CD8 IS REQUIRED DURING POSITIVE SELECTION OF CD4$^-$/CD8$^+$ T CELLS

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The immune response by mature peripheral T cells requires the recognition of antigens presented in the context of self-MHC molecules (1, 2). After antigen presentation by class I or class II MHC molecules, specific antigen recognition involves the TCR with the aid of either CD8 or CD4 molecules, respectively (3). These same components, class I and class II self-MHC, TCR, and CD4, are present and have been implicated in the process of intrathymic T cell development and selection, positive and/or negative (4–25), but the function of the CD8 molecule during any of these developmental events is largely unknown.

Early reports demonstrated that MHC molecules play a crucial role during T cell development. In vivo studies in neonatal mice showed that CD4$^+$ T cells failed to develop in anti–class II–suppressed mice (4), whereas CD8$^+$ T cells failed to develop in anti–class I–suppressed mice (5). Thus, differentiation of precursor T cells into mature T cells involves positive selection on the basis of their MHC specificity. TCR-MHC interactions have been postulated to mediate the recognition events necessary for positive selection (6–9). Findings that support this concept are many and varied. Studies in F$_1$ neonatal mice (Ia-k/Ia-b) established that the appearance of Th cell populations restricted by either Ia-k or Ia-b is specifically inhibited by treatments with anti-Ia-k or anti-Ia-b antibodies, respectively (10). Other experimental support for this notion was provided by studies in transgenic mice (11, 12), and by studies in anti–class I mAb–treated mice (13, 14) or anti-TCR-treated mice (15).

TCR-MHC interactions are not the only ones that are crucial to the process of positive selection. Recently, we showed that other interactions, such as those between MHC and accessory molecules, are essential as well (16). These findings demonstrated that anti-CD4-suppressed mice failed to generate CD4$^+$ T cells, suggesting that CD4 molecules are involved in the positive selection of the T cell repertoire.

The concept that MHC, TCR, and CD4 are involved during positive selection also extends to their participation in negative selection. TCR-MHC interactions are clearly involved in the process of negative selection. A variety of experimental systems

demonstrated that tolerance induction, at least for certain self-class I and class II or class II–associated antigens expressed in the thymus, occurs through clonal deletion of T cells with self-reactive TCRs (18, 22). The idea that CD4 participates in negative selection of class II–reactive T cells is supported by the recent observation that blocking of CD4 allowed self-reactive TCR⁺ T cells to escape clonal deletion at the CD4⁺/CD8⁺ stage, thus permitting the generation of CD8⁺ T cells that otherwise would not have survived (23, 24). No such correlates have been established for the involvement of CD8 during negative selection; however, this problem is compounded by the lack of any defined TCR Vβ region that is negatively selected on class I MHC. Thus, the tools for such an analysis are not yet available. Nonetheless, from studies with TCR-transgenic mice expressing a class I–restricted TCR, it appears that CD8 plays a similar role in deletion of class I–restricted T cells as CD4 does for class II–restricted T cells: the deletion spares cells with low CD8 expression, while those with high levels are deleted (25).

This paper addresses the function of the CD8 molecule during the positive selection of CD4⁻/CD8⁺ T cells. To this end, the effects of blocking CD8 with an anti-CD8 mAb during T cell development were analyzed in vivo. The in vivo experiments involved the treatment of pregnant mice and their progeny for up to 2 wk of age with anti-CD8 mAb. The results from these studies indicate that treatments with intact or divalent, F(ab')₂, anti-CD8 mAbs do not deplete, but rather prevent the generation of CD4⁻/CD8⁺ T cells. CD8 molecules are therefore involved in the positive selection of the T cell repertoire.

Materials and Methods

Mice.   Timed pregnant BALB/c and C3H × BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). The day of a vaginal plug was designated as day 0 of embryonic development.

Antibodies and Treatments.   The anti-CD8.2 mAb, 2.43 (26), was purified from nude mice ascites by ammonium sulfate precipitation and sephadex column size separation. Concentrated antibodies were dialyzed against PBS, filter sterilized, and the concentration of the mAb was determined by spectrophotometric absorbance. The F(ab')₂ fragments were prepared as previously described (27). The F(ab')₂ fragment preparations of anti-CD8 mAb were analyzed by SDS-PAGE and shown to contain no detectable intact anti-CD8 mAb. Anti-CD8 activity of the fragments was demonstrated by their ability to block staining with FITC- or biotin-conjugated anti-CD8 mAb. Absence of intact anti-CD8 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 an acute regimen leading to depletion when intact anti-CD8 mAb is used (data not shown).

In Vivo Treatment with Anti-CD8 mAb.   Pregnant mice were treated from day 16–17 of pregnancy with a daily dose of 0.5 mg of 2.43 mAb, i.p., and treatment was continued on their neonatal offspring at a daily dose of 0.2 mg of 2.43 mAb, i.p., for up to 2 wk. 1 d after treatment termination, thymocyte suspensions were analyzed by FACS. Controls consisted of saline injections of equal volume and regimen. For single-positive (CD4⁺ and/or CD8⁺) T cell enrichment, control and treated mice were injected with hydrocortisone acetate (HC)¹ (0.2 mg, i.p.) 2 d before analysis (28). Before FACS, nonviable cells were removed by density gradient separation (Lympholyte-M; Cedar Lane, Ontario, Canada). The viability of cell suspensions was determined by trypan blue exclusion.

Fluorescence Staining (FACS).   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-step 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 (4). 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-: TCR-α/β (29), CD3 (30), CD4 (31), CD5 (32), CD8 (32), CD8.2 (26), and CD8-β (Lyt-3) (33). For indirect staining, FITC-conjugated goat anti–rat IgG was used as described (34).

Results and Discussion

In Vivo Anti-CD8 Treatment. To test the possible contribution of CD8-ligand (class I MHC) (3) interactions during T cell development, the effect of anti-CD8 treatment in vivo was analyzed. Thymocytes from BALB/c mice that have been treated with anti-CD8 mAb pre- and postnatally became saturated with the specific mAb, and, most importantly, the majority of CD8-expressing cells remained present (data not shown), suggesting that CD8$^+$ thymocytes had not undergone cytotoxic depletion.

To address the phenotypic changes induced by the blocking of the CD8 molecule, a wide spectrum of cell surface markers were analyzed. First, staining for CD8 and CD4 was performed. Fig. 1 *a* shows that in the anti-CD8 mAb–treated thymi, the staining with directly labeled CD8 FITC is blocked, demonstrating total saturation of the CD8 molecule.

The CD8 molecule is composed of two chains, an α chain (Lyt-2) and a β chain (Lyt-3) (33, 35). Our treatments were directed to the α chain; thus, we can visualize the presence of CD8$^+$ T cells by staining for the CD8 β chain. The antibody used for anti-CD8 treatment (2.43, anti-CD8.2, previously known as Lyt-2.2) (26) does not crossblock the staining for CD8-β (53-5.8, anti-Lyt-3) (33) (data not shown). Fig. 1 *b* shows that staining for CD8-β allows for the visualization of CD4$^+$/CD8$^+$ (double-positive) T cells in anti-CD8-treated mice, albeit the CD8 molecules are downmodulated, and also suggests an absence of CD4$^-$/CD8$^+$ T cells.
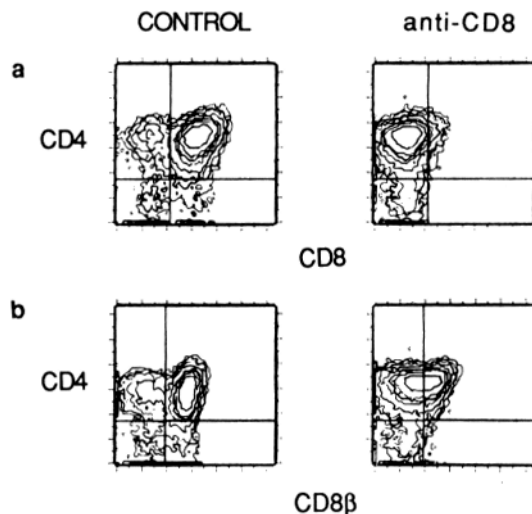

FIGURE 1. Two-parameter flow cytometry analysis of cell surface expression of (*a*) CD4 vs. CD8 (Lyt-2) or (*b*) CD4 vs. CD8-β (Lyt-3) on thymocytes from control and anti-CD8-treated groups. Timed pregnant BALB/c mice and their offspring were treated with 2.43 mAb. Intraperitoneal injections of 0.5 mg were given daily from day 16 of pregnancy and continued at 0.2 mg for 12 d after birth. Thymocytes were analyzed on day 13 after birth.

To further analyze the presence of $CD4^+/CD8^+$ and absence of $CD4^-/CD8^+$ T cells in the anti-CD8-treated thymi, we analyzed the level of CD3 expression on $CD4^+$ and $CD4^-$ T cells. Fig. 2 *a* shows that control thymi contain $CD4^+$ T cells, which are $CD3^-$ (corresponding to $CD4^+/CD8^+/CD3^-$), CD3 dull ($CD4^+/CD8^+/CD3^+$), and CD3 bright ($CD4^+/CD8^-$). These thymi also contain $CD4^-$ T cells that are $CD3^-$ ($CD4^-/CD8^-$) and $CD3^+$ ($CD4^-/CD8^+$, perhaps also some $CD4^-/CD8^-$). Clearly, after anti-CD8 treatment the only population that is altered is the $CD4^-$ T cells that are $CD3^+$ ($CD4^-/CD8^+$). No significant changes are observed in the $CD4^+/CD8^-/CD3^+$ population, nor in the expression of CD3 (dull) on the $CD4^+/CD8^+$ T cells. The first observation is at odds with earlier work in which anti-CD8 prevented the generation of $CD4^+$ T cells (36). However, mixed allogeneic tetraparental chimeras were used in those studies, such that graft-vs.-graft reactions, rather than blocking of development, may have caused this subset's elimination. The second observation shows that contrary to the effects of anti-CD4 on CD3 expression after pre- or postnatal engagement (16, 34), anti-CD8 has no effects on the expression of CD3 on the dull double-positive population. This observation has now been confirmed with saturating doses of three different anti-CD8 mAbs (data not shown).

Next, by using the CD5 marker (Ly-1), we could clearly visualize, in the control thymus, a population of $CD5^+/CD4^-$ T cells (corresponding to $CD8^+$) (37) (Fig. 2 *b*); this population was absent after the anti-CD8 treatment, while the other populations remained unchanged.

Taken together, these findings demonstrate that treatment with anti-CD8 mAb affects only the presence of the $CD3^+/CD4^-/CD5^+/CD8^+$ T cells, and has no effect on the generation of the other major thymic phenotypes (Figs. 1 and 2). Most significantly, double-positive T cells developed normally in the treated groups; thus, these results argue that the absence of $CD4^-/CD8^+$ T cells is not due to a depletion of $CD4^+/CD8^+$ precursor T cells.
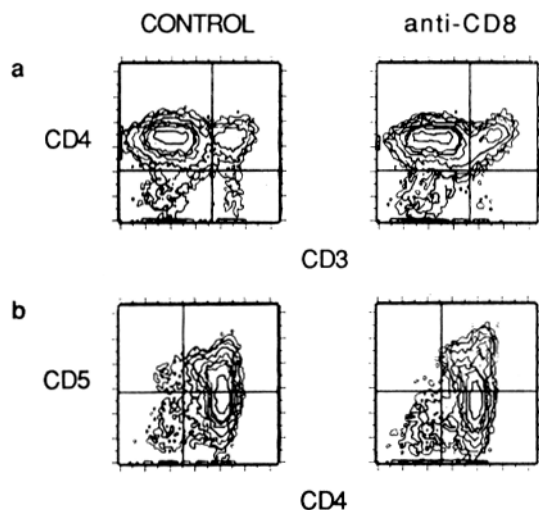

FIGURE 2. Two-parameter flow cytometry analysis of cell surface expression of (*a*) CD4 vs. CD3 or (*b*) CD5 vs. CD4 on thymocytes from control and anti-CD8-treated groups. Timed pregnant BALB/c mice and their offspring were treated with 2.43 mAb. Intraperitoneal injections of 0.5 mg were given daily from day 16 of pregnancy and continued at 0.2 mg for 12 d after birth. Thymocytes were analyzed on day 13 after birth.

To better visualize the presence or absence of the CD8$^+$ T cell population, control and treated groups were subjected to HC treatment 2 d before analysis (28). HC enriches for single-positive mature T cells (Fig. 3 a; reference 38). Again, anti-CD8-treated thymi showed no staining with directly labeled CD8 FITC (Fig. 3 a). This lack of positive staining was not due to saturation and/or blocking of the CD8 molecule, but rather due to a complete absence of the CD8$^+$ T cell population, as shown by the lack of CD8-β^+ T cells as well (Fig. 3 b).

Finally, we examined the mature thymocytes (HC thymus) for the expression of CD5 and TCR on the CD4$^+$ or CD4$^-$ population. This allows for the visualization of the two main populations in an indirect fashion (Fig. 4 a), so as to avoid
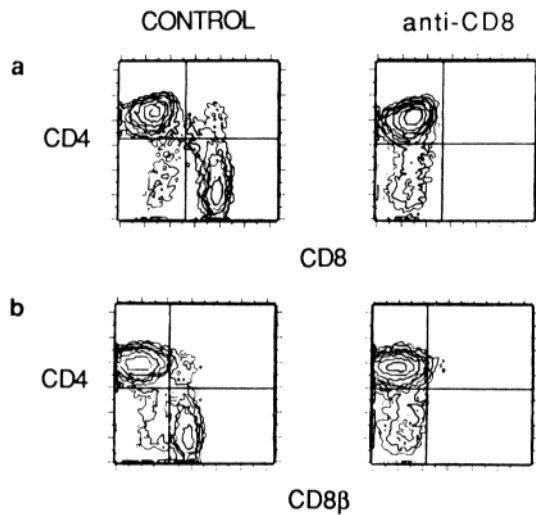
FIGURE 3. Two-parameter flow cytometry analysis of cell surface expression of (a) CD4 vs. CD8 or (b) CD4 vs. CD8-β on thymocytes from HC-treated control and anti-CD8-treated groups. Timed pregnant BALB/c mice and their offspring were treated with 2.43 mAb. Intraperitoneal injections of 0.5 mg were given daily from day 16 of pregnancy and continued at 0.2 mg for 12 d after birth. Thymocytes were analyzed on day 13 after birth. Control and anti-CD8-treated mice were injected with 0.2 mg, i.p., of HC 2 d before analysis.

FIGURE 4. Two-parameter flow cytometry analysis of cell surface expression of (a) CD5 vs. CD4 or (b) CD4 vs. TCR-α/β on thymocytes from HC-treated control and anti-CD8-treated groups. Timed pregnant BALB/c mice and their offspring were treated with 2.43 mAb. Intraperitoneal injections of 0.5 mg were given daily from day 16 of pregnancy and continued at 0.2 mg for 12 d after birth. Thymocytes were analyzed on day 13 after birth. Control and anti-CD8-treated mice were injected with 0.2 mg, i.p., of HC 2 d before analysis.
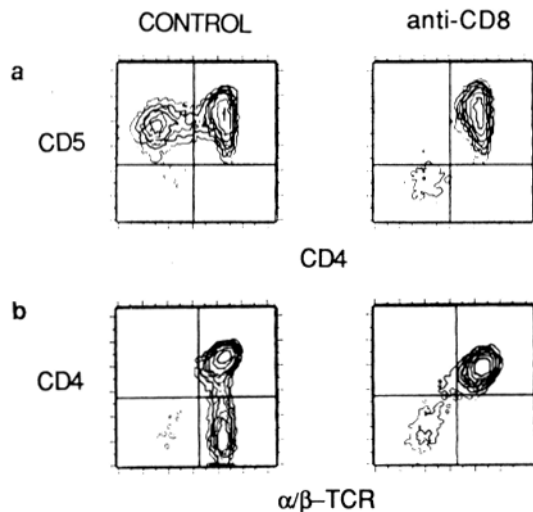
any problems with antibody blocking. In the control thymi, $CD5^+/CD4^-$ correspond to $CD8^+$ T cells, while $CD5^+/CD4^+$ correspond to $CD4^+$ T cells (37). Fig. 5 a shows that the $CD8^+$ T cell population ($CD5^+/CD4^-$) is completely absent in the anti-CD8-treated mice. The failure to generate the $CD8^+$ T cells can also be demonstrated by examining the expression of TCR-α/β on HC thymi. The control thymi contain two well-defined populations of TCR^+ (bright) cells (Fig. 4 b), one in the $CD4^+$ subset and one in the $CD4^-$ subset ($CD8^+$). The $CD4^-/TCR^+$ ($CD8^+$) T cells are clearly absent in the treated group, thus, confirming the failure to generate such T cells when the CD8 molecule is blocked during T cell development.

In Vivo Anti-CD8 F(ab')$_2$ Fragment Treatment. The absence of $CD4^-/CD8^+$ T cells after anti-CD8 treatment suggests a role for the CD8 molecule during the positive selection of $CD8^+$ T cells. Selective removal of $CD4^-/CD8^+$ T cells by the anti-CD8 mAb seems an unlikely explanation for the present results, since $CD4^+/CD8^+$ cells bind the mAb equally well, yet their generation was not affected (Figs. 1 and 2). Nonetheless, we examined the mechanisms by which the anti-CD8 might exert its effects. Most importantly, the possible contribution of Fc-mediated processes needed to be considered. These processes include lysis of cells by direct activation of the complement pathway, and/or removal by macrophages activated through their Fc receptors. The use of F(ab')$_2$ fragments would exclude these possibilities (27). The possible contribution of CD8 multivalent crosslinking can also be obviated by using F(ab')$_2$ fragments, which can only form divalent interactions with the CD8 molecule. Most importantly, the anti-CD8 F(ab')$_2$ fragments used did not cause depletion of $CD4^-/CD8^+$ T cells when injected into adult mice in a short term acute treatment protocol (data not shown; see also references 16 and 27).

In vivo pre- and postnatal treatment with F(ab')$_2$ fragments (>98% purity as determined by SDS-PAGE analysis) saturated and blocked the expression of CD8 on thymocytes (data not shown). More significantly, treatment with anti-CD8 F(ab')$_2$ fragments prevented the development of $CD4^-/CD8^+$ T cells. Fig. 5 a shows a lack of CD8-β^+ T cells in HC thymi from treated mice. The specificity of this effect is demonstrated by the observation that the generation of the other major T cell subsets, i.e., $CD4^+/CD8^-$ and $CD4^+/CD8^+$ cells (data not shown), are not affected (Fig. 5 a). The failure to generate $CD8^+$ T cells after blocking the expression of CD8 can also be demonstrated by using other markers: absence of $CD5^+/CD4^-$ and $TCR^+/CD4^-$ T cells (Fig. 5, b and c) confirms the finding that the $CD4^-/CD8^+$ T cells fail to develop when CD8 is blocked, even under circumstances where Fc-mediated mechanisms can be ruled out.

Finally, in order to distinguish between cytotoxic removal or developmental blockade of $CD4^-/CD8^+$ cells, we took advantage of the existence of two allelic forms of the CD8 molecule (33). BALB/c mice express the CD8.2 allele, while C3H mice express the CD8.1 allele (26), and in heterozygous (C3H × BALB/c)F$_1$ mice, both alleles are codominantly expressed (32, 33; data not shown). Anti-CD8.2 treatment of such (C3H × BALB/c)F$_1$ mice should block only the CD8.2 molecule, but leave the expression of the CD8.1 molecule intact. Indeed, anti-CD8.2 F(ab')$_2$ mAb treatment of perinatal (C3H × BALB/c)F$_1$ mice saturated all CD8.2 molecules on $CD8^+$ cells, while the expression of CD8.1 molecules was not affected (data not shown). It can therefore be predicted that, if interactions between CD8 and its ligand are a requirement for development, the remaining CD8.1 molecules should fulfill these re-
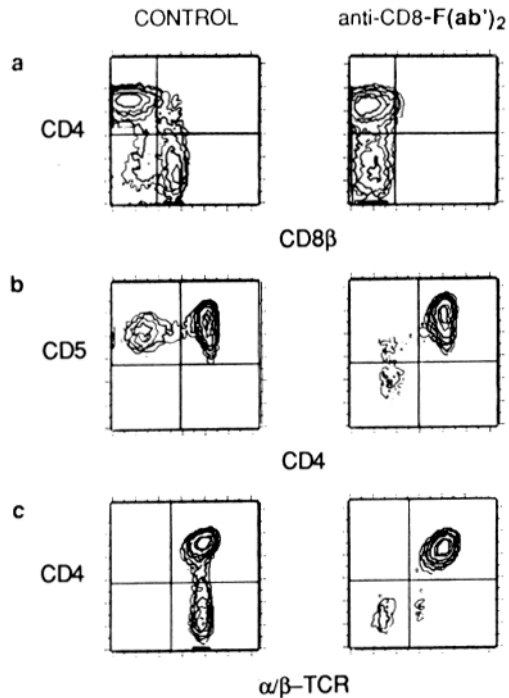
CONTROL   anti-CD8-F(ab')₂

FIGURE 5. Two-parameter flow cytometry analysis of cell surface expression of (a) CD4 vs. CD8-β, (b) CD5 vs. CD4, or (c) CD4 vs. TCR-α/β on thymocytes from HC-treated control and anti-CD8-F(ab')₂-treated groups. Timed pregnant BALB/c mice and their offspring were treated with 2.43 mAb. Intraperitoneal injections of 0.5 mg were given daily from day 16 of pregnancy and continued at 0.2 mg for 12 d after birth. Thymocytes were analyzed on day 13 after birth. Control and anti-CD8-treated mice were injected with 0.2 mg, i.p., of HC 2 d before analysis.

quirements. Indeed, the generation of CD4⁻/CD8⁺ cells in CD8.1/CD8.2 heterozygous mice was mostly unaffected by anti-CD8.2 treatment (Fig. 6, a–c), in striking contrast with the situation in CD8.2 homozygous mice, in which anti-CD8.2 treatment resulted in a complete lack of generation of CD4⁻/CD8⁺ T cells (Figs. 5 and 6). Most likely, the generation of CD4⁻/CD8⁺ cells in the heterozygous mice results from interactions between the unblocked CD8.1 allele and its ligand, which provides the necessary signals for differentiation. These results firmly eliminate the possibility that the absence of CD4⁻/CD8⁺ cells in the anti-CD8-treated homozygous mice was due to direct cytotoxic depletion, since such elimination would also have occurred in heterozygous mice. Additionally, these data imply that the failure to generate CD4⁻/CD8⁺ T cells was not a consequence of negative signals (39, 40), nor over induction of positive signals (39–44), resulting from the mAb engagement of CD8 molecules on differentiating thymocytes.

Taken together, these data clearly indicate that the generation of CD4⁻/CD8⁺ T cells is prevented when CD8 molcules are blocked during early development. In heterozygous F₁ mice, where only one of the two CD8 alleles is blocked, the generation of CD4⁻/CD8⁺ T cells proceeds undisturbed, while treatment with nondepleting F(ab')₂-anti-CD8 mAb does block the development of CD4⁻/CD8⁺ T cells in homozygous mice. These results demonstrate that the absence of CD4⁻/CD8⁺ T cells is not due to a depletion CD8⁺ T cells, but rather is caused by a lack of positive selection. This lack of positive selection is most likely attributable to an obstructed CD8-MHC class I interaction, and is presumably occurring at the double-positive
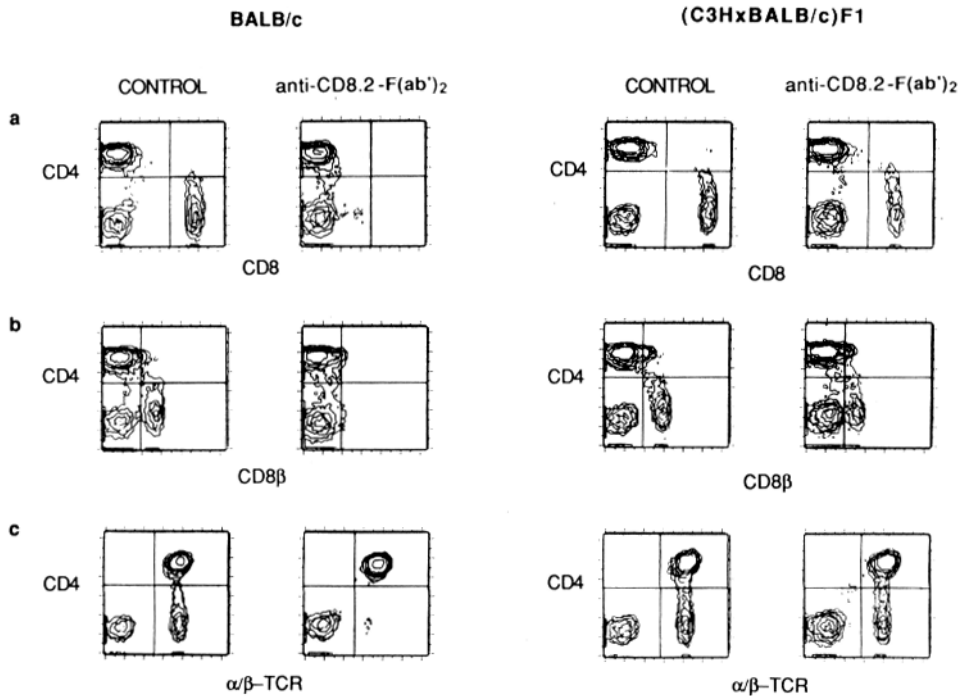
Figure 6.   Two-parameter flow cytometry analysis of cell surface expression of (a) CD4 vs. CD8, (b) CD4 vs. CD8-β, or (c) CD4 vs. TCR-α/β on thymocytes from HC-treated BALB/c or (C3H × BALB/c)F₁ control and anti-CD8-F(ab')₂-treated groups. Timed pregnant BALB/c or C3H mice (mated to BALB/c males) and their offspring, BALB/c or (C3H × BALB/c)F₁, respectively, were treated with 2.43 mAb. Intraperitoneal injections of 0.5 mg were given daily from day 16 of pregnancy and continued at 0.2 mg for 12 d after birth. Thymocytes were analyzed on day 13 after birth. Control and anti-CD8-treated mice were injected with 0.2 mg, i.p., of HC 2 d before analysis. In both treated groups, BALB/c and (C3H × BALB/c)F₁, the same anti-CD8.2 F(ab')₂ mAb batch, schedule, and dosage was used.

transition stage, since the development of CD4⁺/CD8⁺ T cells is not affected (Figs. 1 and 2).

Summary

Interactions between self-MHC molecules and T cells are necessary for the proper development of mature T cells, in part due to an absolute requirement for self-MHC-TCR interactions. Recently, we showed that CD4-mediated interactions also participate in shaping the T cell repertoire during thymic maturation. We now examine the possible role of the CD8 molecule during in vivo T cell development.

Our results demonstrate that perinatal thymi treated with intact anti-CD8 mAb fail to generate CD8 single-positive T cells, while the generation of the other main phenotypes remains unchanged. Most importantly, the use of F(ab')₂ anti-CD8 mAb fragments gave identical results, i.e., lack of generation of CD4⁻/CD8⁺ cells, with no effect on the generation of CD4⁺/CD8⁺. Furthermore, selective blocking of one CD8 allele with F(ab')₂ mAbs in F₁ mice expressing both CD8 alleles did not interfere with the development of CD4⁻/CD8⁺ cells, demonstrating that the ab-

sence of CD8$^+$ T cells in homozygous mice is not due to depletion, but rather is caused by a lack of positive selection. This is most likely attributable to a deficient CD8-MHC class I interaction. Our findings strongly advocate that CD8 molecules are vital to the selection process that leads to the development of mature single-positive CD8 T cells.

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