

Regulation of Thymocyte Development through CD3.

I. Timepoint of Ligation of CD3 ϵ Determines Clonal Deletion or Induction of Developmental Program

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Summary

Several recent observations suggest that successful rearrangement of the T cell receptor (TCR) β locus induces several important events in thymocyte maturation. Allelic exclusion is achieved by interruption of further rearrangement of the β locus, and CD4⁻8⁻ interleukin (IL)-2R⁺ cells enter the CD4⁺8⁺IL-2R⁻ stage. The actual molecular events regulating this important control point are unknown, but may be related to the expression of the TCR- β locus in immature CD4⁻8⁻ thymocytes. It is not clear whether maturation is induced by intracellular appearance of TCR- β chain or by signal transduction through an immature TCR complex on the thymocyte membrane, possibly involving TCR- β chain homodimers and CD3. Here we show that early addition of anti-CD3 mAb to fetal thymic organ cultures induces all known events associated with the acquisition of the CD4⁺8⁺ stage. Expression of CD4 and CD8 is accelerated, IL-2R α is downregulated, and the cells fail to produce TCR- β , possibly based on premature cessation of β gene rearrangement. Upon stimulation with anti-CD3 antibodies, we see calcium mobilization in 15% of all CD4⁻8⁻ thymocytes with no detectable surface TCR expression. These results suggest that functional CD3 is expressed on immature thymocytes at very low concentrations before the appearance of a complete TCR- β chain. Ligation of CD3 at this stage may mimic the maturation signal normally generated by the immature TCR- β homodimer-CD3 complex. The results are consistent with the notion that acquisition of the CD4⁺8⁺ stage involves signal transduction through an immature TCR complex. Later in thymocyte development, ligation of CD3 results in deletion of CD4⁺8⁺ cells. Thus, signal transduction through CD3 may result in entirely different cellular responses, depending on the stage of thymocyte differentiation. These results suggest an involvement of CD3 as a link in signal transduction for at least two different decision points in the development of a thymocyte.

The T lymphocyte lineage is generated from precommitted bone marrow-derived precursor cells that home to the thymus where they enter into a complex developmental program of sequential gene expression and TCR gene rearrangements. Early events include expression of the heat-stable antigen (HSA)¹ (1), followed by the α chain of the IL-2 receptor (IL-2R α) (2, 3). TCR- β chain rearrangement also takes place at this early stage of thymocyte development. Completion of the rearrangement of the β locus is indicated by the appearance of full-length β mRNA and is accompanied by loss of IL-2R α and expression of small amounts of CD4 and CD8 (4, 5). Strong evidence exists that rearrangement of the β locus is critical for the transition to the stage characterized by expression of CD4 and CD8 and loss of IL-2R α

(6–8). This transition does not take place unless in the presence of an intact thymic environment (9). The actual differentiation signals required for further maturation at this important control point in thymocyte development are unknown.

The CD4^{lo}8^{lo}IL-2R⁻ thymocytes will then mature into the CD4⁺8⁺ double-positive (DP) stage (10). The TCR- α genes complete their rearrangement at this stage, the $\alpha\beta$ CD3-TCR is expressed on the cell surface and selection events may now occur. Those thymocytes possessing a TCR that is able to interact with self-MHC are rescued from cell death, a process called positive selection. Negative selection involves deletion of self-reacting thymocytes by means of apoptosis (11–13). The small percentage of cells that survive positive and negative selection increase their TCR expression, lose HSA, and develop into mature CD4⁺ single-positive or CD8⁺ single-positive T cells.

In vivo and in vitro, negative selection of DP thymocytes can be induced by antibodies directed against the TCR. For

¹ Abbreviations used in this paper: DN, double-negative, DP, double-positive, FCM, flow cytometry, FTOC, fetal thymic organ culture, HSA, heat-stable antigen, IC, intracellular, S, surface

example, addition of anti-CD3 or anti-TCR- β chain antibodies to fetal thymic organ cultures (FTOC) can cause deletion of DP TCR⁺ cells by apoptosis (14). However, we and others found that deletion of DP cells by anti-CD3 mAb treatment of FTOC is dependent on the time point of addition (15). In this study, we show that deletion takes place upon delayed addition, but not upon immediate addition of anti-CD3 mAb to day 14 FTOC. In contrast, early addition of anti-CD3 mAb induces accelerated transition of CD4⁻8⁻IL-2R⁺ cells to the CD4⁺8⁺IL-2R⁻ stage, and blocks TCR- β chain production. These data suggest that signaling through CD3, probably complexed with TCR- β chain, regulates transition of CD4⁻8⁻IL-2R⁺ cells to the DP stage possibly associated with cessation of TCR- β chain gene rearrangement. In addition, our data suggest that during development CD3 ϵ can be expressed on the cell surface of thymocytes in the absence of the TCR- β chain.

Materials and Methods

Mice. Newborn mice and 14-d pregnant female BALB/c and C.B.-17 SCID mice were obtained from our own specific pathogen-free breeding facility.

mAb. Anti-TCR- β chain antibody H57-597 (16), anti-TCR- δ chain antibody GL-3 (17), and anti-CD3 ϵ antibody 145-2C11 (18), all hamster IgG; and anti-IL-2R α antibody 5A2 (19) and anti-PGP-1 antibody IM7 (20), both rat IgG, were isolated from culture supernatants by affinity chromatography over protein A columns (Pharmacia, Freiburg, FRG). Purified anti-CD3 ϵ antibody 500A2 was purchased from Pharmingen (San Diego, CA). Flow cytometry employed labeled anti-Lyt-2 (53-6-7), anti-L3T4 (RM-4-5), anti-TCR- β (H57-597), anti-CD3 ϵ (145-2C11), anti-TCR- δ (GL-3) (all purchased from Pharmingen), and fluoresceinated anti-IL-2R α antibody 5A2, using a FACScan[®] flow cytometer (Becton Dickinson & Co., Heidelberg, FRG).

FTOC. Fetal thymic lobes were prepared from BALB/c mice at day 14 of gestation. They were cultured for various time periods on filter discs floating on 1 ml of IMDM supplemented with 10% selected FCS, 1% glutamine, and 1% kanamycin in 12-well dishes (Costar Corp., Cambridge, MA) at 37°C in 7% CO₂/air, similar to the method of Jenkinson et al. (21), as previously described (22). When lobes were cultured with antibodies, the lobes were suspended for 2 h in medium supplemented with anti-CD3 mAb (145-2C11 if not otherwise stated) or anti-TCR- β mAb, and thereafter placed on filter discs floating on the same medium until analysis. Exposure to antibodies was started either at the beginning of culture or after various time intervals, as indicated with each experiment. If not otherwise stated, cultures were terminated on day 9 for analysis. Addition of irrelevant hamster IgG to FTOC at 100 μ g/ml did not show any effect on α/β T cell development compared to FTOC grown in medium without antibodies. Therefore, control lobes were treated in the same way using medium without antibody. Single cell suspensions were prepared and incubated for 6–8 h at 37°C to allow shedding of bound antibodies (23).

Flow Cytometry. Two- and three-color stainings were performed using FITC-, PE-, and biotin-labeled antibodies. As a third color, Tricolor-conjugated Streptavidin (Medac, Hamburg, FRG) was used. Intracellular stainings were performed as described (24), modified to allow two-color combined intracellular (IC) and surface (S) stainings. First, thymocytes were incubated with FITC-labeled antibodies as indicated above. After washing, unlabeled antibodies of the same kind as used for staining the IC antigen were

added to block binding of the labeled antibodies to structures expressed on the cell surface. After incubation and washing, 75 μ l of 0.5% saponin (Sigma Immunochemicals, Deisenhofen, FRG) in PBS/FCS was added together with the PE- or biotin-labeled second antibody. All the following washing steps were performed in 0.1% saponin in PBS/FCS. PE-conjugated Streptavidin was added together with 75 μ l of 0.5% saponin in PBS/FCS to detect biotin-labeled antibodies. Cell suspensions were washed three times before analysis. Efficiency of blocking by the unlabeled antibody was tested by permeabilizing the cell membrane before adding the blocking antibody followed by the labeled antibodies. Because treatment with saponin made distinction of viable cells from dead cells on the basis of cell size or propidium iodide uptake impossible, Tricolor-conjugated Streptavidin was used as a dead cell marker. We found that Tricolor-conjugated Streptavidin specifically penetrates into dead cells and remains in these cells irreversibly through many subsequent washing steps (Levelt, C. N., and K. Eichmann, manuscript in preparation).

Northern Blot Analysis. FTOC were cultured for 9 d in the absence or presence of 100 μ g/ml anti-CD3 mAb. Cells were isolated and total RNA was prepared as described (25), except that the protease digestion step was omitted. Samples were denatured in the presence of ethidium bromide. RNA gels were prepared as described (26). After electrophoresis, gels were photographed and RNA was transferred to membranes (Nylon Hybond, Amersham International, Braunschweig, FRG) and hybridized to ³²P-labeled probes specific for C β 1 and C α (both a kind gift of Dr. H. U. Weltzien, Max Planck Institut für Immunbiologie). Because low RNA yields were expected, quantification was performed by subsequent hybridization with probes specific for α -actin (27).

Mobilization of Intracellular Free Calcium. Calcium mobilization studies were performed as described by Rabinovitch et al. (28). Cells were isolated from thymuses derived from embryos of day 16 gestational age. Thymocytes (5 \times 10⁶/ml) were loaded with Indo-1 by incubation with its acetoxymethyl ester (Molecular Probes Inc., Eugene, OR) (5.3 M) and 0.027% pluronic acid (Molecular Probes Inc.) for 45 min at 37°C. The cells were washed and stained with PE-labeled anti-CD4 mAb and anti-CD8 mAb, FITC-labeled anti-TCR- β mAb and anti-TCR- δ mAb at 4°C. Cells were washed and resuspended in IMDM supplemented with 1% FCS and 1% glutamine. Analysis followed immediately and was performed on a FACStar[®] flow cytometer (Becton Dickinson & Co.). Concentrations of 50 μ g/ml of anti-CD3 mAb and anti-TCR- δ mAb (both hamster IgG) were used for activation.

Results

Early Addition of Anti-CD3 mAb to FTOC Inhibits TCR Expression on CD4⁺8⁺ DP Thymocytes. Thymic lobes from day 14 embryos were cultured for 9 d and analyzed by flow cytometry. 100 μ g/ml of either anti-CD3 mAb or anti-TCR- β chain mAb were added to the cultures at day 0 or 6 of culture. Representative absolute cell numbers recovered from FTOC treated in this way are given in Table 1. A triple-staining experiment monitoring expression of CD4 and CD8 and of TCR- β chain within the CD4⁺8⁺ population upon antibody treatment on day 6 is shown in Fig. 1, A–F. Both antibodies induce partial deletion of the TCR-expressing DP subset. As described earlier, the anti-CD3 mAb has the stronger deleting capacity (29). Fig. 1, G–J shows the effects of the addition of the same antibodies on day 0. Under these

Table 1. Effect of Antibody Treatment of FTOC on Thymocyte Subsets

Thymocyte subset	Percent total				
	Control	Anti-TCR- β Day 0	Anti-CD3 Day 0	Anti-TCR- β Day 6	Anti-CD3 Day 6
CD4 ⁺ CD8 ⁺	45.2	28.8	41.9	33.0	16.4
CD4 ⁺ CD8 ⁺ V β S ⁺	20.4	4.1	1.7	3.8	2.6
CD4 ⁺ V β IC ⁻	11.0	7.0	48.8	9.9	3.5
CD8 ⁺ V β IC ⁻	12.0	8.9	53.0	9.5	5.6
V β S ⁻ V β IC ⁺	37.7	54.3	5.6	42.0	30.1
CD3S ⁻ CD3IC ⁺	41.6	57.2	79.6	45.5	54.5
Absolute cell number per lobe ($\times 10^5$)	1.9	0.9	1.7	0.8	1.0

Proportions of different thymocyte subsets upon antibody treatment of FTOC given in percentage of total FTOC were treated at 0 or day 6 of culture with antibodies to TCR- β or CD3. Control FTOC were cultured in medium without antibodies. Cells were analyzed by FCM on day 9 of culture. Indicated are uncorrected percentages.

conditions treatment with anti-TCR- β mAb also diminishes the CD4⁺8⁺V β S⁺ subset. In contrast, treatment with anti-CD3 mAb does not change the proportion of DP cells but leads to disappearance of TCR expression in this population.

Expression of Intracellular TCR- β Chain in Thymocyte Subpopulations. The results described above could be interpreted either as modulation of the TCR- β chain from the cell surface or as downregulation of the production of the β chain. To distinguish between these possibilities, it was necessary to analyze the expression of the β chain by FACS[®] analysis employing intracellular stainings. To this end, we established several informative staining protocols to analyze intracellular expression of the β chain in thymocyte subsets of untreated organ cultures and in thymuses derived from newborn mice. Depicted in Fig. 2A is a representative experiment analyzing the expression of intracellular β chain (V β IC) and extracellular CD4 in thymocytes derived from FTOC after 9 d of culture. The vast majority of CD4⁺ cells (of which most are DP) show intracellular β chain. It is not clear whether the small group of CD4⁺V β IC⁻ cells are in fact inefficiently stained or really devoid of intracellular β chain. The CD4⁻V β IC⁺ population consists of mature CD8⁺ and immature CD4⁻8⁻ double-negative (DN) cells. Analogous results were obtained by staining for CD8 instead of CD4. TCR- β chain is detected already within TCR⁻ cells (Fig. 2C). Upon surface expression, the concentration of intracellular β chain drops slightly. In thymocytes derived from ex vivo newborn thymus, we find a similar expression of intracellular β chain in the different subsets (Fig. 2, B and D). Differences are only found in the proportions of the populations. Intracellular CD3 is found in 85–95% of all thymocytes. As seen for TCR- β chain, the concentration of intracellular CD3 drops upon surface expression of the TCR (data not shown).

Early Addition of Anti-CD3 mAb to FTOC Leads to Loss of Intracellular β Chain. Organ cultures treated at day 0 with 100 μ g/ml of mAb were examined, and representative original results are given in Fig. 3. In Table 1, the calculated proportions of the most prominent thymocyte subsets are summarized. Anti-TCR- β treatment leads to decreased numbers of CD4⁺ (Fig. 3, A and B) and CD8⁺ cells (see Fig. 1). Whereas the proportions of cells expressing TCR on the surface (V β S) is strongly diminished (Fig. 3, D and E), intracellular β chain expression is not affected by the anti-TCR- β treatment. This is shown by the unchanged ratios of V β IC⁺ to V β IC⁻ cells within the CD4⁺ population (Fig. 3, A and B) and within the V β S⁻ population (Fig. 3, D and E) surviving the anti-TCR- β treatment. In contrast, the anti-CD3 mAb induces a decrease of intracellular β chain in all subpopulations. It is surprising that even the V β S⁻V β IC⁺ subset is strongly diminished, implying that cells on which no surface TCR is detected by flow cytometry are affected by anti-CD3 mAb. Intracellular CD3 expression remains unchanged, accounting for the increase in the CD3S⁻CD3IC⁺ subset (Table 1). Similar results were obtained upon treatment of FTOC with the mAb 500A2, a mAb that binds to a different epitope of the CD3 ϵ chain (30). This excludes the possibility that the effects observed are due to crossreactivity of one anti-CD3 ϵ mAb with an unknown structure on thymocytes. Table 1 also summarizes thymocyte subsets determined by the same protocols upon adding the antibodies to day 6 of culture. Under these conditions, both anti-TCR- β and anti-CD3 antibodies induce deletion of the TCR⁺ DP population, as described above.

Dose-Response Kinetics of the Effect of Anti-CD3 Antibodies. Optimal downregulation of TCR- β chain was obtained by 100 μ g/ml of anti-CD3 mAb, a rather high concentration of mAb. We were therefore worried that nonspecific

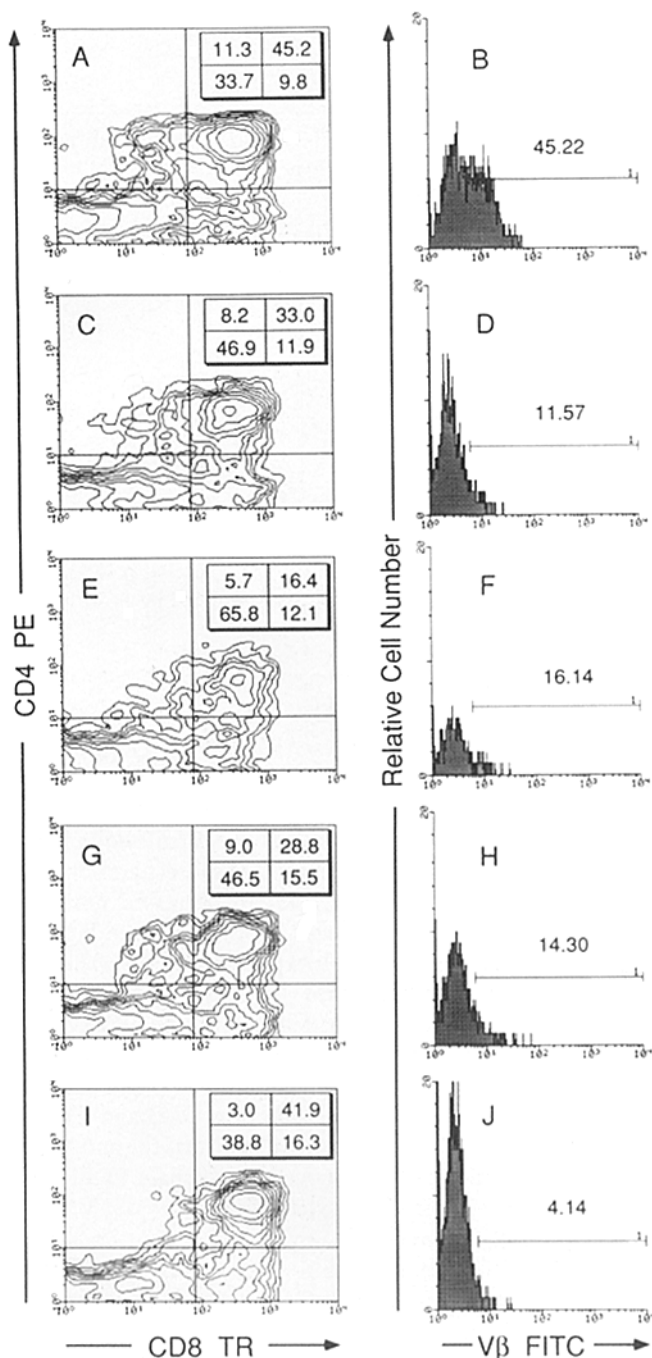


Figure 1. Effect of addition of anti-TCR mAb to FTOC. Antibodies to TCR- β (C, D, G, H) or CD3 ϵ (E, F, I, J) were added at a concentration of 100 $\mu\text{g}/\text{ml}$ to FTOC at day 6 (C–F) or day 0 (G–J) of culture. Control FTOC were cultured without antibody (A, B). Cells were analyzed by three-color flow cytometry (FCM) at day 9. Contour plots (A, C, E, G, I) represent CD4 and CD8 fluorescence intensities. Histograms (B, D, F, H, J) represent TCR- β chain fluorescence intensity of CD4 $^+$ 8 $^+$ cells. The numbers given in each panel represent uncorrected percentages. Background values were insignificant in all cases.

effects of Ig or the small degree of dilution of the culture media contributed to the effect. We therefore tested a series of other Igs at 100 $\mu\text{g}/\text{ml}$, including normal hamster IgG, normal rat IgG, anti-IL-2R α (rat IgG), anti-TCR- δ (hamster

IgG), and anti-PGP-1 (rat IgG). None of these resulted in the disappearance of TCR- β chain in DP thymocytes (data not shown). Furthermore, a dose-response analysis was performed, using concentrations of anti-CD3 mAb between 1 and 100 $\mu\text{g}/\text{ml}$ (Fig. 4). We observe a nearly linear relationship between the antibody dose and the sizes of the V β IC $^-$ CD8 $^+$ and V β IC $^-$ CD4 $^+$ populations. We have also determined, in a different protocol, the degree of saturation of CD3 epitopes by these antibody concentrations. Saturation is reached between 30 and 100 $\mu\text{g}/\text{ml}$. We conclude that anti-CD3 mAb is effective at all concentrations, the maximal response being induced at doses that saturate all available CD3 ϵ molecules.

Time Course of β Chain Shut-down by Anti-CD3 mAb The almost complete loss of intracellular β chain in all cell populations suggested that the shut-down of β chain production by ligation of CD3 takes place before or at the onset of β chain expression. To study this question, we performed time course experiments, adding the antibody at different days of culture and examining the lobes at day 9. In Fig. 5 we show that addition up to day 2 (7 d before analysis) results in constant proportions of CD4 $^+$ and CD8 $^+$ cells devoid of intracellular β chain. Thereafter, β chain shut-down in CD4 $^+$ 8 $^+$ cells decreases and is gradually replaced by a deletional response until day 6. This indicates that shut-down of β chain is inducible until 6–7 d before the thymocytes leave the DP stage, which is at the CD4 $^-$ 8 $^-$ IL-2R $^+$ stage, as can be deduced from the kinetics studies performed by Pearse et al. (4).

Predominance of Short (1.0 kb) TCR- β Chain Transcripts in Anti-CD3 mAb Treated FTOC. We examined expression of TCR- α and - β genes by Northern blot analysis (Fig. 6). A significant difference in TCR- β mRNA is observed between anti-CD3 treated and control FTOC. In the control FTOC, the full-length 1.3-kb (VDJC) transcript of the TCR- β genes is clearly detectable, together with very low amounts of the truncated 1.0-kb (JC or DJC) message. In contrast, in anti-CD3 treated cultures we see a dramatic reduction of the full-length β mRNA and strongly increased expression of the truncated transcript. If β chain shut-down occurred by transcriptional downregulation we would expect a reduction in full-length mRNA and an unchanged or slightly decreased expression of truncated mRNA. The increased level of truncated β mRNA suggests that interruption of β chain rearrangement before V-D joining is the cause of β chain shut-down. Hybridization with the C α probe showed full-length (VJC) 1.6-kb α gene transcripts in anti-CD3 mAb treated and control cultures. A short, 1.3-kb (JC) transcript is barely detectable. Since hybridization with a α -actin probe indicated about 2.5 times more mRNA in the anti-CD3 mAb treated FTOC, TCR- α chain mRNA appears to be diminished. We do not know whether this corresponds to experimental variation or reflects a downregulation of α chain transcription, possibly caused by the inability to express the α chain on the cell surface. Because we do not find a predominance of the truncated α chain transcript in the anti-CD3 mAb treated FTOC, we do not expect rearrangement of the α locus to be inhibited.

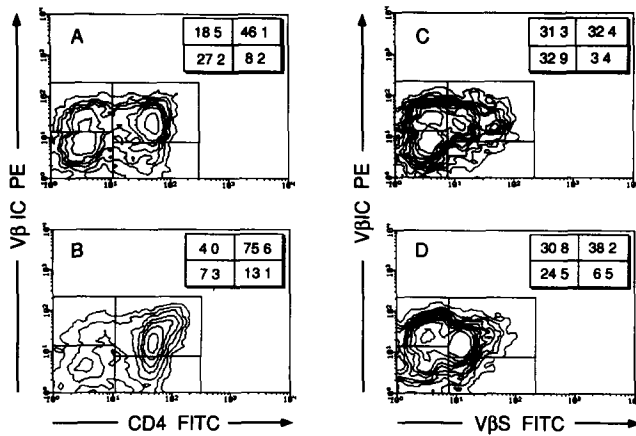


Figure 2. Expression of intracellular TCR- β chain in thymocytes. Thymocytes derived from day 9 FTOC (A, C) and newborn thymus (B, D) were analyzed by FCM. Contour plots represent fluorescence intensity of intracellular TCR- β chain (V β IC) and extracellular CD4 (A, B) and TCR- β (V β S) chain (C, D). Uncorrected percentages are indicated.

Expression of CD4 and CD8 and Loss of IL-2R Is Induced by Anti-CD3 mAb. To investigate the time required for blocking β chain expression, antibodies were added at day 0 of culture and analyses were performed at short time intervals thereafter. The data in Fig. 7 reveal that intracellular β chain is normally found before CD8 (Fig. 7, E and G), and confirm that CD4 appears after CD8 (Fig. 7, A and E). Fig. 7, B and F show that already after 2 d of culture in the presence of anti-CD3 mAb we find reduced numbers of cells with intracellular β chain. This includes the population that

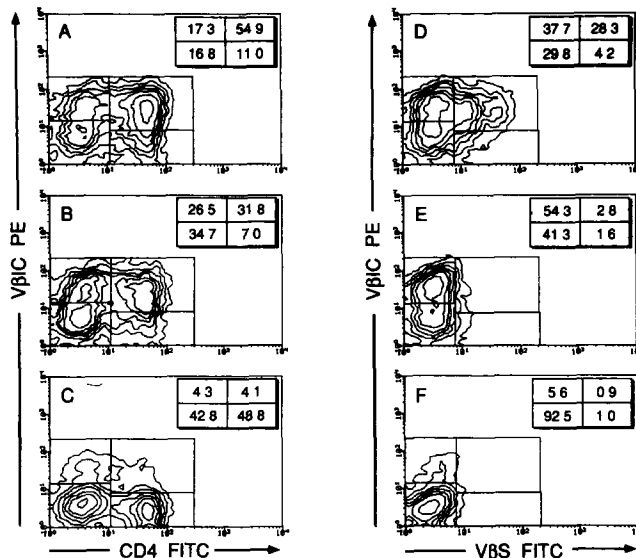


Figure 3. Effect of early addition of anti-TCR antibodies to FTOC on intracellular TCR- β chain expression. FTOC were cultured for 9 d in normal medium (A, D) or medium containing 100 μ g/ml antibodies to TCR- β chain (B, E) or CD3 ϵ (C, F). Cells were analyzed by FCM. Depicted are fluorescence intensities of intracellular TCR- β chain and extracellular CD4 (A-C) and TCR- β chain (D-F). Uncorrected percentages are indicated.

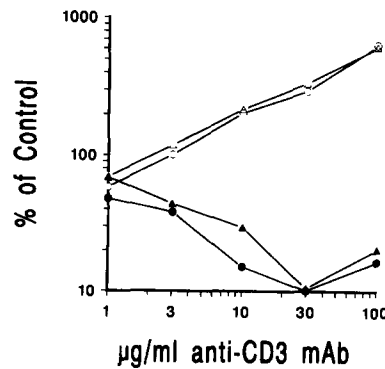


Figure 4. Dose-response kinetics of the effect of anti-CD3 antibodies. Anti-CD3 antibodies were added to FTOC at day 0 of culture at concentrations ranging from 1 to 100 μ g/ml. Expression of intracellular TCR- β chain and extracellular CD4 and CD8 were examined by FCM. The percentages of V β IC⁺ (closed symbols) and V β IC⁻ (open symbols) cells in the CD4⁺ population (circles) and in the CD8⁺ population (triangles) were calculated and are represented as relative proportions (percent control).

does not express CD4 and CD8. Moreover, the antibody treatment results in accelerated induction of CD4 and CD8 (Fig. 7, B, D, F, and H). In another experiment we included the analysis of IL-2R α chain in addition to CD4 and CD8 (Fig. 8). We find that accelerated acquisition of CD4 and CD8 is accompanied by downregulation of IL-2R α chain expression. Because downregulation of IL-2R α seemed only slightly accelerated compared to control FTOC, the results could be due to experimental variation. Therefore we repeated the experiment using FTOC derived from SCID mice, in which development is blocked at the IL-2R α ⁺ stage. Signaling through CD3 is expected to restore the blocked maturation instead of only accelerating normal development. Over 60% of thymocytes derived from untreated SCID-FTOC express IL-2R α (Fig. 9 A). The population showing a low surface

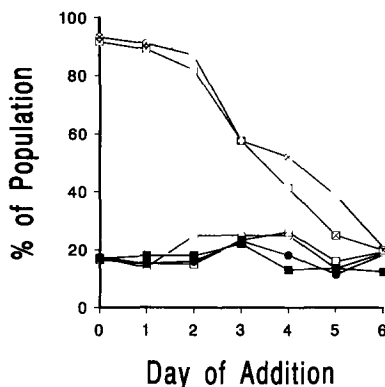


Figure 5. Time course of shut-down of TCR- β production. 30 μ g/ml antibodies to TCR- β (closed symbols), CD3 ϵ (crossed symbols) were added to FTOC at days 0-6 of culture. Control FTOC were cultured without antibodies (open symbols). Cells were analyzed at day 9 of culture by FCM. Represented are the percentages of V β IC⁺ cells in the CD4⁺ population (squares) and CD8⁺ population (circles).

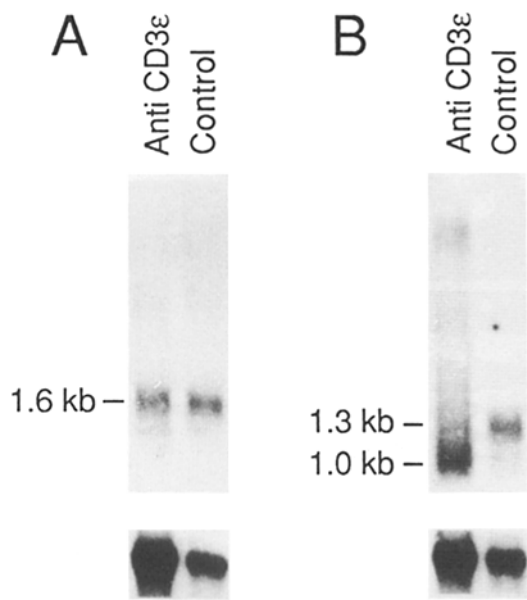


Figure 6. Northern blot analysis of TCR- α and - β mRNA from anti-CD3 treated and control FTOC. FTOC were treated with 100 $\mu\text{g}/\text{ml}$ anti-CD3 antibodies at day 0, and cells were harvested at day 9 of culture. RNA was prepared from $\sim 5 \times 10^6$ cells and hybridized with probes specific for C α (A) and C β 1 (B). For quantification, the membranes were hybridized with α -actin probes (small boxes). By scanning on an LKB Ultrascan XL (LKB, Cambridge, UK) we detected ~ 2.5 times more RNA in the anti-CD3 treated group as compared with the control. The 1.6-kb band detected by the C α probe represents fully rearranged (VJC) α transcripts. The 1.3-kb band detected by the C β 1 probe represents the complete (VDJC) β mRNA, whereas the short transcripts (1.0 kb) derive from incompletely rearranged β loci (DJC or JC).

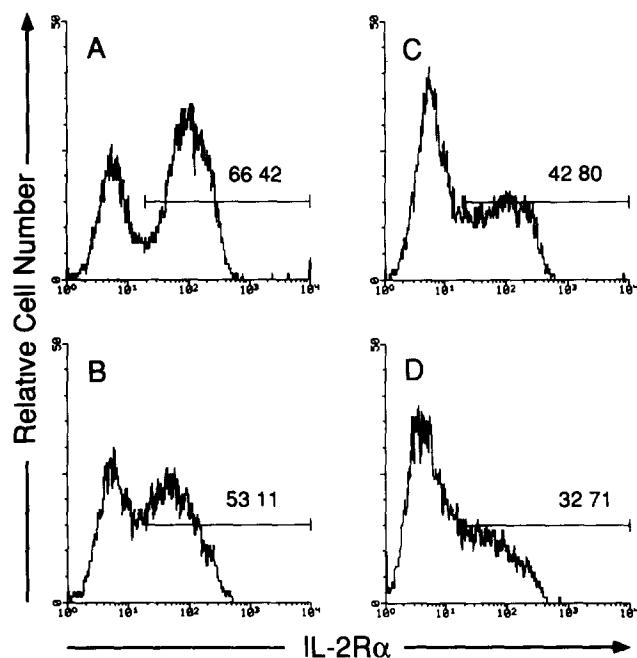


Figure 8. Downregulation of IL-2 receptor α chain (IL-2R α) upon anti-CD3 treatment of FTOC. FTOC were treated with 100 $\mu\text{g}/\text{ml}$ anti-CD3 antibodies at day 0 of culture (B, D) or cultured without antibodies (A, C) and analyzed by FCM at day 2 (A, B) and 3 (C, D) of culture. Histograms show the fluorescence intensity of IL-2R α . Percentages of cells gated positive for IL-2R α are given in the figures. Mean fluorescence channels of positive cells were 145 (A), 88 (B), 127 (C), and 92 (D).

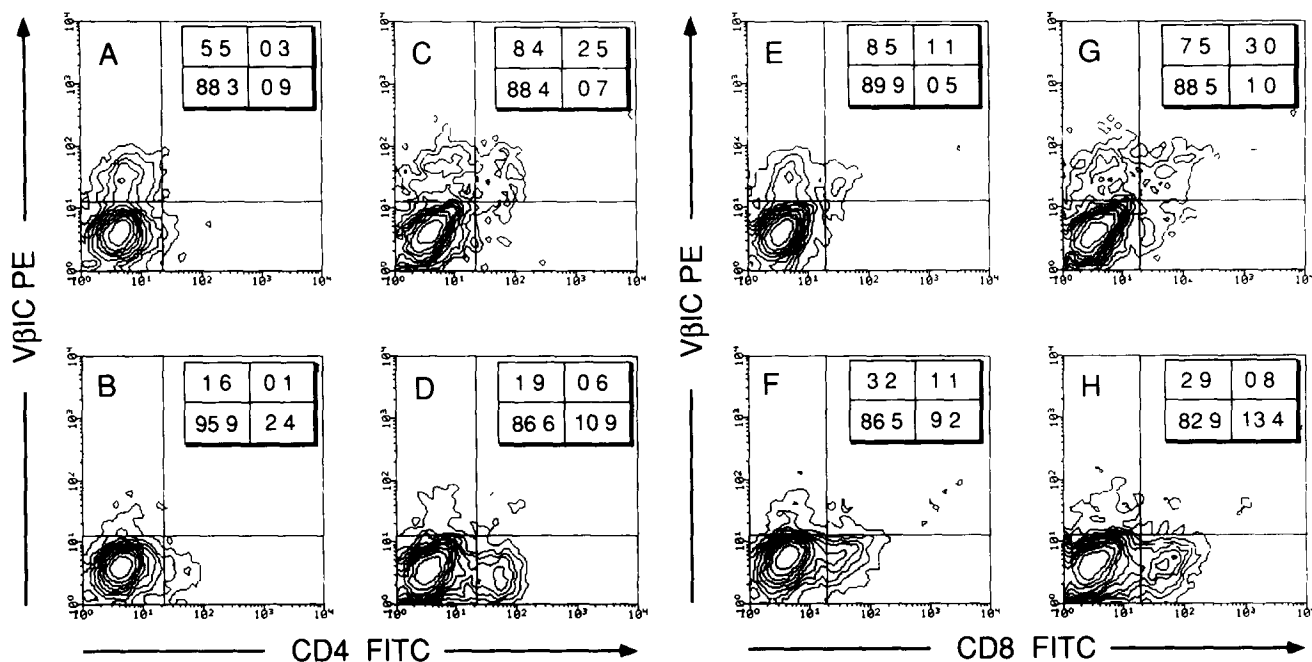


Figure 7. Shut-down of TCR- β production and induction of CD4 and CD8 upon anti-CD3 treatment of FTOC. FTOC were treated with 30 $\mu\text{g}/\text{ml}$ anti-CD3 antibodies at day 0 of culture (B, D, F, H) or cultured without antibodies (A, C, E, G). Analysis by FCM was performed at day 2 (A, B, E, F) and day 3 (C, D, G, H) of culture. Represented are fluorescence intensities of surface CD4 (A-D) and CD8 (E-H) and intracellular TCR- β . Uncorrected percentages are indicated.

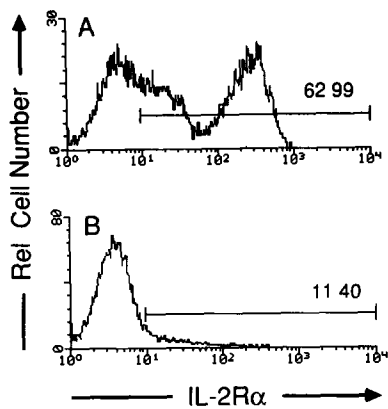


Figure 9. Downregulation of IL-2 receptor α chain (IL-2R α) upon anti-CD3 treatment of FTOC derived from SCID mice. FTOC derived from SCID embryos were treated with 100 $\mu\text{g/ml}$ anti-CD3 antibodies at day 0 of culture (B) or cultured without antibodies (A) and analyzed by FCM at day 6 of culture. Histograms show the fluorescence intensity of IL-2R α . Percentages of cells gated positive for IL-2R α are given in the figures. Background values were <1%.

expression of IL-2R α is also found in the recombinase activation gene (RAG)-2-deficient mouse (8). Treatment with anti-CD3 mAb indeed leads to an almost complete loss of IL-2R α expression (Fig. 9 B). These results reveal that β chain shut-down is not the only consequence of early ligation of CD3 ϵ in thymocytes. Rather, a complete developmental program is initiated which reflects the maturation steps typically observed upon the acquisition of the DP stage.

Calcium Mobilization Can Be Induced by Anti-CD3 mAb in Day 16 Embryonic DN TCR $^-$ Thymocytes. It is generally

accepted that small concentrations of CD3 may be detected on the surface of thymocytes as soon as CD8 is expressed (31). If we assume that our findings are induced by a direct interaction between the antibody and the affected cell, CD3 can transduce a signal even before expression of CD8. To test this hypothesis, we analyzed the capability of DN TCR $^-$ cells derived from ex vivo thymuses from 16-d-old embryos to mobilize Ca^{2+} upon ligation of CD3. The data depicted in Fig. 10, C and D show that indeed Ca^{2+} can be mobilized in 15% of these cells. The response was, however, weaker and shorter than described for mature T cells (32). Thus far, we have no information on whether the immature thymocytes that respond to anti-CD3 mAb with Ca^{2+} mobilization are identical with those that respond by β chain shut-down. Antibodies to TCR- γ/δ did not induce Ca^{2+} mobilization in this cell population (Fig. 10, A and B), excluding that the response is nonspecifically induced by a high concentration of hamster IgG. These results suggest that CD3 ϵ is expressed at low levels in a proportion of DN TCR $^-$ immature thymocytes and represents a functional component in signaling events leading to transition from the DN to the DP stage.

Discussion

Although the developmental sequence of the phenotypes of thymocytes is well described, little is known about the molecular events that drive these cells into each subsequent stage of maturation. An important control point in thymic development is the transition of CD4 $^-$ 8 $^-$ IL-2R $^+$ thymocytes to the CD4 $^+$ 8 $^+$ IL-2R $^-$ stage. Several reports show that

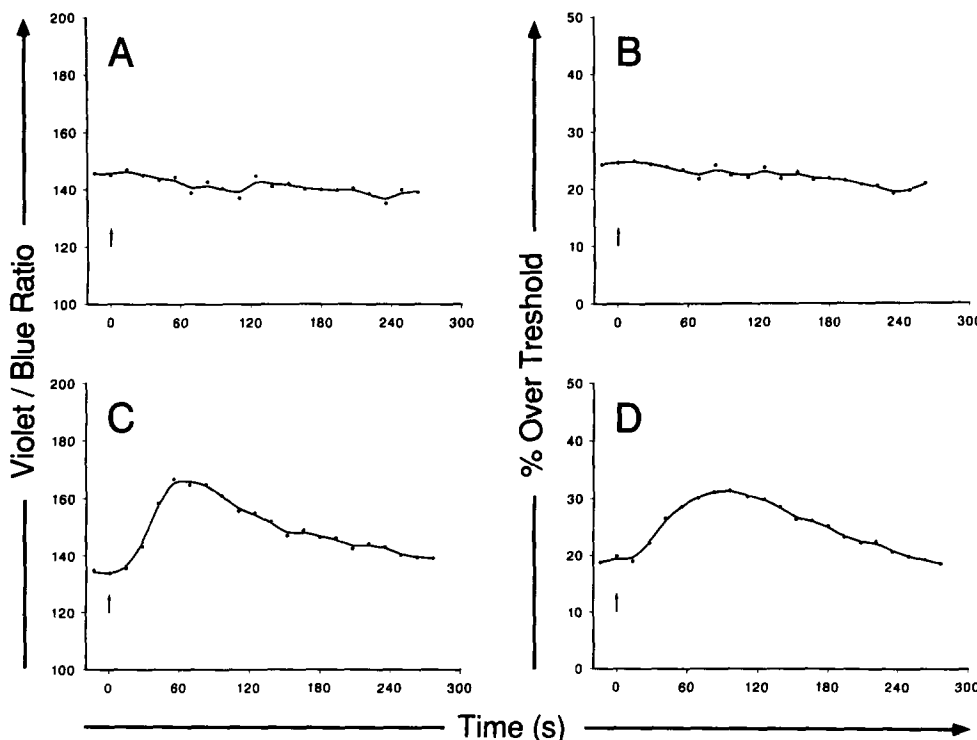


Figure 10. Calcium mobilization induced by anti-CD3 antibodies in DN TCR $^-$ cells. Thymocytes were isolated from embryos of day 16 gestational age and loaded with Indo-1. Subsequently, the cells were stained for CD4, CD8, TCR- β and - δ using antibodies free of azide at 4°C. Gating was for cells negative for all markers. Cells were stimulated at time 0 (arrow) with 50 $\mu\text{g/ml}$ antibodies to CD3 (C, D) or TCR- δ (A, B). A and C give the course of the violet/blue ratio, which is linear to the molarity of mobilized intracellular Ca^{2+} , over time. B and D show the percentage of cells exposing a concentration of intracellular Ca^{2+} above the background level over time.

successful rearrangement and expression of the TCR- β chain plays a critical role in this process. Some years ago it was shown that in SCID mice transgenic for a rearranged TCR- β locus, thymocytes regained the ability to enter the CD4⁺8⁺ stage (6). However, normal numbers of DP cells were only found in SCID mice transgenic for both rearranged α and β loci. Recent results demonstrated that disruption of either RAG-1 (7) or RAG-2 (8) blocks development at the CD4⁻8⁻IL-2R⁺ stage. An arrest in thymocyte development at this point is also found in mice with a disrupted TCR- β locus, in which the very small numbers of CD4⁺8⁺ cells are suspected to belong to the γ/δ lineage. (P. Mombaerts, personal communication). Mice devoid of TCR- α chain, in contrast, show normal numbers of DP thymocytes (33). From these experiments it can be deduced that the complete rearrangement of the β locus is an important prerequisite for further maturation of the α/β lineage. This finding cannot be attributed to intracellular events alone, because the transplantation of normal bone marrow cells into SCID mice gave rise to both donor-derived TCR⁺ thymocytes and host-derived CD4⁺8⁺TCR⁻ SCID thymocytes (34). Recent experiments show that immature thymocytes may express a TCR complex consisting of β - β homodimers in association with CD3. A TCR complex consisting of a β - β chain homodimer, weakly associated with CD3, was detected by Punt et al. (35) on a transformed CD4⁻8⁺ thymocyte. A TCR complex consisting of a β - β homodimer, tightly bound to CD3 δ and CD3 ϵ , but weakly associated with CD3 ζ and CD3 γ was found on SCID T cell lines transfected with a rearranged TCR- β locus (36). It has been suggested that signals generated upon surface expression of this complex induces the expression of the coreceptors CD4 and CD8 and possibly also leads to allelic exclusion, an event that is a consequence of an interruption of TCR- β chain rearrangement (37).

Our findings show that most known events associated with the acquisition of the DP stage can be induced by early treatment of FTOC with anti-CD3 ϵ mAb. CD4 and CD8 expression is induced on DN thymocytes. IL-2R α chain expression is downregulated; the cells fail to produce TCR- β chain. The inhibition of the production of TCR- β protein and of full-length TCR- β mRNA by anti-CD3 may be caused by the same process that, in the physiological situation, results in allelic exclusion, i.e., interrupted rearrangement of TCR- β genes. These findings support the hypothesis that surface expression of and subsequent signal transduction through CD3, perhaps complexed with the TCR- β chain, may be an important event directing the CD4⁻8⁻IL-2R⁺ thymocytes into the next developmental stage. It is not clear, however, whether all of these phenotypic changes are part of a single cellular response or just happen to occur simultaneously.

Alternative explanations for shut-off of TCR- β production have to be considered. Our findings may reflect a novel kind of negative selection involving transcriptional downregulation of TCR- β chain production. Our time course experiments suggest that the window for inhibition of TCR- β chain production is open during the CD4⁻8⁻IL-2R⁺ stage and may be closed before the TCR- α locus has fully rear-

ranged (38). Both a purpose and a pathway for self-tolerance induction at this stage of development are hard to imagine. Furthermore, the increased expression of truncated (1.0 kb) TCR- β message is not easily reconciled with a transcriptional shut-off of TCR- β production. Therefore, we favor the hypothesis that a cessation of rearrangement is the cause of impaired TCR- β chain production. Rearrangement of the TCR- α locus seems unimpaired as can be concluded from the Northern blot analysis showing full-length TCR- α mRNA. This is in line with recent experiments indicating that allelic exclusion of the TCR- α locus takes place at the DP stage and is dependent on positive selection (39).

How could rearrangement of the TCR- β chain genes be interrupted by ligation of CD3? Owen et al. (15) who observed reduced numbers of V β 8IC⁺ cells without reduction of CD4⁺ and CD8⁺ cells upon anti-CD3 treatment of FTOC, suggested that γ/δ cells were necessary for rearrangement of the TCR- β locus and that their function was blocked by anti-CD3 mAb. This can be ruled out because recent experiments show that TCR- δ -deficient mice can nevertheless rearrange the β locus (P. Mombaerts, personal communication). It is also possible that anti-CD3 antibodies block the interaction of an unknown ligand with CD3 which is required for β chain rearrangement to continue to completion. This possibility is unlikely because the blocking of an essential maturation signal would be expected to result in decreased maturation to DP cells. In our experiments, in contrast, accelerated maturation to the DP stage is observed.

We therefore favor the hypothesis that a signal which in the physiological situation is transduced by CD3 complexed with the β - β homodimer, is delivered prematurely upon ligation of CD3 ϵ molecules before rearrangement of the β locus has finished. This implies that CD3 ϵ molecules are expressed on the cell surface of immature thymocytes in the absence of the TCR- β chain and are capable of signal transduction upon crosslinking. This hypothesis is supported by our finding that induction of CD4 and CD8 and downregulation of IL-2R α can be induced in FTOC from SCID mice upon anti-CD3 antibody treatment (manuscript in preparation). The characterization of a thymocyte expressing CD3 on the cell surface in the absence of TCR- α -, β -, γ -, or δ chains (40), and the recent finding that β - β homodimers are expressed on immature thymocytes (35, 36) suggest that the assembly and surface expression of the TCR complex is differently regulated at early stages of thymocyte development and support the hypothesis that surface expression of a partial TCR complex is important for thymocyte maturation.

Our Ca²⁺ mobilization experiments show that a considerable number (15%) of DN TCR⁻ thymocytes derived from embryonic day 16 thymus in fact express functional CD3. It remains to be investigated what the exact phenotype of these cells is. The population appears to be too large to represent immature γ/δ cells expressing undetectable concentrations of TCR. Because TCR- α message is barely detectable at embryonic day 16 (41), the population is also too large to represent a subset of thymocytes that has successfully rearranged both the α and β locus and express still un-

detectable concentrations of TCR. Therefore, we propose that at least part of this population is DN thymocytes expressing some CD3 with or without a TCR- β chain.

It is interesting how the physiological signals for shut-down of β gene rearrangement are generated. A signal may be generated upon formation within the thymocyte membrane of a complex of CD3 with β - β homodimers and perhaps additional molecules. Alternatively, this complex interacts with a ligand on other cells in the thymus, of epithelial or hematopoietic origin. This latter hypothesis is attractive because it provides an explanation for the exclusive localization of T cell development to the thymus.

Taken together, these experiments demonstrate that maturation of DN to DP cells, which appears to be dependent on β chain rearrangement, is a developmental step inducible by signaling through CD3. In addition, the results suggest that CD3 ϵ is expressed on the cell surface of developing thymocytes in the absence of a TCR- β chain. Analysis of the molecular structure of this putative CD3 complex may be of major importance in the understanding of the very early stages of thymocyte development.

We thank Dr R. Carsetti and H. Kohler for assistance with the calcium mobilization experiments, H Goebel for technical assistance, and Drs R. Lamers, H. Burgert, and J. Langhorne for critical reading of the manuscript

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Received for publication 19 August 1992 and in revised form 26 October 1992.

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