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Antigen-Induced Coreceptor Down-Regulation on Thymocytes Is Not a Result of Apoptosis

Maureen A. McGargill and Kristin A. Hogquist

The various stages of T cell development are typically characterized by the expression levels of the two coreceptors, CD4 and CD8. During the CD4^+CD8^+ (double-positive, DP) stage of development, thymocytes that perceive a low avidity signal through the TCR go on to differentiate (positive selection), and ultimately down-regulate one coreceptor to express either CD4 or CD8. Alternatively, thymocytes that perceive a high avidity signal down-regulate both coreceptors and are induced to die via apoptosis (negative selection). However, it has recently been suggested that positively selected thymocytes may also partially down-regulate both coreceptors before up-regulating the one coreceptor that is ultimately expressed. This would imply that coreceptor down-regulation (dulling) is not a consequence of commitment to the death pathway. To explore this possibility, we have utilized an in vitro assay to demonstrate that dulling occurred in response to both positive and negative selecting ligands in vitro, was not a result of nonspecific membrane perturbation, was not dependent on the type of APC, and occurred before death in vitro. Furthermore, when thymocyte apoptosis was blocked, CD4 and CD8 were down-regulated in response to TCR stimulation. These data suggest that dulling in response to TCR ligation is distinct from death, and support a model in which DP dulling occurs during both positive and negative selection. The biological implications of this phenomenon are discussed. The Journal of Immunology, 1999, 162: 1237–1245.

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2 Address correspondence and reprint requests to Dr. Kristin A. Hogquist, Department of Laboratory Medicine and Pathology, University of Minnesota, Box 334 Mayo, 420 Delaware St. SE, Minneapolis, MN 55455. E-mail address: hogquist01@tc.umn.edu

3 Abbreviations used in this paper: DP, double positive; 7AAD, 7-amino actinomycin D; CHX, cycloheximide; FTOC, fetal thymic organ culture; HPRT, hypoxanthine phosphoribosyltransferase; MFI, mean fluorescence intensity; PT, permeability transition; RAG, recombinase-activating gene; SP, single positive.

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CD4 and CD8. However, many of these events also occur during negative selection, and in some systems anti-TCR stimulation induced apoptosis (18–20). Therefore, these data alone do not exclude the possibility that DP dull cells are in the process of being negatively selected.

The most compelling evidence that positively selected cells down-regulate both CD4 and CD8 is that DP dull cells transferred intrathymically do not necessarily undergo apoptosis. In fact, anti-TCR-generated DP dull cells produced mature SP thymocytes (14). Likewise, DP dull cells from transgenic mice that overexpress Bcl-2 generated mature SP T cells upon transfer (21, 22).

These data suggest that the DP dull phenotype may represent an intermediate stage of positive selection, as well as negative selection. Thus, we tested whether CD4 and CD8 down-regulation (dulling) induced by peptide/MHC ligands was an incidental result of cells going through apoptosis. We modified an in vitro dulling assay utilizing the OT-I TCR transgenic system to demonstrate that DP dulling in response to peptide/MHC is not simply a consequence of apoptosis, rather it is a biochemically distinct event, and may reflect an intermediate stage of positive selection.

**Materials and Methods**

**Mice**

C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TAP⁻ is a (129 × C57BL/6)F₁ strain with a targeted disruption of the TAP-1 gene on both chromosomes (gift of Anton Berns, Netherlands Cancer Institute, Amsterdam, The Netherlands). OT-I is a C57BL/6 TCR transgenic strain, expressing a receptor that is specific for the OVA257–295 peptide (OVAp) in the context of the H-2K<sup>+</sup>MHC class I molecule (23). CD8tg is a C57BL/6 strain overexpressing endogenous CD8 (2.43; American Type Culture Collection, Manassas, VA), or transgenic CD8 (1116, a gift from M. J. Bevan).

**Cells and cell lines**

The thymic stromal cell line, 130.8.1, was derived from SV40 T Ag transgenic (B6 × SJL)F<sub>1</sub> mice, and displays surface Ags similar to medullary cells (25). They were grown in RPMI media (RPMI with 10% FCS). 5A.K<sub>2</sub> (a gift of F. Carbone, Monash University, Melbourne, Australia) is a transformed L cell line that has been transfected with H-2K<sup>+</sup> (26). TAP<sup>−</sup> FB (a gift of A. W. Goldrath and M. J. Bevan, University of Washington, Seattle, WA) is a fibroblast cell line derived from TAP<sup>−</sup> embryos that was transfected with SV40.

Peritoneal exudate cells were extracted from B6, TAP<sup>−</sup>, or bm8 mice that were injected 5 days prior with thiglycolate. Macrophages were enriched by adherence to 96-well flat-bottom microtiter plates for 1 h at 37°C, and washed to remove any nonadherent cells.

B cells were enriched from spleen cells, as described (27). In brief, 100 µg/ml goat anti-mouse IgG (Sigma, St. Louis, MO) was adhered to tissue culture-treated plates. Spleen cells isolated from TAP<sup>−</sup> or B6 mice were cultured on the coated plates for 30 min at room temperature, washed four times with PBS, incubated for 1 h in RPMI at 37°C, and removed from the plate. The suspensions contained greater than 75% B cells.

**DP dulling assay**

The DP dulling assay was performed as described (28) with a few modifications. APC (3 × 10⁶/well) were adhered to 96-well flat-bottom microtiter plates for 1 h at 37°C. The monolayers were labeled with 500 nM CellTracker Green CMFDA (Molecular Probes, Eugene, OR) in serum-free media (RPMI) for 15 min, washed twice, and incubated at 37°C for an additional 30 min in RPMI, and then washed two more times. B cells were labeled and washed in suspension according to the same procedure. Thymocytes were isolated from OT-I, TAP<sup>−</sup> or OT-4, TAP<sup>−</sup>, CD8tg mice and cocultured at 5 × 10⁶/well with the labeled APC, with or without 10–100 nM OVAp (SIINFEKL). For anti-Fas Ab treatment, 20 µg/ml of soluble anti-Fas (Jo2; PharMingen, San Diego, CA) was added to suspensions of thymocytes with no APC. In dexamethasone-treated cultures, 1 µM of dexamethasone was added to suspensions of thymocytes with no APC.

To inhibit apoptosis, the thymocytes were preincubated for 2 h at 37°C with 100 µM of the tripeptide, caspase inhibitor, benzoxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone (Z-VAD.fmk; Enzyme Systems Products, Dublin, CA), or DMSO as a solvent control. The thymocytes and inhibitor were then added to the labeled APC, in the presence or absence of OVAp. Likewise, to block new protein synthesis, thymocytes were preincubated for 2 h, at 37°C with 100 µg/ml cycloheximide (CHX; Sigma), or equal amounts of ethanol as a solvent control, and cocultured with the labeled APC, in the presence or absence of OVAp.

After 16–20 h at 37°C, the cells were harvested, stained with mAbs, and analyzed by flow cytometry using a FACS calibur (Becton Dickinson, San Jose, CA). The APC were gated on flow cytometry. The mAbs that were used specific for CD4 (L3T4, RM4-5), CD69 (H1.2F3), CD5 (Ly-1), CD25 (7D4), CD2 (RM2-5) (PharMingen), endogenous CD8 (2.43; American Type Culture Collection, Manassas, VA), or transgenic CD8 (1116, a gift from M. J. Bevan).

The amount of dulling reflects the loss of thymocytes from the CD4<sup>+</sup>CD8<sup>+</sup> DP bright gate. Specific dulling activity is the inverse of the loss of thymocytes from the DP bright gate, and was calculated using the equation (1 – (percentage of DP bright cells in experimental cultures/percentage of DP bright cells in control cultures)) × 100.

**Fetal thymic organ culture (FTOC)**

Fetal thymic lobes were taken on day E16 and cultured for 2 days. On day 2, either 20 µM P815 (HYEFPQQL, 20 µM V- OVA/RGYNYEKLF), or 20 nM OVAp was added to the cultures. The lobes were harvested after 18 h and analyzed by flow cytometry with CD8 APC, CD4 PerCP, and B20-FITC Abs. The dead cells were gated out of this analysis via Annexin V staining (see below).

**Apoposis analysis**

Thymocytes were harvested and stained with mAbs for 30 min, washed twice, and then stained with 100 µg/ml 7-aminotiocynoc D (7AAD; Sigma, St. Louis, MO) for 20 min, and analyzed by flow cytometry. The 7AAD dye stains dead cells brightly, the cells in the process of apoptosis partially, and is excluded from viable cells (29). Because apoptotic thymocytes can be engulfed by APC, we compared the number of live thymocytes remaining after culture. We used the cytometer to count the number of remaining thymocytes by standardizing the volume of each sample analyzed. This was accomplished by adding 40,000 latex beads (5 µm; Interfacial Dynamics, Portland, OR) to each sample before collection, and setting the cytometer to collect 10,000 beads. Therefore, a quarter of each sample was collected, and the amount of specific death was calculated by comparing the number of thymocytes remaining after culture with TAP<sup>−</sup> APC alone, with the number remaining in the presence of OVAp. The following equation was used: (1 – (number of thymocytes remaining in experimental cultures/number of thymocytes remaining in control cultures)) × 100.

Phycoerythrin-conjugated Annexin V (PharMingen) was used to confirm apoptosis by detecting aberrant phosphatidylserine in the outer leaflet of the plasma membrane. After culture, thymocytes were harvested, washed in PBS, and resuspended in binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂). The thymocytes were incubated with 5 µl of Annexin V-phycocerythrin and 100 µg/ml 7AAD for 15 min at room temperature, and then analyzed by flow cytometry. CD16<sup>+</sup> (3) (Molecular Probes) was used to detect mitochondrial membrane permeability transition (PT). After culture, thymocytes were harvested, washed, and resuspended in binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂). The thymocytes were incubated with 1 µl of Annexin V-phycocerythrin and 100 µg/ml 7AAD for 15 min at 37°C, washed twice, incubated at 37°C for an additional 30 min, and then analyzed by flow cytometry. As a positive control, PT was induced by preincubation with the protonophore, carbonyl cyanide m-chlorophenylhydrazide (mCCICP; 50 µM; Sigma) for 15 min at 37°C.

**Reverse-transcriptase PCR**

A total of 1.5 × 10⁶ thymocytes was incubated in a dulling assay with 20 µM P815, 20 nM OVA, or 20 nM OVAp, and harvested after 18 h. The RNA was isolated from these cells with a Micro RNA isolation kit (Stratagene, La Jolla, CA), reverse transcribed with oligo(T) primers (SuperScript; Life Technologies, Gaithersburg, MD), and amplified by PCR at 60°C annealing temperature for 27–33 cycles with CD8β and HPR1 primers (17).

**Results**

Dulling occurs in response to positive and negative selection ligands in vitro

To investigate CD4 and CD8 down-regulation, we modified an in vitro assay that has historically been used to mimic negative selection (6, 8, 28, 30). This approach builds on the observation that during development, thymocytes are induced to die when exposed to the antigenic peptide. Thus, to mimic negative selection, thymocytes were cultured with adherent APC in the presence of the
antigenic peptide, and then the amount of death (via 7AAD uptake) and CD4/CD8 down-regulation (dulling) was determined using flow cytometry. The OT-I TCR transgenic system allowed us to extend this assay to also include positive selection ligands. OT-I thymocytes express a transgenic TCR that is specific for OVAp in the context of the H-2Kb MHC class I molecule (23). Thus, in vivo, OT-I thymocytes are positively selected in the presence of Kb and self peptides, and negatively selected in the presence of Kb and OVAp. Therefore, in vitro, the positive selection ligand was mimicked by B6 APC that express Kb and endogenous self peptides, and the negative selection ligand was supplied by APC that express Kb along with exogenously added OVAp. APC from TAPo mice provided a control for any nonspecific activity that may occur as a result of overnight culture or interactions with cell surface ligands other than class I MHC. TAPo mice do not stably express Kb on the cell surface (31), and therefore cannot stimulate OT-I T cells. However, Kb expression can be induced with the addition of exogenous peptide. Thus, by adding OVAp to TAPo APC, we can directly study the effect of the peptide/MHC by comparing the amount of activity in thymocytes cultured with TAPo APC and no peptide, with the activity in those cultured with TAPo APC and OVAp.

In addition, to study only the relevant preselection DP thymocytes, OT-I mice were bred to TAPo mice. Positive and negative selection of OT-I thymocytes does not occur in these mice due to the lack of the appropriate TCR ligand, and therefore thymocytes accumulate at the DP stage (data not shown).

We used this assay to investigate whether dulling was solely the result of positive selection, or if it also occurred in response to a naturally occurring positive selection ligand. B6 APC were used as a source of the OT-I TCR ligand for positive selection (Kb molecules bearing self peptides), and TAPo APC + the antigenic peptide, OVAp, were used as a source of the OT-I TCR ligand for negative selection. OT-I, TAPo thymocytes were cultured in vitro, with or without APC that had been previously labeled with a fluorescent CellTracker dye. After 18 h of culture, the thymocytes were harvested and stained with Abs to CD4 and CD8. The APC were excluded from this analysis via CellTracker exclusion. The percentage of DP bright thymocytes remaining in response to each ligand was determined (Fig. 1). Interestingly, both positive (B6 APC) and negative (TAPo APC + OVAp) selection ligands induced significant dulling, while the controls did not. The positive selection ligand induced less dulling than the negative selection ligand, which is consistent with the notion that low affinity ligands mediate positive selection, while higher affinity ligands mediate negative selection (32). Additionally, the level of dulling seen in OT-I, TAPo thymocytes exposed to B6 APC in vitro is similar to the level of dulling seen in vivo on the small percentage of CD69+ DP thymocytes that were presumed to be precursors of mature T cells (12). As additional negative controls, neither TAPo APC pulsed with irrelevant peptides, nor Kb Apo APC, which have normal levels of MHC molecules, but do not positively select OT-I thymocytes in vivo (33), induced dulling ([28] and data not shown). Therefore, the dulling seen in this study only occurred in response to specific TCR interactions, including the positive selection ligand. It should be noted that some dulling seen was even in cultures with no APC, the specificity of which is addressed later.

To confirm that this dulling also occurs in the intact thymic environment, we analyzed the level of CD4 and CD8 on thymocytes during positive and negative selection in FTOC. As in the OT-I, TAPo mouse, positive or negative selection of CD8 T cells does not occur in OT-I, TAPo FTOC. However, if a peptide with a low affinity for the OT-I TCR, such as V-OVA, is added to the culture, CD8 T cells will be synchronously positively selected. Conversely, if a peptide with a high affinity is added, OT-I T cells will be deleted (23). Therefore, to analyze dulling in the thymic environment, thymic lobes were taken from day 16 OT-I, TAPo embryos, and cultured in vitro for 2 days. On day 2, either 20 μM P815 (a peptide that binds to Kb, but does not stimulate the OT-I TCR), 20 μM V-OVA, or 20 nM OVAp was added to the cultures. The lobes were harvested the following day, and analyzed by flow cytometry. For this analysis of CD4 and CD8 down-regulation, the

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Both positive and negative selection ligands induce dulling in vitro. A, OT-I, TAPo thymocytes were cultured for 18 h with no APC, TAPo macrophages, B6 macrophages, or TAPo macrophages with 80 nM OVAp. The plots represent total thymocytes with a forward scatter/side scatter gate to eliminate debris. The number indicates the percentage of DP bright thymocytes that remained after culture. The data are representative of at least 10 separate experiments. B, Fetal thymic lobes were taken on day E16 and cultured for 2 days. On day 2, the indicated peptide was added to the cultures, and the lobes were harvested 18 h later. The dead cells were gated out of the FACs analysis via Annexin V staining, and the percentage of DP bright cells (number in the lower left corner) was determined for the remaining live cells.
Dulling is not a result of nonspecific membrane perturbation

Dulling is induced by a variety of cell types

<table>
<thead>
<tr>
<th>Type of APC</th>
<th>Percent of DP Thymocytes Remaining</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No peptide</td>
</tr>
<tr>
<td>Macrophages</td>
<td>76</td>
</tr>
<tr>
<td>Thymic epithelial cells</td>
<td>78</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>72</td>
</tr>
<tr>
<td>B cells</td>
<td>77</td>
</tr>
</tbody>
</table>

* Various APC were labeled with CellTracker dye and cultured with OT-I, TAPo thymocytes, with and without 10 mM OVAp. After 18 h, the thymocytes were harvested and analyzed by flow cytometry for CD4 and CD8 expression. The APC were gated out of the analysis via CellTracker fluorescence. The number indicates the percent of total DP thymocytes that remained after culture. Note that in the data set above, the B cells were tested in a separate experiment. These data are representative of two separate experiments for the thymic epithelial cells, and at least four separate experiments for the remaining APC.

Dead cells were eliminated via Annexin V staining. As shown in Fig. 1A, both V-OVA and OVA induced dulling in FTOC, while P815 did not. Again, the positive selection ligand induced less dulling than the negative selection ligand. This indicated that dulling occurred in response to the naturally occurring positive selection ligands (B6 APC), and a positive selection ligand in the intact thymus (V-OVA in FTOC). Additionally, Barnden et al. showed that OT-I, B6 adult mice exhibit considerable DP dulling, while OT-I, bm8 mice do not (33). Together this suggests that dulling occurs in response to both positive and negative selection ligands.

Dulling is not dependent on the type of APC

The role of costimulators on APC in positive and negative selection is not well characterized, and it is possible that dulling reflects the interaction of a particular costimulator. Thus, to investigate whether dulling was dependent on differentially expressed costimulators, we tested CD4 and CD8 down-regulation in response to a variety of APC. Macrophages were purified by adherence from the peritoneal exudate of H-2Kb TAPo mice and plated at 3 x 10^6 cells per well.

The thymic epithelial cells were a transformed cell line, 1308.1, that was derived from SV40 T antigen transgenic (B6 x SJL)F2 mice (25), and were plated at 3 x 10^6 cells per well.

The fibroblasts, 5AKb cells, were a transformed cell line transfected with H-2Kb and were plated at 1 x 10^6 cells per well.

B cells were enriched from the spleen of H-2Kb TAPo mice by panning with goat anti-mouse IgG and were plated at 3 x 10^5 cells per well.

The kinetics of thymocyte response to Ag

If CD4 and CD8 down-regulation are a consequence of apoptosis, we would expect them to be detectable only after the cells are committed to the apoptotic pathway. Therefore, we looked at the kinetics of dulling and various apoptotic events that occur in response to OVAp. OT-I, TAPo thymocytes were cultured with TAPo APC, in the presence or absence of OVAp, and harvested at various time points. CD4 and CD8 dulling, CD69 up-regulation, mitochondrial membrane PT (an early event thought to be central event that may occur with all cell surface ligands as a result of apoptosis. OT-I, TAPo thymocytes were cultured with TAPo fibroblasts, with and without OVAp for 18 h, and analyzed by flow cytometry for cell surface expression of the indicated molecules. The shaded area represents the expression level on the remaining live thymocytes cultured with TAPo fibroblasts alone. The solid line represents the total remaining thymocytes after culture with TAPo fibroblasts and 10 mM OVAp. The data are representative of four separate experiments.
Dulling after culture (see Materials and Methods). Separate fractions were stained with DiOC6 (3) to measure mitochondrial PT, or 7AAD to measure altered plasma membrane (PM) permeability. The percentage of cells positive for CD69 expression, 7AAD uptake, and DiOC6 (3) uptake is plotted. The percentage of cells positive for dulling is the inverse of the amount of live DP remaining after culture (see Materials and Methods). In all cases, the amount of activity that occurs on freshly isolated thymocytes is subtracted out. These data are representative of two separate experiments.

in initiating apoptosis (35, 36), and altered plasma membrane permeability (indicative of cells late in apoptosis) were measured (Fig. 3). After 3 h in culture with OVAp, the thymocytes had reached maximal CD69 up-regulation, and had just begun to initiate dulling and PT. At 6 h, there was an increase in both the percentage of thymocytes that had down-regulated CD4 and CD8, and the percentage of cells that had undergone PT. However, there was not an increase in the number of cells that had an altered plasma membrane permeability. After 12 h, the amount of dulling was maximal, and there was a further increase in the number of PT+ cells. On the other hand, there was only a small increase in the percentage of 7AAD+ cells, which did not reach maximal levels until 24 h (data not shown). Note that at 12 h not all of the DP dull cells have incurred PT. Thus, dulling occurred concurrent with, or before the earliest detectable apoptotic events.

Dulling occurs in the presence of apoptosis inhibitors

Caspases, a family of cysteine proteases, are important mediators of cell death induced by a variety of stimuli, and are required for apoptosis during negative selection (37, 38). If Ag-induced dulling occurs only as a consequence of apoptosis, then caspases would also be required for dulling. Thus, we next asked whether caspase activation was required for DP dulling. We took advantage of a caspase inhibitor, Z-VAD.fmk, which inhibits thymocyte apoptosis induced by a variety of stimuli (37, 38). OT-I, TAPo thymocytes were preincubated with various concentrations of Z-VAD.fmk or DMSO (a solvent control) for 2 h and then cultured with TAPo macrophages and 10 mM OVAp for 18 h, and analyzed by flow cytometry for CD4, CD8, and 7AAD uptake. The amount of death (A) or dulling (B) that occurred when thymocytes were cultured with TAPo macrophages alone was subtracted out, so that 0% spec. act. includes any nonspecific death and dulling. Z-VAD.fmk effectively blocks nonspecific death, as well as Ag-specific death, and thus generates negative values in graph A. The data are representative of three separate experiments.

Absence of apoptosis under these conditions was confirmed by analysis of mitochondrial PT, as measured by DiOC6 (3) (data not shown). Thus, even in the presence of apoptosis inhibitors, CD4 and CD8 are significantly down-regulated in response to Ag, and therefore dulling is not simply a downstream consequence of cells going through apoptosis.

Only Ag-induced dulling occurs in live cells

CD4 and CD8 down-regulation occurs in thymocytes in response to many distinct apoptotic stimuli (8, 29). To examine dulling in response to other apoptotic stimuli, OT-I, TAPo thymocytes were cultured overnight with either anti-Fas mAb, dexamethasone, TAPo APC + OVAp, or media alone. Each of these stimuli was able to induce both death and dulling in the DP thymocytes (Fig. 5A and data not shown). However, dulling in response to anti-Fas mAb, dexamethasone, or overnight culture was not as pronounced as dulling in response to Ag. Furthermore, exclusive analysis of the remaining live cells revealed that only OVAp could induce dulling in the living cells (Fig. 5B).

To further examine whether there was a difference in Ag-induced dulling and the dulling that occurred in response to the other stimuli, we asked whether the latter also occurred independently of caspases. OT-I TAPo thymocytes were preincubated with Z-VAD.fmk and then cultured with the various stimuli. Z-VAD.fmk blocked apoptosis in response to all stimuli (data not shown). Again, the inhibitor did not block OVAp-induced dulling, but it did block dulling in response to anti-Fas mAb, dexamethasone, and overnight culture (Fig. 5C). Thus, coreceptor dulling can occur in thymocytes as a consequence of apoptosis triggered by a variety of stimuli. However, dulling on viable cells is an exclusive consequence of TCR activation, and this suggests that a distinct biochemical mechanism is involved.

Only OVAp-induced dulling was blocked by CHX

To further distinguish Ag-induced dulling from the nonspecific dulling that occurs as a result of other forms of apoptosis, we tested whether the mechanism in each situation was dependent on new
protein synthesis. OT-I, TAP<sup>+</sup> thymocytes were preincubated with CHX, an inhibitor of new protein synthesis, and then cultured with the various apoptotic stimuli (Table II). CHX stimulates a slight down-regulation of CD4 on both the DP and the CD4 SP subset; therefore, only the CD8 mean fluorescence intensity (MFI) was analyzed. As in the previous experiment, OVAp decreased the CD8 MFI on both the live and dead cells, while Fas only induced dulling on the dead cells. Interestingly, CHX inhibited CD8 down-regulation on the live cells stimulated by OVAp (from 117 to 239 with CHX), but it did not block the dulling seen on the dead cells in response to any of the stimuli. This demonstrates that Ag-induced dulling is distinct from dulling in response to anti-Fas or overnight culture in that it requires new protein synthesis, whereas the latter does not.

Dulling may be transcriptionally regulated

To further investigate the mechanism of dulling, we tested whether CD4 and CD8 down-regulation in response to Ag was due to increased internalization or transcriptional regulation. To accomplish this, we took advantage of transgenic mice that express allelic forms of the CD8<sub>a</sub> and <sub>b</sub> genes under the control of the CD2 promoter (24). If dulling is due to increased CD8 internalization, we would expect to see down-modulation of both the endogenous and transgenic CD8. Conversely, if dulling is transcriptionally regulated, only the endogenous CD8 should be down-regulated. CD8 transgenic mice were bred to OT-I, TAP<sup>+</sup> mice and backcrossed to generate homozygosity at the TAP locus. Thymocytes from OT-I, TAP<sup>+</sup>, CD8 transgenic mice were cultured with TAP<sup>+</sup> fibroblasts, with and without OVAp, and then analyzed for the level of endogenous and transgenic CD8 using allele-specific Abs (Table III). OVAp induced down-regulation of endogenous CD8 from an MFI of 313 to 177. However, the level of the transgenic CD8 under the control of the CD2 promoter did not significantly change (159 versus 158). This suggests that Ag-induced dulling is transcriptionally regulated.

Because CD2 is slightly up-regulated in response to Ag (Fig. 2), it is possible that this up-regulation is compensating for a decrease of the transgenic CD8 expression. Therefore, we directly analyzed

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**Table II. Only OVAp-induced dulling can be blocked by cyclohexamide**

<table>
<thead>
<tr>
<th>CD8 MFI</th>
<th>Live cells</th>
<th>Dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>+ CHX</td>
</tr>
<tr>
<td>Overnight culture</td>
<td>243</td>
<td>249</td>
</tr>
<tr>
<td>TAP&lt;sup&gt;+&lt;/sup&gt; FB + OVA</td>
<td>117</td>
<td>239</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>208</td>
<td>166</td>
</tr>
</tbody>
</table>

* OT-I, TAP<sup>+</sup> thymocytes were pre-incubated with 100 μg/ml cyclohexamide (CHX) for 2 h and then cultured with TAP<sup>+</sup> fibroblasts (FB) + 10 nM OVAp, 20 μg/ml anti-Fas, or in medium alone for 18 h, and analyzed by flow cytometry. The cells were stained with 7AAD to differentiate the live cells from the dead cells, and the mean fluorescence intensity (MFI) of CD8 is given for each subset. The data are representative of three separate experiments.

**Table III. Dulling may be transcriptionally regulated**

<table>
<thead>
<tr>
<th></th>
<th>MFI</th>
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<tbody>
<tr>
<td>TAP&lt;sup&gt;+&lt;/sup&gt; FB</td>
<td>313</td>
</tr>
<tr>
<td>TAP&lt;sup&gt;+&lt;/sup&gt; FB + OVA</td>
<td>177</td>
</tr>
</tbody>
</table>

* OT-I, TAP<sup>+</sup> thymocytes that express a CD8.1 transgene under the control of the CD2 promoter were cultured for 18 h with TAP<sup>+</sup> fibroblasts (FB), in the presence or absence of 10 nM OVAp. The cells were analyzed by flow cytometry with Abs specific for the transgenic CD8.1 and the endogenous CD8.2. The numbers represent the MFIs of Ab staining on the total cells. These data are representative of four separate experiments.
the amount of CD8 mRNA in thymocytes after exposure to OVAp. OT-I, TAP<sup>+</sup> thymocytes were cultured with TAP<sup>+</sup> APC, and either P815, OVAp, or V-OVA. After 18 h, the thymocytes were harvested and analyzed by reverse-transcriptase PCR with primers specific for CD8β or HPRT, as a control.

**Discussion**

The surface level of CD4 and CD8 coreceptors reflects the developmental stage of thymocytes during maturation in the thymus. Before positive and negative selection, thymocytes express both of these coreceptors. During negative selection, thymocytes down-regulate both coreceptors before completing apoptosis. In contrast, positively selected thymocytes complete maturation and permanently down-regulate only one of the coreceptors. However, it has recently been suggested that positively selected thymocytes transit through a stage in which both coreceptors are partially down-regulated before the single coreceptor is ultimately expressed. Therefore, we tested whether CD4 and CD8 down-regulation in response to OVAp, and decreased only slightly in response to V-OVA compared with P815. This confirms that CD8 down-regulation is transcriptionally regulated.

Using a modified in vitro dulling assay, we demonstrated that CD4 and CD8 down-regulation in response to both positive and negative selection ligands. We previously showed that thymocytes that overexpress a CD8 transgene can undergo dulling in response to such ligands (28), but the data presented in this study demonstrate that this phenomenon can occur in cells with normal levels of CD8. It was not noted in the previous work because the assays were performed at a suboptimal APC to thymocyte ratio. Thus, the data in this study are the first demonstration of the use of an in vitro assay that does not rely on CD8 overexpression to detect positive selection ligands. This suggests that such an assay should be applicable for defining relevant self interactions using any TCR transgenic strain. Many groups have used such an in vitro dulling assay to detect negative selection without reporting an effect with cells that bear the positive selection ligand. In most cases, preselection thymocytes were not directly tested with both APC bearing the positive selection ligand, and those without it (6, 8, 9). Thus, the relatively weak dulling that occurs in response to positive selection ligands might have gone unnoticed in the presence of the often significant dulling that occurs in any overnight culture of thymocytes. Moreover, an optimal thymocyte to APC ratio is critical for detecting self MHC peptide interactions (data not shown).

To further distinguish death and dulling, we demonstrated that dulling in response to Ag was not the result of a nonspecific down-regulation of all cell surface molecules that might occur because apoptotic cells have an altered plasma membrane. In fact, the surface expression of certain molecules actually increased in response to Ag, while the level of other molecules remained constant. Additionally, dulling occurred before, or at the same time, as one of the earliest events of apoptosis (mitochondrial membrane PT), and at least 6 h before apoptosis was detected by an altered plasma membrane permeability.

To definitively show that dulling was not simply a consequence of apoptosis, we blocked the death pathway with a caspase inhibitor, and stimulated the thymocytes with Ag. While the caspase inhibitor effectively blocked apoptosis, it did not block dulling, even at the highest concentration. This demonstrates that coreceptor down-regulation in response to Ag is not a consequence of apoptosis.

Dulling occurs in thymocytes undergoing apoptosis in response to a variety of apoptotic stimuli. In contrast to Ag-induced dulling, dulling in response to these other stimuli only occurred on apoptotic cells, and could be blocked with the caspase inhibitor. Thus, only Ag was able to induce dulling in live thymocytes. Based on this, it would seem that dulling in response to Ag is distinct from the nonspecific dulling that occurs as a result of apoptosis. To support this distinction, we showed that only Ag-induced dulling required new protein synthesis. In addition to the in vitro dulling assay, we also demonstrated that CD4 and CD8 down-regulation occurs during positive and negative selection in intact thymic lobes. Together, these data demonstrate that DP dulling is not simply a consequence of apoptosis, and that it occurs in response to positive selection ligands. Along with previous data, this suggests that DP dull cells may be intermediates of positive selection.

It is not clear how a signal through the TCR initiates coreceptor down-regulation. This signal may increase CD8 and CD4 internalization, decrease translation, or decrease transcription of these genes. Our data suggest that Ag-induced coreceptor down-regulation is transcriptionally regulated. Using TCR transgenic thymocytes that expressed a CD8 transgene under the control of the CD2 promoter, we demonstrated that the endogenous CD8 dull in response to OVA, while the transgenic CD8 did not. This suggests that the endogenous CD8 promoter is required for Ag-induced down-regulation. However, it is possible that the modest increase in CD2 expression seen after stimulation (Fig. 2) was due to increased transcription from the CD2 promoter. Such an increase might mask a posttranslational decrease in the transgenic CD8 protein in response to Ag. Thus, we directly assessed the levels of CD8 mRNA in thymocyte cultures and found that the level of CD8 mRNA decreased specifically in response to OVAp (Fig. 6). This is consistent with the work of Merkenschlager et al., which showed that reaggregation of thymocytes with MHC<sup>+</sup> cells caused a decrease in the level of CD8 and CD8 mRNA (17). Our data also suggest that if a posttranslational mechanism for decreasing CD8 exists, such as class I-induced endocytosis, it does not contribute significantly to DP dulling.

These data support a developmental model in which DP thymocytes receive a signal through the TCR, and down-regulate transcription of both CD4 and CD8. In turn, this causes a decrease in the surface levels of the coreceptor proteins, and produces the DP dull phenotype. The signal that induces this may be either a positive or negative selection ligand. Negatively selected thymocytes continue this surface down-regulation until...
apoptosis is complete, and they are engulfed. Positively selected thymocytes, on the other hand, begin up-regulation of the coreceptor that is to be ultimately expressed. This is consistent with a hypothesis in which transcription of the coreceptors during the DP stage utilizes different genetic regulatory elements than at the SP stage. Indeed, recent evidence showed that the cluster III D\textsuperscript{N}ase I-hypersensitive region of the CD8 locus implode parts expression only in the mature CD8\textsuperscript{+} T cells, and not in DP cells (39, 40). Presumably, an element within the CD8 locus (presently unidentified) controls expression at the DP stage. Thus, the CD8 dull phenotype may occur when the cells have begun extinction of CD8 expression via one control element, but expression via the other control element has not yet initiated.

The physiologic relevance of DP dulling may be linked to the fact that during the DP stage, thymocytes are more sensitive to low affinity ligands than mature SP thymocytes (41). This is true despite the higher level of TCR on the latter subset. Therefore, a DP thymocyte that has encountered a low avidity ligand may down-regulate both CD4 and CD8 coreceptors to decrease the overall avidity between the selected thymocyte and the presenting cells. This provides a mechanism for positively selected cells to avoid apoptosis until they have had time to differentiate into less sensitive mature thymocytes. Negative selection ligands, on the other hand, are so strong that CD4 and CD8 down-regulation is not sufficiently to decrease the overall avidity, and the thymocyte is deleted.

This model is supported by the fact that TCR transgenic thymocytes that would normally be positively selected at the DP stage were negatively selected in mice that overexpressed a deleted. This is true despite the higher level of TCR on the latter subset. Therefore, a DP thymocyte that has encountered a low avidity ligand may down-regulate both CD4 and CD8 coreceptors to decrease the overall avidity between the selected thymocyte and the presenting cells. This provides a mechanism for positively selected cells to avoid apoptosis until they have had time to differentiate into less sensitive mature thymocytes. Negative selection ligands, on the other hand, are so strong that CD4 and CD8 down-regulation is not sufficient to decrease the overall avidity, and the thymocyte is deleted.

In conclusion, our experiments demonstrate that DP dulling is not simply a consequence of apoptosis, but that it is a biochemically distinct event that indicates that the thymocyte has received a signal through the TCR. Along with previous data, this advocates that positively selected cells transit through a DP dull stage before completing maturation.

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References
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