Altered functional responsiveness of thymocyte subsets from CD3δ-deficient mice to TCR–CD3 engagement

Vibhuti P. Dave, Robert Keefe, Marc A. Berger, Karel Drbal, Jennifer A. Punt, David L. Wiest, Balbino Alarcon and Dietmar J. Kappes

Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA

1Institute of Molecular Genetics, Academy of Sciences of Czech Republic, Vinenska 1083, 142 20 Prague 4, Czech Republic

2Haverford College, 370 Lancaster Avenue, Haverford, PA 19041, USA

3Centro de Biologia Molecular, CSIC-Universidad Autonoma de Madrid, Cantoblanco, Madrid 28049, Spain

Keywords: CD3δ, thymocyte

Abstract
CD3δ-deficient (δ°) mice are defective in αβ T cell development. Here we explore the capacity of TCR–CD3 signaling complexes expressed on δ° thymocytes to mediate the following functional outcomes in response to antibody cross-linking: (i) the transition from the CD4–CD8– to CD4+FCD8F stage, (ii) the transition from the CD4+FCD8– to CD4+CD8+ or CD4+CD8δ– stages and (iii) the induction of apoptosis. We provide evidence that CD3δε complexes are dispensable for mediating the anti-CD3-mediated CD4–CD8– to CD4+FCD8F transition. On the other hand, CD3δ is critical at the CD4+FCD8F stage. We demonstrate that CD4+FCD8F thymocytes from δ° mice, unlike δ° CD4–CD8– thymocytes and wild-type CD4+FCD8F thymocytes, require prolonged or consecutive stimuli to elicit functional responses. Depending on the nature of the secondary stimulus, δ° thymocytes can be induced to undergo apoptosis or preferential maturation to the CD4+CD8δ– stage. Taken together these results indicate that the signaling capacity of the TCR–CD3 complex is noticeably altered in the absence of CD3δ. The essential role of CD3δ at the CD4+CD8δ– stage of development correlates with the onset of TCRα rearrangement, consistent with a critical structural and/or functional relationship between CD3δ and TCRα.

Introduction
Development of αβ T cells is characterized by three stages defined by expression of the CD4 and CD8 co-receptor molecules. These are in order of increasing maturity, CD4–CD8– [double negative (DN)], CD4+CD8– [double positive (DP)] and CD4+CD8+ or CD4+CD8δ– [single positive (SP)]. The transitions between these stages represent critical functional checkpoints and are dependent on expression of the TCR–CD3 complex. The DN to DP transition is a checkpoint for productive TCRβ rearrangement and is mediated by the pre-TCR complex (1). Pre-TCR comprises TCRβ in association with the invariant surrogate p5δ chain together with all of the CD3 components, it specifically lacks TCRα (2–4). The second transition from DP to SP is a checkpoint for expression of useful TCR specificities and is mediated by complete αβTCR complex. Two opposing processes shape this transition, positive and negative selection. Positive selection leads to rescue from programmed cell death and differentiation to the SP stage, while negative selection leads to suicide by apoptosis.

The molecular basis for these differential outcomes (i.e. positive versus negative selection) following TCR engagement has long been the subject of intense interest. There is mounting evidence for a signal strength model, whereby the outcome is determined primarily by the affinity of the ligand for TCR or by the overall avidity of interaction between a T cell and an antigen-presenting cell. Thus during thymic selection, thymocytes which interact with intrathymic ligands with high affinity or avidity will undergo negative selection, while those that bind more weakly will undergo positive selection. Strong or weak interaction is presumed to translate into strong or weak signal transduction.

Correspondence to: D. J. Kappes
Transmitting editor: C. Martinez-A

Received 4 May 1998, accepted 18 June 1998
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Fig. 1. Induction of DN to DP thymocyte differentiation in RAG°δ° mice by anti-CD3δε/γε. RAG° or RAG°δ° mice were injected with 2C11 or PBS and thymuses isolated after 1–7 days, as indicated. Thymocytes were stained and analyzed by FACS for expression of CD4 and CD8.

The CD3 complex plays a key role in transmitting signals to the interior of the cell following TCR engagement. Each TCR heterodimer is linked to four different CD3 components, CD3ε, γ, δ and ζ. While there are two copies of CD3ζ and ε per complex, the CD3δ and γ components appear to be represented only once and occupy asymmetric positions in the complex, pairing preferentially with TCRα and β respectively (5–8). All of the CD3 components possess extensive intracellular domains that contain one or more copies of a conserved motif responsible for their signaling function, the immunoreceptor tyrosine-based activation motif (ITAM). ITAM are sufficient for mediating signal transduction when expressed as part of single-chain chimeric cell surface receptors (9–12). Engagement of the TCR leads to phosphorylation of the ITAM tyrosine residues by associated kinases (13,14), which in turn leads to recruitment of additional signaling factors via their SH2 domains. ITAM show significant sequence diversity, which is presumably responsible for their differential affinities for intracellular signaling factors (15–18). Differential affinity for intracellular factors could translate into differential signaling. In agreement, stimulation of CD3ε or ζ results in qualitatively distinct patterns of tyrosine phosphorylation, suggesting activation of different pathways (11).

We have generated a mouse model by gene targeting in which expression of the CD3δ component is eliminated, δ° mice. Our initial analysis of the phenotype of δ° mice strongly suggests that CD3δ has a unique and specific function during development. In particular, we found that development of the αβ T cell lineage is almost completely blocked, while that of the alternate γδ T cell lineage is normal (19). This phenotype differs strikingly from knockouts of other CD3 components that have been described, i.e. CD3ε° and ζ° mice, which exhibit blocks in development of both lineages (20–22). In the present study we explore the capacity of δ° thymocytes at different developmental stages to respond to engagement of surface TCR–CD3 complexes. Our data demonstrate the following. (i) DN thymocytes from δ° mice show normal responses to antibody engagement, indicating that CD3δ is dispensable at this stage. (ii) In contrast DP thymocytes from δ° mice are comparatively refractory to TCR-mediated stimuli, requiring prolonged or serial stimuli to achieve apoptosis or further maturation. The specific sensitivity of DP thymocytes to CD3δ deficiency may reflect a higher signaling threshold than for DN thymocytes or impairment of a distinct signaling pathway not required at the DN stage.

Methods

Mice

CD3δ-deficient (δ°) mice and transgenic mice expressing the human CD3δ homologue (hCD3δ° δ° mice) have been described previously (19). RAG2-deficient (RAG°) mice were provided by F. Alt (23) and backcrossed to δ° or δ°hCD3δ° mice to produce doubly deficient RAG°δ° and RAG°δ°hCD3δ° mice. Transmission of the hCD3δ transgene was determined by Southern blot analysis using a specific full-length cDNA probe (obtained by PCR amplification from total thymocyte cDNA of
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Table 1. Numbers of total and DN thymocytes at d7 after anti-CD3 treatment

<table>
<thead>
<tr>
<th></th>
<th>Total thymocytes (x10⁶)</th>
<th>DN thymocytes (x10⁶)</th>
<th>% DN thymocytes</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>RAG⁺</td>
<td>Injected</td>
<td>148 ± 18</td>
<td>13.2 ± 1.4</td>
<td>9 ± 1.5</td>
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<tr>
<td></td>
<td>Uninjected</td>
<td>3.1 ± 1.1</td>
<td>3.1 ± 1.1</td>
<td>100</td>
</tr>
<tr>
<td>RAG⁺ δ⁺</td>
<td>Injected</td>
<td>134 ± 24</td>
<td>4.4 ± 0.9</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Uninjected</td>
<td>2.8 ± 1.3</td>
<td>2.8 ± 1.3</td>
<td>100</td>
</tr>
</tbody>
</table>

δ⁺hCD3δ⁺ mice and sequenced to confirm its identity). δ⁺ mice in the H-2d background were generated by backcrossing our standard δ⁺ line which is on a pure C57Bl/6, H-2b, background for six generations onto BALB/c. Transgenic mice expressing the PCC-specific AND TCR were purchased from Jackson (Bar Harbor, ME) and backcrossed onto δ⁺ lines of either the H-2b or H-2d background.

Antibody injections

Unless otherwise indicated, 8- to 12-week-old mice of the indicated genotype were injected i.p. with 150 µg of antibody (150 µg of each in the case of co-injections) diluted in PBS to a final volume of 500 µl. For assays involving induction of the DN to DP developmental transition, animals were sacrificed at day 7 following antibody injection, except for the time course depicted in Fig. 1. For assays involving induction of apoptosis or maturation of DP thymocytes, animals were sacrificed 72 or 96 h after the initial antibody injection. Annexin V and cell cycle analysis were carried out 20 h following injection. Thymocyte numbers were determined using a hemacytometer. Antibodies used for injection were purified from ascites (2C11, 7D6, MEM-57 and OKT3) or purchased from PharMingen (San Diego, CA) (H57 and anti-CD4).

In vitro antibody stimulations

In vitro stimulations of thymocyte suspensions were carried out essentially as described (24). Briefly, 24-well tissue culture plates were coated overnight at 4°C with the appropriate antibody(ies), each at a concentration of 50 µg/ml. CD4⁺CD8⁺ thymocytes were purified by panning on CD8-coated plates. Purified thymocytes were suspended in RPMI supplemented with 10% FCS and a total of 2.5x10⁶ cells were plated per well in a final volume of 500 µl. Cultures were analyzed 19 h later by staining with 1 mg/ml EtBr and FITC-conjugated anti-CD5.

Assays of apoptosis

For cell cycle analysis, 5x10⁵ cells from untreated or 2C11-injected mice were resuspended in 500 µl of 100 µg/ml propidium iodide (Sigma, St Louis, MO), 0.05% NP-40, 10 µg/ml RNase A in PBS. Cells with a lower DNA content than those in G₁ were considered apoptotic. For analysis of cell death by annexin V, 5x10⁵ thymocytes from untreated or 2C11-injected mice were washed with annexin-staining buffer (140 mM NaCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.5) and incubated for a further 20 min with annexin V–FITC at the concentration suggested by the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). In both cases analysis was by flow cytometry.

Results

CD3δ cannot mediate the DN to DP thymocyte transition

Immature thymocytes express partial clonotype-independent CD3 (CIC) complexes at the cell surface consisting of unlinked CD3δ and γε heterodimers, which are signaling-competent (25,26). Indeed, antibody engagement of CIC expressed on RAG⁺ DN thymocytes (arrested in development due to the
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The absence of TCRβ protein is able to restore their development to the DP stage (26). To test whether CIC complexes lacking CD3δ were competent to signal the DN to DP transition, we treated δ°RAG° double knockouts with anti-CD3 mAb. As expected, δ°RAG° animals are also arrested in development at the DN stage (Fig. 1). Injection of the anti-CD3ε/γ antibody, 2C11, restored development to the DP stage in δ°RAG° mice in 7 days, just as in RAG° animals, giving rise to equivalent numbers of DP thymocytes (Fig. 1); however, the number and proportion of thymocytes belonging to the DN subset after antibody stimulation was consistently 3-fold lower in δ°RAG° than in RAG° mice (Fig. 1 and Table 1). This experiment has been repeated several times with the same result, even when the mAb 7D6 is used to selectively engage only CD3ε complexes on RAG° thymocytes (27) (Fig. 2). The difference in DN cells reflects an increase in the number of DN thymocytes in injected RAG° mice relative to both un.injected RAG° and injected RAG° mice. To test the relative capacities of CD3ε and δε heterodimers to mediate the DN to DP transition, we introduced a human CD3δ (δ°) transgene onto the RAG° background. We have previously shown that the anti-human CD3 antibodies MEM-57 and OKT3 (28,29), which are unreactive with normal mouse T cells, specifically recognize CD3 complexes from δ° mice reconstituted with human CD3δ, indicating that they recognize a new conformational epitope formed by the association of human CD3δ and mouse CD3ε (19). To specifically engage either CD3ε or δε subcomplexes on δ° RAG°δ° mice, we treated them with either 7D6 or MEM-57 respectively. As expected, injection of 7D6 mediated complete restoration of the DP compartment (data not shown). However, administration of MEM-57 (or OKT3) was ineffective, suggesting that immature DN thymocytes are refractory to signaling through CD3ε δε heterodimers (Fig. 2 and data not shown).

Fig. 3. In vivo and in vitro stimulation of δ° DP thymocytes. (a) δ° (−/−) or wild-type (+/+) animals were injected with PBS, 2C11 or dexamethasone as indicated. Thymocytes were isolated 20 h later and subjected to cell cycle analysis by propidium iodide (PI) staining. The percentages of cells in the G1, G2/M and S phases of the cell cycle were measured in segments C, I and D respectively. The percentages of cells with a DNA content lower than those in G1 (sub-G1), and therefore in apoptosis, were recorded in L. (b) Mice of the indicated genotypes were injected with different doses of 2C11 or dexamethasone. After 20 h the percentages of thymocytes in apoptosis were measured by cell cycle analysis or by annexin V staining. (C) DP thymocytes were purified from δ° or wild-type animals and cultured for 20 h in the presence of plate-bound antibodies, as indicated. Cells were stained with EtBr and analyzed by flow cytometry.
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Multiple but not single stimulations with 2C11 can induce apoptosis of CD3δ-deficient DP thymocytes. Animals of the indicated genotype were injected with 2C11 at the indicated times and thymuses isolated 72 h after the initial injection. (a) CD4:CD8 expression profiles of samples indicated. Diagrams at the top of the panel indicate timing of antibody treatment (triangles represent presumed decrease of circulating antibody levels over time). Arrows indicate time of analysis. (b) Absolute number of DP thymocytes present in δ° mice from panel (a).

In a control experiment, injection of MEM-57 or OKT3 into δ°RAG° δ° mice, which show normal thymocyte composition, was able to induce efficient apoptosis of DP thymocytes, confirming the antibodies' reactivity and localization to the thymus (Fig. 2 and data not shown).

**Induction of antibody-mediated apoptosis of δ° DP thymocytes requires prolonged stimulation**

As we have previously described, δ° mice show normal numbers of DP thymocytes, all of the TCRlow phenotype. These cells are refractory to negative selection based on their failure to undergo deletion in HY TCR transgenic male mice expressing self antigen (19). To further test the apoptotic response of δ° DP thymocytes, δ° mice were injected i.p. with the anti-CD3γε/δε antibody 2C11 and the proportion of apoptotic thymocytes was assayed at 20 h by both propidium iodide and annexin V staining. While a single injection of anti-CD3 led to severe thymic atrophy in wild-type mice by 72 h (30), similar treatment of δ° mice with anti-CD3 had no appreciable effect (Fig. 3a and b). Likewise, treatment of δ° mice with doses of anti-CD3 even 8-fold greater than the minimum effective dose for wild-type mice did not increase the fraction of apoptotic cells (Fig. 3a and b).

Since the rapid clearing of injected antibody in vivo is likely to produce only transient TCR engagement (30), we wondered whether prolonging the stimulus could restore the apoptotic response of δ° thymocytes. To test this hypothesis, we treated δ° DP thymocytes in vitro with plate-bound antibodies which are expected to persist for much longer than in vivo. Using this combination of antibodies, we found that prolonged in vitro engagement elicited quite comparable death responses in δ° and wild-type thymocytes (Fig. 3c).

To test whether prolonging antibody engagement could also restore apoptosis in vivo, we injected mice with either two or three 150 µg doses of anti-CD3 at 24 h intervals. Indeed, these treatments resulted in dramatic (>90%) disappearance of DP thymocytes by 72 h following initial injection (Fig. 4a and b). Multiple injections of PBS alone elicited no response, demonstrating that the effect is not stress related.
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(data not shown). Furthermore, administration of multiple anti-CD3 injections into RAG° mice, whose DP compartment was reconstituted by an earlier anti-CD3 injection, failed to induce any disappearance of DP thymocytes, indicating that deletion of DP thymocytes requires rearrangement and presumably surface expression of clonotypic TCR chains (Fig. 4a). Since δ° mice lack mature SP thymocytes and peripheral T cells, we also examined the response of MHC-deficient mice which similarly lack mature T cells. MHC-deficient DP thymocytes underwent efficient apoptosis in response to a single antibody injection, demonstrating that the specific defect in δ° mice cannot be explained by the absence of SP thymocytes or mature peripheral T cells (Fig. 4a). Finally, we note that the effectiveness of sequential injections does not result from a higher final concentration of circulating antibodies, since a single dose of antibody equivalent to the sum of three sequential stimuli is ineffective at inducing apoptosis (Fig. 3a and b).

Alternate secondary stimuli induce further maturation of δ° DP thymocytes

It has been shown that treatment with anti-TCRβ antibody can induce a large proportion of thymocytes in fetal thymic organ culture to differentiate to the SP CD4+ stage (31–33). The capacity of anti-TCRβ to promote maturation rather than apoptosis is apparently due to the ability of this antibody to induce only limited, i.e. bivalent, cross-linking of surface TCR (32). The generation of SP thymocytes is considerably enhanced by the addition of anti-CD4 (31,33). Consequently, we asked whether further maturation could be induced in δ° thymocytes using this antibody combination. Initial experiments showed that in vivo treatment with anti-TCRβ, alone or in combination with anti-CD4, had no effect on the development of δ° thymocytes (Fig. 5, far right panels and data not shown). However, similar treatments of mice ‘primed’ 24 h earlier with anti-CD3 induced marked phenotypic changes. Up-regulation of the activation marker CD5 was seen on virtually all thymocytes within 24 h of the second antibody injection (Fig. 5a and data not shown). Simultaneously CD69 became up-modulated on a substantial fraction of cells. Most significantly we noted the appearance of more mature thymic subsets defined by their co-receptor expression patterns. In particular, secondary injection with anti-TCRβ alone induced the appearance of a distinct population of CD4+CD8dull cells, which resemble transitional cells in the normal DP to SP

![Diagram](image-url)
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Moreover, secondary injection with anti-TCRβ in combination with anti-CD4 resulted in the appearance of substantial numbers of SP CD8+, but not SP CD4+, thymocytes. The kinetics of co-receptor down-modulation are comparable to those seen in antibody-treated cultures of normal DP thymocytes (38). These SP CD8+ thymocytes are relatively long-lived, i.e. persist for 4 days (data not shown), and are clearly generated de novo as they exceed the number of SP CD8+ cells found in anti-CD3 primed δ° mice by 30- to 50-fold (Fig. 5b).

Superantigen-mediated deletion is normal

While the DP to SP transition is severely impeded in δ° mice, a small number of thymocytes, perhaps those expressing TCR with particularly high affinity for selecting ligands, are positively selected and emigrate to the periphery (reaching steady-state numbers of about 10% of normal) (19). This allows us to examine whether negative selection by superantigens leads to the absence of peripheral SP CD4+ T cells bearing particular reactive Vβ segments. We have tested this in two ways. In the first place, we have backcrossed δ° mice onto both H-2b (C57Bl/6) and H-2d (BALB/c) backgrounds, and assayed deletion of T cells expressing the endogenous Vβ11 product. Since the relevant deleting superantigen, Mtv9, is present in both strains, but requires I-E to induce deletion (39,40), Vβ11+ T cells should be present in C57Bl/6 but not in BALB/c mice. This is in fact the case (Fig. 6). In a second test, we have examined deletion of Vβ3+ expressing cells in AND TCR transgenic mice (41). It has previously been demonstrated that in normal, CD3δ+, mice the H-2d background is positively selecting for AND, while the H-2d background is neutral, i.e. neither positively nor negatively selecting (in the absence of relevant superantigen) (42). In δ° mice the H-2d background can no longer support positive selection of AND (19), so that both H-2b and H-2d backgrounds are neutral. Escape of AND+ δ° thymocytes from developmental arrest in either background requires expression of novel TCR specificities. These will mostly utilize endogenous TCRα rather than β chains, since allelic exclusion is much more efficient for the TCRβ locus. Hence most CD4+ T cells that escape to the periphery in the C57Bl/6 (H-2b) background, which lacks relevant superantigen, still express the transgenic Vβ3 chain (Fig. 6). In contrast, in the BALB/c (H-2d) background, which bears the relevant Mtv6-encoded superantigen (39,40), these Vβ3+ CD4+ T cells are largely absent (Fig. 6), indicative of efficient deletion by superantigen. Further consistent with superantigen deletion, the normal peripheral CD4:CD8 ratio is reversed in H-2d AND+ δ° mice (data not shown), reflecting the fact that superantigen deletion affects CD4+ T cells more severely than CD8+ T cells. We conclude that superantigen-mediated negative selection is unperturbed in δ° mice.

Discussion

The present study explores the consequences of CD3δ deficiency on TCR function during thymocyte development. In particular, we demonstrate the following. (i) CD3δ is dispensable for the DN to DP transition. Indeed, specific engagement of CD3δε subcomplexes fails to induce this transition. (ii) The present study explores the consequences of CD3δ deficiency on TCR function during thymocyte development. In particular, we demonstrate the following. (i) CD3δ is dispensable for the DN to DP transition. Indeed, specific engagement of CD3δε subcomplexes fails to induce this transition. (ii) CD3δ plays a critical role at the DP stage. DP thymocytes...
from δ° mice are refractory to TCR-mediated stimulation and require prolonged or consecutive stimuli to overcome this defect. The altered responsiveness of CD3δ-deficient thymocytes is consistent with the hypothesis that individual CD3 components perform essential functions at distinct developmental stages.

It has been previously demonstrated that the DN to DP developmental transition can be restored in RAG° mice by treatment with anti-CD3 (26,43), and that the receptor responsible for mediating this transition consists of CD3γε and δε heterodimers, which are expressed at the cell surface at low levels in the absence of clonotypic TCR α and β chains (25). CD3ζ is not a functionally required component of this complex, since doubly deficient RAG°CD3ζ° mice can be similarly stimulated by anti-CD3 to undergo this transition (44). Other experiments showing that the cytoplasmic domains of both CD3ζ and ε can independently mediate this transition, when expressed as part of chimeric Tac receptors, are less physiologically meaningful, since vast overexpression of the chimeric products is required (45). In the present study we examine the differential capacity of CD3δε and γε heterodimers to induce DN to DP development. We demonstrate that CD3γε heterodimers are sufficient to induce this antibody-mediated transition in RAG°δε mice. Interestingly, while the number of DP thymocytes induced by anti-CD3 treatment is the same in RAG°CD3δε° and RAG°CD3ζ° mice, the number of DN thymocytes is consistently 3-fold higher in RAG°CD3δε° mice. These additional cells may represent DN thymocytes that are refractory to CD3-mediated signaling or that have undergone an alternate developmental transition which does not result in up-regulation of CD4 and CD8, e.g. commitment to the γδ T cell lineage. In keeping with the latter possibility, we find that RAG°CD3δε° mice reconstituted with γδTCR transgenes contain a larger number of DN thymocytes and fewer DP thymocytes than similarly reconstituted RAG°CD3ζ° mice, suggesting that maturation to the γδ lineage is more efficient in the presence than in the absence of CD3ζ (V. P. Dave and D. J. Kappes, unpublished data). CD3δε complexes might influence the DN to DP transition either directly by mediating a distinct signal or indirectly by diluting out the functionally critical signal provided by CD3γε complexes (perhaps by competing with CD3γε heterodimers for expression at the cell surface). The fact that treatment with antibodies that recognize CD38ε° complexes fails to induce the DN to DP transition in RAG°δε° mice reconstituted with human CD38 suggests that CD3δε complexes are inefficient at mediating this transition, in agreement with recent data showing that the DN to DP transition is severely impeded in CD3ε-deficient mice (46). Insufficient affinity of anti-hCD3 antibodies to induce adequate signaling through mixed-species CD3δε° heterodimers complexes could, however, also explain our results, although this seems unlikely given that these antibodies mediate efficient apoptosis of DP thymocytes bearing human CD3δ.

We have tested the ability of DP thymocytes from δ° mice to undergo antibody-mediated apoptosis in vivo and in vitro, as a model of negative selection. Our in vivo data indicate that δ° thymocytes are refractory to a single anti-CD3 stimulus that is sufficient to induce efficient apoptosis of normal DP thymocytes. It has been suggested that in vivo anti-CD3 induced apoptosis of DP thymocytes is partly dependent on cytokines and inflammatory factors released by mature T cells (47). The efficient induction of apoptosis by anti-CD3 in TCRα and MHC-deficient mice, as well as in mice expressing TCR transgenes on a non-selecting background, all of which lack SP thymocytes and peripheral T cells, demonstrates, however, that mature T cells are not required for anti-CD3-mediated apoptosis in vivo (48,49; see Fig. 4). Hence, impaired apoptosis in δ° mice directly reflects altered signaling in DP thymocytes resulting from the absence of CD3δ and is unrelated to the absence of peripheral T cells in these mice. We demonstrate further that the apoptotic response of δ° thymocytes is restored by successive treatments with anti-CD3. Consistent with a requirement for prolonged or consecutive stimulation, δ° thymocytes also undergo efficient apoptosis following persistent stimulation with anti-TCR–CD28 in vitro. The requirement for a 'priming' signal to confer susceptibility to subsequent selection signals might apply in normal mice as well. The purpose of such a checkpoint could be to screen for successful TCRα rearrangement just as the earlier pre-TCR-mediated checkpoint tests for TCRβ rearrangement. The defect in negative selection in δ° mice, which we have previously noted (19), may reflect failure to pass through this checkpoint, rather than a specific requirement for CD3δ in negative selection. This is consistent with the fact that superantigen-mediated deletion is intact in δ° mice.

We further show that different combinations of stimuli can induce changes in δ° DP thymocytes characteristic of positive selection. The requirement for multiple stimuli is consistent with previous reports that complete maturation of DP thymocytes requires continual TCR engagement (50,51). We find that treatment of anti-CD3 primed δ° DP thymocytes with either anti-TCRβ alone or in combination with anti-CD4 induces rapid up-regulation of CD5 and CD69, both characteristic early features of positive selection (33,52). Treatment with anti-TCRβ alone also leads to the generation of a distinct transitional CD4°CD8° population, further consistent with partial positive selection (34–37). Moreover, treatment of δ° thymocytes with a combination of anti-TCRβ and -CD4 anti-CD3 would be expected to induce up-regulation of CD5 and CD69 and to restore the response to anti-CD3.
antibodies gives rise to a substantial population of SP thymocytes. Strikingly, all of these SP thymocytes belong to the CD8 lineage. In contrast, treatment of normal developing thymocytes in fetal thymic organ culture with anti-TCRβ and -CD4 leads to the preferential generation of SP CD8+ thymocytes, resembling the outcome of normal thymic development (31,33). Interestingly, normal TCRγδ thymocytes that have received a positive selection signal, but have not completed maturation, give rise only to SP CD8+ thymocytes when cultured in vitro, but to both SP CD4+ and SP CD8+ subsets upon intrathymic transfer (51). This suggests that the generation of SP CD4+ cells requires additional stimuli provided by thymic antigen-presenting cells. We postulate that antibody-stimulated δ+ thymocytes similarly receive an incomplete signal, in this case due to the altered structure of the TCR signaling complex. The absence of CD3δ may preferentially impair some TCR-mediated signaling pathways but not others leading to imbalances in functional outcome. The protein kinase C pathway, whose activation by phorbol myristate acetate causes rapid down-modulation of CD4 on DP thymocytes (53), might, for instance, be relatively unaffected. In agreement with the data presented here, we have recently found that expression of a truncated CD3δ transgene, lacking a cytoplasmic domain, results in skewed restoration of positive selection in δ+ mice favoring generation of SP CD8+ T cells (V. P. Dave and D. J. Kappes, unpublished data).

In summary, we have shown that the absence of CD3δ alters the functional outcome of signaling through the TCR-CD3 complex at both the DN and DP stages of thymic development. Although CD3δ is already a structural component of the pre-TCR complex (4), our data indicate that it becomes functionally competent only in the context of the complete αβTCR complex at the DP stage. In the absence of CD3δ, DP thymocytes require multiple stimuli to induce either negative or positive selection. Further, antigens inducing positive selection is markedly skewed towards the CD8 lineage. A logical basis for the DP stage-specific functional role of CD3δ is provided by its reported preferential pairing with TCRα (5–8), which is first rearranged and expressed at the DN stage of thymic development. Although CD3δ together with CD8 forms a complex, in this case due to the altered structure of the TCR signal, in this case due to the altered structure of the TCR complex at the DP stage. In the absence of CD3δ, αβTCR complexes can mediate pre-TCR development in vivo. (Eur. J. Immunol. 24:3534).

We thank R. Hardy for critical reading of the manuscript. This work was supported by National Institutes of Health grants CA74620 and AI34472, institutional grant CA06927 from the National Institutes of Health, and also by an appropriation from the Commonwealth of Pennsylvania.

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Abbreviations

CIC clonotype-independent CD3
δ+ CD3δ deficient
DN double negative
DP double positive
SP single positive

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