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

Koji Yasutomo,1* Bruno Lucas,*† and Ronald N. Germain2*

Thymocyte selection involves signaling by TCR engaging diverse self-peptide:MHC molecule ligands on various cell types in the cortex and medulla. Here we separately analyze early and late stages of selection to better understand how presenting cell type, ligand quality, and the timing of TCR signaling contribute to intrathymic differentiation. TCR transgenic CD4+CD8+ thymocytes (double positive (DP)) from MHC-deficient mice were stimulated using various presenting cells and ligands. The resulting CD69hi cells were isolated and evaluated for maturation in reaggregate cultures with wild-type or MHC molecule-deficient thymic stroma with or without added hemopoietic dendritic cells (DC). Production of CD4+ T cells required TCR signaling in the reaggregates, indicating that transient recognition of self-ligands by DP is inadequate for full differentiation. DC bearing a potent agonist ligand could initiate positive selection, producing activated thymocytes that matured into agonist-responsive T cells in reaggregates lacking the same ligand. DC could also support the TCR signaling necessary for late maturation. These results argue that despite the negative role assigned to DC in past studies, neither the peptide:MHC molecule complexes present on DC nor any other signals provided by these cells stimulate only thymocyte death. These findings also indicate that unique epithelial ligands are not necessary for positive selection. They provide additional insight into the role of ligand quality in selection events and support the concept that following initiation of maturation from the DP state, persistent TCR signaling is characteristic of and perhaps required by T cells. The Journal of Immunology, 2000, 165: 3015–3022.

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.
(31–34). 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:MHC molecule ligands and these virgin DP, we have been able to induce in vitro the CD69+ CD4lowCD8low TCRint phenotype typical of thymocytes beginning positive selection (12). By varying the antigenic ligands and presenting cells in this first incubation and then culturing the CD69+ cells in a thymic stromal reaggregate (35), a more precise analysis of the roles of ligand quality, presenting cell type, and signal duration in positive vs negative selection is possible. With this approach, we clearly demonstrate here a requirement for MHC-dependent signals not only for initiation of thymocyte-positive selection, but subsequently for lineage-specific differentiation and survival during development of functional CD4+ T cells. We also document the ability of a strong agonist to initiate selection of T cells that are responsive in the mature state to the same ligand and provide evidence that past failures to observe positive selection potential ligands may be 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 signaling events for functional selection. This is concordant with

Materials and Methods

Mice

Mice with targeted inactivation of both the β2-microglobulin (β2m) gene (38) and Aβ2 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−/− Aβ2−/− mice were provided by B. J. Fowlkes, Laboratory of Cellular and Molecular Immunology, National Institute of Allergy 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 established guidelines.

Flow cytometry

Thymocytes were stained with PE-conjugated anti-CD4, FITC-conjugated anti-CD8, and biotin-conjugated anti-CD49d, anti-CD5, anti-TCRVβ3, anti-heat-stable Ag (HSA), or anti-DNA 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-mAbs, then fixed using 4% paraformaldehyde. After washing, the cells were stained with anti-CD69 mAb followed by PE-conjugated anti-CD4 and FITC-conjugated anti-CD8. 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-mAbs, then fixed using 4% paraformaldehyde. After washing, the cells were stained with anti-CD69 mAb followed by PE-conjugated anti-CD4 and FITC-conjugated anti-CD8.

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 C57BL6/6 or mice with targeted mutations in both the β2m and Aβ2 gene loci were prepared by disaggregating fetal thymic lobes previously cultured for 5 days in 1.35 mM deoxyguanosine (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 10:10:1 (absolute number of DC, 5 × 10^5). 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+ cells 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 promisc treatment

FACS was used to obtain CD69+ CD4lowCD8low thymocytes following in vitro stimulation for 84 h of CD4+CD8− thymocytes from AND TCR transgenic RAG-2−/− Aβ2−/− mice with DC in the presence of 0.01 μM of a synthetic peptide corresponding to pigeon cytochrome c residues 88–104 (PCC88–104). Promisc 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 × 10^5 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 [3H]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/Eβ complexes (12, 57). These studies did not determine whether these agonist-activated, 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 intrathymically can promote effective positive selection (44), and these cells have some epithelial characteristics, these studies with transfectants did not address the issue of whether epithelial vs hemopoietic (especially dendritic) cells had distinct capacities to promote 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 <0.1 μM PCC88–104, presented by DC from B10.BR (I-Ek+) mice, only a fraction of the cells underwent apoptotic cell death (Fig. 1A), and most of the surviving cells became...
CD4<sup>low</sup>CD8<sup>low</sup> and CD69<sup>high</sup> (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 CD69<sup>high</sup> 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<sup>E<sub>k</sub></sup> complexes. Stimulation with PCC<sub>88−104</sub> 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 cells 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<sup>E<sub>k</sub></sup>-expressing DC, CD4<sup>low</sup>CD8<sup>low</sup> thymocytes re-expressed both CD4 and CD8 molecules following treatment by pronase and culture for 14 h at 37°C (Fig. 1, F–H).

CD69<sup>high</sup> DP thymocytes generated by stimulation with agonist-bearing DC or thymic stromal cells show enhanced differentiation into CD4<sup>+</sup> T cells in thymic reaggregate culture

To test whether the CD69<sup>high</sup>CD4<sup>low</sup>CD8<sup>low</sup> 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 PCC<sub>88−104</sub> presented by I<sup>E<sub>k</sub></sup>- expressing DC or CD45<sup>+</sup> TSC. These cells were then cultured for 60 h in reaggregates with TSC expressing I-A<sup>+</sup>, which mediates positive selection without deletion of thymocytes bearing this particular TCR (49). In such reaggregates, unstimulated CD69<sup>low</sup> AND DP gave rise to a modest number of CD4<sup>+</sup> T cells, representing ~12% to a maximum of 20% of the total recovered thymocytes (Fig. 2B). Sorted CD69<sup>−</sup>CD4<sup>low</sup>CD8<sup>low</sup> cells produced in first-step cultures without peptide using DC expressing either the I-A<sup>B</sup> or I-E<sup>k</sup> plus I-E<sup>k</sup>- MHC class II molecules yielded, on the average, about 2- to 3-fold more total CD4<sup>+</sup> mature thymocytes in the reaggregates (Fig. 2, C and D, and Table I). The same degree of enhanced CD4<sup>+</sup> T cell development was seen using equal numbers of CD69<sup>high</sup>CD4<sup>low</sup>CD8<sup>low</sup> thymocytes generated by PCC presentation on I-E<sup>k</sup>- TSC or DC (Fig. 2, E and F). Given the equal input cell number for all reaggregate cultures, this consistently increased output using CD69<sup>−</sup>/high compared with unstimulated CD69<sup>low</sup> 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<sup>+</sup> cells were generated in cultures of DP with MHC class II-deficient DC, and the transfer of such CD69<sup>−</sup> cells into reaggregates with TSC did not yield elevated levels of CD4<sup>+</sup> 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).

CD69<sup>high</sup>CD4<sup>low</sup>CD8<sup>low</sup> thymocytes do not successfully differentiate into CD4<sup>+</sup> 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 CD69<sup>high</sup>
FIGURE 2. AND DP stimulated in vitro by Ag-bearing DC can differentiate into CD4+ T cells in thymic reaggregate culture. AND DP were cultured alone (A) or in reaggregate culture with TSC from C57BL/6 mice for 60 h without prestimulation (B). AND DP were stimulated with C57BL/6 DC (C), B10.BR DC (D), B10.BR TSC plus 0.1 μM PCC88–104 (E), or B10.BR DC plus 0.1 μM PCC88–104 (F) for 20 h. After purification, sorted CD69+ cells were placed in reaggregate culture with TSC from C57BL/6 mice for 60 h (C–F). Recovered cells were stained with anti-CD4 or CD8 mAb and examined by flow cytometry. The box in the upper left quadrant in each two-dimensional histogram (B–F) encloses the CD4+B+ thymocyte population, and the number indicates the percentage of the total recovered thymocytes represented by these cells. The absolute number of recovered viable thymocytes is given below B–F. The results are representative of a large series of experiments in which the recovery of CD4+ cells after reaggregate culture using prestimulated CD69+ cells was consistently between 2- and 3-fold greater than that seen with unstimulated CD69+ cells.

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 se-
tative 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+CD4+CD8low CD8low 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+ T cells developed in the reaggregates containing MHC-negative TSC and H-2b DC (Fig. 3C). Similar results were obtained by using for both the dispersed and reaggregate culture steps 98% pure splenic or thymic CD11C+ 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-2d) in the MHC-negative TSC re-aggregate culture did not rescue CD4+ 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+ cells truly promoted initiation of positive selection. As shown in Fig. 3G and in agreement with previous results (29), addition of unstimulated CD69low DP thymocytes to MHC−/− TSC plus H-2b DC resulted in the recovery of very few CD4+ 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+ cells arising under these reaggregate culture conditions with MHC-deficient TSC and MHC-bearing DC have nearly the same surface phenotype (TCRVB3+ high, MHC class I high, CD5+ high, and HSA+ medium) as CD4+ T cells arising from in vivo selection of AND TCR-bearing cells on I-Ab (Fig. 4, A–D). There is occasionally a small decrease in TCR level among the CD4+ cells that have initiated selection using agonist (data not shown) and a slightly higher level of HSA (Fig. 4D), but these cells are func-
tionally competent, proliferating in response to PCC88–104 presented by I-Ek cells with only a modestly shifted dose-response compared with AND TCR transgenic CD4+ 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 PCC1-I-Ek agonist in the first culture to I-A b in the second culture. I-A b as well as I-Ek class II molecules can support positive selection of AND TCR transgenic thymocytes (49), but I-Ek 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 b DC had a low, but detectable, proliferative response to I-Ek splenic presenting cells (data not shown), consist-
tent with the most responsive cells normally targeted by this de-
letion process surviving in the absence of I-Ek. Therefore, we ex-
amined what effect the presence of I-Ek on DC would have on differentiation in the reaggregates. Development of CD4+ T cells occurs with H-2h DC in the reaggregate (Fig. 5B), but the efficiency is quite low compared with that of H-2b DC (Fig. 5A). To determine whether this is the result of increased negative selection, CD69+CD4+CD8low thymocytes were cultured with a combi-
nation of H-2b and H-2h DC in the presence of MHC-negative TSC.
(Fig. 5C). Under these conditions, the same low number of CD4\(^+\) T cells is seen as with H-2\(^b\) DC and MHC-negative TSC alone, consistent with the H-2\(^b\) 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>
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<th>First-Step Culture</th>
<th>Second-Step Culture</th>
<th>Relative % of CD4(^+) Cells</th>
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<tr>
<td>None</td>
<td>I-A(^b) TSC</td>
<td>11.0 ± 3.4(^*)</td>
<td>2.5 ± 0.2(^*)</td>
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<tr>
<td>I-A(^b) DC</td>
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<td>36.2 ± 4.5(^**)</td>
<td>6.3 ± 1.0(^**)</td>
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<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>
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<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>
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\(\text{data were analyzed using a two-tailed Mann-Whitney U test. The yield of CD4}^+\text{ cells from all prestimulated groups (}\text{**}\text{) was significantly different (}\text{p} < 0.01\text{) from the yield obtained using unstimulated thymocytes (}\text{*}\text{).}

**FIGURE 3.** MHC molecule ligands are required for full development of CD4\(^+\) T cells from AND TCR transgenic CD4\(^{low}\)CD8\(^{low}\)CD69\(^{high}\) cells and can be provided by H-2\(^b\) DC. AND DP were stimulated with DC from B10.BR mice and 0.01 μM PCC 88–104 for 20 h. Purified CD69\(^{high}\)CD4\(^{low}\)CD8\(^{low}\) cells were then cultured with TSC from C57BL/6 mice (A), MHC\(^−/−\) TSC (B), and MHC\(^−/−\) TSC together with enriched DC from C57BL/6 mice (C), purified splenic DC from C57BL/6 mice (D), purified thymic DC from C57BL/6 mice (E), and enriched DC from B10. D2 mice (F). G, CD69\(^{low}\) AND DP thymocytes were added to MHC\(^−/−\) TSC and enriched DC from C57BL/6 mice for 60 h. Recovered cells were stained with anti-CD4 or CD8 Abs and evaluated by flow cytometry. The percentage of cells with a CD4\(^+\) phenotype is given in the upper left quadrant of each panel, and the absolute number of recovered viable thymocytes is given below each panel. As it was not possible to conduct all these reaggregate cultures at one time, the results shown here are a composite from distinct experiments, each repeated two to four times. In each case, positive and negative control groups were included in the original experiment and yielded results similar to those illustrated here in A and/or B.
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 have 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 ligand, 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 CD69low 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, are the only cells capable of completing development upon interaction with MHC expressed only on DC in a reaggregate culture. Initiation of selection on cortical epithelial MHC under normal circumstances apparently narrows the TCR repertoire of cells eventually interacting with other thymic cells types to such an extent that few if any remain able to be specifically selected by these latter interactions, accounting for the results using hemopoietic chimeras.

Although such anatomic considerations argue that peptide:MHC molecules on DC do not participate in the first stage of positive selection under physiologic circumstances, they might nevertheless contribute under normal conditions to the late stages of the selection process, as shown here in the reaggregate cultures. This effect would be unappreciated in the F1→P radiation chimera model. The possible role of thymic DC ligand recognition in prolonging the lifetime of maturing thymocytes is especially intriguing in light of data showing that expression of MHC class II molecules on DC nor any signals provided by other surface or secreted proteins of these cells, including CD80/CD86, stimulate only thymocyte death and, further, that unique epithelial peptide:MHC molecule complexes are not necessarily

FIGURE 4. The surface phenotype and proliferative response of CD4+ T cells selected by agonist and DC. The expression of TCR Vδ3 (A), CD5 (B), MHC class I (C), and HSA (D) on CD4+CD8− T cells positively selected in reaggregate cultures containing MHC−/− TSC by DC from C57BL/6 mice and CD69+ thymocytes generated in a first-step culture using DC from B10.BR mice and 0.01 μM PCC 88–104 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 CD69low T cells generated in a first culture by stimulation for 20 h with H-2b DC (□) or with H-2b DC alone (○).

FIGURE 5. Late negative selection in reaggregate cultures. AND DP were stimulated with DC from B10.BR mice and 0.01 μM PCC 88–104 for 20 h. Purified CD69lowCD4+CD8low cells were then cultured with MHC−/− TSC plus H-2b DC (A), H-2d DC (B), or H-2d and H-2b DC (C) for 60 h. Recovered cells were then stained with anti-CD4 or CD8 Abs and evaluated by flow cytometry. The percentage of cells with a CD4+ phenotype is given in the upper left quadrant of each panel, and the absolute number of recovered viable thymocytes is given below each panel.
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>loig</sup> precursor thymocytes studied by Kisielow and Miazek (17) appear to correspond to CD69<sup>+</sup>CD4<sup>loig</sup>CD8<sup>low</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 throughout 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>low</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 of β<sub>2</sub>m-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>2</sub>m-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 I-EK<sup>+</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 autoimmune 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 hemopoietic 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 vs CD8 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 hemopoietic 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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