Regulation of Thymocyte Development through CD3.
II. Expression of T Cell Receptor β CD3ε and Maturation to the CD4+8+ Stage Are Highly Correlated in Individual Thymocytes

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Summary
Recent studies have shown that maturation of CD4−8− double negative (DN) thymocytes to the CD4+8+ double positive (DP) stage is dependent on expression of the T cell receptor (TCR)-β polypeptide. The exact mechanism by which the TCR-β chain regulates this maturation step remains unknown. Previous experiments had suggested that in the presence of some TCR+ thymocytes, additional DN thymocytes not expressing a TCR-β chain may be recruited to mature to the DP stage. The recent demonstration of an immature TCR-β-CD3 complex on early thymocytes lead to the alternative hypothesis that signal transduction through an immature TCR-CD3 complex may induce maturation to the DP stage. In the latter case, maturation to the DP stage would depend on the expression of TCR-β-CD3 in the same cell. We examined these two hypotheses by studying the expression of the intra- and extracellular CD3ε, CD3γ, and TCR-β polypeptides in intrathymic subpopulations during embryogenesis. CD3ε and CD3γ were expressed intracellularly 2 and 1 d, respectively, before intracellular expression of the TCR-β chain, potentially allowing immediate surface expression of an immature TCR-β-CD3 complex as soon as functional rearrangement of a TCR-β gene locus has been accomplished. Calcium mobilization could be induced by stimulation with anti-CD3ε mAb as soon as intracellular TCR-β was detectable, suggesting that a functional TCR-β-CD3 complex is indeed expressed on the surface of early thymocytes. From day 17 on, most cells were in the DP stage, and over 95% of the DP cells expressed on the TCR-β chain intracellularly. At day 19 of gestation, extremely low concentrations of TCR-β chain and CD3ε were detectable on the cell surface of nearly all thymocytes previously thought to be TCR-CD3 negative. These findings strongly support the hypothesis that maturation to the DP stage depends on surface expression of and subsequent signal transduction through an immature TCR-β-CD3 complex and suggest that maturation to the DP stage by recruitment, if it occurs at all, is of minor relevance.

During embryogenesis, T lineage committed lymphoid precursor cells home to the thymus, where they undergo a developmental program, marked by sequential surface expression of various stage specific glycoproteins and rearrangement of the TCR gene loci. One of the early thymocyte markers is Pgp-1, a molecule that may play a role in homing of precursor cells to the thymus. Further maturation of thymocytes results in the expression of the α chain of the interleukin-2 receptor (IL-2R) and downregulation of Pgp-1 (for review see 1, 2). The IL-2Rα+ stage coincides with a wave of V>D rearrangements of the TCR-β locus (3). An important control point in intrathymic development is the subsequent transition into the CD4+8+ stage, accompanied by a burst of cell divisions, loss of IL-2Rα expression, and arrest of rearrangement, i.e., allelic exclusion, of the TCR-β gene locus (4–7). During the DP stage, rearrangement of the TCR-α locus takes place (for review see 8). Upon production of a functional TCR-α polypeptide chain, a complete TCR-αβ-CD3 complex becomes expressed on the cell surface in low concentration. At this stage repertoire selection takes place, resulting in the production of mature CD4+ and CD8+ single positive (SP) T cells with high TCR-αβ-CD3 expression (9, 10).

Several recent studies collectively suggest that surface expression of and signal transduction through immature forms of the TCR complex may play a decisive role during earlier stages of thymocyte development, i.e., in the transition to the double positive (DP) stage. We have previously reported that treatment of fetal thymic organ cultures with anti-CD3ε antibodies accelerated the appearance of DP cells and induced
several other phenotypic changes accompanying this maturation step (11). Mice that are unable to produce a TCR-β chain, either by a deficiency in the rearrangement activity (SCID or RAG-1 or -2 mutants) or by mutation of the TCR-β locus itself, show a block in development at the CD4-8- double negative (DN) stage (12-15). Development to the DP stage is restored by introduction of a functionally rearranged TCR-β transgene in these mice strains (15-17) or by cross-linking of CD3ε on the cell surface of TCR-β-deficient thymocytes (17a). An immature TCR complex consisting of a TCR-β homodimer and CD3γ, δ, ε, and ζ has been detected on immature thymus thymoma cell lines (18, 19) and a similar complex was found on thymocytes from TCR-β transgenic RAG-2 mutant mice, however, without detectable CD3ζ (17). These findings lead to the hypothesis that surface expression of and signal transduction through an incomplete TCR-β-CD3 complex is required for a thymocyte to mature into the DP stage. This hypothesis predicts that all DP cells show a productive rearrangement of the TCR-β locus. On the other hand, an earlier study showed that transplantation of normal bone marrow into SCID mice gave rise to both donor-derived TCR+ thymocytes and host-derived CD4+8- thymocytes (20). It was hypothesized that the presence of TCR+ thymocytes would recruit SCID-derived DN cells into the developmental process, even though these cells do not themselves show functional rearrangement of the TCR-β locus. In this article we examined these two alternative pathways of development to the DP stage by studying the intra- and extracellular expression of the TCR-β chain and of components of the CD3 complex and their signaling capacity in normal mice during embryogenesis. We find that DP cells almost exclusively arise from precursors that express intracellular TCR-β chain together with CD3ε and ζ. Low-level surface expression of a functional CD3 complex precedes acquisition of the DP stage. Our results suggest that signal transduction through an immature TCR-β-CD3 complex induces a developmental response within the same cell, and that recruitment plays a minor role, if any.

Materials and Methods

*Mice.* Pregnant female BALB/c mice were obtained from our specific pathogen-free breeding facility.

*MAb.* Anti-TCR chain antibody H57-597, anti-TCR-δ chain antibody GL-3, anti-CD3ε antibody 500A2, and all hamster IgG, were purchased from Pharmingen (San Diego, CA). Flow cytometry was performed on a FACScan® flow cytometer (Becton Dickinson and Co., Heidelberg, FRG) using labeled anti-Lyt-2 (53.6-7), anti-CD3ε antibody 500A2, and all hamster IgG, fluorescedinated anti-ID2Rca antibody 5A2 (21) and biotin-labeled anti-CD3ε antibody H146-968 (18). The MAb 2.4G2 (22) was used to block FcyRII binding.

*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 (11), but modified to minimize background or inefficient staining and to increase sensitivity. First, thymocytes were incubated with anti-FcyRII mAb and normal hamster, mouse, and rat IgG to inhibit unspecific staining. Then, the cells were washed and stained with FITC-labeled antibodies as indicated above. After incubation and washing, 20 µl of 0.25% Saponin (Sigma Chemical Co., 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. The intracellular staining was performed twice to exclude inefficient staining of intracellular structures. PE-conjugated Streptavidin was added together with 20 µl of 0.25% Saponin in PBS/FCS to detect biotin-labeled antibodies. Cell-suspensions were washed three times before analysis. Before the last centrifugation step, the cells were suspended in washing medium and incubated for 30 min on a roller. 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 (22a). Sensitve staining for the detection of TCR-β chain and CD3ε on the cell surface of thymocytes were performed as follows: cells were incubated with supernatant from the hybridomas 500A2 or H57–597, control samples were incubated with normal hamster IgG; the cells were washed and then incubated with FITC-labeled rabbit anti-hamster IgG Ab; washed again; and then incubated with FITC-labeled goat anti-rabbit IgG Ab.

*Mobilization of Intracellular Free Calcium.* Calcium mobilization studies were performed as described by Rabinovitch (23). Cells were isolated from thymuses derived from fetuses of day 16 gestational age. Thymocytes (5 x 10⁶/ml) were loaded with indo-1 by incubation with its acetoxy-methyl ester (5 .3 M) and 0.027% pluronic acid (Molecular Probes Inc., Eugene, OR) for 45 min at 37°C. The cells were washed and stained with PE-labeled anti-CD4 mAb, anti-CD8 mAb, FITC-labeled anti-IL-2Rα mAb, and biotin-labeled anti-Pgp-1 mAb followed by Tricolor-labeled Streptavidin at 4°C. Cells were washed and resuspended in Iscoves' modified Dulbecco medium supplemented with 1% FCS and 1% glutamine. Analysis followed immediately and was performed on a FACStar® flow cytometer (Becton Dickinson and Co.). Concentrations of 3 µg/ml of anti-CD3 mAb, anti-TCR-δ mAb, anti-TCR-β (all hamster IgG), and normal hamster IgG followed by 30 µg/ml rat anti-hamster IgG antibodies were used for stimulation.

**Results**

Almost All of the DP Thymocytes Express Intracellular TCR-β Chain. Thymocytes derived from 14- to 19-d fetuses were examined for intracellular expression of the TCR-β chain by flow cytometry. In Figs. 1-3 representative experiments are depicted showing intracellular expression of the TCR-β chain and surface expression of CD4 (Fig. 1), CD8 (Fig. 2), and IL-2Rα (Fig. 3) during embryonic development. Day 15 is the first day at which we detected the TCR-β chain (Fig. 1 B, 2 B, and 3 B). The cells expressing the TCR-β chain were still CD4-8-. Expression of CD8 began one day later, predominantly in the TCR-β intracellular (IC) population (Fig. 2 C). At days 17-19 over 80% of all thymocytes entered the DP stage (data not shown). The data demonstrate that over 95% of all CD4+ or CD8+ thymocytes possessed a functionally rearranged TCR-β locus (Figs. 1, D-F and 2, D-F). A minor subpopulation of CD4+ and CD8+ cells did not express the TCR-β chain. It is unlikely that this is due to inefficiency of intracellular staining, for all CD8+ thymocytes derived from 18-d-old fetuses could be stained.
intracellularly with PE-labeled anti-CD3\(\epsilon\) mAb 500A2, and also hamster IgG (Fig. 4 A). Neither is it due to background staining by the anti-CD8 mAb, as isotype control mAb did not show significant staining (Fig. 4 B). Some of these thymocytes may have been in the γδ lineage, because ~1% of the CD8+ cells and 0.5% of the CD4+ cells stained for intracellular TCR-\(\beta\) chain (data not shown). These data show that the percentage of CD4+ or CD8+ cells having a functional rearrangement of the TCR-\(\beta\) locus clearly exceeded the 56% (one minus the probability of two out of frame rearrangements = 1 - (2/3)^2 = 5/9 \approx 56\%) predicted if the TCR-\(\beta\) chain did not play a critical role in maturation of individual thymocytes to the DP stage (8).

No TCR-\(\beta\) IC+ cells were found in the Pgp-1hi population at any timepoint tested (not shown). The majority of the IL-2Rα+ cells were devoid of the TCR-\(\beta\) chain, and the few IL-2Rα+ cells expressing the TCR-\(\beta\) chain appeared to be in the process of losing the IL-2Rα chain (Fig. 3). Since acquisition of IL-2Rα and loss of Pgp-1 coincide (2), these results indicate that functional TCR-\(\beta\) locus rearrangement is first accomplished at the IL-2Rα+ stage.

CD3\(\epsilon\) and CD3\(\gamma\) Are Expressed at the IL-2Rα+ Stage. In addition to the induction of the DP stage, surface expression of the TCR-\(\beta\) chain, and signal transduction through the TCR-\(\beta\) CD3 complex may regulate allelic exclusion by arresting TCR-\(\beta\) locus rearrangement. Such a mechanism would function optimally if the components of the CD3 complex were expressed before functional rearrangement of the TCR-\(\beta\) locus, in order to warrant immediate surface expression as soon as a functional TCR-\(\beta\) polypeptide has been made. Therefore, we examined the intracellular expression of the CD3\(\epsilon\) and \(\gamma\) chains, starting at day 13 of gestation. Representative results are shown in Figs. 5–8. We detected CD3\(\epsilon\) already at day 13 of gestation (Fig. 5 A), i.e., 2 d before expression of the TCR-\(\beta\) chain. CD3\(\epsilon\)+ cells were detected in the Pgp-1hi population, but not all Pgp-1hi cells showed staining for CD3\(\epsilon\), independently of the day of analysis (Fig. 5). All cells in the IL-Rα+ stage expressed the CD3\(\epsilon\) chain (Fig.
Expression of intracellular TCR-β chain and surface expression of IL-2Rα. Thymocytes derived from fetuses at days 14-17 of gestation were analyzed by FCM. Contour plots represent fluorescence intensity of intracellular TCR-β chain and extracellular IL-2Rα.

6). Cells remained CD3ε IC+ during further maturation (not shown). The CD3ζ chain was detected first on day 14 in cells in the process of losing Pgp-1 (Fig. 7) and in IL-2Rα+ cells (Fig. 8 B). Pgp-1+ cells were CD3ζ IC- on all days of gestation. These data suggest that expression of the CD3ζ chain coincides with downregulation of Pgp-1 and maturation to the IL-2Rα+ stage. Furthermore, the data show that components of the CD3 complex are expressed well before the appearance of the TCR-β chain.

**TCR-β and CD3ε Are Expressed on the Surface of Most DP Thymocytes.** In the thymus, surface expression of the TCR complex has been observed at three different levels; nil, low, and high (for review see 1, 2). If surface expression of the TCR-β chain would be a prerequisite for maturation to the DP stage one should expect TCR-β and CD3ε to be present on the surface of all DP thymocytes, i.e., that the apparent TCR-β+ cells are in fact TCR-βlow. We analyzed thymocytes derived from fetuses at day 19 of gestation by flow cytometry, using a sensitive double layer indirect staining protocol. Over 85% of these cells were in the CD4+8+ stage. In Fig. 9, A and C, conventional staining profiles of thymocytes with directly PE-labeled anti-CD3ε mAb (Fig. 9 A) or anti-TCR-β

**Figure 3.** Expression of intracellular TCR-β chain and surface expression of IL-2Rα. Thymocytes derived from fetuses at days 14-17 of gestation were analyzed by FCM. Contour plots represent fluorescence intensity of intracellular TCR-β chain and extracellular IL-2Rα.

**Figure 4.** Controls for surface and intracellular staining protocols. Thymocytes derived from fetuses at day 18 of gestation were analyzed by FCM. Contour plots represent fluorescence intensity of intracellular CD3ε and extracellular CD8 (A) and background fluorescence intensities using FITC-labeled IgG2a (surface) and SA-PE (intracellular) (B).

**Figure 5.** Expression of intracellular CD3ε and surface expression of Pgp-1 in thymocytes at days 13-16 of gestation. Thymocytes derived from fetuses at days 13-16 of gestation were analyzed by FCM. Contour plots represent fluorescence intensity of intracellular CD3ε and extracellular Pgp-1.

**Figure 6.** Expression of intracellular CD3ε and surface expression of IL-2Rα in thymocytes at days 13-16 of gestation. Thymocytes derived from fetuses at days 13-16 of gestation were analyzed by FCM. Contour plots represent fluorescence intensity of intracellular CD3ε and extracellular IL-2Rα.
Figure 7. Expression of intracellular CD3ε and surface expression of Pgp-1 in thymocytes at days 13–16 of gestation. Thymocytes derived from fetuses at days 13–16 of gestation were analyzed by FCM. Contour plots represent fluorescence intensity of intracellular CD3ε and extracellular Pgp-1.

(Fig. 9C) are shown. Fig. 9, B and D, represents thymocytes stained by unlabeled anti-TCR-β mAb (Fig. 9D) or anti-CD3ε mAb (Fig. 9B), followed by FITC-labeled rabbit anti-hamster antibodies, followed by FITC-labeled goat anti-rabbit antibodies. Hamster IgG was used as a control and is represented by the light shaded histogram. Stainings with anti-CD3 or anti-TCR-β mAb showed three levels of positive staining instead of the two previously described; low, medium, and high. The low staining cells correspond to the subset of DP cells previously thought to be TCR negative. The cells showing a medium level of staining correspond to those DP cells previously defined as TCR low. Cells staining at a high level correspond to mature SP cells.

Calcium Mobilization by Anti-CD3ε Correlates with Expression of Intracellular TCR-β. To examine whether the TCR at its lowest level is capable of signal transduction, we determined calcium mobilization in thymocytes at different stages of development upon stimulation with anti-CD3ε, anti-TCR-β, or anti-TCR-δ mAb. For this purpose, thymocytes at day 16 of gestation were used, because there is only marginal expression of rearranged TCR-α genes at this age (24). Cells were stained for Pgp-1, IL-2Rα, and CD4 plus CD8 and intracellular calcium levels of the different subpopulations after stimulation with the three different antibodies were examined. Fig. 10, A–C, show that with anti-CD3ε anti-

Figure 8. Expression of intracellular CD3ε and surface expression of IL-2Rα in thymocytes at days 13–16 of gestation. Thymocytes derived from fetuses at days 13–16 of gestation were analyzed by FCM. Contour plots represent fluorescence intensity of intracellular CD3ε and extracellular IL-2Rα.

Figure 9. Surface expression of CD3ε and TCR-β on thymocytes. Thymocytes from fetuses at day 19 of gestation were analyzed FCM using directly labeled mAb (A and C) or using a sensitive double layer indirect staining protocol (B and D). Histograms represent surface expression of CD3ε (A and B) and TCR-β (C and D). Hamster IgG control stainings are represented by the light shaded histograms (B and D).

Figure 10. Calcium mobilization in thymocytes at different stages of development upon stimulation with anti-CD3ε, anti-TCR-β, and anti-TCR-δ mAb. A: Day 16, B: Day 15, C: Day 14, D: Day 13.
bodies calcium mobilization could be induced in 76% of the CD4+ or CD8+ cells and in 19% of all CD4+ IL-2Rαβ cells, but not in Pgp-1h' cells. This correlates well with intracellular TCR-β expression in these subpopulations, indicating that a functional TCR-CD3 complex is expressed on the cell surface of thymocytes as soon as intracellular TCR-β chain is detected. Only marginal calcium responses could be induced using anti-TCR-β mAb (Fig. 10, D–F). A similar difference in response to cross-linking with anti-TCR-β or anti-CD3e mAb has been described for an immature cell line expressing a TCR consisting of TCR-β and CD3 in the absence of TCR-α (18). Anti-TCR-β mAb antibodies induced significant calcium influx but only in few cells (Fig. 10, G and H). Normal hamster IgG did not result in calcium mobilization in any of the subpopulations (not shown). In adult thymus, calcium mobilization could be induced over 80% of the cell using anti-CD3e mAb, but only in 50% using anti-TCR-β mAb (not shown). Therefore, these data indicate that calcium mobilization can be induced in cells expressing the TCR complex at its lowest level, but only with anti-CD3e mAb and not with anti-TCR-β mAb.

**Figure 10.** Calcium mobilization in intrathymic subpopulations induced by anti-CD3 or TCR antibodies. Thymocytes were isolated from fetuses at day 16 of gestation and loaded with indo-1. Subsequently, the cells were stained for CD4, CD8, IL-2Rα, and Pgp-1 using antibodies free of azide at 4°C. Three-dimensional plots represent the course of the violet/blue ratio, which is linearly related to the molarity of intracellular Ca2+, over time. Cells were gated for expression of CD4 or CD8 (A, D, G), low expression of IL-2Rα (B, E, H) or high expression of Pgp-1 (C, F, I). Addition of antibodies against CD3e (A–C), TCR-β (D–F) or TCR-δ (G–H) is indicated by the first arrow, addition of the anti-hamster Ig by the second. The maximal percentage of thymocytes showing a concentration of intracellular Ca2+ above background upon anti-CD3e mAb stimulation was 75% for the CD4+/CD8+ population, 19% for the IL-2Rαβ population, and 4% for the Pgp-1h' population.

**Discussion**

Recent studies have shown that functional rearrangement of the TCR-β locus plays an important role in the development of DP thymocytes (12–17, 25). However, it remains unknown how the TCR-β chain regulates this maturation step. The finding that thymocyte cell lines and thymocytes derived from TCR-β transgenic mice can express an immature TCR-β–CD3 complex on the cell surface without a TCR-α chain lead to the hypothesis that thymocytes require surface expression of and signal transduction through an immature TCR complex in order to mature to the DP stage (15, 17, 19). This hypothesis was supported by the finding that a block in thymocyte development at the DN stage was found in mice deficient for p56lk (26) or overexpressing dominant-negative forms of lck (27) and in CD3γ mutant mice (Terhorst, C., personal communication). Furthermore, in mice overexpressing lck (28), maturation to the DP stage was normal, but a block of rearrangement of the TCR-β locus was detected, probably reflecting the mechanism for allelic exclusion. The latter results corresponded to our observation that addition of anti-CD3e mAb to fetal thymic organ cultures inhibited functional rearrangements of the TCR-β locus while it accelerated induction of DP cells (11).

On the other hand, other previous experiments had indicated that maturation to the DP stage could be induced in TCR-β-deficient thymocytes by transfer of syngeneic TCR+ cells (20). These latter results suggested that maturation to the DP stage did not require direct induction through TCR-β–CD3 but occurred by recruitment as soon as some TCR+ cells had been generated in the thymus.

The studies in TCR-deficient mouse strains like the TCR-δ−, TCR-β−, RAG-1−, or RAG-2− mutant mice did not exclude maturation by recruitment. A complete block in the production of DP cells was observed only in mice without any TCR surface expression (RAG-1− or RAG-2− mutants and the TCR-δ− × TCR-β− double mutant mice); development of some DP cells was observed in mice in which certain types of TCR complexes could be expressed on the cell surface (TCR-α− or β− mutant) (12–17, 29).

In order to study the relative importance of the two possible maturation pathways, we examined the expression of TCR/CD3 components and their signaling capacity in the intrathymic subpopulations during embryogenesis of normal mice. If the surface expression of an immature TCR-β–CD3 complex is required for every thymocyte in order to mature to the DP stage, all DP thymocytes should produce a TCR-β chain. Furthermore, one would expect that all DP cells express TCR-β together with the CD3 complex on the cell surface, and that signal transduction through this immature TCR complex is possible. On the other hand, thymocytes that have matured to the DP stage without a functionally rearranged TCR-β gene locus should be demonstrable by a CD4+ TCR-β IC− or CD8+ TCR-β IC− phenotype.

We detected a sequential expression of components of the CD3 complex before maturation to the DP stage. CD3e was detected already in cells at the Pgp-1h' stage. Because the antibody used in these experiments can only detect CD3e in the presence of a CD3γ or δ chain (30), at least one of these
chains is also expressed at this early stage of maturation. Expression of the CD3\(\beta\) chain was found to coincide with the downregulation of Pgp-1 and acquisition of IL-2R\(\alpha\). We found expression of TCR-\(\beta\) protein in IL-2R\(\alpha^+\) and IL-2R\(\alpha^-\) cells, but not in Pgp-1\(^{1}\) cells. These results indicate that the TCR-\(\beta\) chain is first produced at the IL-2R\(\alpha^+\) stage, and that the components of the CD3 complex are already present at that stage.

From day 17 to 19 of gestation, nearly all CD4\(^+\) or CD8\(^+\) cells expressed the TCR-\(\alpha\) chain intracellularly, stressing the importance of expression of the TCR-\(\beta\) chain for the individual thymocyte to mature to the DP stage. However, we did find some CD4\(^+\) TCR-\(\beta\) IC\(^-\) (2–3\%) and CD8\(^+\) TCR-\(\beta\) IC\(^-\) (2–4\%) cells at all days of gestation tested. We detected a smaller population of TCR-\(\delta\) IC\(^+\) cells in the CD8\(^+\) or CD4\(^+\) populations, suggesting that at least some of the CD4\(^+\) TCR-\(\beta\) IC\(^-\) and CD8\(^+\) TCR-\(\beta\) IC\(^-\) cells are in the \(\alpha\beta\) lineage. These cells may have lost the expression of the TCR-\(\beta\) chain, for example, due to secondary rearrangements, or have matured to the DP stage in the absence of a TCR-\(\beta\) chain. In the latter case, our data indicate that TCR-\(\alpha\) chain expression may not be an absolute requirement for a thymocyte to mature to the DP stage. We envisage that expression of the TCR-\(\beta\) chain induces a new wave of proliferations in the individual thymocyte (3, 26, 27, 31), leading to further differentiation. Spontaneous low-level proliferation may also lead to further maturation, but less effectively so.

In the atrophied thymic environment of rearrangement-deficient mice, spontaneous proliferation and differentiation may be negligible. The presence of TCR-\(\alpha\) cells may contribute to an improved development of the thymic stroma (32, 33), allowing the development of some DP TCR-\(\beta\) IC\(^-\) thymocytes. Reconstitution experiments in the RAG-1- or RAG-2-deficient mice should give a definitive answer to this question.

Using a double layer indirect staining protocol, we detected CD3\(\varepsilon\) and TCR-\(\beta\) on the surface of most thymocytes at day 19 of gestation. A similar finding was previously obtained in DP thymocytes derived from adult mice (34). Because in situ hybridization studies have shown that only \(\sim 50\%\) of all DP cells express the TCR-\(\alpha\) gene (35), this finding indicates that a TCR-\(\beta\)-CD3 complex is expressed on a greater number of DP, including those not yet producing the TCR-\(\alpha\) chain. Furthermore, the calcium mobilization experiments show that a functional CD3 complex is already expressed on the surface of thymocytes at day 16 of gestation in subpopulations expressing intracellular TCR-\(\beta\) chain, i.e., the CD4\(^+\) 8\(^-\) IL-2R\(\alpha^+\) and the CD4\(^+\)/CD8\(^+\) populations. Because at this timepoint expression of the TCR-\(\alpha\) genes is barely detectable (24), it is very likely that the inducible thymocytes express an immature TCR-CD3 complex without the TCR-\(\alpha\) chain.

Anti-TCR-\(\beta\) chain antibodies did only induce a minimal calcium influx in these early thymocyte populations. A similar observation was made in a thymoma cell line expressing an immature TCR-\(\beta\)-CD3 complex (18). This may suggest that induction of calcium mobilization by anti-CD3\(\varepsilon\) mAb requires a TCR-\(\beta\)-CD3 complex whereas induction by anti-TCR-\(\beta\) mAb requires a TCR-\(\alpha\)-CD3 complex. This explains the observation in adult mice, in which we could induce calcium mobilization in most DP thymocytes using anti-CD3\(\varepsilon\) antibodies, but only in 50\% when using anti-TCR-\(\beta\) antibodies, correlating well with the percentage of \(\alpha\beta\)-TCR-\(\beta\) thymocytes. However, in a SCID thymoma cell line transfected with a functionally rearranged TCR-\(\beta\) locus, calcium influx could be induced with anti-TCR-V\(\beta\) mAb (19). Because this cell line shows a higher surface density of the immature TCR-\(\beta\)-CD3 complex, it is more likely that the difference simply depends on the surface density of the TCR complex. In the frame of our hypothesis that the DP stage is induced by ligation of TCR-\(\beta\) we have to assume this occurs by a cellular response not involving calcium influx.

In conclusion, the data demonstrate that nearly all CD4\(^+\) or CD8\(^+\) cells show functional rearrangement of the TCR-\(\beta\) locus. Therefore, we can exclude a major role for recruitment in maturation into the DP stage, initiated by the presence of a few TCR-\(\alpha\) cells. Furthermore we show that apparent TCR-\(\beta\) DP cells in fact express the TCR-\(\beta\) chain on the cell surface together with CD3, and that this TCR complex is capable of signal transduction. Collectively, our data support the hypothesis that surface expression of an immature TCR complex and subsequent signal transduction through this complex regulates maturation to the DP stage.

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