

ROLE OF CD4 IN THYMOCYTE SELECTION AND MATURATION

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Bone marrow-derived precursor T cells differentiate intrathymically into mature T cells under selective forces that are largely unknown. Thymic differentiation is believed to require both "negative" and "positive" selection (1-3). The TCR is clearly involved in the process of negative selection or tolerance induction (1, 4-6). Recently, results from a variety of experimental systems demonstrated that tolerance induction, at least for certain self-antigens expressed in the thymus, occurs through clonal deletion of T cells with self-reactive TCRs (1, 5, 6). Such T cells with TCR specificity for self-antigen appear to be deleted after the double-positive stage ($CD4^+/CD8^+$) of thymocyte development (7-9).

One proposed model used to describe the process of positive selection argues that only T cells with low avidity for self-MHC glycoproteins expressed in the thymus are allowed to become functional and to be exported to the periphery; thus, positively selected (2, 3). This model is largely based on studies with radiation-induced bone marrow chimeras and thymus-engrafted mice, which, given the complexities of such experimental systems, have generated some controversy with respect to the model of positive selection (10, 11).

Additional support for this model came from *in vivo* studies in neonatal mice that demonstrated that MHC glycoproteins play a crucial role during T cell development. $CD4^+$ T cells failed to develop in anti-class II-suppressed mice (12); whereas $CD8^+$ T cells failed to develop in anti-class I-suppressed mice (13). Thus, differentiation of precursor T cells into mature T cells involves selection on the basis of their MHC specificity. Subsequent studies in F_1 neonatal mice (Ia-k/Ia-b) established that the appearance of Th cell precursors restricted by either Ia-k or Ia-b is specifically inhibited by treatment with anti-Ia-k or anti-Ia-b antibodies, respectively (14). These results were consistent with the notion that TCR-MHC interactions mediate the recognition events necessary for positive selection (14). Other experimental support for this notion was provided by studies in transgenic mice (9, 15, 16), and by studies in anti-class I mAb-treated mice (17, 18), or anti-TCR-treated mice (19).

While TCR-MHC interactions are indeed crucial in the positive selection pro-

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cess, other interactions, such as those between MHC antigens and accessory molecules (20, 21), may be essential as well. The present study analyzes the possible role of CD4-ligand interactions in early T cell development. The role of CD4 in T cell activation is by no means clear, but its contribution to the process of T cell selection is suggested by the observation that CD4 is involved in aiding class II MHC-restricted recognition of antigen by mature (CD4⁺) T cells; either through a direct binding of class II MHC (22–26), or by providing an additional signal (27–31).

The concept that CD4 participates at least in negative selection is supported by the recent observation that blocking of CD4 allowed self-reactive T cells to escape clonal deletion at the CD4⁺/CD8⁺ stage, thus permitting the generation of CD8⁺ cells with self-reactive TCRs, which otherwise would not have survived (7, 8). Further evidence for the involvement of accessory molecules during negative selection comes from experiments using transgenic mice, in which autoreactive T cells were shown to be deleted at the double-positive stage (9). Our recent findings show that engagement of the CD4 molecule on thymocytes results in the modulation of the expression of CD3/TCR (32), suggesting a function for CD4 as a signal-transducing molecule in thymocytes. Both avidity enhancement and signal transduction functions of the CD4 molecule may add to the selective forces that T cells encounter during their journey through the thymus.

This paper examines the role of the interactions between CD4 and its ligand in the generation of the T cell repertoire. To this end, the effects of blocking CD4 with an anti-CD4 mAb during T cell development were analyzed *in vivo* and *in vitro*. The *in vivo* experiments involved the treatment of pregnant mice with anti-CD4 mAb, while the fetal thymus organ culture (FTOC)¹ system was used to further analyze the developmental function of the CD4 molecule during intrathymic selection *in vitro* by comparing the effects of monovalent and divalent anti-CD4 mAbs. The results from these studies indicate that treatment with intact, bivalent, or monovalent anti-CD4 mAbs prevent the generation of CD4⁺/CD8⁻ T cells; thus, strongly indicating that CD4 molecules are involved in the positive selection of the T cell repertoire.

Materials and Methods

Mice. Timed pregnant C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The day of observation of a vaginal plug was designated as day 0 of embryonic development. Pregnant female mice were killed by cervical dislocation and embryos dissected from the uterus. Thymus lobes were dissected from the embryos using a dissecting microscope and fine watchmaker's forceps.

Fetal Thymus Organ Culture. Intact fetal thymus lobes were removed from day 15 embryos and placed in organ culture as previously described (33, 34). Briefly, four to six individual fetal thymus lobes (~2–4 mm in between each lobe) were placed on the surface of 0.8- μ m Nucleopore filters (Thomas Scientific, Philadelphia, PA), that had been boiled for 30 min in distilled water before use. Filters were supported by a Gelfoam gelatin sponge (sterile, size 20 × 60 × 7 mm; Upjohn Co., Kalamazoo, MI). Each sponge was placed in a well of a tissue culture plate (No. 3506; Costar, Cambridge, MA) (six wells/plate) and soaked in 3 ml of culture medium. The culture medium used was Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM glutamine, 10 U/ml penicillin, 100 μ g/ml streptomycin, 100 μ g/ml gentamicin, 0.11 mg/ml sodium pyruvate, 5 × 10⁻⁵ M 2-ME, and 10 mM Hepes.

¹ Abbreviation used in this paper: FTOC, fetal thymus organ culture.

Cultures were incubated for 7–9 d at 37°C in a humidified incubator containing 7.5% CO₂ in air. At the end of the culture period, the thymic lobes were mechanically disrupted by placing them on a fine nylon mesh and were squeezed by scraping the nylon mesh surface with the tip of a syringe plunger. The cell suspension was passed through a second fine nylon mesh to ensure a single cell suspension; cells released were free of connective tissue. After washing in cold PBS, cells were resuspended for FACS analysis. Viability of cell suspensions was determined by the trypan blue exclusion test.

Antibodies and Treatments. The anti-CD4 mAb, GK1.5 (35), and the anti-class II mAb, Y3P specific for Ia-b (36), were purified from nude mice ascites by ammonium sulfate precipitation and size separation. For in vitro treatments, either intact, F(ab')₂ or Fab anti-CD4 mAb, or intact anti-class II mAb were added every day during the culture period, in a final volume of 30 µl applied on top of the lobes at a concentration of 0.2–2 mg/ml, depending on the experiment (see legends). F(ab')₂ fragments were prepared as previously described (37), and Fab fragments were prepared according to standard papain digestion protocols (38). The F(ab')₂ and Fab fragment preparations of anti-CD4 mAb were analyzed by SDS-PAGE and shown to contain no detectable intact anti-CD4. Anti-CD4 activity of the fragments was demonstrated by their ability to block staining with FITC- or biotin-conjugated anti-CD4 mAb. Absence of intact anti-CD4 mAb was further demonstrated by the failure of the F(ab')₂ fragments to cause depletion of CD4⁺/CD8⁻ T cells when injected into adult mice, in a regimen leading to depletion when intact anti-CD4 mAb is used (data not shown; see also references 37 and 39). Finally, Fab fragments were shown to be also “functionally” free of intact anti-CD4, by their inability to cause modulation of cell surface CD4; intact anti-CD4, in contrast, causes rapid modulation of CD4 at 37°C (data not shown; see also reference 30).

Controls consisted of saline, once it had been demonstrated that equal doses of irrelevant rat mAb had no effect (data not shown). For in vivo treatments with anti-CD4 mAb, pregnant mice were treated from day 15 of pregnancy with a daily dose of 1 mg GK1.5, i.p., and thymocyte suspensions from babies were analyzed on the day of birth. Controls consisted of saline, once it had been demonstrated that irrelevant rat mAb had no effect (data not shown).

Sorting for CD4⁺/CD8⁺ T Cells. Thymic cell suspensions were enriched for T cells expressing CD4⁺/CD8⁺ by three cycles of panning with mAbs to CD4 and CD8 adsorbed onto a plastic dish surface, as previously described (40); adherent cells (CD4⁺/CD8⁺; >98% purity) were analyzed by flow cytometry for the coexpression of CD4 and CD8.

Fluorescence Staining. Cell suspensions were prepared in HBSS (without phenol red) containing 1% BSA and 0.1% sodium azide (FACS buffer). Cells (10⁶/100 µl buffer) were incubated on ice for 30 min with 10 µl of the appropriate antibody, and washed twice after each incubation. Control staining of cells, either stained with irrelevant antibody or with second antibody alone, were used to obtain background fluorescence values. The samples were analyzed on a FACS 440 (Becton Dickinson & Co., Mountain View, CA) interfaced to a PDP 11/24 computer as previously described (12). Data were collected on 50,000 cells and are shown as contour diagrams, with a three-decade log scale of green fluorescence on the x-axis, and a three-decade log scale of red fluorescence on the y-axis. Reagents used for direct staining were FITC- or biotin-conjugated anti-CD3 (41), -CD4 (35), -CD8 (42), and -CD5 (43). For indirect staining, FITC-conjugated goat anti-rat-IgG was used as described (32).

Results and Discussion

Class II Suppression in FTOC. The FTOC system allows for the in vitro differentiation of immature, CD3⁻/CD4⁻/CD8⁻, day 15–16 murine fetal thymocytes into T cells with a mature phenotype (Fig. 1; control FTOC as compared with fresh thymus; see also reference 44). To analyze whether T cell development in FTOC is subject to selective forces similar to those affecting T cell development in vivo, we examined the effects of blocking class II MHC antigens in vitro. Neonatal class II suppression in vivo results in the failure to generate CD4⁺/CD8⁻ mature T cells (12). FTOCs treated with anti-class II mAb also fail to develop the CD4 single-positive mature

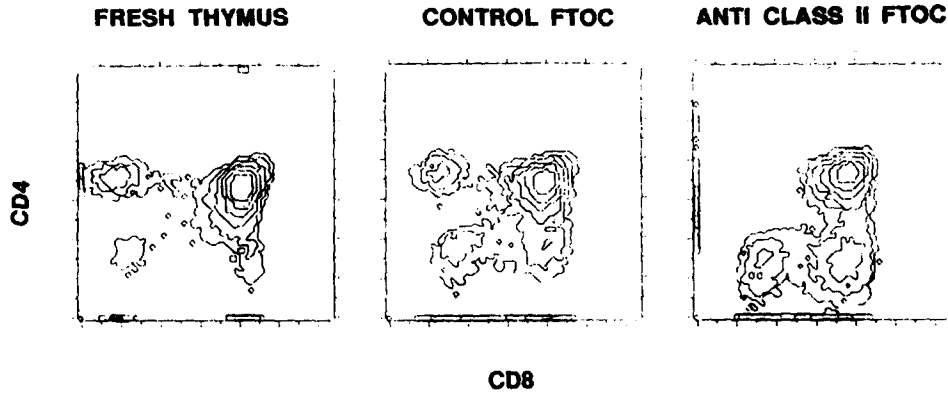


FIGURE 1. Two-parameter flow cytometry analysis of cell surface CD4 vs. CD8 expression on FTOC treated with anti-class II mAb. *Left*, untreated young adult thymus; *middle*, control (untreated) FTOC; *right*, anti-class II-treated FTOC. Day 15 fetal thymuses from C57BL/6 mice were cultured for 8 d; anti-class II-treated cultures received daily additions of 30 μ l of a solution of 2 mg/ml purified anti-Ia(b) mAb (final concentration 20 μ g/ml); control cultures received irrelevant mAb.

T cell population (Fig. 1). These results show a perfect correlation with the results previously obtained in *in vivo* anti-class II-treated mice (12); that is, only the CD4⁺/CD8⁻ T cells fail to mature, and the generation of the other main T cell subsets (CD4⁻/CD8⁻, CD4⁻/CD8⁺, and CD4⁺/CD8⁺) remains intact.

Anti-CD4 Treatment of FTOC. The above observations raise the question of whether these results are a reflection of blocked TCR-class II interaction, blocked CD4-class II interaction, or both. To test the possible contribution of CD4-ligand interactions to T cell development, the effect of anti-CD4 treatment *in vitro* was analyzed. Control FTOCs give rise to bright CD5⁺(Ly1)/CD8⁻ T cells (Fig. 2), which correspond to the presence of CD4⁺/CD8⁻ cells (45). In contrast, organ cultures treated with anti-CD4 mAb fail to develop the bright CD5⁺/CD8⁻ T cell population (Fig. 2). Direct staining for CD4 is complicated by the presence of anti-CD4 mAb in

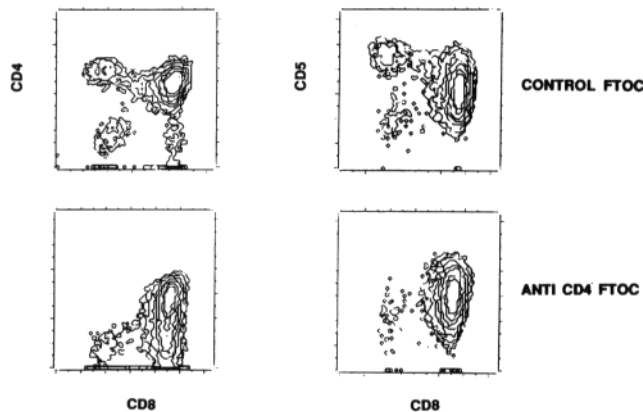


FIGURE 2. Two-parameter flow cytometry analysis of cell surface CD4 (*left*) or CD5 (*right*) vs. CD8 expression on FTOC treated with anti-CD4 mAb. *Top panels*, control (untreated) FTOC; *bottom panels*, anti-CD4-treated FTOC. Day 15 fetal thymuses from C57BL/6 mice were cultured for 8 d; anti-CD4-treated cultures received daily additions of 30 μ l of a solution of 1 mg/ml purified anti-CD4 mAb (final concentration 10 μ g/ml); control cultures received irrelevant mAb.

the FTOC, but still reveals identical findings: no effects on the generation of CD4⁻/CD8⁺ and CD4⁺/CD8⁺ cells are observed. Indirect staining demonstrated the presence of the CD4⁺/CD8⁺ subset, in which CD4 is both blocked and down-modulated due to the presence of the mAb; indirect staining also showed the absence of the CD4⁺/CD8⁻ subset (data not shown). Anti-CD4-treated, as well as control, FTOCs contain a dull CD5⁺/CD8⁻ population that corresponds to CD4⁻/CD8⁻ T cells (45). Additionally, CD5⁺/CD8⁺ cells, corresponding to CD4⁺/CD8⁺ T cells, and CD5⁻/CD8⁺ (CD4⁻/CD8⁺ single positive), are also present in both control and treated FTOC. It therefore appears that the anti-CD4 treatment selectively blocks the development of only the CD4 single-positive T cells. Anti-CD8 mAb treatment has no effect on the generation of CD4⁺/CD8⁻ cells (data not shown), and its effect on other parameters of T cell development is currently being investigated. Selective removal of CD4⁺/CD8⁻ cells by the anti-CD4 mAb seems an unlikely explanation for the present results, since CD4⁺/CD8⁺ cells bind the mAb equally well, yet their generation was not affected.

Anti-CD4 F(ab')₂ or Fab Treatment of FTOC. We next examined the mechanisms by which the anti-CD4 mAb might exert its effects. First, the possible contribution of Fc-mediated processes needed to be considered. The Fc portion may be responsible for the direct activation of the complement system, or removal by macrophages activated through their Fc receptors. The use of F(ab')₂ fragments would exclude these possibilities. Second, the possible contribution of CD4 crosslinking can be tested by comparing the effects of intact vs. Fab mAb treatment. Effective treatment with Fab fragments could not easily be achieved in vivo because the antibody fragment was rapidly cleared before accomplishing its function (data not shown); the FTOC system allowed us to specifically overcome this problem.

Organ-cultured fetal thymic lobes treated with F(ab')₂ fragments (>95% purity as determined by SDS-PAGE analysis) did not give rise to single-positive CD4 T cells (Fig. 3). The results were analogous to those obtained with the intact anti-CD4 mAb (Fig. 2); therefore, Fc-mediated mechanisms cannot be responsible for the absence of the CD4⁺ T cells. Most importantly, the F(ab')₂ fragments used did not cause depletion of CD4⁺/CD8⁻ T cells when injected into adult mice (data not shown), in keeping with reports by others (37, 39). Thus, these results indicate that the effects of the anti-CD4 treatment are either due to the engagement or blocking of CD4, rather than to the direct removal of CD4⁺/CD8⁻ T cells, and are compatible with the observation that CD4⁺/CD8⁺ cells are not removed (see above).

Furthermore, FTOC treatment with Fab fragments of anti-CD4, containing no detectable intact anti-CD4 (>99% purity by SDS-PAGE), generates a population of thymocytes in which the CD4⁺/CD8⁻ are likewise lacking (Fig. 3). The use of Fab fragments precludes the possible effects generated by CD4 crosslinking; indeed, Fab fragments did not modulate CD4, while intact anti-CD4 did (not show). These observations also provide further functional evidence for the lack of contaminating intact anti-CD4 mAb in the Fab fragment preparations. Taken together, these observations suggest that blocking of CD4 results in the failure to generate CD4 single-positive T cells. Also, the observed effects are not related to crosslinking, but, rather, to steric hindrance of crucial interactions requiring the CD4 molecule. Such interactions might involve either an activation-induced physical association between the CD4 molecules and the CD3/TCR complex (27–29, 31), or binding of CD4 to class

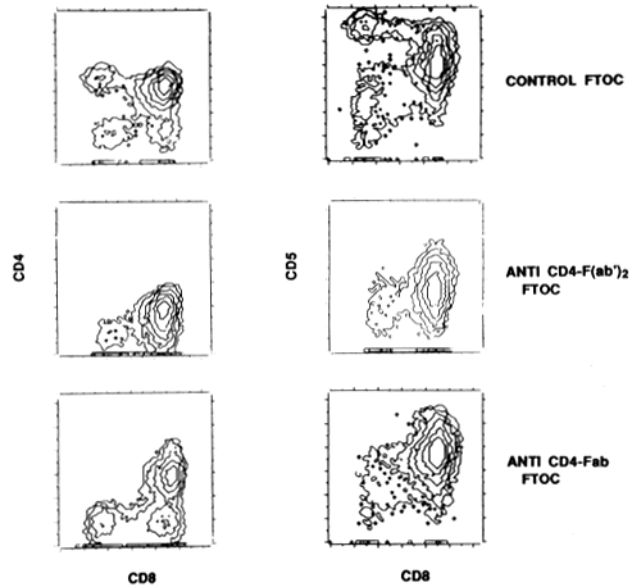


FIGURE 3. Two-parameter flow cytometry analysis of cell surface CD4 (left) or CD5 (right) vs. CD8 expression on FTOC treated with anti-CD4 F(ab)₂ or Fab mAb fragments. *Top panels*, control FTOC; *middle panels*, anti-CD4-F(ab)₂-treated FTOC; *bottom panels*, anti-CD4-Fab-treated FTOC. Day 15 fetal thymuses from C57BL/6 mice were cultured for 8 d; anti-CD4-treated cultures received daily additions of 30 μ l of a solution of 1 mg/ml purified anti-CD4 F(ab)₂ or Fab mAb fragments (final concentration 10 μ g/ml); control cultures received irrelevant mAb.

II MHC (22–26). Our results suggest that anti-CD4-induced inhibition of the appearance of CD4⁺/CD8⁻ cells is related to the anti-CD4 mAb's effect on the function of the CD4 molecule during T cell development. This may result not only in a decrease in avidity between T cells and selecting elements, but also lead to an interference with other yet unknown signals that affect the selection process. While the present data does not distinguish between prevention of positive signals or induction of negative signals, it is clear that such signaling would not require cross-linking. Effects of monovalent anti-CD4 may reflect the prevention of an activation-induced physical association between CD4 and the TCR complex (27–29, 31). Regardless of the mechanism for the observed perturbation in the development of CD4⁺/CD8⁻ cells, these findings provide strong evidence for the conclusion that interactions requiring the CD4 molecule are crucial to the generation of mature single-positive CD4 T cells.

Anti-CD4 Treatment of Pregnant Mice. The above results strongly suggest that engagement of the CD4 molecule plays a major role in the positive selection of CD4⁺/CD8⁻ T cells in vitro. To address the veracity of these in vitro results, pregnant mice were subjected to anti-CD4 treatment.

Clearly, in vivo anti-CD4 treatment generated results similar to those obtained with the FTOC system (Fig. 4, compared with Figs. 2 and 3). Pregnant mice treated with anti-CD4 mAb gave birth to neonates that lacked CD4 single-positive or bright CD5⁺/CD8⁻ T cells in their thymi (Fig. 4); the other phenotypes remained unchanged. Again, it should be emphasized that double-positive T cells develop normally in the treated mice. Together with the observation that, as with the FTOC, F(ab)₂ fragments had identical effects as intact anti-CD4 mAbs (data not shown), these findings exclude the possible effects of Fc-mediated events. Since F(ab)₂ fragments, when injected into adult mice, do not deplete CD4⁺/CD8⁻ T cells (see

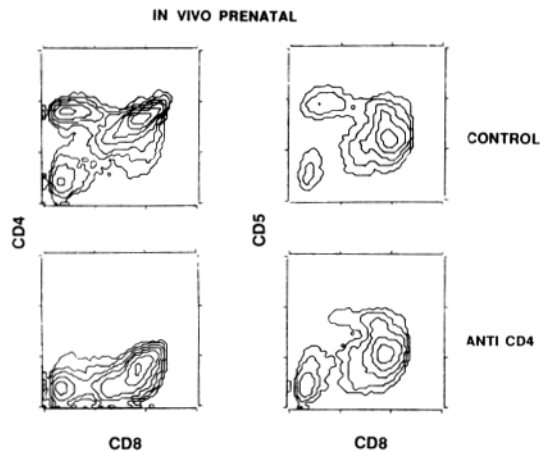


FIGURE 4. Two-parameter flow cytometry analysis of cell surface CD4 (left) or CD5 (right) vs. CD8 expression on neonatal thymocytes from C57BL/6 pregnant mice treated with anti-CD4 mAb. *Top panels*, neonatal thymocytes from control (untreated) pregnant mice; *bottom panels*, neonatal thymocytes from anti-CD4-treated pregnant mice. Day 15 pregnant mice were treated with a daily dose of 1 mg purified anti-CD4 mAb; control mice received irrelevant mAb. Thymocytes were analyzed on the day of birth.

above), these results indeed demonstrate that the absence of CD4 single-positive T cells in fetal thymi subjected to anti-CD4 treatment is not due to depletion, but rather, is caused by a failure of positive selection, attributable to blocked CD4-ligand interactions.

Our results indicate that, *in vitro* and *in vivo*, the CD4 molecule is critically involved in the selection process that leads to the development of mature CD4 single-positive T cells.

CD3/TCR Modulation in Anti-CD4-treated FTOC and Pregnant Mice. The observed effects of anti-CD4 mAb may reflect either its ability to decrease the avidity of the interaction between developing thymocytes and class II MHC-expressing cells (22–27), and/or reflect its ability to provide additional signals upon exposure of thymocytes to anti-CD4. Whether CD4 can also function as a signaling molecule in developing thymocytes is unknown, but our recent findings (reference 32 and below) suggest that it may.

Occupancy of the CD4 molecule by an anti-CD4 mAb results in a substantial increase in CD3 expression in treated fetal thymic organ cultures (Fig. 5 *a*). The CD3 upregulation occurs also *in vivo*; in pregnant mice, anti-CD4 treatments lead to an increased expression of both CD3 and the TCR- α/β (data not shown; see reference 32) in the thymus of the fetus, indicating once more that the FTOC and *in vivo* systems correlated well. Expression of several other cell surface molecules (CD8, Thy-1, and J11D) was not affected, in keeping with our recent report on anti-CD4 effects in neonatal mice (32). As reported before, the increase in CD3 expression occurs primarily in the double-positive (CD4⁺/CD8⁺) cells (data not shown; see reference 32).

We next investigated whether this increase in CD3 is a reflection of a “feedback” mechanism operating to compensate for the loss of bright CD3⁺/CD4⁺/CD8⁻ T cells. Failure to generate CD4⁺/CD8⁻ T cells can be achieved either by anti-class II mAb treatment (Fig. 1, and reference 12), or by anti-CD4 mAb treatment (Figs. 2 and 3). However, treatment with anti-class II mAb did not result in a significant change in CD3 expression (Fig. 5 *d*), while treatment with anti-CD4 mAb does up-

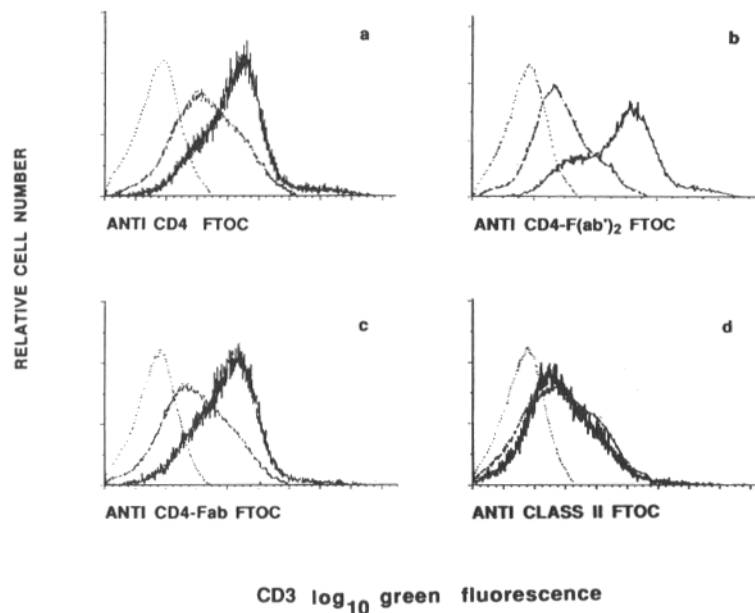


FIGURE 5. Flow cytometry analysis of cell surface CD3 expression on FTOC. (a) Anti-CD4-treated FTOC (solid line), control FTOC (dashed line); (b) anti-CD4 F(ab')₂-treated FTOC (solid line), control FTOC (dashed line); (c) anti-CD4 Fab-treated FTOC (solid line), control FTOC (dashed line); (d) anti-class II-treated FTOC (solid line), control FTOC (dashed line). (a-d) Dotted line indicates staining with irrelevant mAb. Day 15 fetal thymuses from C57BL/6 mice were cultured for 8 d; cultures were treated with 30 μ l of a solution of purified mAb (see Figs. 1-3).

regulate CD3 (Fig. 5 a). Thus, the upregulated CD3 expression is not a consequence of the removal of CD4⁺/CD8⁻ T cells or blocking of thymocyte-class II MHC interactions by anti-class II mAb. Rather, these results indicate that the upregulated CD3 expression is a direct consequence of the binding of anti-CD4 mAb to double-positive thymocytes.

Finally, we compared the effects of intact F(ab')₂ and Fab fragments of anti-CD4 mAb on CD3/TCR upregulation in FTOC. Absence of intact anti-CD4 mAb in these fragments was verified by SDS-PAGE analysis, and by the inability of these preparations to cause depletion of CD4⁺/CD8⁻ T cells through repeated administrations of the fragments to adult mice (data not shown; see also references 37 and 39). Surprisingly, we observed that not only the bivalent anti-CD4, F(ab')₂, mAb, but also the monovalent anti-CD4, Fab, mAb (Fig. 5, b and c) caused CD3/TCR upregulation. This effect was seen with three different preparations of anti-CD4 Fab fragments, all of which contained no detectable intact anti-CD4 mAb, and did not modulate CD4 expression (data not shown). Thus, the CD3/TCR upregulation does not require crosslinking of CD4, but appears to be solely a consequence of blocked CD4-ligand interactions. Alternatively, monovalent (noncrosslinking) engagement of CD4 transduces a signal resulting in CD3 upregulation. Studies are currently in progress to determine whether other parameters reflecting CD4 signaling require crosslinking.

A role for CD4 as a signaling molecule in mature T cells was already suggested

in previous studies (27–31); it has now been extended to neonatal (32) and prenatal (present study) T cells. To what extent this upregulated TCR/CD3 level contributes to the failure to generate CD4 cells is not clear. If the TCR/CD3 complex is functional, one can postulate that CD4⁺/CD8⁻ T cells are not generated because precursors with high levels of TCR/CD3 are “negatively selected.” Alternatively, the failure to generate CD4⁺/CD8⁻ T cells may be solely a consequence of the blocked physiological CD4–class II interactions, and occurs regardless of the increased TCR level. In this interpretation, the immature CD4⁺/CD8⁺ T cells have upregulated their CD3/TCR (after CD4 engagement by the mAb), yet it would appear that this response is insufficient and cannot override the lack of CD4–class II interactions, resulting in a failure to positively select the CD4⁺/CD8⁻ subset. Our data also suggest that positive selection is a consequence of coordinate signalling through a TCR/CD3–CD4 complex. If either component of the complex is blocked (i.e., with anti-TCR, -class II, or -CD4), positive selection fails to occur. Either hypothesis would argue that CD4 signals are crucial to T cell development, and that positive selection is not solely a consequence of TCR–MHC interactions with an appropriate affinity.

Taken together, our results indicate that the effects of the anti-CD4 treatment are twofold; a selective failure to develop CD4⁺ single-positive T cells, under conditions where direct depletion of CD4⁺/CD8⁻ T cells can be excluded, and a CD3 upregulation in double-positive T cells. These results extend the evidence for CD4 as an avidity-enhancing and signal-transducing molecule in mature T cells to a crucial role in the differentiation of immature T cells and the selection of the T cell repertoire.

Summary

We examined the possible role of CD4 molecules during *in vivo* and *in vitro* fetal thymic development. Our results show that fetal thymi treated with intact anti-CD4 mAbs fail to generate CD4 single-positive T cells, while the generation of the other phenotypes remains unchanged. Most importantly, the use of F(ab')₂ and Fab anti-CD4 mAb gave identical results, i.e., failure to generate CD4⁺/CD8⁻ T cells, with no effect on the generation of CD4⁺/CD8⁺ T cells. Since F(ab')₂ and Fab anti-CD4 fail to deplete CD4⁺/CD8⁻ in adult mice, these results strongly argue that the absence of CD4⁺/CD8⁻ T cells is not due to depletion, but rather, is caused by a lack of positive selection, attributable to an obstructed CD4–MHC class II interaction. Furthermore, we also observed an increase in TCR/CD3 expression after anti-CD4 (divalent or monovalent) mAb treatment. The TCR/CD3 upregulation occurs in the double-positive population, and may result from CD4 signaling after mAb engagement, or may be a consequence of the blocked CD4–class II interactions. One proposed model argues that the CD3 upregulation occurs in an effort to compensate for the reduction in avidity or signaling that is normally provided by the interaction of the CD4 accessory molecule and its ligand. As a whole, our findings advocate that CD4 molecules play a decisive role in the differentiation of thymocytes.

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