Evidence for Extrathymic Generation of Intermediate T Cell Receptor Cells in the Liver Revealed in Thymectomized, Irradiated Mice Subjected to Bone Marrow Transplantation

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Summary

In addition to the major intrathymic pathway of T cell differentiation, extrathymic pathways of such differentiation have been shown to exist in the liver and intestine. In particular, hepatic T cells of T cell receptors or CD3 of intermediate levels (i.e., intermediate T cell receptor cells) always contain self-reactive clones and sometimes appear at other sites, including the target tissues in autoimmune diseases and the tumor sites in malignancies. To prove their extrathymic origin and self reactivity, in this study we used thymectomized, irradiated (B6 × C3H/He) F_1 mice subjected to transplantation of bone marrow cells of B6 mice. It was clearly demonstrated that all T cells generated under athymic conditions in the peripheral immune organs are intermediate CD3 cells. In the case of nonthymectomized irradiated mice, not only intermediate CD3 cells but also high CD3 cells were generated. Phenotypic characterization showed that newly generated intermediate CD3 cells were unique (e.g., interleukin 2 receptor α^-/β^+ and $CD44^+L$-selectin$^-$) and were, therefore, distinguishable from thymus-derived T cells. The precursor cells of intermediate CD3 cells in the bone marrow were $Thy-1^+CD3^-$. The extrathymic generation of intermediate CD3 cells was confirmed in other combinations of bone marrow transplantation, C3H → C3H and B10.Thy1.1 → B6.Thy1.2. The generated intermediate CD3 cells in the liver contained high levels of self-reactive clones estimated by anti-Vβ monoclonal antibodies in conjunction with the endogenous superantigen minor lymphocyte–stimulating system, especially the combination of B6 → (B6 × C3H/He) (graft-versus-host situation). Moreover, these self-reactive clones were not anergic but functional in in vitro cultures. These results reveal that intermediate CD3 cells are truly of extrathymic origin and that only such intermediate CD3 cells comprise self-reactive forbidden T cell clones.

It has been proposed that extrathymic pathways of T cell differentiation exist in the liver (1–4). These hepatic T cells have unique properties as primitive lymphocytes; e.g., they have TCR of intermediate (int)[1] levels (termed int TCR cells) and contain double-negative (DN) $CD4^-8^-$ cells (5, 6). They consist of a high proportion of γ/δ T cells as well as of α/β T cells and always contain self-reactive forbidden clones as estimated by anti-Vβ mAbs in conjunction with the endogenous superantigen Mls system. A similar population was also identified by other investigators (7–9). int TCR

cells constitutively express IL-2Rβ, similar to NK cells. The majority of $CD8^+$ cells among int TCR cells carry the α/α homodimer of CD8 antigens (6). Although intermediate CD3 cells are very few in youth, they become prominent with aging (5, 10). Even in youth, intermediate CD3 cells increase in number at the target organ in autoimmune diseases (11, 12) and at tumor sites in malignancies (13–15). Since all T cells in the peripheral organs, except the intestine, in athymic nude mice are int TCR cells, we speculate that they are of extrathymic origin (5). However, because of the genetic abnormality of these athymic nude mice, it is unclear whether all T cells generated in normal mice under athymic conditions are int TCR (or CD3) cells.

We used here thymectomized, irradiated mice subjected to bone marrow transplantation (Tx-RBMT) to overcome the above situation. To identify the origin of newly gener-

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[1] *Abbreviations used in this paper:* BMT, bone marrow transplantation; DN, double negative; int, intermediate; MNC, mononuclear cells; mRNA, messenger RNA; RAG-1, recombination-activating gene 1; Tx(−), nonthymectomized; Tx(+), thymectomized; Tx-RBMT, thymectomized, irradiated, and subjected to bone marrow transplantation.

ated T cells, thymectomized, irradiated (B6 × C3H/He) F_1 mice were subjected to transplantation of bone marrow cells (BMT) of B6 origin. The expression of H-2K antigens enabled us to determine whether they were of recipient F_1 origin (H-2K^{b+k+}) or donor B6 origin (H-2K^{b+}). The use of these F_1 mice had two other important advantages. Recently, NK1.1$^+$ T cells with TCR of intermediate levels have been identified in the thymus and peripheral immune organs (16–31). Since many properties (e.g., TCR of intermediate levels and the expression IL-2Rα^-/β^+ and CD44$^+$) are shared by intermediate CD3 cells and NK1.1$^+$ T cells (32, 33), we directly compared their relationships in F_1 mice expressing NK1.1 alloantigens in this study. Moreover, B6 mice and B10 congenic mice do not have self-reactive forbidden clones estimated by anti-Vβ mAbs in conjunction with the Mls system. These mice lack I-E molecules, which present Mls antigens to T cells. However, F_1 mice carry I-E molecules; specifically, they receive Mls-1^{b}2^a antigens from their C3H/He mother. Since this combination of BMT has a potential to induce GVHD, the extrathymic generation of int CD3 cells was also confirmed in other combinations: C3H/He → C3H/He and B10.Thy1.1 → B6.Thy1.2.

It was demonstrated that all T cells generated in the liver and other organs under athymic conditions were int CD3 cells. More importantly, self-reactive forbidden clones were confined to these int CD3 cells, and such newly generated clones were not anergic but functional, especially in the combination of B6 → (B6 × C3H/He) F_1 (GVH situation). Taken together with data from recent studies (32), it would appear that extrathymic int CD3 (or TCR) cells are involved in many immunological phenomena because of their autoreactivity.

Materials and Methods

Mice. Male C57BL/6 (B6), B6-*nu/nu*, C3H/He, and (B6× C3H/He) F_1 mice at 7–15 wk of age were used. Mothers of the F_1 mice were female C3H/He (Mls-1^{b}2^a) mice (34) from which the F_1 mice received the superantigen, Mls-1^{b}2^a. They were originally obtained from Jackson Laboratories Japan (Tokyo, Japan) and were maintained in the animal facility of Niigata University (Niigata, Japan). B10.Thy1.1 mice (35), which were kindly provided by Dr. T. Sato (National Institute of Radiological Science, Chiba, Japan), were also used. This strain of mice was genetically identical to B6.Thy1.2 mice except for the expression of Thy1.1 antigens.

Cell Preparations. Hepatic mononuclear cells (MNC) were isolated by a previously described method (36). Briefly, mice anesthetized with ether were killed by total exsanguination by cardiac puncture. To obtain MNC, the liver was removed, pressed through a 200-gauge stainless steel mesh, and suspended in PBS (0.1 M, pH 7.2). After one washing with PBS, MNC were isolated from hepatocytes and hepatocyte nuclei by Ficoll-Isopaque density (1.090) gradient centrifugation. To avoid selective cell loss by the gradient centrifugation method (37), sufficient dilution of mashed liver samples with the medium (i.e., 30 ml for two livers) was important before it was overlaid on the gradient cushion. MNC collected from the interface were then suspended in MEM supplemented with 2% FCS. The preparations of hepatic MNC contained <4% Kupffer cells (36). Spleen cells were also collected by the Ficoll-Isopaque method, while thymocytes were obtained by forcing the thymus through a 200-gauge steel mesh.

Immunofluorescence Test. The surface phenotype of cells was analyzed using mAbs in conjunction with two- or three-color immunofluorescence tests (5). FITC-conjugated anti-CD3 (145-2C11) mAb was obtained from Pharmingen (San Diego, CA). Biotin-conjugated anti–IL-2Rβ (TM-β1) mAb (38) was also used (kindly provided by Dr. T. Tanaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Biotin-conjugated reagent was developed with PE-conjugated avidin (Caltag Laboratories, San Francisco, CA). Each population of Vβ^+ cells was identified by three-color staining for CD3 (FITC), IL-2Rβ (PE), and corresponding Vβ (Red 613) (36). Red 613–conjugated streptavidin was obtained from Becton Dickinson and Co. (Mountain View, CA). All biotin-conjugated reagents of anti-Vβ mAbs against Vβ3, 6, 8, and 11 were obtained from Pharmingen. Other FITC- or PE-conjugated mAbs against H-2K^k, H-2K^b, IL-2Rα, NK1.1, TCR-α/β, TCR-γ/δ, CD4, CD8, Pgp-1 (CD44), Mel-14 (L-selectin), Thy1.1, and Thy1.2 antigens were also obtained from Pharmingen. The fluorescence-positive cells were analyzed with a FACScan® (Becton Dickinson and Co.). 10,000 cells were analyzed.

DNA Synthesis Assay. 40,000 MNC from the liver, spleen, and bone marrow (0.2 ml of 2 × 10^5 cells/ml) were cultured for 3 d with or without each immobilized mAb in a 96-well flat-bottomed microculture plate at 37°C in a CO_2 incubator. RPMI 1640 medium supplemented with 1% autologous mouse sera, 5 × 10^{-5} M 2-ME, and antibiotics was used. In some cultures, recombinant mouse IL-2 (Sionogi Seiyaku Co., Osaka, Japan) was added at a concentration of 10 U/ml. DNA synthesis was examined by [^{3}H]thymidine uptake assay, as shown previously (1). 0.5 μCi of [^{3}H]thymidine was added in the final 16 h of the culture. The mean ± 1 SD was determined in triplicate cultures.

Reverse Transcription-PCR Assay for the Detection of Messenger RNA (mRNA) of RAG-1 Gene. To detect mRNA of recombination-activating gene 1 (RAG-1) gene, RNA was reversely transcribed using the primers of these genes, and such cDNA was further amplified by the PCR method as previously described (9). Briefly, RNA was prepared from MNC of various organs by an acid guanidinium thiocyanate–phenol-chloroform method. cDNA was synthetized using 10 μg RNA, Moloney murine leukemia virus reverse transcriptase (200 U; GIBCO BRL, Gaithersburg, MD), random primer (Takara Shuzo Co., Tokyo, Japan), reverse transcriptase buffer (5×; GIBCO BRL), dithiothreitol (10 mM), dNTP (1 μM), and RNase inhibitor (50 U; Takara Shuzo Co.) in a final volume of 20 μl for 60 min at 42°C.

For PCR amplification, 5 μl of cDNA was transferred to individual tubes that contained 10 pM of the primers for RAG-1, Taq DNA polymerase (2.5 U; Toyobo Co., Osaka, Japan), and dNTP (200 μM) in 10 × PCR buffer (Toyobo Co.) and $MgCl_2$ (2 mM). For RAG-1, 5' primer = 5'-GTCTCCAGTAGTTCCAGA (276–294); 3' primer = 5'-CTAGCCTGAGTTCTCTTG (841–859), yielding a 603-bp fragment, were used. The samples were overlaid with light mineral oil (Sigma Chemical Co., St. Louis, MO), heated to 94°C for 5 min to denature DNA/RNA duplexes, and then subjected to 30 amplification cycles of 50 s at 94°C, 30 s at 55°C, and 2 min at 72°C, in a thermal cycler (Takara Shuzo Co.). After amplification, 33% of the PCR products were electrophoresed on 3.0% agarose gel, stained with ethidium bromide, and visualized under UV illumination. Primers for β-actin were also used to assess the integrity of the RNA preparation.

Results

All T Cells Generated under Athymic Conditions Are int CD3 Cells. To identify lymphocyte subsets, including NK cells, int CD3 (or TCR) cells, and high CD3 cells (5, 36), two-

color staining for CD3 and IL-2Rβ was performed (Fig. 1 A). When MNC in the liver and spleen of control B6 mice (9 wk old) were examined, clear peaks of CD3$^-$IL-2Rβ^+ (mainly asialo GM$_1^+$ NK) cells, CD3-int$^+$IL-2Rβ^+ cells (i.e., int CD3 cells), and CD3-high$^+$IL-2Rβ^- cells (i.e., high CD3 cells) were identified. CD3$^-$IL-2Rβ^+ NK cells and int CD3 cells (arrows) were more abundant in the liver than in the spleen of the control B6 mice (Fig. 1 A, upper panels). Thymocytes in the B6 mice consisted of CD3$^-$, CD3-low$^+$, and CD3-high$^+$ cells, all lacking the expression of IL-2Rβ. In the case of B6 nude mice, all T cells in both the liver and spleen were int CD3 cells (Fig. 1 A, lower panels). We postulated that high CD3 cells and int CD3 cells might be of thymic origin and of extrathymic origin, respectively.

To directly prove this possibility, we used thymectomized (Tx($+$)) (B6 $\times$ C3H/He) F$_1$ mice (Fig. 1 B). These F$_1$ mice were thymectomized at the age of 7 wk and irradiated with 10 Gy 2 wk later. Nonthymectomized (Tx($-$)) F$_1$ mice were used in parallel. These Tx($-$) or Tx($+$) irradiated mice were immediately injected with 10^7 bone marrow cells of B6 mice. 2 wk later, MNC obtained from the liver and spleen of these BMT mice were examined. Surprisingly, all newly generated T cells, both in the liver and spleen of all these BMT mice, were IL-2Rβ^+ int CD3 cells at 2 wk after treatment, whether they were Tx($+$) or Tx($-$). In the case of Tx($-$) mice (Fig. 1 B, upper panels), a small but significant proportion of high CD3 cells were generated in the liver and spleen 4 and 8 wk after the treatment. In sharp contrast, Tx($+$) irradiated mice did not acquire such high CD3 cells in either organ at any time after treatment (Fig. 1 B, second row). This was true even 50 wk or more after the treatment (data not shown).

To determine the phenotype of the precursors for int CD3 cells in the bone marrow, further experiments were conducted (Fig. 1 B, third and fourth rows). For this purpose, we injected mice with Thy1.2$^+$ cell–depleted or CD3$^+$ cell–depleted bone marrow cells (10^7 cells/mouse). Interestingly, Thy1.2$^+$ cell–depleted bone marrow cells did not reconstitute int CD3 cells even at 8 wk after treatment. On the other hand, CD3$^+$ cell–depleted bone marrow cells reconstituted int CD3 cells in both organs, similar to the case of whole bone marrow cells. In a subsequent study, we also used liver MNC, splenocytes, and lymph node cells to reconstitute int CD3 cells in Tx($+$) irradiated mice (data not shown). Among these cells, 10^8 liver MNC, but not 10^7 liver MNC cells, reconstituted int CD3 cells, whereas 10^8 splenocytes or 10^8 lymph node cells did not. All mice injected with 10^7 or 10^8 splenocytes or lymph node cells died within 4 d after treatment.

To determine whether int CD3 cells were of donor or recipient origin, H-2K expression was investigated in terms of K^{b+k+} (F$_1$ recipient) or K^{b+} (B6 donor) (Fig. 1 C). Since these T cells were k$^-$b$^+$, they were of donor B6 origin. This result was derived from the liver of BMT mice, and the result from the spleen confirmed it.
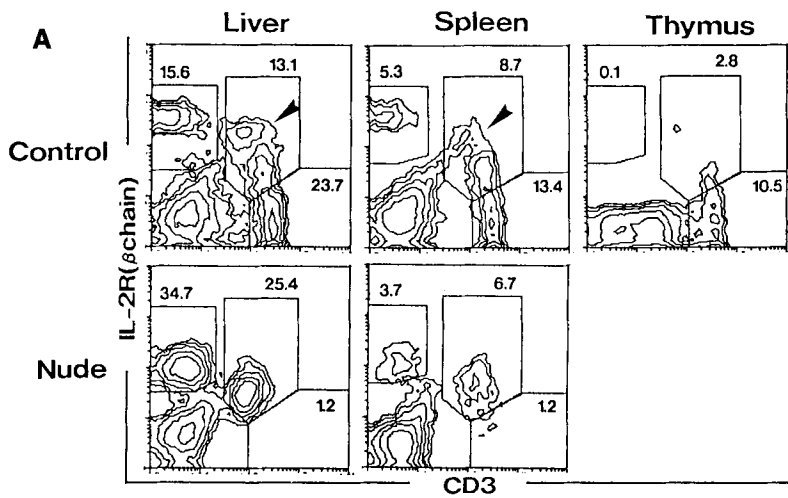
Phenotypic Characterization of Newly Generated int CD3 Cells. Further phenotypic characterization of int CD3 cells appearing in the liver of BMT (B6 → F$_1$) mice was carried out (Fig. 2). IL-2Rβ^+ int CD3 cells were all IL-2Rα^-. This phenotype was unique when compared with that of resting or activated high CD3 cells. Thus, resting high CD3 cells were IL-2Rβ^-IL-2Rα^-, while activated high CD3 cells were IL-2Rβ^+IL-2Rα^+ (36). One-third of the population of int CD3 cells coexpressed NK1.1 antigens, and NK1.1$^+$ T cells were confined to a population of int CD3 cells, concerning their intensity of CD3. All generated int CD3 cells were TCR-α/β^+ and expressed mainly CD8 antigens. DN CD4$^-$8$^-$ cells were very few in number, as shown by two-color staining for CD3 (green) and a mixture of CD4 and CD8 (red). The adhesion molecules, were CD44$^+$(Pgp-1)L-selectin$^-$(Mel-14). This phenotype of adhesion molecules on int CD3 cells was also unique, because high CD3 cells are CD44$^-$L-selectin$^+$ (33).
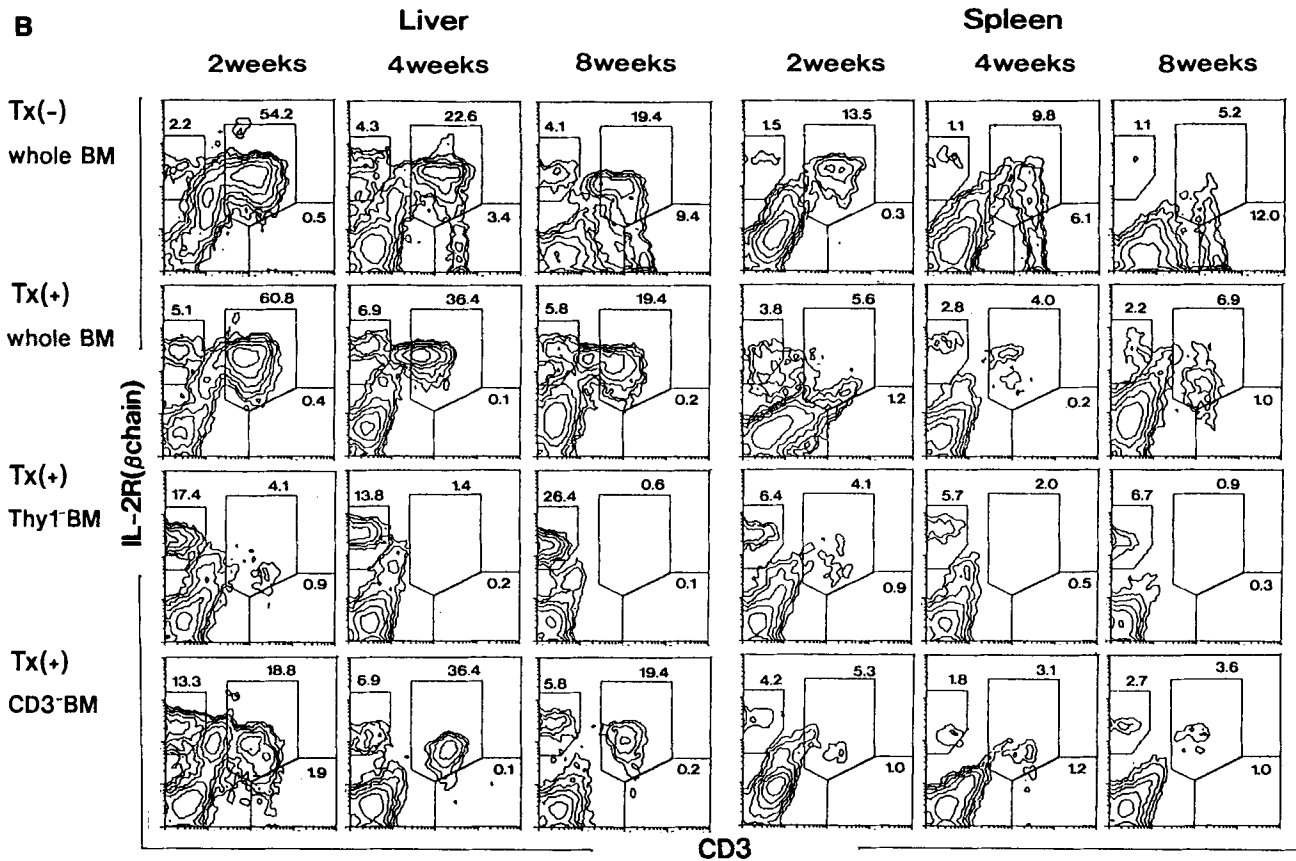
Confirmation of Extrathymic Generation of int CD3 Cells in Different Combinations of BMT. In a previous experiment, we used the combination of BMT, parent → F$_1$, to determine whether the recovered T cells were of donor origin. However, this combination induces GVHD. In this regard, we further examined the extrathymic generation of int CD3 cells in different combinations, i.e., C3H/He → C3H/He and B10.Thy1.1 → B6.Thy1.2 (Fig. 3). All recipient mice with or without thymectomy were irradiated (10 Gy) and were immediately injected with 10^7 bone marrow cells (depleted of CD3$^+$ cells) of the donor. 1 mo after such treatments, mice were examined on the levels of int CD3 cells and high CD3 cells. In both combinations, Tx($+$) mice produced only int CD3 cells, whereas Tx($-$) mice produced not only int CD3 cells but also high CD3 cells. In the latter combination, we confirmed that the expanding int CD3 cells in Tx($+$) mice were of donor origin (i.e., Thy1.1$^+$Thy1.2$^-$).

Identification of Self-reactive Clones among int CD3 Cells. As shown previously (2), int CD3 cells are comprised of self-reactive forbidden clones as estimated by anti-Vβ mAbs in conjunction with the endogenous superantigen Mls system. In this experiment, we investigated whether the newly generated int CD3 cells in BMT mice contained such self-reactive clones (Fig. 4). We used two combinations of BMT, i.e., B6 → (B6 $\times$ C3H/He) F$_1$ and C3H/He → C3H/He. B6 mice did not have self-reactive forbidden clones estimated by anti-Vβ mAbs in conjunction with the Mls system, because they lack I-E molecules, which undergo the antigen presentation. However, (B6 $\times$ C3H/He) F$_1$ mice have such clones, V$\beta3^+$ and V$\beta11^+$. Mls-1^{b}2^a superantigens are derived from parental female C3H/He mice. Control values in high CD3 cells and int CD3 cells were derived from either (B6 $\times$ C3H/He) F1 and C3H/He mice. int CD3 cells in the liver of BMT (Tx-RBMT) mice (F$_1$) showed extremely high levels of forbidden clones, both V$\beta3^+$ and V$\beta11^+$ (Fig. 4 A). On the other hand, high CD3 cells seen in the liver of control F$_1$ mice did not contain self-reactive clones V$\beta3^+$ and V$\beta11^+$, while int CD3 cells in the liver of control F$_1$ mice contained a few such clones. The nonforbidden clones V$\beta6^+$ and V$\beta8^+$ were found to be distributed in all cell fractions tested.

As already mentioned, the combination of BMT, B6 → (B6 $\times$ C3H/He) F$_1$, induces GVHD. In this regard, we also examined the levels of V$\beta3^+$ and V$\beta11^+$ forbidden

A

Liver Spleen Thymus

Control

IL-2R(β chain)

| 15.6 | 13.1 |
| | 23.7 |

| 5.3 | 8.7 |
| | 13.4 |

| 0.1 | 2.8 |
| | 10.5 |

Nude
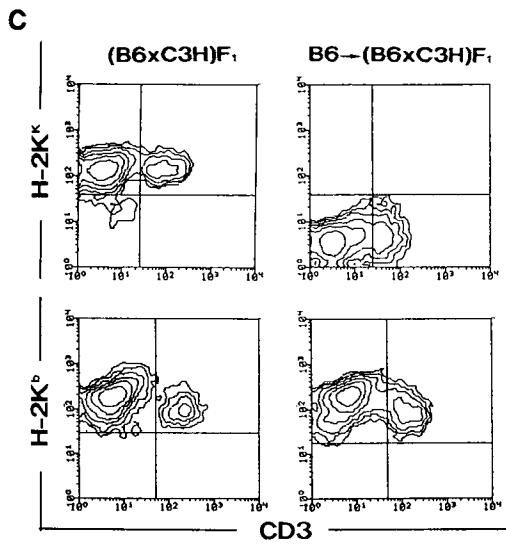
| 34.7 | 25.4 |
| | 1.2 |

| 3.7 | 6.7 |
| | 1.2 |

CD3

Figure 1. Expansion of int CD3 cells in Tx-RBMT mice. (*A*) Identification of int CD3 cells in the liver of control and athymic nude mice by two-color staining for CD3 and IL-2Rβ. (*B*) Expansion of int CD3 cells in Tx-RBMT mice. (*C*) int CD3 cells expanding in the liver are of donor origin. In experiment *A*, male C57BL/6 (B6)-+/+ and B6-*nu/nu* mice at the age of 15 wk were used. In experiments *B* and *C*, (B6×C3H/He) F₁ mice at the age of 7 wk were thymectomized. 2 wk later, these F₁ mice were irradiated (10 Gy), and 10⁷ bone marrow cells of B6 mice were immediately intravenously injected. Some Tx(−) F₁ mice were also used. As sources of bone marrow cells, whole cells, Thy-1⁺ cell–depleted cells, and CD3⁺ cell–depleted cells were used. Two-color staining for CD3 and IL-2Rβ was carried out to identify a population of int CD3 cells. By this staining, int CD3 cells were estimated to be CD3-int⁺IL-2Rβ⁺; high CD3 cells (i.e., T cells of thymic origin) were estimated to be CD3-high⁺IL-2Rβ⁻; and NK cells were estimated to be CD3⁻IL-2Rβ⁺. To determine the origin of T cells expanding in BMT mice, two-color staining of CD3 and H-2Kᵏ (or H-2Kᵇ) was also performed. Numbers in the figure indicate the percentages of fluorescence-positive cells in the corresponding area.

B

Liver Spleen

2 weeks 4 weeks 8 weeks   2 weeks 4 weeks 8 weeks

IL-2R(β chain)

Tx(−) whole BM

| 2.2 | 54.2 | | 4.3 | 22.6 | | 4.1 | 19.4 | | 1.5 | 13.5 | | 1.1 | 9.8 | | 1.1 | 5.2 |
| | 0.5 | | | 3.4 | | | 9.4 | | | 0.3 | | | 6.1 | | | 12.0 |

Tx(+) whole BM

| 5.1 | 60.8 | | 6.9 | 36.4 | | 5.8 | 19.4 | | 3.8 | 5.6 | | 2.8 | 4.0 | | 2.2 | 6.9 |
| | 0.4 | | | 0.1 | | | 0.2 | | | 1.2 | | | 0.2 | | | 1.0 |

Tx(+) Thy1⁻BM

| 17.4 | 4.1 | | 13.8 | 1.4 | | 26.4 | 0.6 | | 6.4 | 4.1 | | 5.7 | 2.0 | | 6.7 | 0.9 |
| | 0.9 | | | 0.2 | | | 0.1 | | | 0.9 | | | 0.5 | | | 0.3 |

Tx(+) CD3⁻BM

| 13.3 | 18.8 | | 6.9 | 36.4 | | 5.8 | 19.4 | | 4.2 | 5.3 | | 1.8 | 3.1 | | 2.7 | 3.6 |
| | 1.9 | | | 0.1 | | | 0.2 | | | 1.0 | | | 1.2 | | | 1.0 |

CD3

clones in the combination of BMT, C3H/He → C3H/He (Fig. 4 *B*). Although Tx-RBMT mice of this combination also contained Vβ3⁺ and Vβ11⁺ forbidden clones in the liver, the levels were extremely low compared with Tx-RBMT

mice of B6 → (B6 × C3H/He) F₁ combination. This raises the possibility that the high levels of forbidden clones seen in F₁ recipient mice might be responsible for the onset of GVHD in this combination.

C

(B6xC3H)F₁   B6→(B6xC3H)F₁

H-2Kᴷ

H-2Kᵇ

CD3

C3H→C3H

B10.Thy1.1
↓
B6.Thy1.2

Tx(+)   Tx(-)

IL-2R(βchain)

CD3

Thy1.1

Thy1.2

CD3   CD3

Expanding Forbidden Clones in F₁ Recipient Mice Could Respond to Immobilized Anti-Vβ3 mAb It was then investigated whether such self-reactive clones identified in BMT mice of the combination of B6 → (B6 × C3H/He) F₁ were in anergic states or in nonanergic states. Hepatic MNC isolated from either BMT mice (F₁) or control C3H/He mice were stimulated with immobilized anti-Vβ3 mAb (Fig. 5). Since anergic, self-reactive Vβ⁺ cells are known to proliferate in response to anti-Vβ mAb in the presence of a low dose of
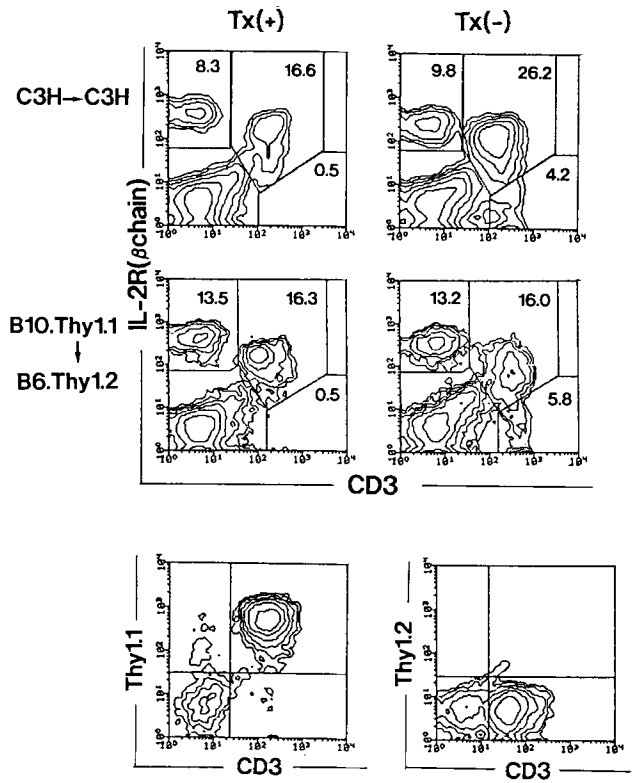
Figure 3. Confirmation of the extrathymic generation of int CD3 cells in other combinations of BMT. Two other combinations of BMT, C3H/He → C3H/He, and B10.Thy1.1 → B6.Thy1.2, were performed under athymic and thymic conditions. Mice were examined 1 mo after treatments. In both combinations, only int CD3 cells were generated in Tx(+) mice, whereas not only int CD3 cells but also high CD3 cells were generated in Tx(-) mice. In the combination of B10.Thy1.1 → B6.Thy1.2, the generated T cells were confirmed to be of donor origin (Thy1.1⁺1.2⁻).

IL-2R(βchain)

IL-2R(αchain)

NK1.1

γδ-TCR

CD4

CD4,CD8

Pgp-1

Mel-14

CD8   CD3   CD3   CD3
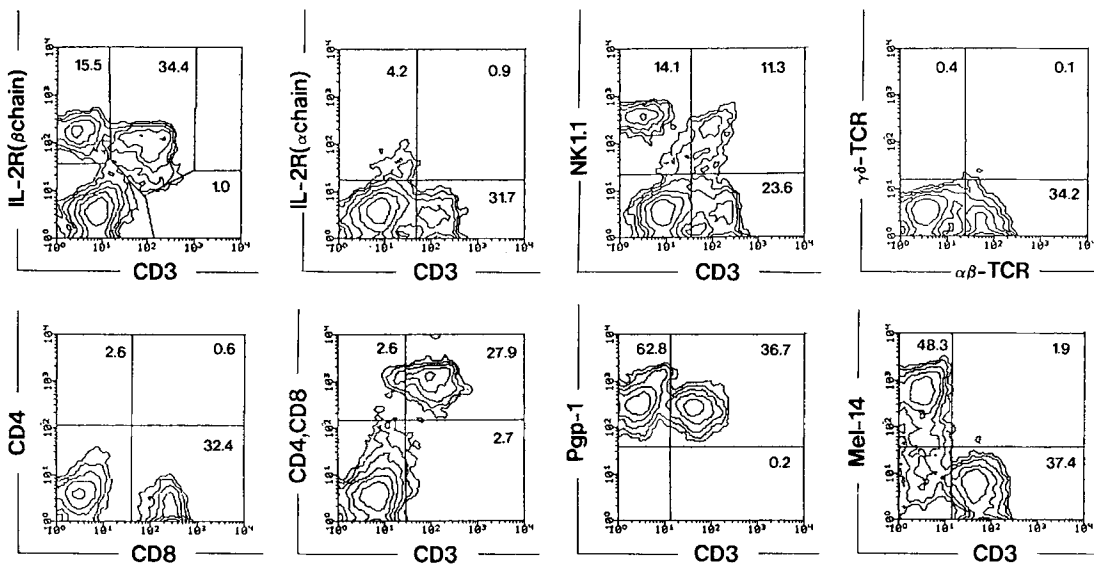
Figure 2. Further phenotypic characterization of int CD3 cells expanding in the liver of Tx-RBMT mice. Two-color stainings for various combinations, as indicated in the figure, were performed. Numbers in the figure indicate the percentages of fluorescence-positive cells in the corresponding area. int CD3 cells were estimated to be IL-2Rα⁻/β⁺, and one-third of this population expressed NK1.1 antigens. Almost all int CD3 cells generated were α/β T cells with CD8⁺ and Pgp1⁺Mel-14⁻ phenotypes.
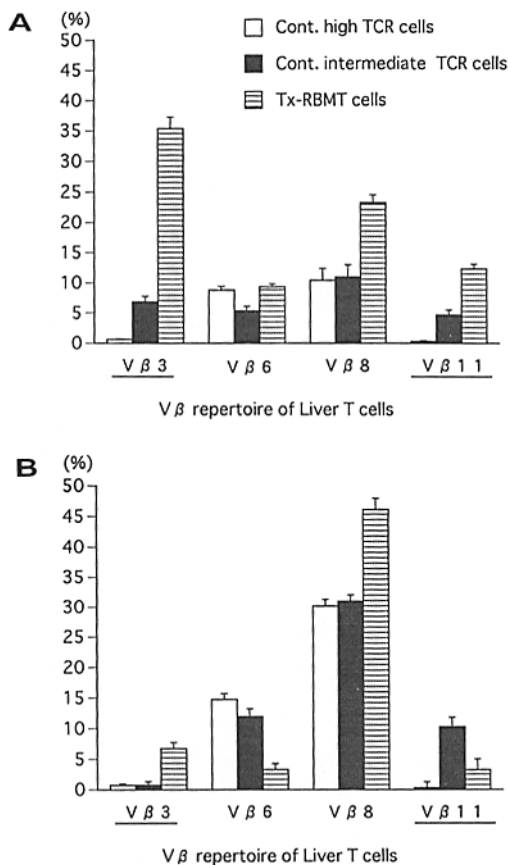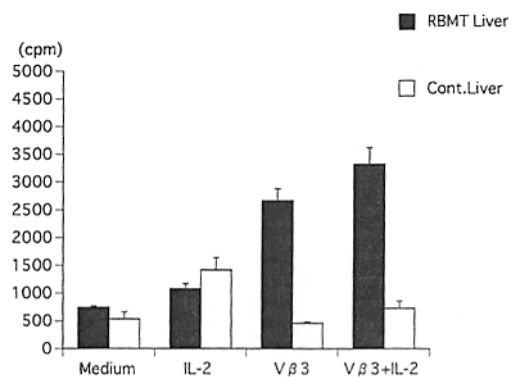
Figure 4. Identification of self-reactive forbidden clones among int CD3 cells. (A) B6 → (B6 × C3H/He) F₁; (B) C3H/He→C3H/He. Each population of Vβ⁺ cells was identified by three-color staining for CD3 (FITC), IL-2Rβ (PE), and corresponding Vβ (Red 613). Vβ3⁺ and Vβ11⁺ cells (*underlined*) are self-reactive forbidden clones, while Vβ6⁺ and Vβ8⁺ cells are nonforbidden clones in (B6×C3H/He) F₁ mice and C3H/He mice (Mls-1ᵇ2ᵃ). The mean ± 1 SD was determined from three experiments.

Figure 5. Proliferative response of newly generated int CD3 cells to immobilized anti-Vβ3 mAb in in vitro culture (*ordinate*, counts per minute of [³H]thymidine). Tx-RBMT mice were used 1 mo after BMT of the combination, B6 → (B6 × C3H/He) F₁. To examine whether newly generated int CD3 cells were in anergic states or in nonanergic states, the proliferate response of liver MNC in Tx-RBMT mice and control mice to the immobilized anti-Vβ3 mAb was analyzed. The mean ± 1 SD was determined by triplicate cultures.

IL-2 (10 U/ml), such culture conditions were applied in parallel. Newly generated hepatic T cells isolated from BMT mice were found to vigorously proliferate in response to anti-Vβ3 mAb, irrespective of the presence of IL-2 (i.e., nonanergic states). On the other hand, hepatic MNC in control C3H/He mice did not respond to anti-Vβ3 mAb even in the presence of IL-2 (i.e., highly anergic states).

The Expression of mRNA of RAG-1 by Liver MNC in BMT Mice. We then examined whether MNC obtained from the liver and spleen of BMT mice and control mice expressed mRNA of RAG-1 (Fig. 6). It was demonstrated that liver MNC in BMT mice highly expressed mRNA of RAG-1, possibly reflecting their active generation. At this amplification level (30 cycles), the expression of mRNA of RAG-1 in liver MNC of control mice was minor, and that in splenic MNC of both BMT and control mice was at an undetectable level.

Discussion

In the present study, using Tx(+) irradiated mice subjected to BMT, we directly demonstrated that all T cells generated

in the liver and spleen under athymic conditions were int CD3 (or TCR) cells with unique properties. As already shown in our previous studies (5, 6), all T cells seen in the liver and other peripheral immune organs except the intestine in congenitally athymic nude mice are int TCR cells. Moreover, mRNA of RAG-1 and RAG-2 was detectable in liver MNC in control mice (9). In light of these findings, we proposed the possibility that int TCR cells are generated extrathymically and that the liver is the major site of their differentiation (5, 6). However, various criticisms have been raised: (a) Congenitally athymic nude mice still have a remnant of the thymus, and these mice are not athymic; and (b) the expression of RAG-1 and RAG-2 is only high at the precursor cell level, and, therefore, it is difficult to estimate which type of precursors for T and B cells express mRNA of RAG-1 and RAG-2.
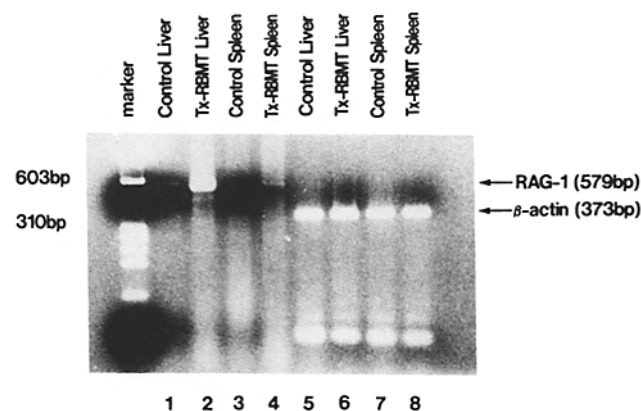
Figure 6. Expression of mRNA of RAG-1 by liver MNC isolated from Tx-RBMT mice. To detect mRNA of RAG-1 gene, RNA was reversely transcribed using the primers of these genes, and cDNA was further amplified by the PCR method. A clear band of RAG-1 was observed in liver MNC of Tx-RBMT mice.

To answer these questions, we used Tx-RBMT mice. By using this protocol, we were also able to investigate the influence of the thymus on the generation of T cell subsets and the phenotype of precursor cells of extrathymic T cells in the bone marrow. Since these BMT mice produced only int CD3 cells, and Tx(−) mice with BMT always produced not only int CD3 cells but also high CD3 cells, we estimate that int CD3 cells are of extrathymic origin and high CD3 cells are of thymic origin. The phenotype of precursors of extrathymic T cells, i.e., Thy-1⁺CD3⁻, was also a very interesting finding of this study. The properties of int CD3 cells generated under athymic conditions in this experimental protocol coincided with those of int CD3 cells seen in the liver of normal mice and congenitally athymic mice (5, 6, 32, 33). Specifically, both of them expressed CD3 of intermediate levels, both had the phenotype of IL-2Rα⁻/β⁺ and CD44⁺L-selectin⁻, and both contained self-reactive clones. The only difference was that newly generated int CD3 cells in this study showed a predominance of CD8⁺ cells (very few DN CD4⁻8⁻ cells), and self-reactive clones were not in anergic states, especially the combination of parent and F₁ mice. In a recent study, we reported that nonanergic, active self-reactive T cells became effector cells for autoimmune states seen in chronic GVHD (35). It has previously been shown that int CD3 cells in normal mice are usually in anergic states (7, 39). In a syngeneic combination (C3H/He → C3H/He), self-reactive clones among int CD3 cells were very low (Fig. 4) and were at anergic state (data not shown). Taken together, the elevated levels of forbidden clones among int CD3 cells are important indicators predicting the onset of GVHD. In any case, forbidden clones were always confined to int CD3 cells but not at all to high CD3 cells.

In this study, we investigated the expression levels of mRNA of RAG-1 of newly generated T cells in Tx-RBMT mice. Although we were not able to determine which subsets expressed high levels of mRNA, it was true that liver MNC in these mice expressed higher levels of mRNA than did liver MNC in control mice. It can be speculated that some T or B cells intensively differentiated to produce a mature repertoire of TCR or immunoglobulins under this condition. Several questions regarding int CD3 cells arise; e.g., how different are int CD3 cells from activated, thymus-derived T cells? Thymus-derived T cells acquire IL-2Rβ and CD44 antigens after activation. However, activated, thymus-derived T cells always acquire IL-2Rβ in parallel with the highest expression of IL-2Rα (36). Only NK cells and extrathymic T cells

constitutively express IL-2Rβ but lack IL-2Rα (5). Similarly, activated thymus-derived T cells acquire CD44 antigen but do not lose the expression of L-selectin at that time (33). More importantly, we have not observed that high CD3 cells down-regulate their expression of TCR or CD3 after activation by any antigenic and mitogenic stimulations (32, 33).

Several investigators have recently focused attention on NK1.1⁺ α/β T cells with DN or CD4⁺ phenotype (16–24). These cells correspond to the int TCR cells in our studies. There are some int CD3 cells lacking the expression of NK1.1 antigens, as shown in this study. Such NK1.1⁺ T cells or int CD3 cells are generated extrathymically. However, they are also generated through an alternative pathway of T cell differentiation in the thymus, independent from the main stream of the major intrathymic pathway (21–24). Many functional studies on NK1.1⁺ T cells with DN CD4⁻8⁻ phenotype revealed that this population was very important to understanding the mechanism involved in hybrid resistance (25) and autoreactivity (26–30). NK1.1⁺ T cells preferentially used invariant Vα14 chain (9, 31). We have recently investigated the relationship between int CD3 cells and NK1.1⁺ T cells in various immune organs of B6 mice in detail (manuscript in preparation). All NK1.1⁺ cells are confined to IL-2Rβ⁺ int CD3 cells, and both NK1.1⁺ and NK1.1⁻ subsets are present among intermediate CD3 cells. Such NK1.1⁺ subsets mainly comprise DN CD4⁻8⁻ cells and CD4⁺ cells, whereas such NK1.1⁻ subsets comprise CD8⁺ cells (predominantly) as well as DN and CD4⁺ cells.

We propose the possibility that int TCR cells generated in the liver and thymus should be categorized as a common lineage of T cells. Because of their primitive phenotype and functions, they may be called "primitive or primordial" T cells. These primitive T cells generated in the primitive pathways of T cell differentiation (TCR⁻ → TCRⁱⁿᵗ) might be a fundamental immune system phylogenetically developed earlier than the major intrathymic pathway (i.e., TCR⁻ → TCRˡᵒʷ → TCRʰⁱᵍʰ). In a series of recent studies (32, 35), we reported that these primitive T cells are quite important to the pathogenesis of autoimmune diseases or autoimmune states induced by chronic GVHD because of their autoreactivity of self-reactive clones. In the present study we provide evidence that int TCR cells are extrathymically generated in the liver and other immune organs of normal mice under athymic conditions. However, we do not deny the independent generation of int CD3 cells in the alternative intrathymic pathway.

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References

1. Ohteki, T., S. Seki, T. Abo, and K. Kumagai. 1990. Liver is a possible site for the proliferation of abnormal CD3$^+$4$^-$8$^-$ double-negative lymphocytes in autoimmune MRL-*lpr/lpr* mice. *J. Exp. Med.* 172:7–12.

2. Abo, T., T. Ohteki, S. Seki, N. Koyamada, Y. Yoshikai, T. Masuda, H. Rikiishi, and K. Kumagai. 1991. The appearance of T cells bearing self-reactive T cell receptor in the livers of mice injected with bacteria. *J. Exp. Med.* 174: 417–424.

3. Seki, S., T. Abo, T. Ohteki, K. Sugiura, and K. Kumagai. 1991. Unusual $\alpha\beta$-T cells expanded in autoimmune *lpr* mice are probably a counterpart of normal T cells in the liver. *J. Immunol.* 147:1214–1221.

4. Okuyama, R., T. Abo, S. Seki, T. Ohteki, K. Sugiura, A. Kusumi, and K. Kumagai. 1992. Estrogen administration activates extrathymic T cell differentiation in the liver. *J. Exp. Med.* 175:661–669.

5. Iiai, T., H. Watanabe, S. Seki, K. Sugiura, K. Hirokawa, M. Utsuyama, H. Takahashi-Iwanaga, T. Iwanaga, T. Ohteki, and T. Abo. 1992. Ontogeny and development of extrathymic T cells in mouse liver. *Immunology.* 77:556–563.

6. Ohtsuka, K., T. Iiai, H. Watanabe, T. Tanaka, M. Miyasaka, K. Sato, H. Asakura, and T. Abo. 1994. Similarities and differences between extrathymic T cells residing in mouse liver and intestine. *Cell. Immunol.* 153:52–66.

7. Goossens, P.L., H. Jouin, G. Marchal, and G. Milon. 1990. Isolation and flow cytometric analysis of the free lymphomyeloid cells present in murine liver. *J. Immunol. Methods.* 132:137–144.

8. Murosaki, S., Y. Yoshikai, A. Ishida, T. Nakamura, G. Matsuzaki, H. Takimoto, H. Yuuki, and K. Nomoto. 1991. Failure of T cell receptor Vβ negative selection in murine intestinal intra-epithelial lymphocytes. *Int. Immunol.* 3:1005–1013.

9. Makino, Y., N. Yamagata, T. Sasho, Y. Adachi, R. Kanno, H. Koseki, M. Kanno, and M. Taniguchi. 1993. Extrathymic development of Vα14-positive T cells. *J. Exp. Med.* 177:1399–1408.

10. Ohteki, T., R. Okuyama, S. Seki, T. Abo, K. Sugiura, A. Kusumi, T. Ohmori, H. Watanabe, and K. Kumagai. 1992. Age-dependent increase of extrathymic T cells in the liver and their appearance in the periphery of older mice. *J. Immunol.* 149:1562–1570.

11. Masuda, T., T. Ohteki, T. Abo, S. Seki, M. Nose, H. Nagura, and K. Kumagai. 1991. Expansion of the population of double negative CD4$^-$8$^-$ T$\alpha\beta$-cells in the liver is a common feature of autoimmune mice. *J. Immunol.* 147:2907–2912.

12. Tsuchida, M., H. Hanawa, H. Hirahara, H. Watanabe, Y. Matsumoto, H. Sekikawa, and T. Abo. 1994. Identification of CD4$^-$ CD8$^-$ $\alpha\beta$ T cells in the subarachnoid space of rats with experimental autoimmune encephalomyelitis. A possible route by which effector cells invade the lesions. *Immunology.* 81:420–427.

13. Seki, S., T. Abo, K. Sugiura, T. Ohteki, T. Kobata, H. Yagita, K. Okumura, H. Rikiishi, T. Masuda, and K. Kumagai. 1991. Reciprocal T cell responses in the liver and thymus of mice injected with syngeneic tumor cells. *Cell. Immunol.* 137:46–60.

14. Ohmori, K., T. Iiai, H. Watanabe, T. Tanaka, M. Miyasaka, and T. Abo. 1993. Activation of extrathymic T cells in the liver of mice bearing syngeneic tumors. *Biomed. Res. (Tokyo).* 14:65–79.

15. Iiai, T., H. Watanabe, T. Iwamoto, I. Nakashima, and T. Abo. 1994. Predominant activation of extrathymic T cells during

16. Budd, R.C., G.C. Miescher, R.C. Howe, R.K. Lees, C. Bron, and H.R. MacDonald. 1987. Developmentally regulated expression of T cell receptor β chain variable domains in immature thymocytes. *J. Exp. Med.* 166:577–582.

17. Fowlkes, B.J., A.M. Kruisbeek, H. Ton-That, M.A. Weston, J.E. Coligan, R.H. Schwartz, and D.M. Pardoll. 1987. A novel population of T-cell receptor $\alpha\beta$-bearing thymocytes which predominantly expresses a single Vβ gene family. *Nature (Lond.).* 329:251–254.

18. Crispe, I.N., M.W. Moore, L.A. Husmann, L. Smith, M.J. Bevan, and R.P. Shimonkevitz. 1987. Differentiation potential of subsets of CD4$^-$8$^-$ thymocytes. *Nature (Lond.).* 329:336–339.

19. Takahama, Y., A. Kosugi, and A. Singer. 1991. Phenotype, ontogeny, and repertoire of CD4$^-$CD8$^-$ T cell receptor $\alpha\beta^+$ thymocytes. Variable influence of self-antigens on T cell receptor Vβ usage. *J. Immunol.* 146:1134–1141.

20. Arase, H., N. Arase-Fukushi, R.A. Good, and K. Onoe. 1993. Lymphokine-activated killer cell activity of CD4$^-$CD8$^-$ TCR$\alpha\beta^+$ thymocytes. *J. Immunol.* 151:546–555.

21. Egerton, M., and R. Scollay. 1990. Intrathymic selection of murine TCR$\alpha\beta^+$CD4$^-$CD8$^-$ thymocytes. *Int. Immunol.* 2:157–162.

22. Suda, T., and A. Zlotnik. 1993. Origin, differentiation, and repertoire selection of CD3$^+$CD4$^-$CD8$^-$ thymocytes bearing either $\alpha\beta$ or $\gamma\delta$ T cell receptors. *J. Immunol.* 150:447–455.

23. Seki, S., D.H. Kono, R.S. Balderas, and A.N. Theofilopoulos. 1994. Vβ repertoire of murine hepatic T cells. Implication for selection of double-negative $\alpha\beta^+$ T cells. *J. Immunol.* 153:637–646.

24. Ohteki, T., and H.R. MacDonald. 1994. Major histocompatibility complex class I related molecules control the development of CD4$^+$8$^-$ and CD4$^-$8$^-$ subsets of natural killer 1.1$^+$ T cell receptor-α/β^+ cells in the liver of mice. *J. Exp. Med.* 180:699–704.

25. Takeda, K., M.W. Moore, and G. Dennert. 1994. Acute rejection of grafts in mice. Dependence on and independence of functional TCR in the rejection process. *J. Immunol.* 152:4407–4416.

26. Prud'homme, G.J., D.C. Boccarro, and E.C.H. Luke. 1991. Clonal deletion and autoreactivity in extrathymic CD4$^-$ CD8$^-$ (double negative) T cell receptor-$\alpha\beta$ T cells. *J. Immunol.* 147:3314–3318.

27. Troutt, A.B., and A. Kelso. 1992. Enumeration of lymphokine mRNA-containing cells *in vivo* in a murine graft-versus-host reaction using the PCR. *Proc. Natl. Acad. Sci. USA.* 89:5276–5280.

28. Martínez-A., C., M.A.R. Marcos, I.M. de Alboran, J.M. Alonso, R. de Cid, G. Kroemer, and A. Coutinho. 1993. Functional double-negative T cells in the periphery express T cell receptor Vβ gene products that cause deletion of single-positive T cells. *Eur. J. Immunol.* 23:250–254.

29. Arase, H., N. Arase, Y. Kobayashi, Y. Nishimura, S. Yonehara, and K. Onoé. 1994. Cytotoxicity of fresh NK1.1$^+$ T cell receptor α/β^+ thymocytes against a CD4$^+$CD8$^+$ thymocyte population associated with intact Fas antigen expression on the target. *J. Exp. Med.* 180:423–432.

30. Sakamoto, H., J. Michaelson, W.K. Jones, A.T. Bhan, S. Abhyankar, M. Silverstein, D.E. Golan, S.J. Burakoff, and J.L.M.

Ferrara. 1991. Lymphocytes with a CD4⁺CD8⁻CD3⁻ phenotype are effectors of experimental cutaneous graft-versus-host disease. *Proc. Natl. Acad. Sci. USA.* 88:10890–10894.

31. Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I–specific CD4⁺ and CD4⁻CD8⁻ T cells in mice and humans. *J. Exp. Med.* 180:1097–1106.

32. Abo, T., H. Watanabe, T. Iiai, M. Kimura, K. Ohtsuka, K. Sato, M. Ogawa, H. Hirahara, S. Hashimoto, H. Sekikawa, and S. Seki. 1994. Extrathymic pathways of T-cell differentiation in the liver and other organs. *Int. Rev. Immunol.* 11:61–102.

33. Ohtsuka, K., K. Hasegawa, K. Sato, K. Arai, H. Watanabe, H. Asakura, and T. Abo. 1994. A similar expression pattern of adhesion molecules between intermediate TCR cells in the liver and intraepithelial lymphocytes in the intestine. *Microbiol. Immunol.* 38:677–683.

34. Okada, C.Y., B. Holzmann, C. Guidos, E. Palmer, and I.L. Weissman. 1990. Characterization of a rat monoclonal antibody specific for a determinant encoded by the Vβ7 gene segment. Depletion of Vβ7⁺ T cells in mice with Mls-1ª haplotype. *J. Immunol.* 144:3473–3477.

35. Ikarashi, Y., T. Abo, K. Kawai, T. Iiai, H. Watanabe, K. Suzuki,

Y. Matsumoto, S. Omata, and M. Fujiwara. 1994. Expansion of intermediate T-cell receptor cells in mice with autoimmune-like graft-versus-host disease. *Immunology.* 83:205–212.

36. Watanabe, H., T. Iiai, M. Kimura, K. Ohtsuka, T. Tanaka, M. Miyasaka, M. Tsuchida, H. Hanawa, and T. Abo. 1993. Characterization of intermediate TCR cells in the liver of mice with respect to their unique IL-2R expression. *Cell. Immunol.* 149:331–342.

37. Huang, L., K. Sye, and I.N. Crispe. 1994. Proliferation and apoptosis of B220⁺CD4⁻CD8⁻TCRαβ^intermediate T cells in the liver of normal adult mice: implication for *lpr* pathogenesis. *Int. Immunol.* 6:533–540.

38. Tanaka, T., M. Tsudo, H. Karasuyama, F. Kitamura, T. Kono, M. Hatakeyama, T. Taniguchi, and M. Miyasaka. 1991. A novel monoclonal antibody against murine IL-2 receptor β-chain. Characterization of receptor expression in normal lymphoid cells and EL-4 cells. *J. Immunol.* 147:2222–2228.

39. Tsuchida, M., T. Iiai, H. Watanabe, and T. Abo. 1992. Relative resistance of intermediate TCR cells to anti-CD3 mAb in mice *in vivo* and their partial functional characterization. *Cell. Immunol.* 145:78–90.