+CD8^-coreceptor down-modulation, either alone or with the help of 0.5 ng/ml PMA (Fig. 3). All other peptides that act as TCR antagonists but do not promote positive selection, including but not limited to Ube1x509−517K1A, Ube1x509−517Q7A, Ube1x509−517F8A, and Ube1x509−517K1M, also fail to induce assisted dulling (Fig. 4).

We analyzed the correlation between the capacity of a peptide to induce positive selection and its antagonist or assisted dulling activity. Our data suggest that there is a positive correlation between
assisted dulling and positive selection \((r = 0.729)\). Assisted dulling is also more sensitive than antagonist assays in the detection of peptides that promote positive selection \((r = 0.65)\). However, this difference in sensitivity between assisted dulling and antagonism is reduced when only strong antagonists are included in the analysis \((r = 0.69)\). Therefore, assisted dulling detects peptides that promote positive selection as well as those with strong antagonist activity. These results suggest that strong, but not weak, antagonist activity is associated with thymocyte positive selection in the H-Y TCR transgenic model.

Preserved pattern of TCR engagement in the presence of suboptimal phorbol ester stimulation

We have recently demonstrated that the pattern of TCR contacts used to induce positive selection by Ube1x509–517 is different from that required to induce negative selection by Smcy738–746 \((22)\). Positive selection requires TCR contacts at the N terminus (positions 1 and 2) and the C terminus (positions 6, 7, and 8) of the peptide. Negative selection does not require contacts at the N terminus of the peptide, but, instead, uses contacts at central position...
It was therefore of interest to determine whether assisted dulling induced by Ube1x509–517 required N-terminal or central TCR contacts. To address this question we used variants of the Ube1x509–517 peptide with single substitutions of original amino acids by alanine at each position of the peptide except positions 5 and 9, which are anchor positions for peptide binding to MHC. All variant peptides bind H-2D^d (22). As shown in Fig. 4, positions 1, 2, 6, 7, and 8 were essential for assisted dulling, whereas position 4 was not required. As was the case for induction of positive selection in FTOCs (22), only conservative replacement at position 1 (K to R) preserved the functional activity of the peptide. In contrast, the presence of amino acid that has a poor capacity to form noncovalent bonds (M) disabled the activity of the peptide. Thus, TCR contacts required to induce assisted dulling are same as those required for positive selection.

Signal enhancement by suboptimal phorbol ester stimulation does not lead to thymocyte apoptosis

The assisted coreceptor down-modulation may represent an initial stage of positive selection that cannot be completed in vitro. Alternatively, increased strength of the signal could convert a positively selecting ligand into a negatively selecting one. To test the latter possibility, we determined whether apoptosis is induced in thymocytes stimulated with Ube1x509–517 and suboptimal PMA. Although ~50% of H-Y thymocytes stimulated with Smcy738–746 stained positively in the TUNEL assay (Fig. 5A), cells stimulated with combination of Ube1x509–517 and PMA did not differ from control thymocytes (Fig. 5B). Thus, the synergistic actions of PMA and positively selecting peptide do not lead to negative selection.

Suboptimal phorbol ester treatment does not impair positive selection

We next wanted to determine how the presence of suboptimal concentrations of PMA affected the ability of Ube1x509–517 to induce positive selection. H-Y TCR transgenic thymocytes were treated with Ube1x509–517 and PMA in suspension culture, as described above, and were then used for RTOCs with TAP1−/− macrophages. After 3 days of culture, the RTOCs were analyzed for the presence of CD4^+ CD8^+ T cells (Fig. 6). This is probably due to the effect of endogenous self-peptides, as the same result occurred in a similar study using dendritic cells as APCs (23). Importantly, a significant increase in the number of CD4^+ CD8^+ thymocytes was observed in RTOCs that used cells treated with Ube1x509–517 alone. Ube1x509–517 and PMA, when added together, acted synergistically to produce an additional increase in the number of CD4^+ CD8^+ thymocytes, although the contribution of PMA was relatively minor over that of the peptide alone. To eliminate the effect of endogenous self-peptides and observe the isolated effects of Ube1x509–517 and suboptimal PMA, we used TAP1−/− macrophages during the primary thymocyte culture. As expected, TAP1−/− macrophages promoted selection of CD4^+ CD8^+ thymocytes only if loaded with Ube1x509–517 (Fig. 6). PMA acted synergistically to produce an additional increase in the number of CD4^+ CD8^+ thymocytes, albeit the contribution of PMA was again relatively minor. Thus, suboptimal concentrations of PMA during primary culture do not convert peptide-induced positive selection into negative selection.

Stronger signals during positive selection are known to raise the threshold of activation by Ag (24, 25). To determine whether coretreatment with PMA may affect the responsiveness of selected cells to Ag in a similar manner we tested proliferative responses of thymocytes derived from RTOC to Smcy738–746. However, to date, we have not observed functional responses to Ag by CD4^+ CD8^+ thymocytes isolated from RTOCs treated either with Ube1x509–517 and PMA or with Ube1x509–517 alone (data not shown). These findings suggest that positive selection in suspension culture, although effective for phenotypic maturation, may not be optimal for generating functionally competent cells in this experimental model.

Discussion

Coreceptor down-modulation characterizes the initial stages of both positive and negative selection. Hence, induction of coreceptor down-modulation in vitro suspension cultures is used as a surrogate assay for both of these processes. Under these culture conditions, ligands that induce positive selection induce none or very weak coreceptor down-modulation unless the signal is amplified in a nonspecific manner. Amplification of the signal can be achieved by coreceptor overexpression (14). However, the requirement for TCR and coreceptor transgenes as well as for H-2 background that cannot induce positive selection results in a complex
and time-consuming breeding. It would be advantageous, therefore, if at least one breeding component could be replaced with an externally applicable component. Obvious candidates that could replace the need for CD8 transgene are pharmacological agents that could strengthen TCR signaling pathways known to be involved in positive selection. We showed that amplification can be achieved by the use of suboptimal concentrations of phorbol esters. We also found that signal amplification does not convert positive selection into negative selection, but only makes the productive interaction of thymocytes with positively selecting ligands visible. We demonstrated that induction of assisted coreceptor down-modulation correlated better with positive selection than with antagonist activity. However, signal amplification did not significantly improve the completion of thymocyte maturation in MHC-deficient RTOCs, suggesting that the efficiency of positive selection does not depend on the degree of coreceptor down-modulation.

Our results using RTOCs demonstrate that positive selection can be split into at least two events: one early event that is MHC-dependent but apparently cell type-independent, and a late event that is apparently MHC-independent but cell type-dependent. Similar conclusions have been reached in previous studies using RTOCs, where positive selection was completed in two phases (23). However, some minor differences can also be noted between the two sets of results. Although, in our studies, the second stage was apparently MHC-independent, MHC was required in some, but not all, experimental conditions for phenotypic maturation in studies by Yasutomo et al. (23). Some differences in experimental protocols exist that could potentially explain different findings. First, in contrast to Yasutomo et al. (23), we did not purify CD69higth thymocytes after the first culture. Triggering CD69 initiates signaling (26), and it is possible that the anti-CD69 treatment used for sorting interfered with or modulated signaling in thymocytes. Also, we used macrophages instead of dendritic cells. These two cell types may differ in the levels of MHC molecules, adhesion/costimulatory molecules, and adherence to the plastic. Finally, different types of MHC class I-deficient cells were used for secondary cultures. The residual levels of MHC class I in TAP1-deficient cells (this study) are higher than those in β2-m-deficient cells (27). In this case it would have to be postulated that the MHC signals in the second phase need not be strong and most likely do not depend on the presence of specific peptides that initiated selection.

The two-signal selection described above is reminiscent of requirements for stimulation of naive cells. Two signals are required for full T cell activation: an Ag presented by MHC molecules, and costimulatory molecules (28). Thymic epithelial cells can readily promote positive selection (29–34), and although there are reports of positive selection induced by other cell types, these are always less efficient than thymic epithelial cells (35–39). The separation of two events required for thymocyte maturation can explain these apparent discrepancies in the literature. MHC-dependent signal can be delivered by any cell type, whereas thymic epithelial cells offer an equivalent of costimulatory signal. In this scenario, the signal provided by self-peptide/MHC complexes need not be presented by the same cell that provides the equivalent of costimulatory signal. Thus, the equivalent of costimulation can be provided...
in trans, as is the case with the classical costimulation (40). And because costimulation of mature T cells in trans is less efficient (41), we expect the costimulation equivalent provided by thymic epithelial cells also to be less efficient when provided in trans. Hence, we postulate that in all experimental settings where cell types other than thymic epithelial cells were found to promote positive selection, these cells provided the MHC-dependent signal, whereas thymic epithelial cells provided costimulation equivalent in trans. This scenario requires relative anatomical vicinity of MHC donor cells and thymic epithelial cells. Because of the lower efficiency of trans-delivered costimulatory signals (41), all other cell types showed reduced efficiency in selection compared with thymic epithelium. The nature of the epithelial cell ligand is at present unknown, but classical costimulatory interactions (provided by CD86/CD80 molecules) have been excluded (42, 43).

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References


