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TCR Signaling for Initiation and Completion of Thymocyte Positive Selection Has Distinct Requirements for Ligand Quality and Presenting Cell Type


Generation of an effective αβ T cell repertoire is achieved during intrathymic development by a three-part process: 1) death of precursor thymocytes that receive an inadequate TCR signal from self-ligands, 2) differentiation of thymocytes that receive a suitable TCR signal into mature CD4+ and CD8+ T cells, and 3) apoptosis of thymocytes that recognize more potent or higher density ligands, eliminating many cells capable of developing effector activity in response to self-peptide:MHC ligands in the periphery (1, 2). Yet despite the wealth of evidence supporting this “neglect, select, and eliminate” model, key aspects of the paradigm remain unresolved.

Very low concentrations of agonist in some studies, but only of partial agonist/antagonist ligands in others, can promote maturation without death in fetal thymus organ cultures, and in several studies when mature cells do develop upon exposure to agonists/ partial agonists they are unresponsive to the same ligand (3–9). In contrast, several groups have reported generation of responsive mature T cells using agonist ligand (10, 11), and recent work from this (12) and other laboratories (13) has shown that immature thymocytes signal in an agonist-like fashion when confronted with ligands that are partial agonists/antagonists of mature T cells with identical TCR. This raises questions about whether agonist quality can itself be a determining factor in thymocyte selection and leaves open the issue of whether TCR ligand quality or quantity makes the major contribution to determining cell fate.

The required duration and anatomic locus of MHC-dependent TCR signaling for full maturation are other unresolved questions. Several groups have suggested that double-positive (DP) thymocytes sample peptide:MHC molecule ligands on cortical epithelial cells and, provided that the signals received from the engaged TCR are neither too weak nor too strong, initiate a TCR-independent process leading to extended survival and lineage-specific differentiation into CD4+ or CD8+ medullary T cells (14, 15). Other experiments have instead provided evidence for a more extensive role of positive signaling through the TCR for completion of T cell development (16–18).

Even when the existing observations themselves are more consistent, important issues of interpretation remain. Many, but not all (19–22), experiments indicate that only TCR signaling occurring in response to peptide:MHC molecule ligands displayed by cortical epithelial cells can promote allele-specific positive selection, lineage choice, and export of functional mature T cell maturation of DP thymocytes, whereas MHC molecules on hemopoietic cells are ineffective in this regard (23–29). MHC ligands on dendritic cells (DC) in particular have been associated with negative, not positive, selection (30), a property most often attributed to a special capacity of these cells to induce thymocyte apoptosis in conjunction with high levels of TCR and possibly CD28 signaling.

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3 Abbreviations used in this paper: DP, double positive; DC, hemopoietic dendritic cell; β2m, β2-microglobulin; RAG-2, recombinase-activating gene-2; HSA, heat-stable Ag; PCC 88–104, synthetic peptide corresponding to pigeon cytochrome c residues 88–104; PI, propidium iodide; TSC, thymic stromal cells.

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However, whether this difference reflects distinctive ligands and signals provided by epithelial vs hemopoietic presenting cells or is simply a consequence of intrathymic cell location and access to precursor T cells has not been carefully addressed.

By studying DP precursors from recombinase-activating gene-2 (RAG-2)−/− mice with transgenic TCR expression but lacking selecting MHC molecules, it is possible to avoid contamination of the input population with any cells having already received TCR-MHC-dependent differentiation signals. Using specific peptide: the input population with any cells having already received TCR-lecting MHC molecules, it is possible to avoid contamination of heat-stable Ag (HSA), or anti-D b Abs followed by avidin-allophycocyanin.

2 FACScan or a FACStarPlus (Becton Dickinson, Mountain View, CA). List- hamster IgG in medium containing 0.1% saponin. All Abs were purchased were stained with anti-Bcl-2 mAb followed by FITC-conjugated anti- mAbs, then fixed using 4% paraformaldehyde. After washing, the cells using allophycocyanin-conjugated anti-CD4 and PE-conjugated anti-CD8

In the case of staining for intracellular Bcl-2, the thymocytes were stained

positive immunological role, rather than only posing a problem to

signals from self-peptide:MHC complexes on thymic DC may play a

due to unappreciated late stage deletion. Most surprisingly, we find that MHC molecules on DC can mediate all the required TCR signaling events for functional selection. This is concordant with data showing a possible role for DC-borne MHC molecules in peripheral T cell homeostasis (36, 37) and suggests that TCR sig- nals from self-peptide:MHC complexes on thymic DC may play a positive immunological role, rather than only posing a problem to be overcome by negative selection.

Materials and Methods

Mice

Mice with targeted inactivation of both the β2-microglobulin (β2m) gene (38) and ApoB gene loci (39) were obtained from Taconic Farms (Taconic, NY) and are referred to as MHC−/− throughout. B10.BR, B10.D2, and C57BL/10 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The AND TCR transgenic (40) RAG-2−/− (41) mice and AND TCR transgenic RAG-2−/− ApoB−/− mice were provided by B. J. Fowlkes, Lab- oratory of Cellular and Molecular Immunology, National Institute of Al- lergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). All mice were bred and maintained in a National Institute of Allergy and Infectious Diseases Research Animal Facility in accordance with es- tablished guidelines.

Flow cytometry

Thymocytes were stained with PE-conjugated anti-CD4, FITC-conjugated anti-CD8, and biotin-conjugated anti-CD49d, anti-CD5, anti-TCR V3, anti-heat-stable Ag (HSA), or anti-D P Abs followed by avidin-allophycocyanin. In the case of staining for intracellular Bcl-2, the thymocytes were stained using allophycocyanin-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs, then fixed using 4% paraformaldehyde. After washing, the cells were stained with anti-Bcl-2 mAb followed by FITC-conjugated anti- hamster IgG in medium containing 0.1% saponin. All Abs were purchased from PharMingen (San Diego, CA). Flow cytometry was performed on a FACSscan or a FACSstar® (Becton Dickinson, Mountain View, CA). List- mode data files were analyzed using CellQuest software (Becton Dickin- son, Mountain View, CA).

Thymocyte stimulation, analysis, and preparation of CD69high cells

Stimulation of DP thymocytes in dispersed culture and the analysis of death and CD69 up-regulation responses were performed as previously described in detail (12), except that the source of presenting cells was varied. Viable CD69high cells for transfer into reaggregate cultures were obtained by FACS after stimulation of CD4+CD8+ thymocytes from 1- to 2-wk-old AND TCR transgenic RAG-2−/− MHC class II−/− mice using these published culture conditions.

Thymic reaggregate culture

Thymic stromal cells (TSC) from C57BL/6 or mice with targeted muta- tions in both the β2m and ApoB gene loci were prepared by disaggregating fetal thymic lobes previously cultured for 5 days in 1.35 mM deoxy- guanosine (Sigma, St. Louis, MO) using 0.05% trypsin (Life Technologies, Grand Island, NY) and 0.02% EDTA. Reaggregates were formed by mixing together the desired TSC and thymocytes at a 1:1 cell ratio (absolute number, 5 × 10^6 of each cell type) or TSC, thymocytes, and DC at a cell ratio of 10:1:1 (absolute number of DC, 5 × 10^6). After pelleting the cells by centrifugation, the cell mixture was placed as a standing drop on the upper membrane surface of a Transwell culture well containing RPMI 1640 supplemented by 10% FCS and cultured for 60 h at 37°C.

Preparation of DC

DC were purified from disrupted, collagenase-treated spleens of C57BL/6 or B10.BR mice as described previously (42). Briefly, low density cells obtained by BSA gradient centrifugation were cultured in plastic dishes for 1–2 h, after which the nonadherent cells were washed away. The adherent cells were detached from the plate using 10 mM EDTA. The CD4+ , CD8−, or B220+ cells were removed from this population by incubation with each Ab followed by depletion of Ab-coated cells using sheep anti-rat IgG- coupled magnetic beads (Dynabeads, Dynal, Oslo, Norway). The resultant population contained 80–85% CD11c+ cells and is referred to as DC in this study. Highly purified splenic DC were obtained by FACS using CD11c staining after bead depletion. The resulting cells were 98% CD11c+. Highly purified thymic DC were prepared in a similar manner, except that bead depletion after the adherence step was omitted.

Surface protein re-expression after promose treatment

FACS was used to obtain CD69+CD4+CD8+ thymocytes following in vitro stimulation for 84 h of CD4+CD8+ thymocytes from AND TCR transgenic RAG-2−/− B2m−/− mice with DC in the presence of 0.01 μM of a synthetic peptide corresponding to pigeon cytochrome c residues 88–104 (PCC88–104). Promose treatment of these cells and analysis of coreceptor re-expression were performed as previously described (43).

Proliferative response of T cells

CD4+ thymocytes (1 × 10^5) from reaggregate cultures or thymus from mice of the indicated type were incubated with 5 × 10^6 mitomycin C-treated spleen cells in the presence or the absence of PCC for 60 h, after which the cultures were pulsed with 1 μCi of [H]^thymidine for another 12 h before harvesting and measurement of incorporated label.

Results

CD69 and Bcl-2 expression are up-regulated by surviving DP thymocytes stimulated in vitro using agonist-bearing DC

In dispersed cultures of AND TCR transgenic RAG-2−/− thymocytes from mice lacking selecting MHC class II molecule expression (AND DP), CD69 up-regulation can be induced in a cohort of cells without concomitant cell death using transfected ICAM-1+ L cells bearing agonist cytochrome c peptide/E α complexes (12, 57). These studies did not determine whether these agonist-acti- vated, viable thymocytes are capable of further differentiation and, if so, whether any resulting mature cells would be reactive to the selecting ligand. Also, because L cell transfectants injected intra- thymically can promote effective positive selection (44), and these cells have some epithelial characteristics, these studies with trans- fectants did not address the issue of whether epithelial vs hemo- poietic (especially dendritic) cells had distinct capacities to pro- mote such early differentiation without death or whether DC could support further T cell maturation.

To investigate these issues, we examined the ability of splenic DC to induce CD69 up-regulation without concomitant cell death across a range of agonist peptide concentrations. When AND DP were stimulated with 0.01 μM PCC88–104 presented by DC from B10.BR (I-E^b+) mice, only a fraction of the cells underwent ap- optotic cell death (Fig. 1A), and most of the surviving cells became...
CD69 high DP thymocytes generated by stimulation with pronase and culture for 14 h at 37°C (Fig. 1, F–H). These findings indicate that DC presenting agonist ligand can stimulate AND DP to acquire the phenotype of thymocytes after stimulation in vitro by DC with or without added agonist peptide. CD4lowCD8low and CD69high (Fig. 1, B–D; data shown for 0.01 μM peptide). The extent of coreceptor down-regulation (data not shown) or up-regulation of CD69 was much less and involved fewer cells without added peptide (Fig. 1D). Because CD69 expression levels reflect the strength of TCR signaling (45, 46), these latter results indicate that the CD69high cells surviving stimulation with PCC have undergone TCR signaling in response to this agonist ligand and do not correspond to cells responding only to self-peptide-I-Ek complexes. Stimulation with PCC88–104 also led to readily detectable up-regulation of Bcl-2 protein levels in most cells (Fig. 1E). These findings indicate that DC presenting agonist ligand can stimulate AND DP to acquire the phenotype of thymocytes initiating positive selection (47, 48) without committing all the same signaled cells to death. However, this initial activation is not adequate to promote lineage-specific differentiation, as assessed by regulation of coreceptor expression. After stimulation with 0.01 μM PCC presented by I-Ek expressing DC, CD4lowCD8low thymocytes re-expressed both CD4 and CD8 molecules following treatment by pronase and culture for 14 h at 37°C (Fig. 1, F–H).

CD69high DP thymocytes generated by stimulation with agonist-bearing DC or thymic stromal cells show enhanced differentiation into CD4+ T cells in thymic reaggregate culture

To test whether the CD69highCD4lowCD8low thymocytes generated in these dispersed cell cultures had received signals promoting further maturation, cells with this phenotype were sorted from cultures stimulated for 20 h with 0.01 μM PCC88–104 presented by I-Ek+ DC or CD45− TSC. These cells were then cultured for 60 h in reaggregates with TSC expressing I-Ak, which mediates positive selection without deletion of thymocytes bearing this particular TCR (49). In such reaggregates, unstimulated CD69low AND DP gave rise to a modest number of CD4+ T cells, representing ~12% to a maximum of 20% of the total recovered thymocytes (Fig. 2B). Sorted CD69+CD4lowCD8low cells produced in first-step cultures without peptide using DC expressing either the I-Ak+ or I-Ek+ MHC class II molecules yielded, on the average, about 2- to 3-fold more total CD4+ mature thymocytes in the reaggregates (Fig. 2, C and D, and Table I). The same degree of enhanced CD4+ T cell development was seen using equal numbers of CD69highCD4lowCD8low thymocytes generated by PCC presentation on I-Ek+ TSC or DC (Fig. 2, E and F). Given the equal input cell number for all reaggregate cultures, this consistently increased output using CD69high compared with unstimulated CD69low DP suggests that the stimulated cells from the first culture had initiated positive selection that continued in the reaggregates regardless of whether the activating ligand was self-peptide/MHC class II or foreign agonist complexes. No CD69+ cells were generated in cultures of DP with MHC class II-deficient DC, and the transfer of such CD69+ cells into reaggregates with TSC did not yield elevated levels of CD4+ mature cells. This also argues for a specific effect of TCR stimulation in the first culture on initiation of differentiation, rather than an unspecific effect of cell coculture (data not shown).

CD69highCD4lowCD8low thymocytes do not successfully differentiate into CD4+ T cells in dispersed culture or reaggregate culture with MHC molecule-negative TSC

Despite prolonged culture with presenting cells bearing the appropriate MHC molecule and peptide ligand, stimulated CD69high
thymocytes that are not reaggregated with TSC fail to show evidence of lineage-specific coreceptor expression (Fig. 1, F–H). Thus, although they appear to have received signals initiating selective events, CD69<sup>high</sup>/CD4<sup>low</sup>/CD8<sup>low</sup> thymocytes still require the support of TSC for lineage-specific differentiation and maturation. To examine whether MHC molecule interactions are necessary for this further differentiation or if other molecules expressed by TSC are sufficient, CD69<sup>high</sup>/CD4<sup>low</sup>/CD8<sup>low</sup> cells were combined with αββ<sub>2</sub>m-deficient (MHC-negative) TSC in reaggregate culture. In contrast to the efficient development of CD4<sup>+</sup> T cells in the presence of MHC-positive TSC (Fig. 3A), few if any CD4<sup>+</sup> cells could be recovered from the cultures with MHC-neg-

ative TSC, and the absolute cell yield was also markedly reduced (Fig. 3B). These results indicate that once thymocytes have initiated positive selection, additional MHC-dependent interactions are necessary for further survival and differentiation.

To determine whether these late-stage positive selection signals can also be provided by MHC molecules on DC and whether TCR or only CD4 coreceptor signaling was involved, CD69<sup>high</sup>/CD4<sup>low</sup>/CD8<sup>low</sup> cells were placed in reaggregate cultures composed of MHC-positive TSC or of MHC-negative TSC mixed with class II-positive DC. A substantial number of CD4<sup>+</sup> T cells developed in the reaggregates containing MHC-negative TSC and H-2<sup>b</sup> DC (Fig. 3C). Similar results were obtained by using for both the dispersed and reaggregate culture steps 98% pure splenic or thymic CD11C<sup>+</sup> DC obtained by cell sorting (Fig. 3, D and E). The MHC molecule requirement in the second culture involves TCR and not just coreceptor engagement, as inclusion of DC from a nonselecting haplotype (H-2<sup>b</sup>) in the MHC-negative TSC reaggregate culture did not rescue CD4<sup>+</sup> T cell development (Fig. 3F). The ability to meet the requirement for TCR-MHC interaction in the reaggregate cultures using DC rather than TSC allowed us to confirm that the preactivation step giving rise to CD69<sup>high</sup> cells truly promoted initiation of positive selection. As shown in Fig. 3G and in agreement with previous results (29), addition of unstimulated CD69<sup>low</sup> DP thymocytes to MHC<sup>−/−</sup> TSC plus H-2<sup>b</sup> DC resulted in the recovery of very few CD4<sup>+</sup> T cells. This clearly shows that the first stimulation induces differentiation changes necessary for progression to maturity in the second culture, particularly in the context of MHC display by DC.

The CD4<sup>+</sup> cells arising under these reaggregate culture conditions with MHC-deficient TSC and MHC-bearing DC have nearly the same surface phenotype (TCRβ<sup>high</sup>, MHC class I<sup>high</sup>, CD5<sup>high</sup>, and HSA<sup>int</sup>) as CD4<sup>+</sup> cells that were never exposed to PCC during differentiation (Fig. 4, A–D). There is occasionally a small decrease in TCR level among the CD4<sup>+</sup> cells that have initiated selection using agonist (data not shown) and a slightly higher level of HSA (Fig. 4D), but these cells are functionally competent, proliferating in response to PCC<sub>88–104</sub> presented by I-E<sup>k</sup> cells with only a modestly shifted dose-response compared with AND TCR transgenic CD4<sup>+</sup> cells that were never exposed to PCC during differentiation (Fig. 4E).

Late-stage negative selection can obscure early signals capable of initiating effective positive selection

To investigate why these results showing generation of Ag-responsive cells using agonist ligand differ from those reporting either only death or unresponsiveness of cells exposed to agonist during thymic development (5, 6, 8, 9), we considered the change from PCC-I-E<sub>k</sub> agonist in the first culture to I-A<sub>b</sub> in the second culture. I-A<sub>b</sub> as well as I-E<sub>k</sub> class II molecules can support positive selection of AND TCR transgenic thymocytes (49), but I-E<sub>k</sub> associated with self-peptides also induces in vivo deletion of a fraction of the cells that begins selection on this MHC class II molecule (50). In agreement with these data, the cells emerging from two-stage cultures involving I-A<sub>b</sub> DC had a low, but detectable, proliferative response to I-E<sub>k</sub> splenic presenting cells (data not shown), consistent with the most responsive cells normally targeted by this deletion process surviving in the absence of I-E<sub>k</sub>. Therefore, we examined what effect the presence of I-E<sub>k</sub> on DC would have on differentiation in the reaggregates. Development of CD4<sup>+</sup> T cells occurs with H-2<sup>b</sup> DC in the reaggregate (Fig. 5B), but the efficiency is quite low compared with that of H-2<sup>b</sup> DC (Fig. 5A). To determine whether this is the result of increased negative selection, CD69<sup>high</sup>/CD4<sup>low</sup>/CD8<sup>low</sup> thymocytes were cultured with a combination of H-2<sup>b</sup> and H-2<sup>k</sup> DC in the presence of MHC-negative TSC.
Under these conditions, the same low number of CD4\(^+\) T cells is seen as with H-2\(^k\) DC and MHC-negative TSC alone, consistent with the H-2\(^k\) DC triggering substantial deletion at a late stage of thymocyte maturation despite being fully competent to initiate positive selection among DP (see Fig. 2D).

### Discussion

Even the most well-studied aspect of αβ T cell development, namely positive and negative selection based on TCR interaction with self-peptide/MHC molecule complexes, is incompletely understood. Although the application of transgenic and gene targeting technology has contributed greatly to our understanding of the role of specific MHC ligands in repertoire selection (2), both the timing of the TCR-ligand interactions contributing to the two opposing selection events as well as the consequences of TCR signaling in response to ligands of various quality on distinct cell types remain controversial. Using a two-stage in vitro model system, we provide evidence here of a requirement for MHC-dependent TCR signaling throughout thymocyte development from the DP to the single-positive stage, for the capacity of peptide:MHC ligands on hemopoietic DC to provide these signals, and for the ability of transient exposure to stimulatory (agonist) ligands to initiate differentiation of mature T cells that remain reactive with the same stimulus.

One surprising result of these experiments is the ability of DC to provide TCR stimulation suitable for initiation and completion of positive selection. A large body of data generated using radiation bone marrow chimeras or thymus-grafted animals argues that under normal conditions, radiation-resistant, nonhemopoietic cells dictate the MHC allele-restricted function of peripheral mature T cells (20–23, 26). Some previous experimental results argue against a unique capacity of thymic epithelial cells to promote

**Table I. Relative efficiency of positive selection**

<table>
<thead>
<tr>
<th>First-Step Culture</th>
<th>Second-Step Culture</th>
<th>Relative % of CD4(^+) Cells</th>
<th>Total CD4(^+) Cells (×10(^5))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>I-A(^b) TSC</td>
<td>11.0 ± 3.4*</td>
<td>2.5 ± 0.2*</td>
</tr>
<tr>
<td>I-A(^b) DC</td>
<td>I-A(^b) TSC</td>
<td>36.2 ± 4.5**</td>
<td>6.3 ± 1.0**</td>
</tr>
<tr>
<td>I-E(^k) DC</td>
<td>I-A(^b) TSC</td>
<td>32.9 ± 5.5**</td>
<td>6.7 ± 1.1**</td>
</tr>
<tr>
<td>I-E(^k) TSC + PCC</td>
<td>I-A(^b) TSC</td>
<td>38.8 ± 3.2**</td>
<td>6.8 ± 1.4**</td>
</tr>
<tr>
<td>I-E(^k) DC + PCC</td>
<td>I-A(^b) TSC</td>
<td>34.5 ± 5.9**</td>
<td>6.6 ± 1.1**</td>
</tr>
</tbody>
</table>

* Data were analyzed using a two-tailed Mann-Whitney U test. The yield of CD4\(^+\) cells from all prestimulated groups (**) was significantly different (p < 0.01) from the yield obtained using unstimulated thymocytes (*).
positive selection, as intrathymic injection of transfected L cells can induce the development of functional T cells restricted to MHC molecules present on these injected cells but absent from the thymic stroma (44). Furthermore, limited evidence for even hemopoietic cell contributions to positive selection has been reported in chimeric mice (19–22). However, with the sole exception of the unconfirmed results of Longo et al., none of these prior studies suggests that DC in particular can contribute to thymocyte selection in anything but a negative manner (28, 29, 41).

How can these data be reconciled with the present observations? Two major differences between these previous investigations and those detailed here are our initiation of positive selection in dispersed culture and the presence of different peptides in the MHC molecules on the DC used to initiate and to complete selection. In the normal thymus, thymocytes first expressing the αβ TCR involved in initiating selection interact with cortical epithelial cells in the subcapsular region. Cells failing to receive adequate TCR signals in this location do not leave the cortex (51) and hence are unable to interact with DC localized at the cortico-medullary junction or in the medulla. In reaggregate cultures, the admixed cells may not remain randomly positioned (52, 53), and therefore the input DP may also fail to associate at a significant frequency with DC. Only in the dispersed culture are the DP thymocytes able to interact effectively with the Ag-bearing DC. As the present data show, such interaction, even involving full agonist peptide-MHC molecule complexes, can stimulate DP thymocytes to undergo the phenotypic changes characteristic of cells initiating positive selection in vivo, and at least a fraction do so without undergoing apoptosis. This differentiation without death only occurs at a certain ligand density, consistent with many studies on thymocyte development. It is the CD69high cells surviving this initial signaling event that are able to mature most effectively upon subsequent introduction into a suitable stromal environment, and they, but not CD69low T cells, positively selecting H-2b AND DP thymocytes generated in a first-step culture using DC from B10.BR mice and 0.01 μM PCC88–104 for 20 h (solid line) or isolated from the thymus of positively selecting H-2b AND TCR transgenic RAG-2−/− mice (dotted line) was examined by flow cytometry. Background staining is shown by the bold lines. E, The proliferative responses of various CD4+ T cell populations to different concentrations of PCC presented by mitomycin C-treated spleen cells from B10.BR mice. CD4+ T cells were generated by reaggregate culture of unstimulated DP with H-2b TSC (●) or by reaggregate culture with MHC−/− TSC plus H-2b DC of CD69high cells generated in the first culture by stimulation for 20 h with H-2b DC (□) with 0.1 μM PCC88–104 plus H-2b DC (△), or with H-2b DC alone (○).
required for positive selection, in contrast to the conclusion of Nakagawa et al. (54).

The results obtained in this two-step culture system help clarify conflicting data obtained with cells isolated by cell sorting from normal thymuses. The CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>low</sup> precursor thymocytes studied by Kisielow and Miazek (17) appear to correspond to CD69<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells (42), and thus the results presented here are in full accord with the prior data reported by these investigators indicating a need for continued TCR signaling through-out maturation. Barthlott et al., in contrast, reached a different conclusion using reaggregate culture and cells sorted from P14 TCR transgenic mice (14), as did Hare et al. in a related system (15). However, the sorting gates used by Barthlott et al. to isolate CD4<sup>+</sup>CD8<sup>+</sup> cells clearly include cells with fully mature levels of TCR expression, and a similar lack of separation between thymocytes in the early and late stages of maturation appears likely in the studies of Hare et al. Therefore, it is not surprising that these more mature cells show less obvious dependence on the selecting MHC allele for terminal differentiation into CD8<sup>+</sup> cells, as the data reported here, our additional unpublished observations, and the findings of Kisielow and Miazek (17) indicate that these cells have passed the checkpoint for lineage-specific coreceptor regulation.

A last point involves the ability to induce the maturation of CD4<sup>+</sup> T cells responsive to the same agonist peptide:MHC molecule agonist ligand as that used to initiate positive selection. In fetal thymic organ culture β<sub>2m</sub>-deficient TCR transgenic cells, Hogquist et al. were unable to obtain mature CD8<sup>+</sup> T cells using agonist peptides, and partial agonist peptides for the TCR donor clone stimulated positive selection of cells with low CD8 levels that were completely unresponsive to these ligands (5, 6). Others could obtain mature CD8<sup>+</sup> TCR transgenic T cells in TAP-deficient and β<sub>2m</sub>-deficient organ culture, respectively, using agonist peptide (4, 7–9), but where tested, these cells were unresponsive to this same ligand. Our data argue that exposure to even strong agonist ligand at concentrations able to activate mature cells does not prevent generation of thymocytes still reactive with the same ligand, provided that this or other highly stimulatory ligands for the TCR are eliminated from the environment of the thymocyte during the late stages of maturation. The fetal thymic organ culture experiments arguing that agonist cannot positively select functional cells all involve the continuous presence of ligand throughout the thymus during development, exposing cells that successfully initiated selection on such ligands to additional stimuli that can induce death among more differentiated cells, as shown here in reaggregates containing 1-E<sup>k+</sup> DC (Fig. 5). In accord with this model, direct in vivo evidence that late negative selection of thymocytes can play a major role in eliminating cells that could otherwise undergo effective positive selection and maturation has been reported (55). Agonist ligands primarily or exclusively present on cortical epithelial cells might well initiate selection of T cells that can complete their selection on less potent ligands of medullary cells. Such cells could be relevant in epithelial-specific autoimmunity in the periphery.

The use of the Jenkinson and Owens reaggregate method as modified here to separately probe the signals and cell types involved in early and late thymocyte development provides a powerful tool for analysis of this differentiation process. The results reported here using this approach emphasize that a single, discrete TCR signaling event involving peptide:MHC molecules on cortical epithelial cells does not suffice to promote effective thymocyte-positive selection, lineage-specific differentiation, and cell survival. Nor are the roles of epithelial and hematopoietic cells, especially DC, what have been traditionally assumed from radiation chimera and transgenic studies of the past. We have recently used this model system to dissect the signaling requirements for CD4<sup>+</sup> vs CD8<sup>+</sup> lineage commitment as well as the role of Notch-1 in lineage-specific progression; the appreciation of the need for different signals in the initiation and completion of selection as demonstrated here was key to the success of this study (56). Finally, the idea that self-MHC recognition on hematopoietic cells may contribute in a positive rather than only a negative manner to thymocyte development alters the view we have of the relationship of different cell types to life and death decisions of T cells.

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