Thymocyte Differentiation in Fetal Thymus Organ Culture

胎仔胸腺臓器培養法による胸腺細胞分化の解析

松橋信行

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ABSTRACT

During the course of differentiation in the thymus, precursor T-cells are negatively selected by self tolerance mechanisms or positively selected to acquire restriction specificity to self major histocompatibility complexes. In the present study the process of T-cell differentiation and those selections were investigated by means of fetal thymus organ culture (FTOC). Effects of cyclosporin A (CsA) on thymocyte differentiation were first investigated because CsA is known to block the signaling cascade initiated by TCR cross-linking. Effects of IL-7 on FTOC were then investigated because IL-7 has recently been reported to have a co-stimulatory activity on T-lineage cells, although the agent was identified as a pre-B cell growth factor. Finally, self-tolerogenicity of thymocytes was examined using FTOC. The first and second series of the experiments were concerned with the positive selection of thymocytes, while the second and third ones focused on the mechanism of negative selection.

CsA inhibited the development of $CD4^+8^-$ cells, but not of $CD4^-8^+$ cells. However, those $CD4^-8^+$ cells were shown to be immature $CD4^-8^+$ cells which had developed from $CD4^-8^-$ double negative (DN) cells and would develop into $CD4^+8^+$ double positive (DP) cells. Thus, CsA blocked the maturation step from DP cells to mature $CD4^-8^+$ or $CD4^+8^-$ single positive (SP) cells. On the other hand, the agent did not inhibit the development of $CD3^+4^-8^-TCR\alpha\beta^-$ cells, which were supposed to be T-cells bearing $\gamma\delta$-TCR chains.

These results suggest that the development of αβ- and γδ-thymocytes differ in the requirement for thymocyte-stromal cell interactions. IL-7 supported the proliferation of a wide range of T-lineage cells. FTOC in the presence of IL-7 generated DN cells, CD4 or CD8 SP cells, and very few DP cells. The DN cells developing in the presence of IL-7 were $CD3^+4^-8^-\alpha\beta^-$, and were supposed to be γδ-thymocytes. CD8 SP cells developing therein were mature $CD3^+4^-8^+\alpha\beta^+$ cells, which suggested that IL-7 acts as a lineage-specific growth/differentiation factor of $CD8^+$ αβ-T cells. The mechanism of the reduction of DP cells was investigated, but the reduction was not found to be due to induction of non-specific killer cells which could have developed in the presence of IL-2. Three possibilities for this were considered. First, IL-7 may block the maturation step from immature $CD4^-8^+$ to $CD4^+8^+$ cells. Second, IL-7 may facilitate the process of the maturation process from DP to SP cells. And third, it may facilitate apoptosis of DP cells. In order to elucidate whether thymocytes have tolerizing ability in the thymus, thymic chimeras were constructed in vitro by colonizing Thy-1 bright fetal thymocytes of A mice into 2'-deoxyguanosine-treated fetal thymus of B mice. Allo-killer activity of thymocytes developing in those lobes were examined. These cells were tolerant of class I, but not of class II MHC of A mice. This indicates that Thy-1-positive cells can tolerize thymocytes themselves to self class I MHC.

ABBREVIATIONS: FTOC, fetal thymus organ culture; CsA, cyclosporine A; IL, interleukin; Con-A, Concanavalin-A; LPS, lipopolysaccharide; PHA, phytohemagglutinin; MHC, major histocompatibility complex; TCR, T cell receptor; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PE, phycoerythrin; CTL, cytotoxic T lymphocyte; DN, double negative; DP, double positive; SP, single positive; GD, gestational day; GD15+n, GD 15 fetal thymus lobes organ-cultured for n days; dGuo, 2'-deoxyguanosine; dGuo-BALB/c$\leftarrow$ Thy-1^{+}-B10, dGuo-treated BALB/c fetal thymuses colonized with Thy-1 bright B10 fetal thymocytes.

INTRODUCTION

Thymus, which is composed of stromal cells and precursor T-cells, that is, thymocytes, is known to play a crucial role in the T-cell development. During the development in the thymus, self-reactive thymocytes are deleted and self-MHC-restricted thymocytes are positively selected. Little is known about the mechanism underlying this action. However, accumulating evidence has indicated that a signal triggered by the interaction between the T-cell receptor (TCR) on the thymocyte and the major histocompatibility complex (MHC) molecule on thymic stromal cells is essential in the differentiation process of thymocytes.

To investigate the mechanism of the T-cell development in the thymus, we utilized the fetal thymus organ culture (FTOC) established by Jenkinson et al (1). Phenotypically and functionally normal thymocyte differentiation have been demonstrated to proceed in FTOC (2,3). FTOC has three principal advantages in such investigations. First, it can be performed in the presence of a desired concentration of agents that may affect T-cell development. Second, since no stem cells migrate in, synchronized differentiation of thymocytes can be observed. Third, it can be repopulated with desired cell populations.

Several investigators including us have utilized antibodies specific for MHC class I (4), class II (2,5,6), or CD4 (7) to demonstrate that these molecules are involved in T-cell development. Since cyclosporine A (CsA) is known

to block the signal transduction cascade (8,9) and to induce thymus atrophy when administered in vivo (10,11), we used CsA in FTOC in an attempt to block the T-cell differentiation. The agent has been reported to abrogate the generation of $CD4^+8^-$, but not that of $CD4^-8^+$ thymocytes in FTOC (2). Since both CD4 and CD8 single positive (SP) cells are known to be positively selected in the thymus (12-16), we wished to elucidate the reason why $CD4^-8^+$ cells remained in the thymus in the presence of CsA.

What factor is essential in the thymocyte-stromal cell interaction has not yet been elucidated. Known factors such as IL-2, IL-4, IL-1, IL-6, or IFN-γ, alone or in combination, cannot induce the normal thymocyte differentiation. In 1988, interleukin 7 (IL-7) was identified and cloned from a bone marrow stromal cell line as a pre-B cell growth factor (17,18). A Northern blot analysis revealed that the thymus presents an abundant IL-7 message (18). This implies that IL-7 may play a role in T-cell development, which is produced by the thymic stromal cells. We therefore investigated the effects of IL-7 on T-lineage cells.

Little is also known about the mechanism behind negative selection. The negative selection has been demonstrated to take place at, or immediately before or after the $CD4^+8^+$ double positive (DP) cell stage (7,19,20). However, it remains controversial as to which cell population in the thymus can tolerize thymocytes to self-antigens. It has been reported that 2'-deoxyguanosine

(dGuo)-sensitive cells are responsible for the negative selection, while dGuo-resistant cells induce positive selection (21-25). Since dGuo deletes hematopoietic cells such as thymocytes, macrophages, or dendritic cells (1, 26), those cells, especially macrophages or dendritic cells, are assumed to be the tolerizing cells. In a recent report, Matzinger et al. have demonstrated that negative selection was carried out by dendritic cells (27). However, the possibility is not ruled out that other cell populations among dGuo-sensitive cells may induce self-tolerance. In the present report, we colonized Thy-1 bright DN thymocytes into allogeneic thymus lobes, and evaluated the tolerogenicity of class I molecules on the donor cells.

MATERIALS AND METHODS

Mice

C57BL/10 (B10), B10.BR, B10.D2, and BALB/c mice were bred in our colony at the National Institute of Radiological Science, Chiba, Japan. Each strain is known to express an F23.1 determinant of TCR.

Medium

Alpha MEM medium was supplemented with penicillin-streptomycin, 2mM L-glutamine, 15mM HEPES, $5x10^{-5}$M 2-mercaptoethanol, 1% anti-PPLO agent (Gibco, Chargin Falls,

Ohio), and 10% heat-inactivated fetal calf serum (complete medium).

Reagents

CsA (Sandoz Co. Ltd, Basel, Switzerland) was dissolved in methanol at $1x10^{-3}$ M, and at the time of use, it was further diluted with medium to $1x10^{-7}$M, a concentration sufficient to inhibit allospecific cytotoxic T lymphocyte (CTL) induction or alloreactive mixed lymphocyte reaction (8). As a control, methanol was diluted at the same concentration and added into FTOC. Monoclonal antibodies (mAb) RL172.4 (rat mAb to CD4, reference 28), GK1.5 (rat mAb to CD4, reference 29), F23.1 (mouse mAb to $V\beta 8$, reference 30), 145-2C11 (hamster mAb to $CD3_{\varepsilon}$, reference 31), and J11d (rat mAb to J11d, reference 32), H57-597 (hamster mAb to all TCR-$\alpha\beta$ chains, reference 33), 10-2.16 (mAb to $I\text{-}A^{k}$, reference 34), and M5/114 (mAb to $I\text{-}A^{b}$ and cross-reactive to $I\text{-}E^{k}$, reference 35) were used. Murine recombinant IL-7 was supplied by Dr. N. Minato, and was used at the concentration of 200 u/ml. 2'-deoxyguanosine (dGuo) was purchased from Sigma Chemical Co. (St. Louis, MO).

Fetal thymus organ culture

Organ culture was performed according to the methods of Jenkinson et al (1), with slight modification. In brief, thymus lobes from fetuses of 15th or 16th gestational day (GD15 or 16) were cultured on polycarbonate filters (0.8

micrometer pore size, Nuclepore, Pleasanton, CA) floated on 4 ml of culture medium in a 100-mm tissue culture dish. Medium was exchanged every 4 days.

Depletion of CD4 positive cells

In order to obtain $CD4^-8^+$ and $CD4^-8^-$ cells, thymocytes were cytotoxically treated with anti-CD4 monoclonal antibodies (GK1.5 and RL172.4) and guinea pig complement (Cedarlane Co., Hornby, Canada). Cells were incubated with anti-CD4 for 30 minutes on ice, washed, and then incubated in complement solution (1:6 dilution) without serum for 60 minutes. These procedures were repeated twice in order to deplete $CD4^+$ cells effectively.

Cell surface marker analysis

For CD8/CD4 analysis, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 and phycoerythrin (PE)-conjugated anti-CD4 antibodies (Becton Dickinson, Mountain View, CA). For CD8/TCR-$\alpha\beta$ chains or CD8/CD3 analysis, cells were sequentially incubated with either F23.1, H57-597, or 145-2C11, biotin-conjugated goat anti-mouse IgG (Cappel, Westchester, PA), normal mouse serum to saturate goat anti-mouse IgG reactivity, and a mixture of PE-conjugated streptavidin (Becton Dickinson) and FITC-conjugated anti-CD8. We used biotin-conjugated goat anti-mouse IgG as the second reagent for staining with 145-2C11 or H57-597 because anti-mouse IgG cross-reacted well with

hamster IgG. For CD8/J11d analysis, J11d, goat anti-rat IgG biotin (E.Y Lab. San Mateo, CA), and normal rat serum were used. Stained cells were analyzed on FACStar (Becton Dickinson). Positive-negative demarcations were made according to an autofluorescence profile of the same cells as a negative control.

Allospecific CTL assay

Allospecific CTL were induced as follows. Thymocytes were harvested from FTOC, and resultant cells ($3x10^5$cells/well) were cultured for 5 days in the presence of 30Gy-irradiated allogeneic spleen cells ($3x10^6$/well) and 1.25% of EL-4 culture supernatant (interleukin 2 source) in a 24-well plate. As class I-expressing allogeneic targets, Con-A-induced T-cell blasts were prepared by culturing spleen cells of allogeneic mice for two days in the presence of 3 μg/ml of Con-A. Class II-expressing B cell targets were prepared as follows. Allogeneic spleen cells were depleted of T-cells by treating them with anti-BAT (brain-associated theta) antibody (reactive with all T-cells, in ascitic form, 1:40 dilution, supplied by Dr. Tomio Tada, University of Tokyo) and guinea pig complement (1:6 dilution), and the resultant cells were cultured for two days in the presence of 10μg/ml of lipopolysaccharide (LPS). Responder cells were harvested, and recovered live cells were mixed with ^{51}Cr-labeled targets in round-bottomed 96-well plates for 4 hours. The ^{51}Cr activity released from the target cells was measured by a gamma-counter, and %

specific ^{51}Cr release was calculated as follows: 100x(E-S)/(M-S), where E, S, and M represent experimental, spontaneous, and maximal ^{51}Cr release, respectively. Maximal ^{51}Cr release was induced by adding 0.2% sodium deoxycholate. Blocking experiments were performed by adding anti-Ia^k (10-2.16 (anti-I-A^k) plus M5/114 (anti-I-E^k), each in ascitic form, at the dilution of 1:200) or anti-I-E^k alone at the effector phase of the killer assay.

Cell proliferation assay

Thymocytes of 4-day-old mice were cultured in the presence of PHA and IL-2, IL-4, or IL-7, and pulse labeled with 10 μCi of ^{3}H-thymidine. The cells were harvested with an automated cell harvester, and the ^{3}H-thymidine incorporation was measured. The cell number was counted in another aliquot of cell suspension.

PHA-mediated cytotoxicity assay

Thymocytes recovered from IL-7-added FTOC were examined for their killer activity by a PHA-mediated cytotoxicity assay. EL-4 and YAC-1 cells were used as targets in the presence of 1% PHA. Percent specific Cr-release was calculated as mentioned above.

FTOC of chimeric thymuses

GD15 BALB/c fetal thymuses were cultured in FTOC in

the presence of 1.35mM dGuo for 3 days. Such a treatment partially deletes hematopoietic cells such as macrophages, dendritic cells, and thymocytes (data not shown). Then the lobes were cultured in the absence of dGuo for 24 hours. Unsorted allogeneic fetal thymus cells or sorted Thy-1 bright cells were colonized into the dGuo-treated BALB/c thymus lobes by a hanging drop method ($2x10^4$cells per lobe: 36). One BALB/c lobe was dropped onto 25μl of complete medium containing $2X10^4$ allogeneic cells in a well of a Terasaki plate, and the plate was incubated overnight in an upside down position. On the next day the lobes were transferred onto a polycarbonate filter and cultured. 14 days later, thymocytes were harvested and MLC was set up against B10.BR, B10, or BALB/c spleen cells. Con-A-induced T-cell blasts and LPS-induced B-cell blasts were used as class I- and class II-expressing targets, respectively. B10.D2 B-cell blasts were used as $H-2^d$ B-cell targets, because BALB/c LPS-blasts developed very poorly. % specific Cr release was calculated as mentioned above. Sorting of Thy-1 bright cells was performed as follows. Thymocytes were stained with FITC-anti-Thy1.2 mAb (Meiji Seika, Tokyo, Japan), washed, and brightly positive cells were sorted under gating for lymphocyte fraction. Resultant cells were analyzed on FACStar, and more than 99% of them were shown to be brightly positive for Thy-1.2 (data not shown).

RESULTS

Thymocyte differentiation proceeded in a synchronized way in FTOC

Almost all thymocytes from fetuses of 15th gestational day (GD15) consisted of $CD4^-8^-$ double negative (DN) cells (Fig. 1A). Within one day of culture (GD15+1), $CD4^-8^+$ cells and, shortly after, $CD4^+8^+$ double positive (DP) cells developed (Fig. 1B). By GD15+2, almost all cells were $CD4^+8^+$ (Figs. 1C and D). On GD15+5, CD8 and CD4 SP cells started to develop simultaneously (Fig. 1E). At the same time, DN cells re-appeared as a second wave (Table 1). Inasmuch as thymocytes differentiated in a synchronized way and since maturated SP cells did not emigrate from the thymic lobes in FTOC, both CD4 and CD8 SP cells accumulated and DP cells decreased in proportion at later periods (Figs. 1F and G).

As described above, the first wave of thymocyte differentiation generated SP cells on GD 15+5, but they were accompanied by a second wave of DN cells. Thereafter, we could detect all 4 phenotypes of thymocytes in FTOC as far as the culture was carried out. The increase in the proportion of DN cells in GD15+5 thymus lobes (Fig. 1E) was not due to loss of cells of other phenotypes, because Table 1 shows that the absolute number of DN cells in GD15+5 lobes (3.4×10^4) was more than that in GD15+3 lobes (1.0×10^4).

In the presence of CsA, $CD4^-8^+$ cells developed, but $CD4^+8^-$ cells did not

Addition of CsA into the culture did not affect the differentiation steps up to the accumulation of DP cells (Figs. 1D and H, Table 1). CsA failed to inhibit the development of DP cells even in GD14 thymuses, too (data not shown). However, CsA inhibited the generation of $CD4^+8^-$ cells (Figs. 1I, J, and K). On the contrary, $CD4^-8^+$ cells, though fewer than in CsA-free culture, appeared in the presence of CsA. In CsA-free thymic lobes, SP cells accumulated during the culture period. In contrast, CsA-added culture provided some 15% of $CD4^-8^+$ SP cells, which did not increase at least up to GD16+13. Regarding absolute cell numbers, the number of DN and DP cells did not show marked difference between CsA-free and CsA-added FTOC, while CsA-added FTOC generated significantly decreased number of CD4 or CD8 SP cells.

DP cells in the FTOC consisted of two populations, one was large in cell size and the other was small. Approximately 17% of DP cells were larger in size. The cell size was measured by forward scatter on FACS analysis. Since large DP cells are reported to be in cell cycle, it was thought possible that the difference in cell size might reflect different maturation stage or activation state among DP cells. However, addition of CsA did not change the proportion of small vs. large DP cells (data not shown).

$\underline{CD4^-8^+}$ $\underline{\text{cells developing in the presence of CsA were phenotypically immature}}$

In order to further investigate the nature of the $CD4^-$

8^+ cells in CsA-added culture, we examined whether they bore J11d, CD3, or TCR $\alpha\beta$ chains. Thymocytes were depleted of $CD4^+8^-$ and $CD4^+8^+$ cells by cytotoxic treatment, and were stained with monoclonal antibodies specific for those determinants (Figs. 2 and 3). In CsA-free culture, most of $CD4^-8^+$ cells were $CD3^+$ and approximately 15% of the $CD4^-8^+$ cells expressed F23.1 determinant at high density. On the contrary, most of the $CD4^-8^+$ cells generated in the presence of CsA were $TCR/CD3^-$ and $J11d^+$, which were the same phenotype as that of the early $CD4^-8^+$ cells (37). Thus, they were assumed to be immature $CD4^-8^+$ cells which had developed from DN cells in the second wave. These results suggest that CsA inhibited the generation of not only $CD4^+8^-$, but also mature $CD4^-8^+$ cells.

$CD4^-8^+$ cells generated in the presence of CsA did not contain functionally mature precursor CTL

In order to examine whether the $CD4^-8^+$ cells generated in the presence of CsA were functionally mature, allospecific CTL activity was investigated. $CD4^-8^+J11d^+$ cells in adult mouse thymus are known to be defective in the ability to generate allospecific CTL (37). Cells from CsA-free thymic lobes exhibited allospecific cytotoxicity, but cells from CsA-added lobes did not (Table 2). This was not due to carrying-over of CsA, because CsA had been excluded from the FTOC 24 hours before preparation of the responder cells.

$CD3^+4^-8^-$ cells generated in the presence of CsA in FTOC

In the presence of CsA, about two thirds of DN cells were $CD3^+$, but no cells were $F23.1^+$, and most of the DN cells were $J11d^-$ (Fig. 2). Staining with mAb H57-597, reactive with all mouse $\alpha\beta$-TCR, confirmed that these $CD3^+4^-8^-J11d^-$ cells did not bear TCR-$\alpha\beta$ chains (Fig. 3). These DN cells were assumed to be $\gamma\delta$-thymocytes. The same cell population was thought to be present in CsA-free culture, but addition of CsA into the FTOC increased the proportion of the cell population and made it easy to detect it. Thus, CsA failed to inhibit the generation of $CD3^+4^-8^-J11d^-\alpha\beta^-$ cells, possibly possessing TCR-$\gamma\delta$ chains.

Thymocytes proliferate in the presence of IL-7

As a preliminary experiment, we investigated the proliferating response of thymocytes to IL-7. IL-7, as well as IL-2 or IL-4, in combination with PHA, supported the proliferation of thymocytes from mature thymus (data not shown). On the other hand, cultivation of thymocytes with IL-7 alone induced very poor proliferating response (data not shown). These positive data prompted us to perform further experiments.

Effects of IL-7 on FTOC

In order to investigate on what stage of thymocyte development IL-7 can act, we performed FTOC with IL-7 added

in different periods. As shown in Table 3, the effects of IL-7 did not differ largely according to periods in which IL-7 was added. FTOC in the presence of IL-7 produced a slightly decreased number of thymocytes. Among them, DP cells were markedly decreased, DN cells were increased in proportion, and among SP cells, the CD8/CD4 ratio was somewhat increased. The total cell number was slightly decreased in IL-7-added FTOC, but a further decrease was observed in FTOC with IL-7 added during half of the culture period. This may be interpreted to mean that in the presence of IL-7, a decrease in DP cells is observed first, followed by expansion of mature $\alpha\beta$- and $\gamma\delta$-thymocytes. Thus, IL-7 seems to act on a wide range of thymocytes, except for DP cells. The cell size was larger in IL-7-added FTOC than in the control, which was thought to be due to the increase in the DN cell population (data not shown).

The phenotypes of the thymocytes in those FTOC were further analyzed (Fig. 4). As shown in the figure, most of the $CD4^-8^-$ cells were $CD3^+J11d^-F23.1^-$, which suggested they were mainly $\gamma\delta$-T cells. The ratios of $F23.1^+$ cells within CD4 or CD8 SP cells were 2.2/11.8 (19%) and 6.7/29.7 (22%), respectively, which implied that the CD4 and CD8 SP cells were mature $\alpha\beta$-T cells.

To confirm these findings, the phenotypes were analyzed after depletion of $CD4^+$ cells by anti-CD4 and complement treatment (Fig. 5). The figure shows that most of the $CD8^+4^-$ cells are $CD3^+\alpha\beta^+$, and that most of the DN cells are $CD3^+\alpha\beta^-$.

IL-7 does not induce non-specific killer cells

IL-2 is known to induce non-specific killer cells that can kill thymocytes (38). In order to ascertain whether the decrease in DP cells in FTOC with IL-7 was due to such killer cells, we performed a PHA-mediated cytotoxicity assay. Thymocytes developing in the presence of IL-7 did not have cytotoxic activity on any targets (data not shown).

Unfractionated thymus cells can induce self class I tolerance

In Table 4, BALB/c fetal thymuses were treated with dGuo and were repopulated with unfractionated thymus cells from GD15 B10 and/or B10.BR fetuses (dGuo-BALB/c←B10, etc.) As shown in the table, thymocytes from dGuo-BALB/c←B10 or from dGuo-BALB/c←B10.BR killed B10.BR or B10 T-cell blasts, respectively (lines 2 and 3), which showed that allo-MHC specific CTL against a third party could be induced from the dGuo-treated BALB/c thymuses repopulated with allogeneic thymus cells. However, they were tolerant of the donor MHC (lines 1 and 4). When dGuo-treated BALB/c lobes were repopulated with B10 and B10.BR thymus cells at the same time, cytotoxic activities against B10.BR and B10 were gone (lines 5 and 6). In short, unfractionated thymus cells had tolerogenic ability upon repopulating allogeneic thymuses.

Unfractionated fetal thymus cells can tolerize thymocytes to

their own class I and class II MHC

Since unfractionated thymus cells are composed of many kinds of cells including Ia-expressing macrophages and dendritic cells, it was expected that class II tolerance would be achieved in such chimeric thymuses. Therefore, class I and class II reactivities of thymocytes from dGuo-BALB/c←B10.BR were examined (Fig. 6). They could not kill B10.BR T cell blasts (A) nor B cell blasts (B), though did kill a third party, B10 cells (C). The result indicates that unfractionated thymus cells, which contains both thymocytes and stromal cells, can tolerize thymocytes to their own class I and class II MHC.

Split tolerance in chimeric thymuses constructed with Thy-1 bright cells from allogeneic fetal thymuses

To evaluate the tolerogenic ability of thymocytes, Thy-1-bright cells from GD15 B10.BR fetal thymuses were sorted out and were colonized into dGuo-treated BALB/c thymuses (dGuo-BALB/c←Thy-1^{+}B10.BR). The cells were stimulated with B10.BR, B10, or B10.D2 (H-2^{d}, same MHC as BALB/c). As shown in Fig. 7, allo-MHC specific CTL against a third party was inducible from the chimeric thymuses (C and F). When CTL was induced against the donor (B10.BR) MHC, they failed to kill B10.BR-T-cell blasts (A). However, they did kill B10.BR-B-cell blasts (D). Furthermore, this killing was specifically inhibited by adding anti-I-A^{k} and anti-I-E^{k} antibodies in combination into the effector phase

of the CTL assay, but not by adding anti-I-E^k alone (D). The killing activity was MHC specific, as B10.D2 and B10 LPS blasts were not lysed (E and F). Anti-B10 CTL cross-reacted with B10.BR B-cell but not T-cell blasts (A and D), demonstrating that class I tolerance against the donor haplotype was valid even in cross-priming condition.

In (B) and (E), cytotoxicity against B10.D2 targets were very limited. This was thought to be due to incomplete dGuo-treatment of BALB/c thymuses, because such a weak treatment allows certain numbers of hematopoietic cells alive.

These split reactivities against Con A blasts and LPS blasts were in sharp contrast to the previous results, in which CTL generated from dGuo-BALB/c←B10.BR failed to kill both B10.BR T- and B-cell blasts (Fig. 6). These results indicate that Thy-1-bright cells and/or their descendants have a tolerogenic ability to induce class I tolerance, but not class II tolerance.

DISCUSSION

Accumulating evidence indicates that interaction between TCR and MHC molecules is involved as a key element not only in the negative selection, but also in the positive selection of thymocytes. Such evidence includes in vitro or in vivo blocking experiments using anti-CD4 (7), anti-class II (2,5,6), or anti-class I mAb (4), experiments with TCR-transgenic mice (14-16,19), or experiments showing deletion

of T-cells expressing TCR with specific Vβ regions (20, 39-41), and a recent experiment using β_2-microglobulin deficient mice (42).

It has been indicated that anti-MHC class I antibodies block the development of $CD4^-8^+$ cells, and that anti-class II or anti-CD4 antibodies block that of $CD4^+8^-$ cells (2, 4-7). Of note is that in both cases the development of DP cells is not inhibited (2, 4-7). In vivo administration of CsA has already been reported to induce atrophy of thymic medulla (10, 11), where mature SP cells are the predominant thymocyte population. We attempted to further scrutinize the cell kinetics by virtue of FTOC in the presence or absence of CsA.

CsA is known to exert many kinds of suppressive effects on the immune system, including blockage of the signal transduction cascade in T-cells triggered by perturbation of the TCR/CD3 complex (8, 43, 44), which leads to pretranscriptional inhibition of lymphokine production. The exact mechanism of this blockage is not known yet, but previous reports suggest that CsA does not affect the signal cascade from perturbation of the TCR/CD3 complex to calcium influx or turnover of phosphatidyl inositol (45-49). Therefore, the agent may block the signal cascade at a later step, possibly by binding to cyclophilin and by inhibiting the peptidyl-prolyl cis-trans isomerase activity of cyclophilin (48). On the other hand, the agent is also known to disrupt the thymic stromal cell function including Ia expression (10). At any rate, the agent is expected to

have suppressive effects on thymocyte development either by interfering with the signal cascade or by impairing the stromal cell function.

FTOC has a certain advantage in investigating which stage of T-cell differentiation CsA would inhibit. If CsA is administrated in vivo, one cannot rule out the possibility that some cells escape the effects of the agent. Indeed, it is reported that cells escaping the effects of CsA exhibit autoreactivity in vivo (10,49). On the other hand, if too much CsA is administered, one cannot discriminate the inhibitory effects of CsA on lymphopoiesis from the toxic effects of the agent. In the organ culture, however, a pharmacologically effective concentration of CsA can be obtained throughout the culture period. In addition, in FTOC, no stem cells migrate into the thymus during the culture period, and cells differentiate in a synchronized manner. Moreover, mature SP cells do not emigrate from the thymic lobes. All of these factors made it possible to analyze the fine differentiation kinetics of thymocytes.

CsA has been reported to inhibit the development of CD4, but not CD8 SP cells in vitro (2). The present study demonstrated that the CD8 SP cells developing in the presence of CsA revealed an immature phenotype of $J11d^{+}\alpha\beta^{-}$ (Figs. 2 and 3). The CD8 SP cells were also shown to be functionally immature. Moreover, they were also less in number than appeared via CsA-free FTOC. Thus, they were early $CD4^{-}8^{+}$ cells which developed from DN cells and would develop into DP cells (Fig. 1). Hence, CsA inhibits the

differentiation step from DP cells not only to CD4, but also to CD8 mature SP cells.

The question then arises: What is the mechanism of this inhibitory effect? Two explanations are possible. One is that CsA inhibits the positive selection of thymocytes by blocking the signal transduction cascade initiated by perturbation of the TCR/CD3 complex (8,9). The other is that CsA impairs thymic stromal cell functions which, in turn, leads to failure of mature SP cell generation. CsA has been reported to suppress MHC expression induced by LPS or allogeneic tumor cells, but not that induced by IFN α/β or viral infection (48,50). In the previous study, we could not demonstrate a decrease in the expression of the Ia molecule on thymic stromal cells in CsA-added thymic lobes in our culture condition (2). Although it is not a formal proof, these results suggest that the positive selection takes place at the transition step from DP cells to SP cells. And this hypothesis is consistent with the result of a recent report in which DP cells, but not SP cells could develop in the absence of appropriate MHC in TCR gene-transgenic mice (16).

Interestingly, CsA inhibited the differentiation of mature SP cells, but not of $CD3^+4^-8^-TCR\alpha\beta^-J11d^-$ cells, which were assumed to be cells bearing TCR-$\gamma\delta$ chains. This result agrees with a recent report where CsA was administered in vivo (51,52). In chicken, it has been reported that thymocytes bearing the TCR $\gamma\delta$-homologue at high density are present in the subcapsular zone of the thymic cortex, where

cells bearing $\alpha\beta$-homologue are rare (53). This indicates that the requirement for interaction between thymocytes and thymic stromal cells is different in the development of $\alpha\beta$- and $\gamma\delta$-T cells. Further investigations will be necessary to elucidate the positive selection of TCR-$\alpha\beta$ and TCR-$\gamma\delta$-bearing cells and its requirement for stromal elements.

Although IL-7 was detected and isolated as a pre-B cell growth factor produced by bone marrow stromal cells, it has been suspected that IL-7 would have a role in T-cell development, because IL-7 mRNA is abundant in the thymus. Actually, IL-7 alone or in combination with other reagents was observed to promote the growth of a wide range of T-lineage cells: immature DN cells, $\gamma\delta$ T-cells, and mature CD4 or CD8 $\alpha\beta$ T-cells (54-58). On the other hand, an anti-CD3 treatment in vivo, but not in vitro, induced IL-7 responsiveness in T-cells, indicating the necessity of cell-to-cell interaction for the induction of functional IL-7 receptor (54). Considering that T-cell development depends on complicated thymocyte-stromal cell interaction, and that IL-7 may be an essential factor in such interactions, it is important to understand the physiological effects of IL-7 upon developing thymocytes. To elucidate such effects, we added recombinant IL-7 into the medium of FTOC, in which phenotypically and functionally normal thymocyte differentiation takes place.

The data presented above show that IL-7 supports the growth of a wide range of T-lineage cells. Both $\alpha\beta$ and $\gamma\delta$ thymocytes responded to IL-7. By contrast, in B-cells, IL-7

is reported to act as a growth factor only on immature B-cells, and not on mature B-cells (55). The marked decrease in DP cells in IL-7-added FTOC was not due to the killing of thymocytes, and was observed even in FTOC with IL-7 added from day 5, when most of the thymocytes were DP (Table 3). Thus IL-7 was suggested to promote apoptosis of DP cells and/or differentiation of DP cells into SP cells. It is noteworthy that both CD4 and CD8 mature $\alpha\beta$ cells proliferate in response to IL-7, but that CD8 $\alpha\beta$ cells selectively develop in FTOC with IL-7. This indicates that the IL-7 responsiveness is acquired by CD8 SP cells or their direct precursor cells, but not by CD4 SP cells. IL-7 is considered to act as a lineage-specific growth/differentiation factor of CD8 $\alpha\beta$ cells. Further investigations will be necessary to determine the exact role of IL-7 in thymocyte development.

Experiments using chimeric thymuses showed that thymus cells colonized into allogeneic fetal thymuses can induce class I and class II tolerance in an allo-CTL assay. It is appropriate to ask, which cell population of the colonized cells induced this tolerance? This tolerance induction may be attributed to Ia-positive cell populations including macrophages/dendritic cells. In a preliminary study, we saw that Thy-1 negative peritoneal cells can induce tolerance to their own class I antigens when colonized into allogeneic fetal thymuses (data not shown). As described above, dendritic cells are reported to be able to induce class I tolerance (27).

Then, can thymocytes tolerize thymocytes themselves? Indeed, in the periphery, pCTL reactive to class I or class I-restricted minor histocompatibility antigens can be tolerized by veto cells, which belong to Thy-1$^-$ or Thy-1$^+$ cell lineage (59-63). Regarding thymocytes, Shimonkewitz et al. (64) constructed chimeric mice by injecting allogeneic Thy-1$^+$ cells intrathymically, and showed that class I tolerance can be induced in the thymus by the thymocytes themselves. In a previous report, we constructed "parabiosis" of the thymus in FTOC, where two H-2 disparate thymus lobes were cultured in contact with each other (3). Upon contact, co-migration of cells occurred and mutual class I tolerance was achieved. Histochemical analysis revealed that cells rapidly migrating between the parabiotic thymus lobes were Thy-1$^+$. Migration of Ia$^+$ cells was limited and slow. Since murine thymocytes are known to lack Ia expression, these findings indicated that only thymocytes, not dendritic cells or macrophages, had migrated between the thymus lobes before thymocytes became immunocompetent. Thus, the class I antigen expressed on thymocytes was thought to act as a tolerogen in pCTL differentiation.

In order to investigate the tolerogenicity of thymocytes directly, we constructed thymic chimeras by colonizing purified Thy-1 bright cells into allogeneic thymic lobes. As shown in Fig. 7, Thy-1 bright thymus cells induced class I, but not class II CTL tolerance when colonized into allogeneic thymuses. Since thymocytes are

the sole Thy-1 bright cell population, the result listed above indicates that thymocytes can act as tolerizing cells to their class I MHC. Thus, Thy-1 bright cells, namely, thymocytes, tolerized thymocytes themselves to the donor's class I MHC in the absence of Ia-positive cells of the donor haplotype. Whether every thymocyte can induce the class I tolerance or a certain subpopulation of thymocytes can do so will be a subject of further investigation. Our results do not determine to what extent thymocytes, dendritic cells, or macrophages act as tolerizing cell in vivo, nor do they clarify the mechanism of the tolerance induction: clonal deletion or clonal anergy. However, noting the tolerizing ability of thymocytes will be of benefit.

In summary, several aspects of thymocyte differentiation were elucidated in this study using FTOC (Fig. 8). CsA has previously been reported to inhibit the appearance of CD4, but not of CD8 SP cells. This study, however, demonstrated that the development of not only CD4-, but also the CD8-expressing mature -thymocyte is inhibited by the agent. It is widely known that CsA blocks the signal cascade triggered by the perturbation of the TCR/CD3 complex, and that interaction between TCR on the thymocyte and MHC on the thymic stromal cell is essential in the positive selection of both CD4 and CD8 SP cells. Thus, CsA is suggested to interfere with the positive selection of $\alpha\beta$-thymocytes by blocking the signaling via the TCR/CD3 complex. In addition, the study demonstrates that $\gamma\delta$-

thymocytes do develop in the presence of CsA, which indicates that the requirement for the thymocyte-stromal cell interaction is different in αβ- and γδ-T cells. The exact mechanism of the positive selection is unknown, but this study raises the possibility that IL-7 acts not only as a growth factor for T-cells, but also as a lineage-specific differentiation/growth factor for CD8 αβ-T cells. Concerning self-tolerance induction, the study is demonstrates that thymocytes in neonate-equivalent cultured thymus, which consists mostly of DP thymocytes, can be tolerized to self-class I MHC by Thy-1 bright cells. This indicates that the thymocyte-thymocyte interaction is sufficient in inducing self-class I tolerance. In all these experiments, FTOC was very useful in investigating the mechanism of thymocyte development in the thymus.

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REFERENCES

1. Jenkinson, E. J., Franchi, L. L., Kingston, R., and Owen, J. J. T., Eur. J. Immunol. **12**, 583, 1982.

2. Takeuchi, Y., Habu, S., Okumura, K., and Suzuki, G., Immunology **66**, 362, 1989.

3. Suzuki, G., Kawase, Y., and Hirokawa, K., Eur. J. Immunol. **19**, 1525, 1989.

4. Marusic-Galesic, S., Stephany, D. A., Longo, D. L., and Kruisbeek, A. M., Nature **333**, 180, 1988.

5. Kruisbeek, A. M., Mond, J. J., Fowlkes, B. J., Carmen, J. A., Bridges, S, and Longo, D. L., J. Exp. Med. **161**, 1029, 1985.

6. DeLuca, D., J. Immunol. **136**, 430, 1986.

7. Fowlkes, B. J., Schwartz, R. H., and Pardoll, D. M., Nature **334**, 620, 1988.

8. Sawada, S., Suzuki, G., Kawase, Y., and Takaku, F., J. Immunol **139**, 1797, 1987.

9. Colombani, P. M., Robb, A., and Hess, A. D., Science **228**, 337, 1985.

10. Cheney, R. T. and Sprent, J., Transpl. Proc. **17**, 528, 1985.

11. Beschorner, W. E., Di Gennaro, K. A., Hess, A. D., and Santos, G. W., Cell. Immunol. **110**, 350, 1987.

12. Bevan, M. J., Nature **269**, 417, 1977.

13. Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P. A., and Klein, J., J. Exp. Med. **147**, 882, 1978.

14. Teh, H. S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Bluthmann, H., and von Boehmer, H., Nature **335**, 229, 1988.

15. Kisielow, P., Teh, H. S., Bluthmann, H., and von Boehmer, H., Nature 335, 730, 1988.

16. Sha, W. C., Nelson, C. A., Newberry, R. D., Kranz, D. M., Russel, J. H., and Loh, D. Y., Nature 336, 73, 1988.

17. Namen, A. E., Schmierer, A. E., March, C. J., Overell, R. W., Park, L. S., Urdal, D. L., and Mochizuki, D. Y., J. Exp. Med 167, 988, 1988.

18. Namen, A. E., Lubton, S., Hjerrild, K., Wignall, J., Mochizuki, D. Y., Schmierer, A. Mosley, B., March, C. J., Urdal, D., Gillis, S., Cosman, D., and Goodwin, R. G., Nature 333, 571, 1988.

19. Kisielow, P., Bluthmann, H., Staerz, U. D., Steinmetz, M., and von Boehmer, H., Nature 333, 742, 1988.

20. Kappler, J. W., Roehm, N., and Marrack, P., Cell 49, 273, 1987.

21. Lo, D., Sprent, J., Nature 319, 672, 1988.

22. von Boehmer, H. and Schubiger, K., Eur. J. Immunol. 14, 1048, 1984.

23. von Boehmer, H. and Hafen, K., Nature 320, 626, 1986.

24. Jenkinson, E. J., Jhittay, P., kingston, R., and Owen, J. J. T., Transplantation 331, 331, 1985.

25. Suzuki, G., Moriyama, T., Takeuchi, Y., Kawase, Y., and Habu, S., J. Immunol 142, 1463, 1989.

26. Owen, J. J. T. and Jenkinson, E. J., Am. J. Anat. 170, 301, 1984.

27. Matzinger, P. and Guerder, S., Nature 338, 74, 1989.

28. Ceredig, R., Lowenthal, J. W., Nabholz, M., and MacDonald, H. R., Nature 314, 98, 1985.

29. Dialynas, D. P., Wilde, D. B., Marrack, P., Pierres, A., Wall, K. A., Harvan, W., Otten, G., Loken, M. R., Pierres, M., Kappler, J., and Fitch, F. W., Immunol. Rev. 74, 29, 1983.

30. Staerz, U. D., Rammensee, H. G., Benedetto, J. D., and Bevan, M. J., J. Immunol. 134, 3994, 1985.

31. Leo, O., Foo, M., Sachs, D. H., Samelson, L. E., and Bluestone, J. A., Proc. Natl. Acad. Sci. U.S.A. 84, 1374, 1987.

32. Bruce, J., Symington, F. W., McKearn, T. J., and Sprent, J., J. Immunol. 127, 2496, 1981.

33. White, J., Herman, A., Pullen, A. M., Kubo, R., Kappler, J. W., Marrack, P., Cell 56, 27, 1989.

34. Oi, V. T., Jones, P. P., Goding, J. W., and Herzenberg, L. A., Curr. Topics Microbiol. Immunol. 81, 115, 1978.

35. Bhattacharya, A., Dorf, M. E., and Springer, T. A., J. Immunol. 127, 2488, 1981.

36. Kingstom, R., Jenkinson, E. J., Owen, J. T. T., Nature 317, 811, 1985.

37. Crispe, N. and Bevan, M. J., J. Immunol. 138, 2013, 1987.

38. Skinner, M., Le Gros, G., Marbrook, J., and Watson, J. D., J. Exp. Med 165, 1481, 1987.

39. MacDonnald, H. R., Schneider, R., Lees, R. K., Howe, R. C., Acha-Obera, H., Festenstein, H., Zinkernagel, R. M., Hengartner, H., Nature 332, 40, 1988.

40. Kappler, J. W., Staerz, U., White, J., Marrack, P. C., Nature 332, 35, 1988.

41. Pullen, A. M., Marrack, P., Kappler, J. W., Nature 335, 796, 1988.

42. Zijlstra, M., Bix, M., Simister, N.E., Loring, J.M., Raulet, D.H., Jaenisch, R., Nature 344, 742, 1990.

43. Shevach, E.M., Ann. Rev. Immunol. 3, 397, 1985.

44. Britton, S. and Palacios, R., Immunol. Rev. 65, 5, 1982.

45. Kay, J.E., Benzie, C.R., and Borghetti, A.F., Immunology 50, 441, 1983.

46. Metcalfe, S., Transplantation 39, 161, 1984.

47. Bijsterbosch, M.K. and Klaus, G.G.B., Immunology 56, 435, 1985.

48. Feutren, G., Curr. Opin. Immunol 2, 239, 1989.

49. Sorokin, R., Kimura, H., Schroder, K., Wilson, D.H. and Wilson, D.B., J. Exp. Med. 164, 1615, 1986.

50. Halloran, P.F., Urmson, J., van der Meide, PH, Autenreid, P., J. Immunol 142, 4241, 1989.

51. Jenkins, M.K., Schwartz, R.H., and Pardoll, D.M., Science 241, 1655, 1988.

52. Gao, E., Lo, D., Cheney, R., Kanagawa, O., and Sprent, J., Nature 336, 176, 1988.

53. Bucy, R.P., Chen, C.H., Losch, U., and Cooper, M.D., J. Immunol. 141, 2200, 1988.

54. Morrissey, P.J., Goodwin, R.G., Nordan, R.P., Anderson, D., Grabstein, K.H., Cosman, D., Sims, J., Lupton, S., Acres, B., Reed, S.G., Mochizuki, D., Eisenman, J., Conlon, P.J., and Namen, A.E., J. Exp. Med 169, 707, 1989.

55. Lee, G., Namen, A.E., Gillis, S., Ellingsworth, L.R., and Kincade, P.W., J. Immunol. 142, 3875, 1989.

56. Conlon, P.J., Morrissey, P.J., Nordan, R.P., Grabstein, K.H., Prickett, K.S., Reed, S.G., Goodwin, R.,

Cosman, D., and Namen, A. E., Blood 74, 1368, 1989.

57. Watson, J. D., Morrissey, P. J., Namen, A. E., Conlon, P. J., and Widmer, M. B., J. Immunol 143, 1215, 1989.

58. Okazaki, H., Ito, M., Sudo, T., Hattori, M., Kano, S., Katsura, Y., and Minato, N., J. Immunol 143, 2917, 1989.

59. Muraoka, S., Miller, R. G., J. Immunol 131, 45, 1983.

60. Muraoka, S., Miller, R. G., J. Exp. Med 152, 54, 1980.

61. Fink, P. J., Weissman, I. L., and Bevan, M. J., J. Exp. Med 157, 141, 1983.

62. Claesson, M. H., Miller, M. G., J. Exp. Med 160, 1702, 1984.

63. Fink, P. J., Rammensee, H. G., and Bevan, M. J., J. Immunol 133, 1775, 1984.

64. Shimonkevitz, R., and Bevan, M. J., J. Exp. Med. 168, 143, 1988.

Table 1

Course of cell number of each phenotype of thymocytes in FTOC

		GD15+0	15+3	15+5	15+8	15+12～15
CsA free	4^-8^-	78	10	34	12	12
	4^-8^+	1	4	28	28	38
	4^+8^-	2	12	28	22	25
	4^+8^+	1	193	127	85	9
	total	81±10	220±10	215±15	147±15	83±32
CsA added	4^-8^-		20	37	20	21
	4^-8^+		2	17	11	8
	4^+8^-		4	5	3	1
	4^+8^+		182	86	37	30
	total		210±30	145±5	72±4	60±21

Note. Numbers of thymocytes of each phenotype in various conditions are listed. Each value represents the mean of three or four experiments and is described as (cell number)x10^{-3}/lobe. Total numbers are described as mean±S.E.

Table 2

Thymocytes from CsA-added FTOC did not contain alloreactive precursor CTL

Responder cells from B10.BR	FTOC	stimulator	E/T	%specific Cr-release from BALB/c	from B10.BR
CsA	0 M	BALB/c	20	57	3.2
			10	52	2.5
	0 M	B10.BR	20	4.8	0
			10	0	3.0
CsA	10^{-7}M	BALB/c	20	5.5	0
			10	2.1	0
	10^{-7}M	B10.BR	20	N.D.	N.D. (a)
			10	0	0

Note. $3x10^5$ B10.BR thymocytes from CsA-free or CsA-added FTOC were incubated with $3x10^6$ 30Gy-irradiated spleen cells from syngeneic (B10.BR) or allogeneic (BALB/c) mice in 2 ml of medium containing 1.25% of EL-4 culture supernatant. After 5 days, cells were harvested and % specific Cr-release from ^{51}Cr-labeled Con A blasts from B10.BR or BALB/c spleen cells was measured in a killer assay. (a) N.D. : not done

Table 3

Number and percentage of each thymocyte population in FTOC with or without IL-7

	IL-7(-)/(-)	(-)/(+)	(+)/(-)	(+)/(+)
$CD4^-8^-$	28(13%)	30(22%)	32(17%)	55(28%)
$CD4^-8^+$	28(13%)	24(17%)	31(17%)	51(25%)
$CD4^+8^-$	20(9%)	12(8%)	22(12%)	21(10%)
$CD4^+8^+$	144(65%)	74(53%)	104(55%)	73(37%)
total	220	140	190	200

Note. GD15 fetal thymus lobes were cultured in the presence (+) or absence (-) of IL-7 for initial five days (left parentheses), and further five days (right parenthesis). Cells were harvested and were analyzed for CD4/CD8 expression on FACStar. Each value represents (cell number)$\times 10^{-3}$/lobe in one of three similar experiments.

Table 4

Allospecific CTL activity of thymocytes from chimeric thymuses.

line	BALB/c thymuses colonized with	stimulator in MLC	%specific Cr-release from B10-T	from B10.BR-T
1.	B10	B10	0	20
2.	B10	B10.BR	3	70
3.	B10.BR	B10	70	11
4.	B10.BR	B10.BR	N.D. [a)]	0
5.	B10.BR+B10	B10	4	0
6.	B10.BR+B10	B10.BR	2	10

Note. 2'-deoxyguanosine-treated GD15 fetal BALB/c thymus lobes were repopulated with unfractionated B10 and/or B10.BR fetal thymocytes (10^4 cells/lobe for each parental cells) (dGuo-BALB/c←B10, etc.). Fourteen days later, allo-MLC was set up in the presence of exogenous IL-2. Killer activity of the resultant cells against B10 and B10.BR T-cell blasts was determined by a 4-hour ^{51}Cr release assay at the E/T ratio of 10:1. Killing activity is expressed as % specific Cr-release.

a) N.D.: not done.

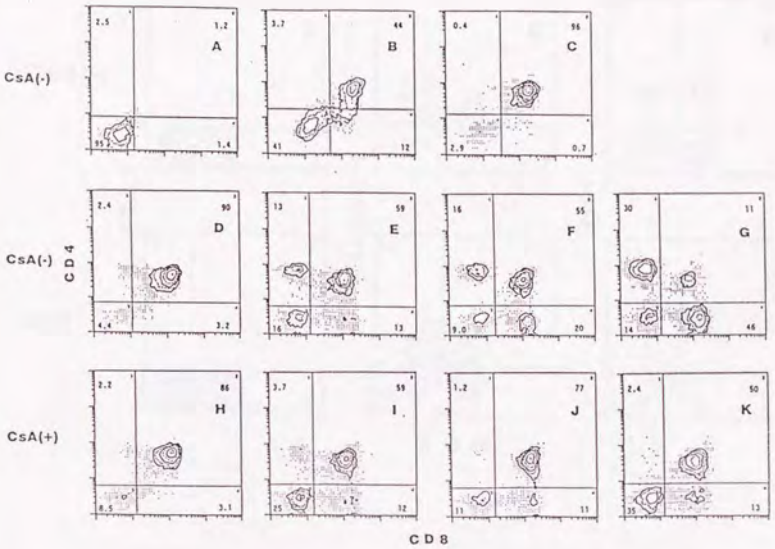


Fig. 1. CsA inhibited the development of $CD4^{+}8^{-}$ cells, but not that of $CD4^{-}8^{+}$ cells.

Thymocytes recovered from thymus lobes of GD15 fetuses (A), or lobes organ-cultured for 1 day (B), 2 days (C), 4 days (D,H), and 5 days (E,I) were stained with PE-anti-CD4 and FITC-anti-CD8 antibodies. Similarly, thymocytes from GD16 fetal thymuses cultured for 9 (F,J) or 13 days (G,K) were stained with those antibodies. CsA was added (H-K) at the final concentration of $1x10^{-7}M$, or not added (A-G).

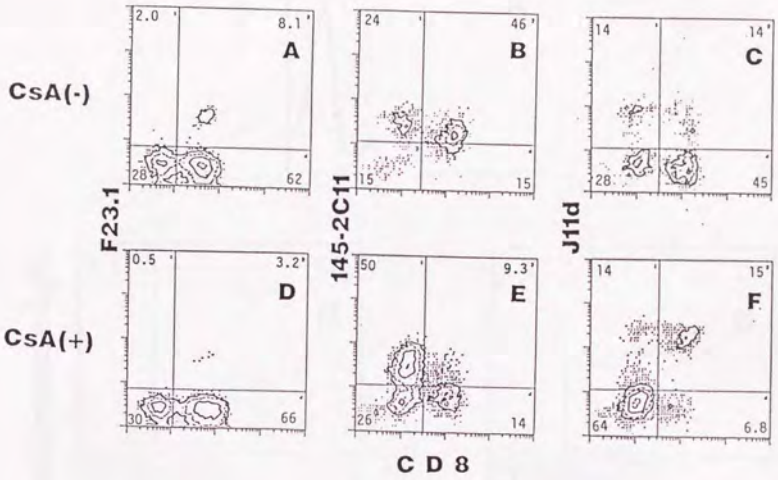


Fig. 2. CsA inhibited the development of TCR$\alpha\beta^+$/$CD3^+J11d^-$ mature SP cells, but not that of TCR$\alpha\beta^-CD3^+$ DN cells.

FTOC was set up in the presence (D,E,F) or absence (A,B,C) of CsA. Thymocytes from 9 days (A,D) or 13 days (B,C,E,F)-cultured GD16 thymus lobes were cytotoxically treated with anti-CD4 antibodies and complement to delete $CD4^+8^-$ and $CD4^+8^+$ cells. Resultant cells were dual-stained with FITC-CD8 and F23.1, 2C11 or J11d antibodies, as indicated.

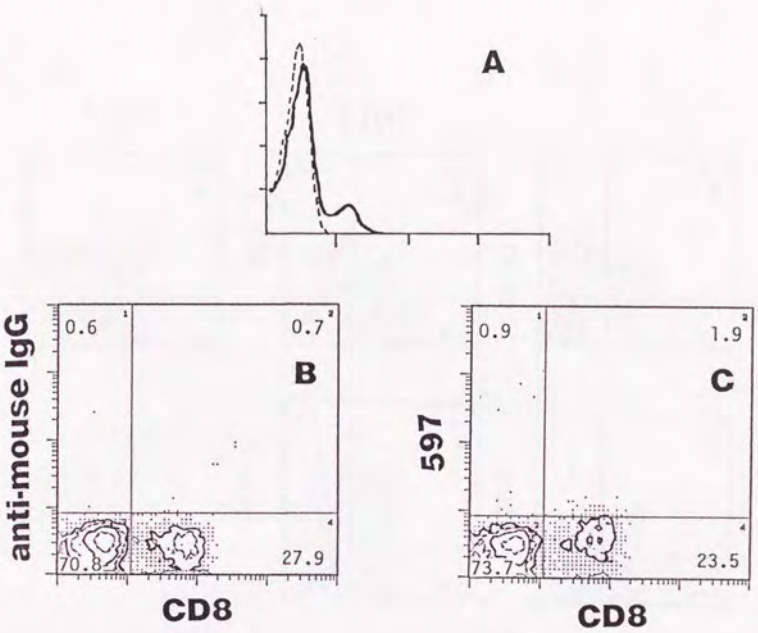


Fig. 3. The $CD4^{-}8^{-}$ cells developing in the presence of CsA did not bear $\alpha\beta$-TCR chains.

Positive control for H57-597 staining is shown using adult thymocytes (3A). Dotted line and solid line indicate autofluorescence and H57-597-staining profiles, respectively. Thymocytes from CsA-added FTOC (GD15+15) were treated with anti-CD4 and complement. Resultant cells were stained with anti-CD8 and 597 (3C). 3B shows a control using anti-CD8 and the second antibody alone.

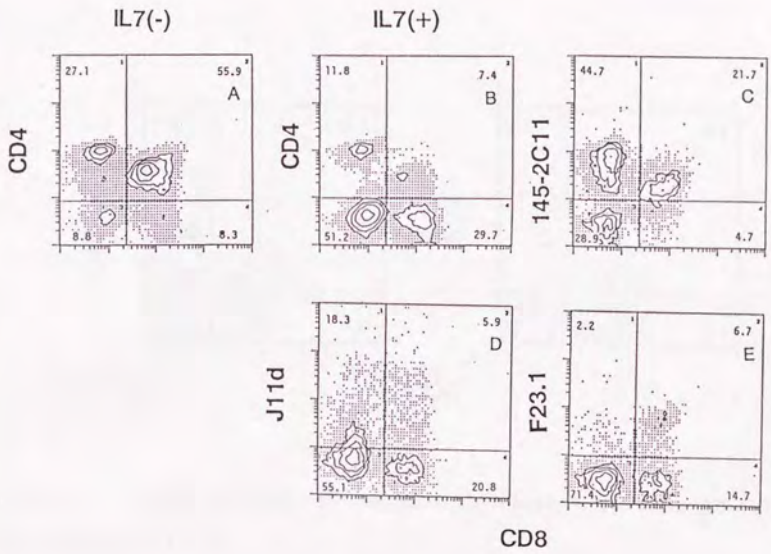


Fig. 4. Effects of IL-7 on FTOC.

GD15 fetal thymus lobes were cultured in the presence (B-E) or absence (A) of IL-7. On day 13, cells were harvested and were stained for CD8 and CD4, CD3, $V\beta_8$, or J11d. Recovered cell numbers were 8.5×10^4/lobe in control FTOC, and 4.9×10^4/lobe in IL-7 added FTOC.

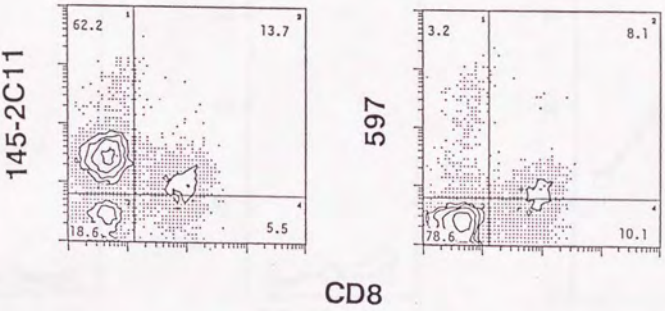


Fig. 5. Both αβ- and γδ- mature thymocytes develop in the presence of IL-7.

Cells from GD15+13 IL-7-added FTOC were depleted of CD4 positive cells by treating with anti-CD4 and complement, and were stained for CD8 and CD3 (A) or TCR-αβ chains (B).

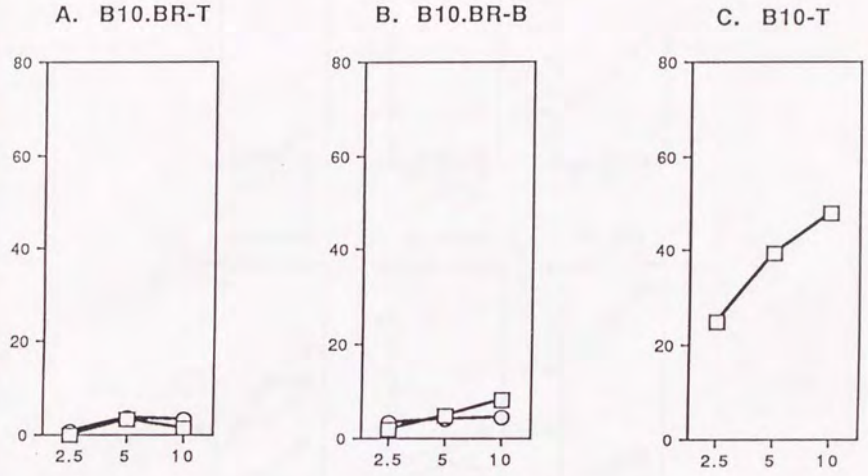


Fig. 6. Unfractionated fetal thymus cells can induce class I and class II tolerance.

Thymocytes developing in dGuo-BALB/c←B10.BR thymuses were stimulated with B10 (□)or B10.BR (○) spleen cells, and the resultant cells were examined for their killer activity against B10.BR-T, B10.BR-B, or B10-T cell blasts. This figure illustrates the results of a series of three similar experiments.

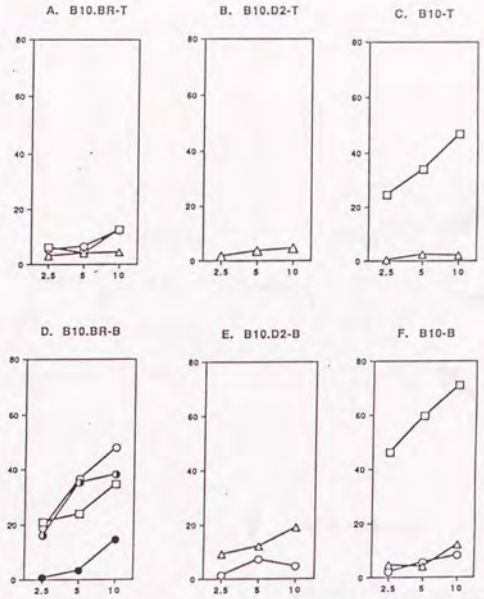


Fig. 7. Thy-1 positive fetal thymocytes can induce split tolerance.

Thy-1 bright positive B10.BR fetal thymocytes were sorted on FACStar and were colonized into dGuo-treated BALB/c thymuses. Cells developing therein were stimulated with B10 (□), B10.BR (○), or B10.D2 (△) spleen cells, and were examined for their killer activity against T- or B-cell blasts of B10.BR, B10.D2 (H-2^d, same as BALB/c), or B10. In (D), anti-I-A^k plus anti-I-E^k (●) or anti-I-E^k alone (◑) was added to the wells containing B10.BR-stimulated responder cells at the time of killer assay. The figure illustrates the results of one of three similar experiments. B10.D2 B-cell blasts were used as H-2^d class II targets, because BALB/c LPS-blasts developed poorly.

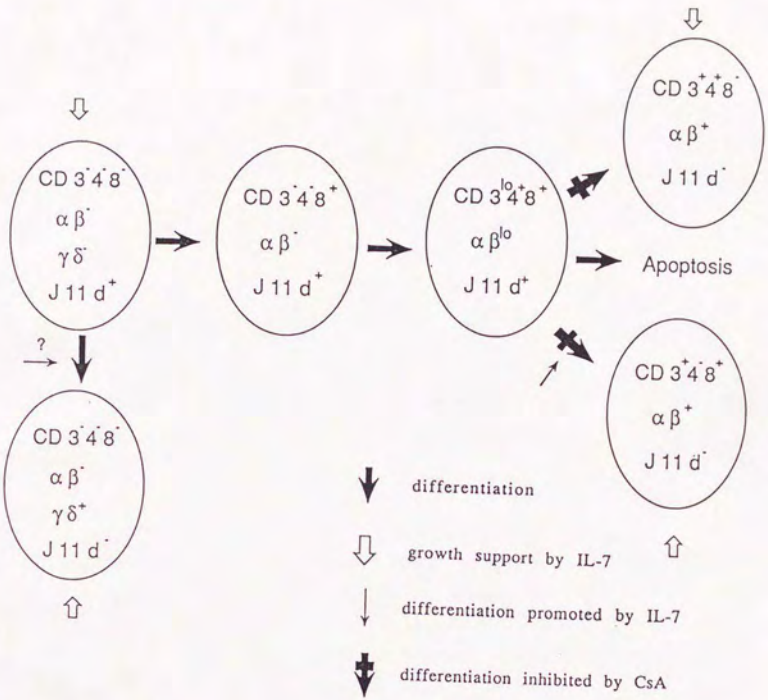
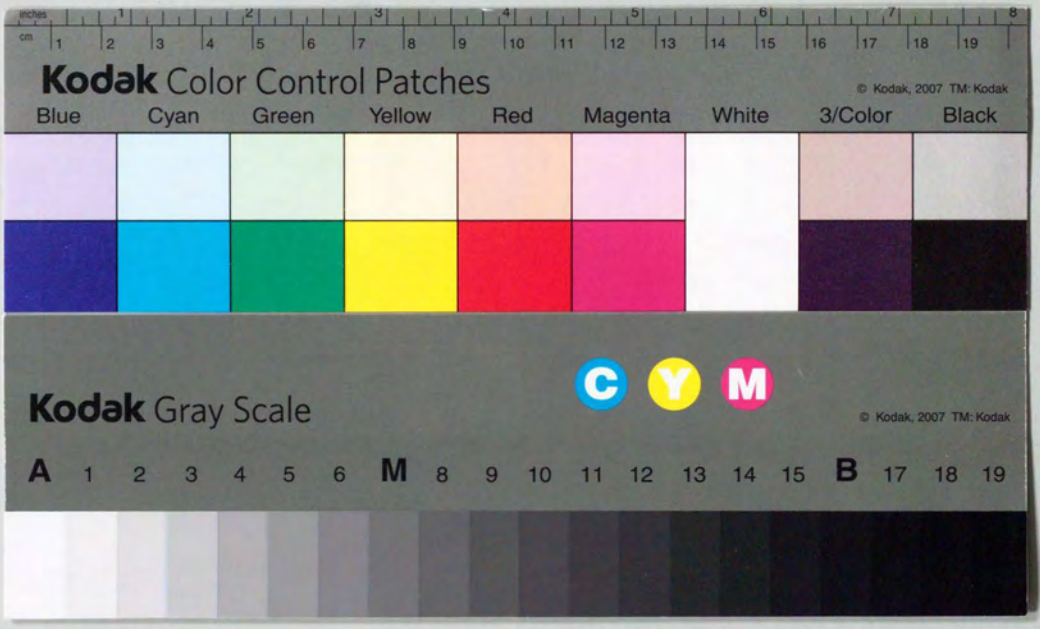


Fig. 8. Thymocyte differentiation studied in this work. The course of thymocyte differentiation and effects of agents elucidated in this study are indicated.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