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Delineation of Signals Required for Thymocyte Positive Selection

Fabio R. Santori* and Stanislav Vukmanović 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. The Journal of Immunology, 2004, 173: 5517–5523.

*Michael Heidelberger Division of Immunology, Department of Pathology and New York University Cancer Center, New York University School of Medicine, New York, NY 10016; and †Center for Cancer and Immunology Research, Children’s Research Institute, Children’s National Medical Center, Washington, DC 20010

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Address correspondence and reprint requests to Dr. Stanislav Vukmanovic, Center for Cancer and Immunology Research, Children’s Research Institute, Children’s National Medical Center, 111 Michigan Avenue NW, Washington, DC 20010-2970. E-mail address: svukmano@cnmc.org

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

**Coreceptor down modulation dulling assay**

C57BL/6 mice were injected s.c. 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<sup>3</sup> cells/well. Macrophages were incubated for at least 1 h at 37°C, then they received 4 × 10<sup>3</sup> thymocytes/well derived from either male or female HYB10.D2 RAG<sup>−/−</sup> 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 and CD8-CyChrome Abs (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<sup>−/−</sup> 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<sup>β</sup> 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 V<sub>J</sub>8-FITC (BD Pharmingen) to ascertain expression of H-Y TCR and anti-H-2<sup>K</sup>-PE (BD Pharmingen) to ascertain for TAP1<sup>−/−</sup> status. Fetal lobes were further analyzed by triple FACS staining with anti-mouse CD4-PE, CD8-CyChrome and CD24-FITC mAbs (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<sup>−/−</sup> 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<sup>5</sup> stromal cells were mixed with 1 × 10<sup>5</sup> HY thymocytes derived from HY<sup>+</sup> mice on the B10.D2 RAG<sup>−/−</sup> background that were previously incubated in suspension cultures with TAP1<sup>−/−</sup> macrophages, 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<sub>m</sub>, β<sub>2</sub>-microglobulin; RTOC, reaggregation thymus organ culture.
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.
4. 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\(^b\) (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 Smyc738–746 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 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.

**Discussion**

Coreceptor down-modulation characterizes the initial stages of both positive and negative selection. Hence, induction of coreceptor down-modulation in 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 RTOC, suggesting that the efficiency of positive selection does not depend on the degree of coreceptor down-modulation.

Our results using RTOC 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 CD69<sup>high</sup> 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 β<sub>2</sub>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.

**FIGURE 4.** TCR contacts essential for assisted dulling. Peritoneal exudate cells from thioglycolate-treated C57BL/6 mice were cocultured with H-Y TCR transgenic, B10.D2, RAG<sup>2</sup>−/− thymocytes in the presence of 0.5 ng/ml PMA alone or in combination with 10 μM of the original (KSNL-NRQFL) or one of the indicated variants of Ube1<sub>x</sub>509–517. After overnight culture, thymocytes were stained with CD4- and CD8-specific mAbs. Shown are the mean percent dulling and SD calculated relative to the number of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in cultures treated with medium only.

**FIGURE 5.** Induction of apoptosis of H-Y thymocytes. H-Y TCR transgenic, B10.D2, RAG<sup>2</sup>−/− thymocytes cultured overnight in the absence (MC) or the presence of Smcy738–746 were analyzed for DNA breaks using a TUNEL assay (A). The same cells were cultured in the absence or the presence of 0.5 ng/ml PMA (line overlaps for the most part with the MC line) or in the presence of Ube1<sub>x</sub>509–517 and PMA (B).
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**


