Acta Medica et Biologica
Vol. 41, No. 2, 59-79, 1993

Multiplicity of Sites for Extrathymic T-cell Differentiation

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Received June 15, 1993

Summary. **In addition to an intrathymic pathway of T-cell differentiation, extrathymic pathways of T-cell differentiation have been found to exist at multiple sites in the living bodies of both mice and humans. Such sites include the sinusoids in the liver, intraepithelial sites in the intestine, the splenic red pulp, the thymic medulla, the decidua in the uterus, and the omentum in the peritoneal cavity. Although extrathymic pathways are minimal in youth, they become predominant with aging and under conditions of bacterial infections, pregnancy, malignancies and autoimmune diseases. Since T cells with extrathymic properties are present even in the thymic medulla, and all of them carry many properties of primitive T cells irrespective the sites where they exist, their differentiation pathways should be termed "primitive pathways of T-cell differentiation" rather than extrathymic pathways. In this review, we introduce the phenotypic and functional properties of these primitive T cells and discuss why such primitive T cells exist at multiple sites.**

I. Introduction

It is well known that T cells differentiate in the thymus,[1-4] where positive and negative selections of T cell clones take place. As a result, most mature T cells after maturation in the thymus comprise cells that recognize foreign antigens in the context of major histocompatibility complex (MHC) antigens. On the other hand, recent studies have demonstrated that extrathymic pathways of T-cell differentiation exist in the liver[5-15] and intenstine.[16-19]

Extrathymic T cells at these sites have common and distinct properties as primitive lymphocytes. For example, both T cells in the liver and intestine display a morphology of large granular lymphocytes (LGL),[6,18] contain a considerably large proportion of $\gamma\delta$ T cells as well as $\alpha\beta$ T cells,[20-22] and comprise a significant proportion of self-reactive forbidden clones estimated by a system of Mls and anti-Vβ monoclonal antibodies (mAb).[8,12,23] Similar to NK cells, all extrathymic T cells in the liver and approximately half of those in the intestine have been found to constitutively express IL-2 receptor β-chain (IL-2Rβ).[24] Extrathymic T cells contain double-negative (DN) $CD4^-8^-$ cells (one-third of the population) as well as single-positive $CD4^+$ or $CD8^+$ cells.[5,9]

It is conceivable that extrathymic T cells are more primitive than regular T cells of thymic origin.[14,25] The order of lymphocyte development in phylogeny may be as follows:

NK cells → extrathymic T cells → regular T cells.

Because extrathymic T cells preferentially comprise self-reactive V$\beta 8^+$ cells and forbidden T cell clones, they might be beneficial for the survey of atypical cells generated in the body. T cells of thymic origin and extrathymic T cells seem to form two major immune systems for the recognition of foreign antigens and self-antigens, respectively. We feel that, without understanding the functions of these primitive, extrathymic T cells, many immunological phenomena involved in incurable diseases and other conditions including malignancies, autoimmune diseases, chronic Graft-versus-Host (GVH) disease, infections by intracellular pathogens, aging and pregnancy, can not be properly understood.

II. Identification of intermediate TCR cells at multiple sites

In a series of recent studies, we revealed that extrathymic T cells express a T cell receptor (TCR) of intermediate intensity and IL-2Rβ of high intensity in mice.[13,15,26] In this regard, we have termed this population "intermediate TCR cells".[9,14]

As well established, regular T cells differentiate in the thymus, passing through a stage at which dull TCR is carried, and then acquiring bright TCR at

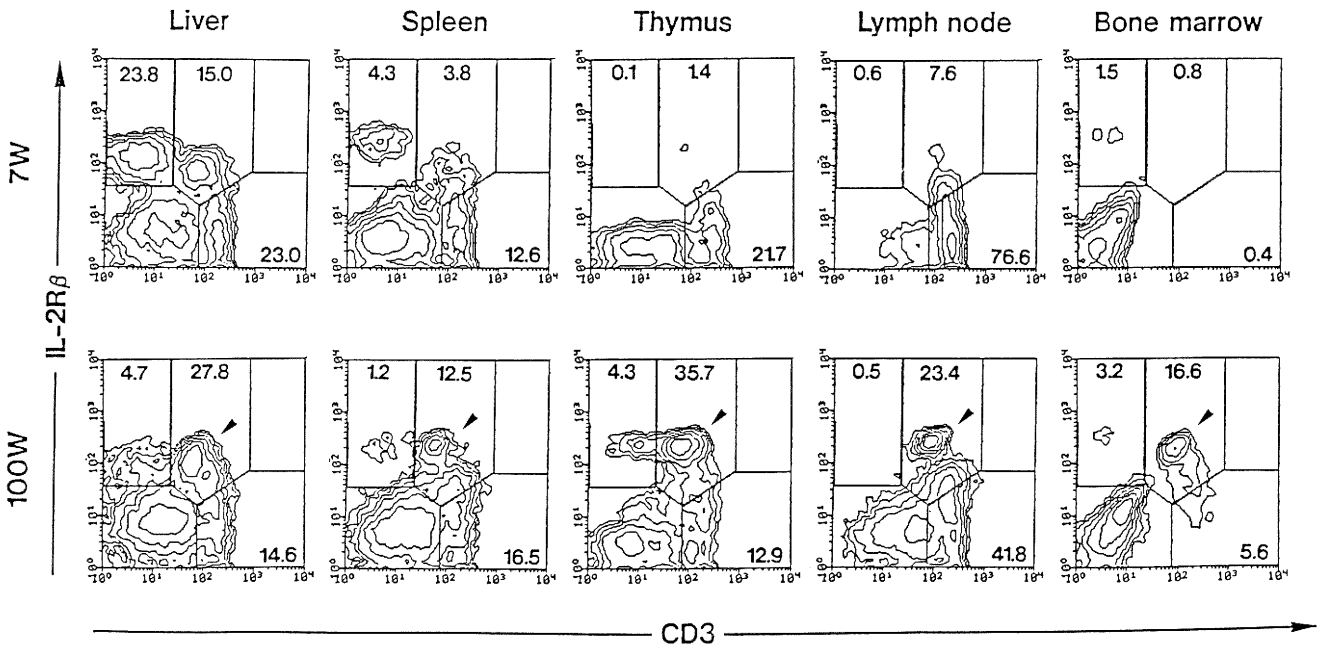

Fig. 1. Identification of intermediate TCR cells in the various immune organs in mice. Mice at ages of 7 (young) and 100 wks (old) were examined. Intermediate TCR cells are identified by two-color immunofluorescence staining for CD3 and IL-2Rβ. Numbers in the squares show the percentages of fluorescence positive cells in respective positions. CD3-intermediate$^+$ IL-2Rβ^+ cells (indicated by arrowheads) increased throughout the organs in older mice.

maturity.[1-4] The intensity of intermediate TCR on extrathymic T cells in the liver is at an intermediate position between those of dull and bright TCR on thymocytes.[9] As shown in Fig. 1, intermediate TCR cells, as well as other lymphocyte populations, could be indentified by two-color immunofluorescence tests using anti-TCR (or CD3) and anti-IL-2Rβ mAbs. Here, mononuclear cells (MNC) were isolated from various organs of mice at the ages of 7 wks and 100 wks. When MNC in the liver of mice aged 7 wks were observed, four lymphocyte populations were clearly identifiable: namely, CD3$^-$IL-2Rβ^- (mainly B cells), CD3$^-$IL-2Rβ^+ (NK cells), CD3-intermediate$^+$ IL-2Rβ^+ (extrathymic T cells) and CD3-bright$^+$ IL-2Rβ^- (regular T cells of thymic origin).

As already reported,[27,28] NK cells were confirmed to be most abundant in the liver. This is true even in the case of intermediate TCR cells. In other words, primitive lymphocytes, i.e., both NK and extrathymic T cells, are primarily present in the liver (i.e., hepatic sinusoids). As shown in the figure, B cells were most abundant in the spleen, whereas regular T cells were most abundant in the lymph nodes. Neither extrathymic T cells nor regular T cells existed in the bone marrow.

As for the thymus, almost all cells lacked the expression of IL-2Rβ and consisted of null, dull and bright CD3 (or TCR) cells. However, it was noteworthy that intermediate TCR cells became prominent throughout the organs tested in fully mature mice aged 100 wks. Even in the thymus, a considerably large proportion of intermediate TCR cells were demonstrated. As shown later, such intermediate TCR cells localize in the thymic medulla but not in the cortex.[29] It is suggested in this section that intermediate TCR cells expand with aging when the thymus becomes involuted.

Several investigators have recently reported minor populations of T cells with unique phenotypes in the thymus, spleen and other peripheral organs. Arase et al. demonstrated the existence of CD4$^+$8$^-$ cells expressing TCR of lower intensity in the thymus.[30] In contrast to regular thymic T cells, these T cells were NK1.1$^+$ CD44$^+$ (Pgp-1), heat-stable antigen$^-$(HSA), and Mel-14$^-$. As touched upon previously,[9,13] these properties correspond to those intermediate TCR cells in our studies.

Kikly and Dennert found that NK1.1$^+$ CD3$^+$ cells with a DN CD4$^-$8$^-$ phenotype became prominent in the spleen of mice during acute marrow graft rejection.[31] These T cells showed extrathymic development and were responsible for F_1 hybrid resis-

tance. The data showed that these T cells had CD3 of intermediate intensity. Mieno et al. also reported that DN $\alpha\beta$ T cells became detectable in the spleen during allogeneic tumor rejection, especially when $CD8^+$ cytotoxic T cells were deleted *in vivo*.[32] Similarly, a small but significant proportion of DN $\alpha\beta$ T cells with TCR of low density were also reported in the thymus,[33-42] spleen[31,32,42,43] lymph nodes[44] and bone marrow[45] by other investigators. It is conceivalbe that all these T cells with important biological functions may belong to some of the intermediate TCR cells described here. Thus far, investigators have not categorized the above populations as a similar group of T cells. Detailed comparisons of the properties of intermediate TCR cells among the organs, and of their origin, i.e., questions as to whether they are independently generated from earlier precusors *in situ* or migrate from place to place, remain to be further investigated.

To determine the expression of CD4 and CD8 antigens on intermediate TCR cells in various organs, three-color stainings for IL-2Rβ, CD4 and CD8 were performed in mice injected with anti-asialo GM_1 antibody *in vivo*. Since all NK cells in these mice were eliminated, all IL-2Rβ^+ cells were intermediate TCR cells. The gated analysis of IL-2Rβ^+ intermediate TCR cells was carried out with respect to the expression of CD4 and CD8 (Fig. 2). It was clearly demonstrated that all intermediate TCR cells in the organs tested contained DN $CD4^-8^-$ cells as well as single-positive $CD4^+$ or $CD8^+$ cells. In particular, almost all IL-2Rβ^+ cells in the bone marrow were of the DN phenotype.

In this experiment, T cells in various organs, including the liver, spleen, lymph nodes and peripheral blood, of congenitally athymic nude mice were characterized (Fig. 3). To identify intermediate and bright TCR (CD3) cells, two-color staining for CD3 and IL-2Rβ was performed. Since TCR and CD3 molecules exist at a ratio of 1 : 1 on the cell surface, either staining produces the same pattern.[8,9] It was demonstrated that only intermediate CD3 cells, but not bright CD3 cells, were present throughout the organs tested in the case of athymic nude mice. In this regard, it is conceivable that intermediate TCR cells are definitely of extrathymic origin. NK cells (i.e., $CD3^-$IL-2Rβ^+) are normally present in these mice. Two-color staining for TCR-$\alpha\beta$ and TCR-$\gamma\delta$ also revealed that intermediate TCR cells were comprised

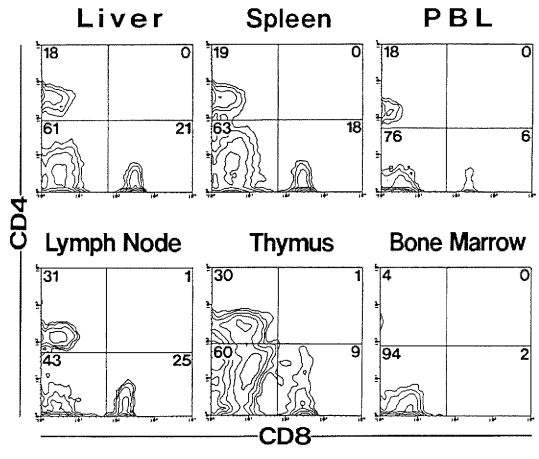

Fig. 2. The expression of CD4 and CD8 antigens on intermediate TCR cells in the various organs. Mice treated *in vivo* with anti-asialo GM_1 antibody were used. MNC were stained with different three colors of anti-IL-2Rβ, CD4 and CD8 mAbs. The gated analysis of IL-2Rβ^+ intermediate TCR cells was performed to determine the expression of CD4 and CD8 antigens. Numbers in the squares show the percentages of fluorescence positive cells in respective positions. Intermediate TCR cells contained DN $CD4^-8^-$ cells in every tested organ.

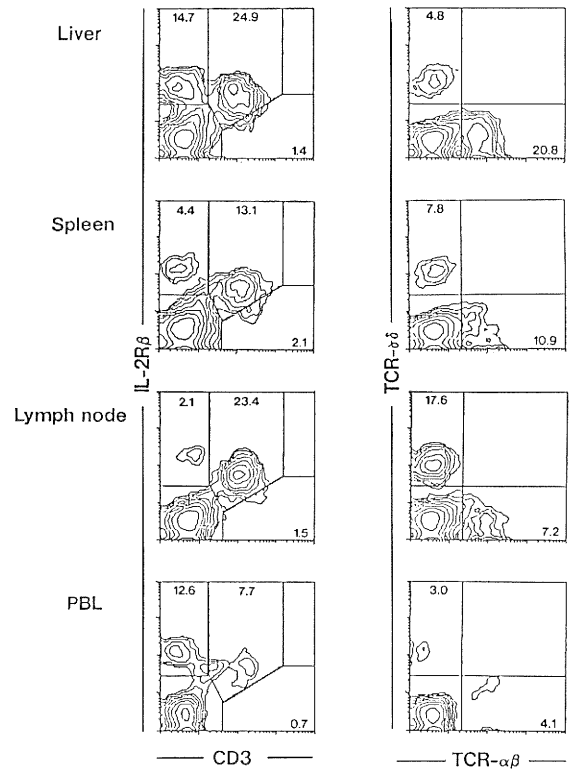

Fig. 3. All T cells in athymic nude mice are intermediate TCR cells. Two-color staining for CD3 and IL-2Rβ reveals that all T cells in athymic nude mice are intermediate TCR cells comprised of both $\alpha\beta$ and $\gamma\delta$ T cells.

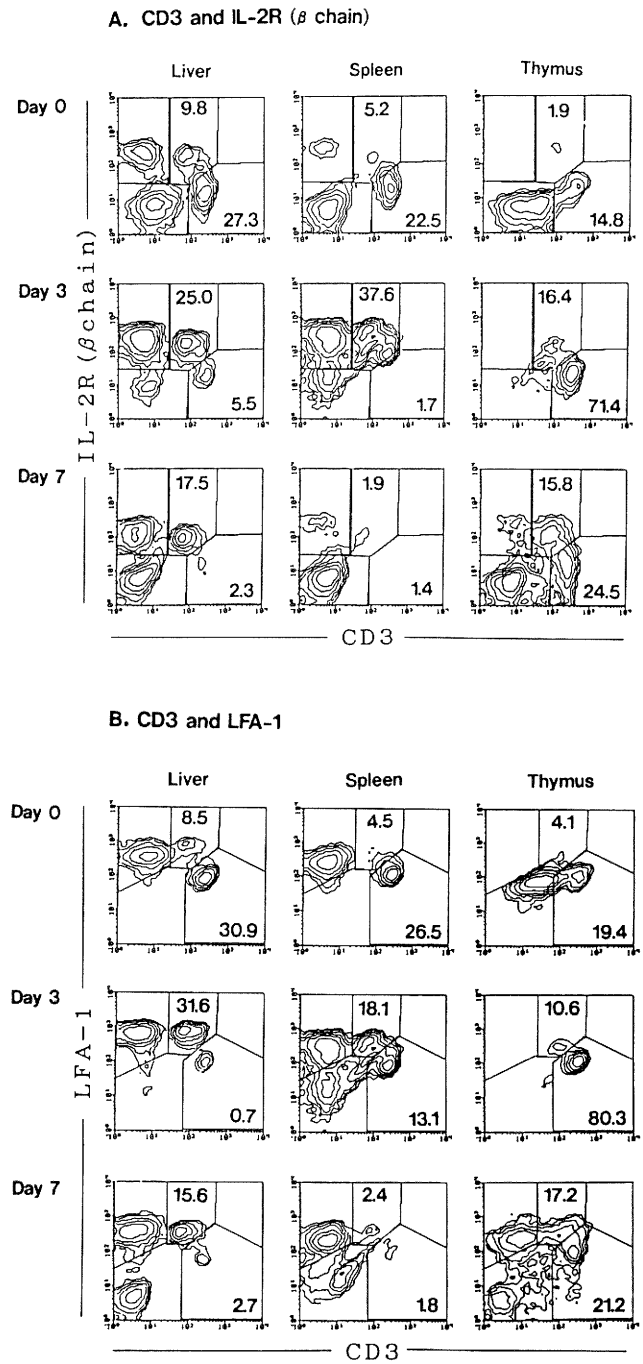

Fig. 4. Radioresistance of intermediate TCR cells in mice. Mice were irradiated with 9Gy and immediately injected with 10^7 syngeneic bone marrow cells. The two-color stainings (A and B) were performed on the indicated days. Intermediate TCR cells became prominent throughout the organs after treatment.

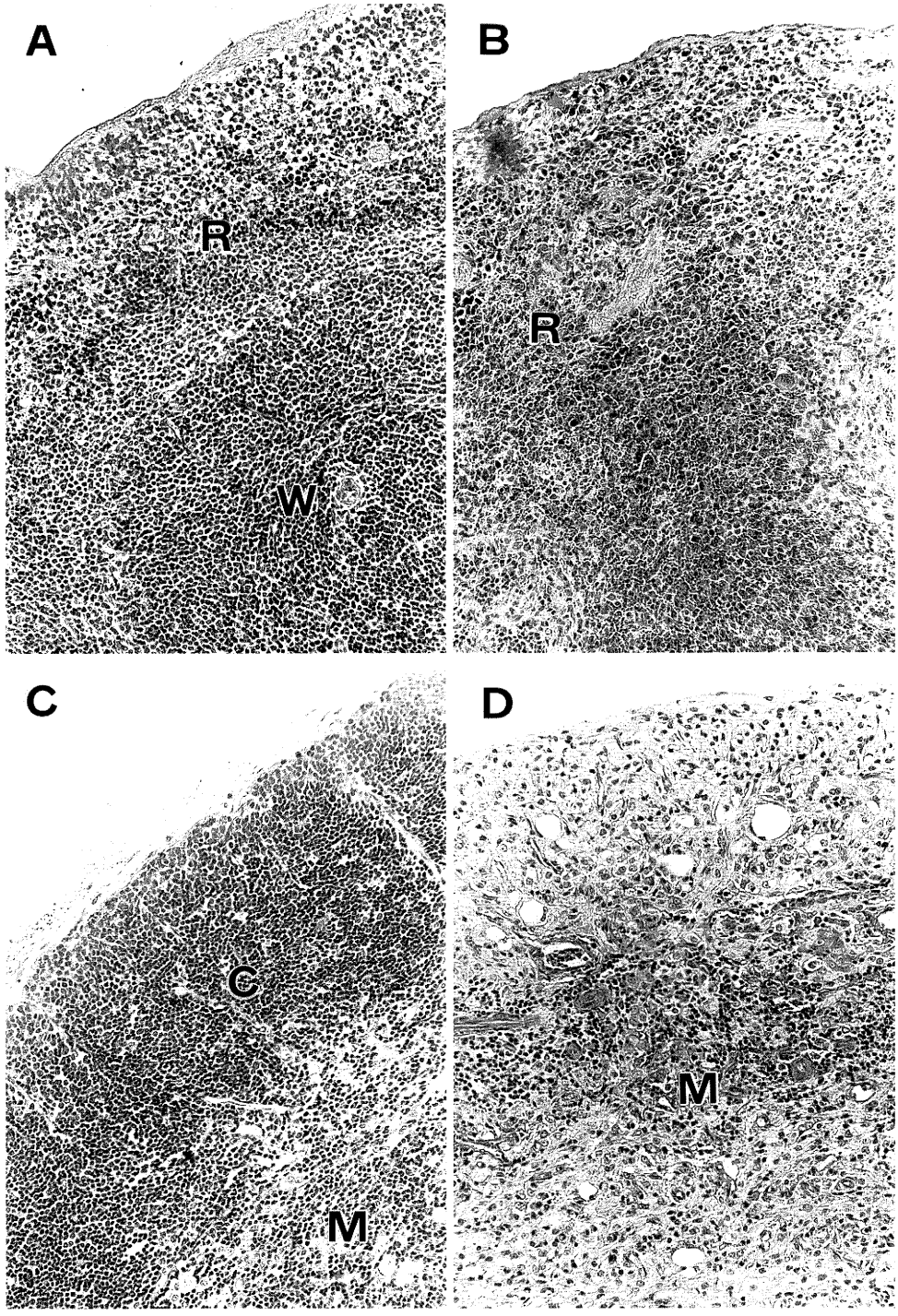

Fig. 5. Radioresistant cells localized in the splenic red pulp and the medulla of the thymus. **A.** Spleen of control mice (R = red pulp, W = white pulp), **B.** Spleen of irradiated mice, **C.** Thymus of control mice (C = cortex, M = medulla), **D.** Thymus of irradiated mice. The morphology of the spleen and thymus (×100) in the treated mice (see Fig. 4) is represented (H-E staining). The splenic medulla and the thymic medulla became prominant after treatment.

of both $\alpha\beta$ T cells and $\gamma\delta$ T cells. These results are compatible with previous results in these mice: namely, that T cells in athymic nude mice have TCR of relatively dull intensity, comprise self-reactive forbidden T cell clones, and are functionally mature in some respects.[46-48]

III. Radioresistance of intermediate TCR cells

In the course of studies on intermediate TCR cells, we discovered that intermediate TCR cells were considerably radioresistant, while bright TCR cells were radiosensitive.[29] When mice were irradiated (9Gy) and immediately injected with 10^7 syngeneic bone marrow cells, a unique feature of cell distribution was observed in the various organs of these mice (Fig. 4). Two-color staining for CD3 and IL-2Rβ showed that intermediate TCR cells became prominent in all organs tested both on day 3 and day 7 after treatment. This was due to the radioresistance of intermediate TCR cells. Since null and dull TCR cells in the thymus were completely eradicated on day 3 after treatment, these immature thymocytes were found to be the most radiosensitive. Such immature thymocytes again appeared as early as 7 days after treatment. All these variations were confirmed by two-color staining for CD3 and LFA-1. Because intermediate TCR cells expressed a higher level of LFA-1 than did bright TCR cells, intermediate TCR cells were clearly identified. In this staining, the peaks of NK and B cells were not separate.

This irradiation method demonstrated actual sites where intermediate TCR cells localized in the spleen and thymus (Fig. 5). After irradiation, the remaining

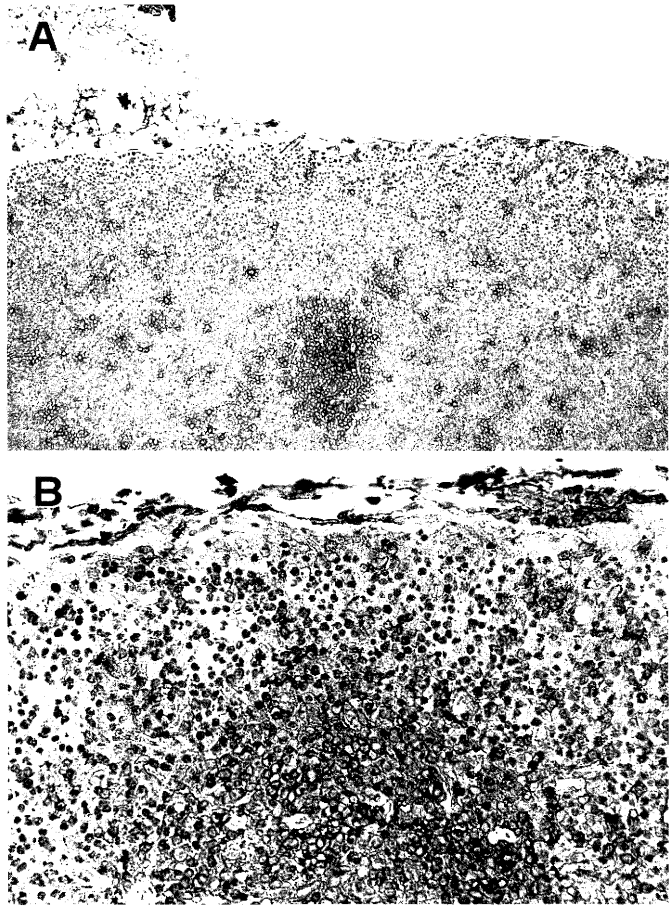

Fig. 6. Localization of CD44$^+$ cells in the thymic medulla. **A.** Thymus of control mice ($\times$100), **B.** Thymus of irradiated mice (day 7) ($\times$200). Since intermediate TCR cells highly express CD44 antigen, immuno-peroxidase staining for CD44 was perfomed to identify intermediate TCR cells in the thymus. CD44$^+$ cells became prominant after treatment due to their radioresistance.

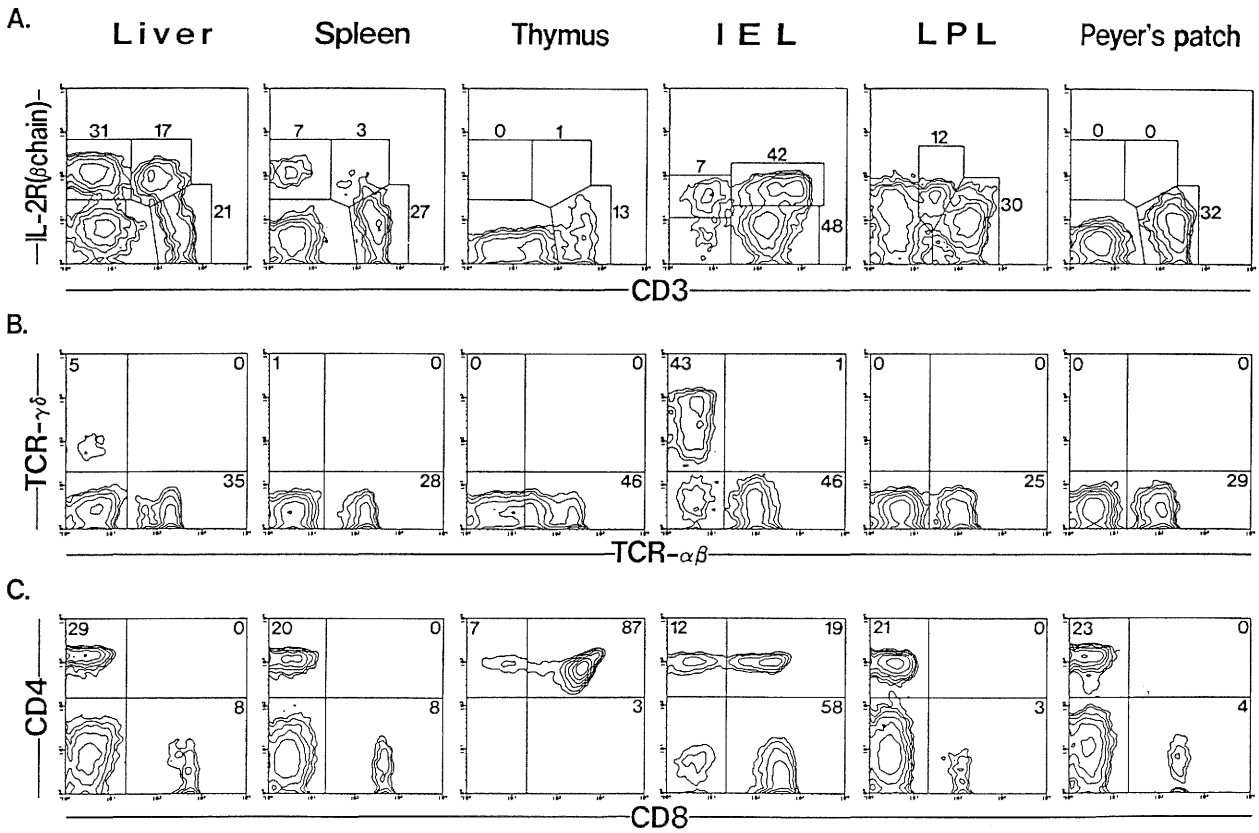

Fig. 7. Comparison of the phenotypes among MNC isolated from the various immune organs in mice. **A.** Two-color staining for CD3 and IL-2Rβ, **B.** Two-color staining for TCR-$\alpha\beta$ and -$\gamma\delta$, **C.** Two-color staining for CD4 and CD8. IEL showed a unique staining pattern, showing both IL-2Rβ^+ and IL-2Rβ^- population. The staining pattern of LPL was silmilar to that of liver MNC. $\gamma\delta$ T cells were mainly present in hepatic MNC and IEL in the intestine.

cells localized in the red pulp of the spleen and in the medulla of the thymus. Many of these remaining cells still had the properties of intermediate TCR cells, showing the high expression of IL-2Rβ, LFA-1 and CD44. This staining (Fig. 6) was conducted because intermediate TCR cells highly express CD44 whereas bright TCR cells almost lack it. As shown in the figure, cells localized in the thymic medulla of normal mice were CD44$^+$, while the majority of cells in the cortex were CD44$^-$. After irradiation, only CD44$^+$ cells localized in the medulla remained. In short, the multiplicity of the sites for extrathymic T cells was revealed by irradiation. Since the remaining T cells at the these sites showed the morphology of lymphoblastic lymphocytes with a larger light scatter,[29] it is speculated that T-cell differentiation (at least expansion) may occur at multiple sites in the body.

IV. A comparison of the phenotypes between intermediate TCR cells in the liver and intraepithelial lymphocytes (IEL) in the intestine

It had been suspected that some Thy-1$^+$ cells might be generated at intraepithelial sites of the intestine in a thymus-independent manner.[16-19] After the introduction of a TCR gene, TCR, and mAbs against TCR, it was confirmed that such population eventually comprises T cells, including both $\alpha\beta$ and $\gamma\delta$ T cells, and is generated extrathymically. Subsequently, T cells existing in the lamina propria were also determined to be of extrathymic origin, while T cells localized in the Peyer's patches were found to be of thymic origin.[49] In this section, we directly compare the phenotypes of intermediate TCR cells and IEL and lamina propria lymphocytes (LPL) in the intestine (Fig. 7). MNC isolated from the representative immune organs were examined in parallel.

As already mentioned, intermediate TCR cells

were clearly demonstrated to be in the liver, but generally not in the spleen and thymus. However, MNC in the intestine showed rather unique patterns depending on the isolated sites.

Two-color staining for CD3 and IL-2Rβ was first applied. In the case of IEL, major populations were superbright CD3$^+$ IL-2Rβ^+ and bright CD3$^+$ IL-2Rβ^-. Both of them were of extrathymic origin as shown elsewhere. On the other hand, the staining pattern of LPL slightly mimicked that of liver MNC, although these intermediate CD3 cells had a lower intensity of IL-2R. As expected, all CD3$^+$ cells in the Peyer's patches were regular, bright CD3 cells lacking IL-2Rβ. Two-color staining for TCR-$\alpha\beta$ and -$\gamma\delta$ demonstrated that $\gamma\delta$ T cells were abundant only in the liver and at intraepithelial sites of the intestine.

The results of two-color staining for CD4 and CD8 were interesting, in that not only thymocytes but also IEL comprised CD4$^+$8$^+$ double-positive (DP) T cells. As has been established, DP cells of thymocytes are immature T cells with dull TCR, while those of IEL are mature T cells *per se* with bright TCR. It is conceivable that both intermediate TCR cells in the liver and IEL in the intestine are generated as primitive T cells in early phylogeny. However, they may subsequently develop independently at these different sites.[14,25]

It is of interest why extrathymic T cells are generated both in the liver and intestine (Fig. 8). Phylogenetically, a primitive liver is known to develop as projections from the intestinal tissue. Reflecting this situation, IEL and submucosal lymphocytes, which might originally develop from macrophages or NK cells existing at the sites of endodermal cells, are common to the liver and intestine in early phylogeny.

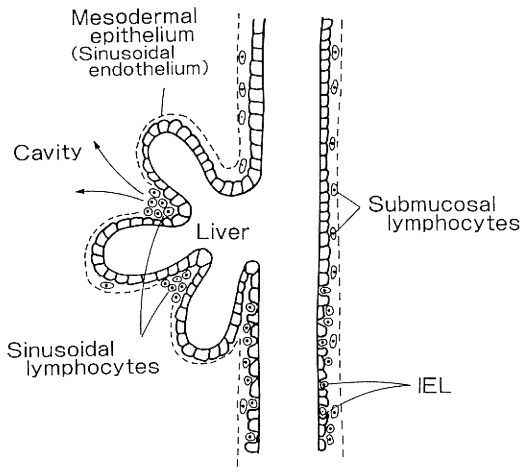

Fig. 8. A schema representing why IEL in the intestine and sinusoidal lymphocytes in the liver have certain properties in common.

Since extrathymic T cells in the liver are located under, but not on, the sites of endodermal cells (i.e., parenchymal hepatocytes), it is quite possible that the properties of extrathymic T cells in the liver are similar to those of LPL rather than IEL. LPL are located under the epithelial cells, while IEL are on the epithelial cells. It is known that sinusoidal lumens are open to the peritonal cavity in invertebrates with an open vessel system.[14] In this regard, it is suspected that extrathymic T cells produced in the hepatic sinusoids have previously functioned in the peritoneal cavity to survey atypical cells generated *in vivo* at those phylogenetic stages. In fact, we were able to obtain numerous lymphoid cells from the peritoneal cavity in arthropods (e.g., crab and shrimp).

V. Recognition system of extrathymic T cells

Although a few $\gamma\delta$ T cells exist in the thymus, intermediate TCR cells in the liver and IEL in the intestine are comprised of a considerably large proportion of $\gamma\delta$ T cells as well as $\alpha\beta$ T cells. Since the introduction of $\gamma\delta$ T cells, several cell lines of $\gamma\delta$ T cells in mice have been demonstrated to recognize certain antigens in the context of monomorphic class I MHC antigens, such as TL and Qa (i.e., TLA complex) in mice.[50,51] These MHC antigens are known to be less polymorphic than regular MHC (e.g., H-2K and D in mice) and, possibly, appear earlier phylogenetically.

In light of these findings, it is noteworthy that TL-transgenic mice, which were recently produced by Y. Obata et al., display an abnormal development of the thymus and have an abnormally high proportion of $\gamma\delta$ T cells in various immune organs.[52] In a subsequent collaborative study with Y. Obata, we demonstrated that not only $\gamma\delta$ T cells but also intermediate TCR-$\alpha\beta$ cells were highly expanded throughout the organs of these mice, including the liver. Some such data on TL-transgenic mice are herein presented (Fig. 9 to 11). As shown in Fig. 9, all T cells, but not other types of lymphocytes, expressed a high level of TL antigens. On the other hand, C3H/He background mice did not show such expression.

To identify intermediate TCR cells and others, two-color staining for CD3 and IL-2Rβ was then performed (Fig. 10A). An increase in the proportion of intermediate TCR cells was demonstrated throughout the organs tested except for IEL. In the cases of the thymus and spleen, some CD3-intermediate$^+$ IL-2Rβ^- cells were identified. There was a possibility that such cells were abnormally activated

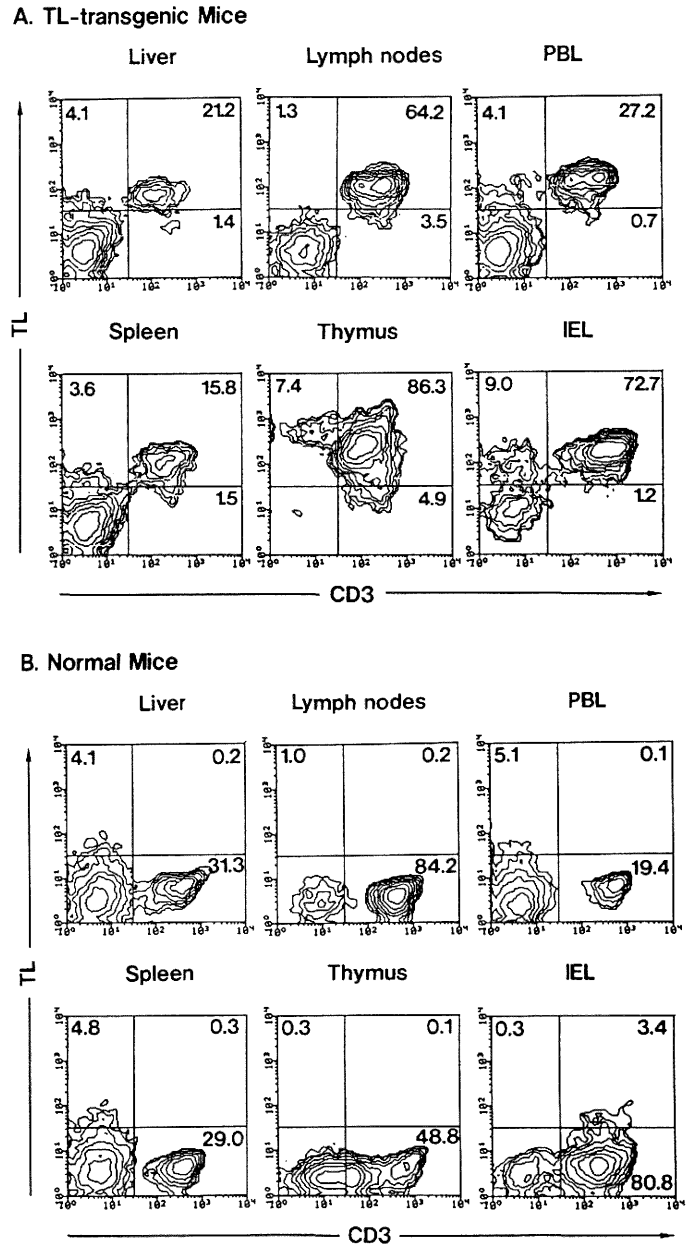

Fig. 9. Phenotypic characterization of MNC in TL-transgenic mice (I). TL expression was confined to the T cells in TL-transgenic mice.

intermediate TCR cells. Interestingly, IEL showed an almost normal staining pattern. In other words, the abnormality of extrathymic T cells was confined to the intermediate TCR cells and was not found in IEL in the intestine. It was confirmed that $\gamma\delta$ T cells, which are intermediate TCR cells, also increased throughout the organs (Fig. 10B).

The two-color staining for CD4 and CD8 antigens also demonstrated that the distribution of this population is quite unique, especially in the thymus of TL-transgenic mice (Fig. 11A). Almost all DP $CD4^+8^+$ cells disappeared. This implies that regular intrathymic T-cell differentiation was arrested. Since intermediate TCR cells occupied the whole thymus, it can be estimated that primitive T-cell differentiation took place in the thymus of these mice. This notion is compatible with the fact that in the morphology of the thymus of TL-transgenic mice, the thymic cortex

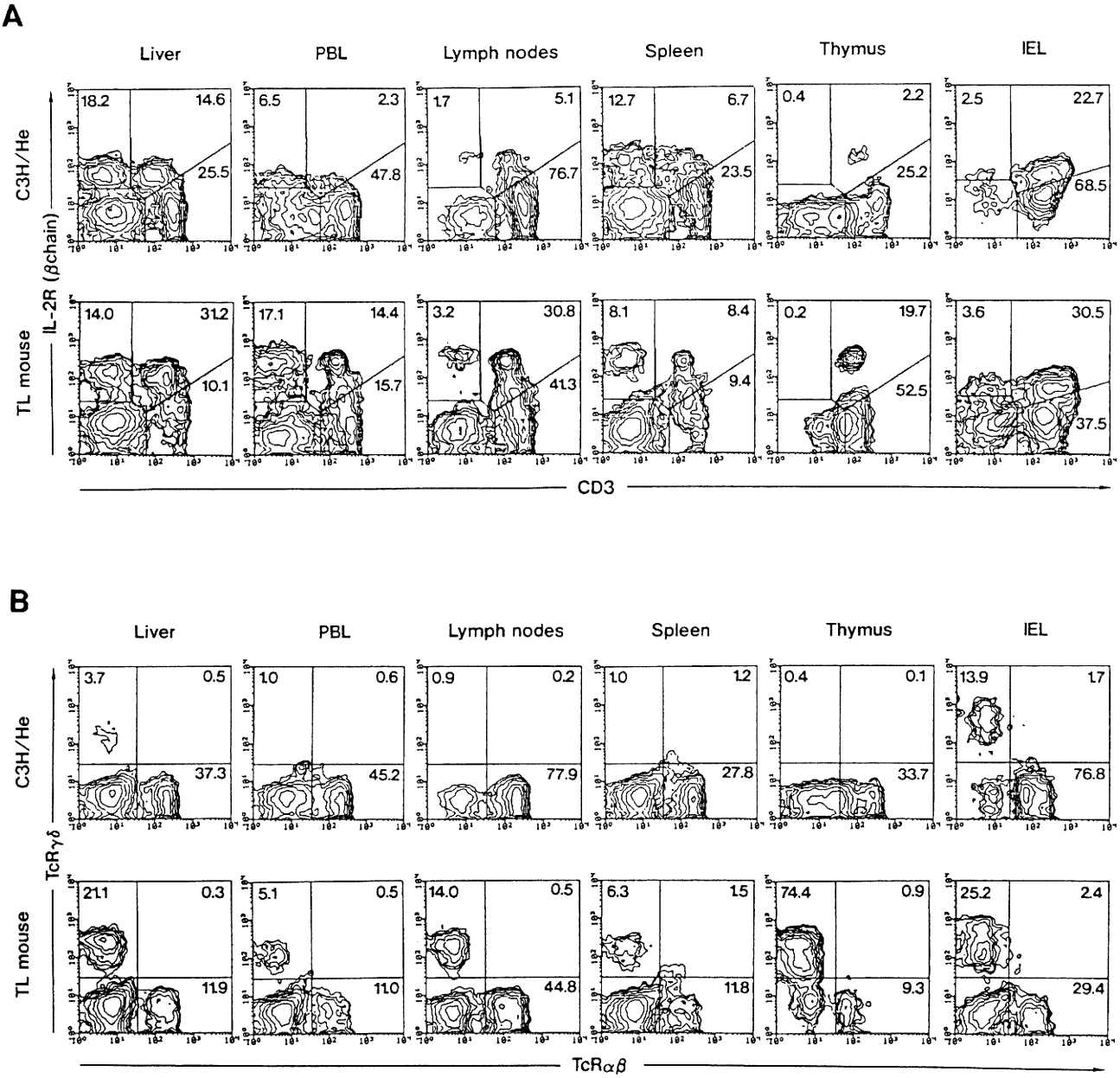

Fig. 10. Phenotypic characterization of MNC in TL-transgenic mice (II). **A.** Two-color staining for CD3 and IL-2Rβ, **B.** Two-color staining for TCR-$\alpha\beta$ and -$\gamma\delta$. Intermediate TCR (CD3) cells and $\gamma\delta$ T cells greatly increased throughout the organs tested.

disappeared and, instead, only the medulla-like architecture remained.

To determine whether a DN CD4$^-$8$^-$ cell population existed in some organs of these mice, two-color staining with anti-CD3 mAb and a mixture of anti-CD4 and -CD8 mAbs was then performed (Fig. 11B). As shown in the figure, a large proportion of DN CD4$^-$8$^-$ cells was identified throughout the organs. It is therefore conceivable that an abnormally high expression of monomorphic MHC antigens in the body only accelerates the development of the primitive T cells, mainly DN CD4$^-$8$^-$ intermediate TCR

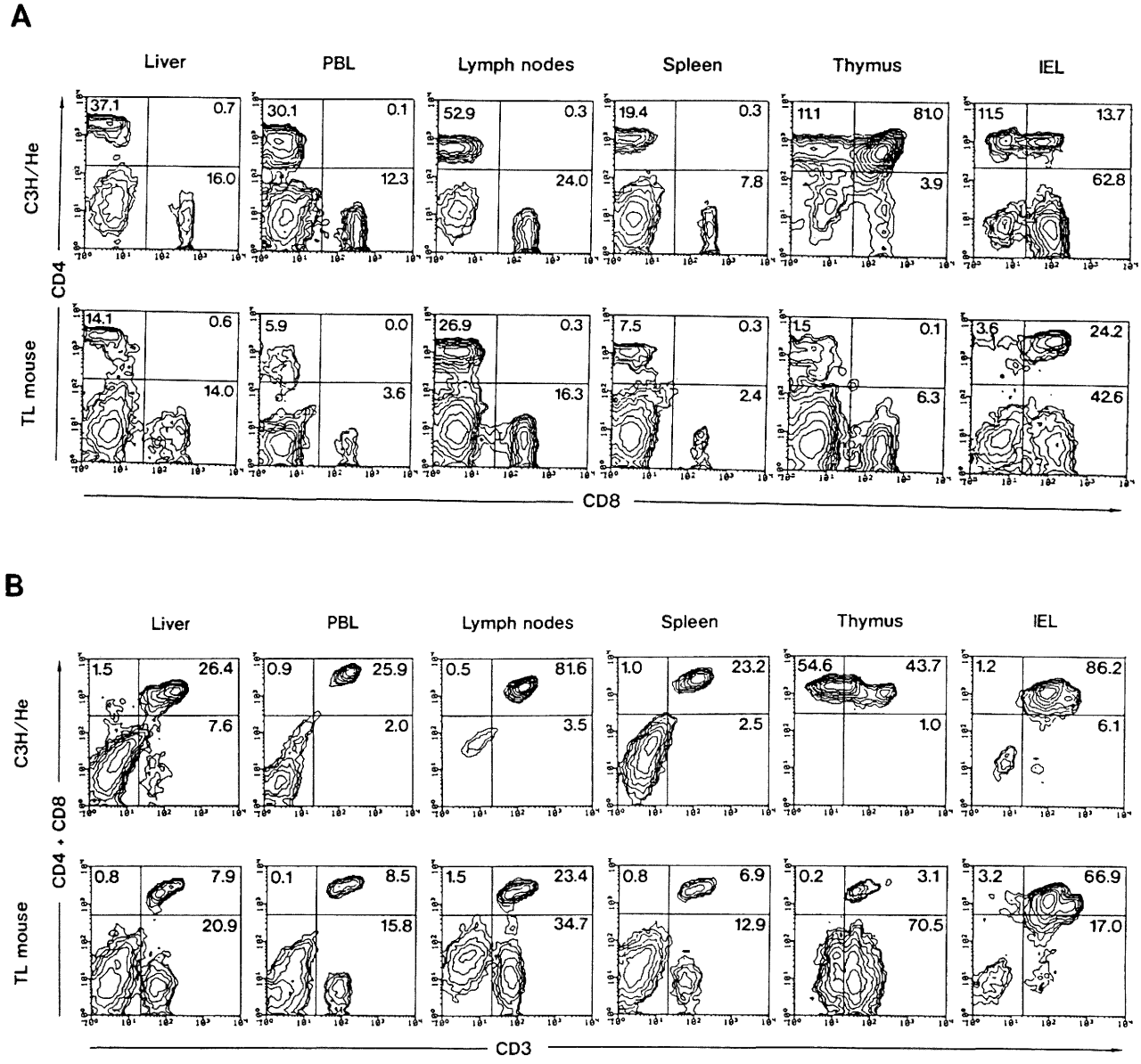

Fig. 11. Phenotypic characterization of MNC in TL-transgenic mice. (III). **A.** Two-color staining for CD4 and CD8, **B.** Two-color staining for CD3 and a mixture of CD4 and CD8. The staining for (B) was performed to identify DN $CD4^-8^-$ T cells. DN $CD4^-8^-$ T cells increased throughout the organs in TL-transgenic mice.

cells. In this case, it is still unknown what kinds of self-antigens are recognized by these T cells in the context of TL antigens.

VI. Conditions under which activation of primitive T cells occurs

To date, our experiments have confirmed that primitive, extrathymic T cells are activated with aging. Even in youth, these primitive T cells are highly activated under the following conditions (Table 1). Almost all of the conditions tested here might be rational responses of the living body. It seems, however, that some autoimmune diseases and chronic GVH responses might be the result of an overreaction of primitive T cells.

We previously reported that tumor-bearing mice were found to be at the activation stage of primitive T cells, at least, at an early phase after tumor inoculation.[6,10,53] A well-known phenomenon, profound

Table 1. Activation states of primitive T-cell differentiation

1. Aging
2. Pregnancy
3. Infection by intracellular pathogens
4. Malignancies
5. Autoimmune diseases
6. Chronic GVH diseases
7. Administration of lymphotoxin or estrogen
8. Non-classical MHC antigens
9. Superantigens
10. H-Y antigen
11. Earliest fetal thymus

thymic atrophy seen in tumor-bearing individuals[54] might appear as a reciprocal response to the suppression of the intrathymic T-cell differentiation pathway. Not only malignancies, but also the active regeneration of benign cells seem to be recognized and surveyed by primitive T cells. During the regeneration of hepatocytes after partial hepatectomy, intermediate TCR cells in the liver and other organs were highly activated, accompanied thymic atrophy.[55]

An interesting case similar to the above is represented in Fig. 12. When mice were transgened with *jun* or *fos* (i.e., onco-gene or cell activation gene) (these mice were produced by Dr. Takeshi Tokuhisa at the Kobe University School of Medicine), an activation of intermediate TCR-$\alpha\beta$ cells was seen throughout the organs tested (Fig. 12A). This was more striking in F_1 (*jun* × *fos*) mice. In these mice, all activated intermediate TCR cells were $\alpha\beta$ T cells, but were not $\gamma\delta$ T cells (Fig. 12B). In contrast to the case of TL-transgenic mice, thymic atrophy was sometimes less severe and the decrease in the proportion of DP $CD4^+8^+$ cells in the thymus was not striking (Fig. 13A). However, the appearance of DN $CD4^-8^-$ cells was demonstrated in the liver and other organs in these mice, especially in F_1 (*jun* × *fos*) mice (Fig. 13B).

It is well known that MRL-*lpr*/*lpr* mice have the *lpr* gene, which has recently been shown to be the Fas gene with an unexpected transposon,[56] and display a spontaneous onset of autoimmune disease similar to human systemic lupus erythematosus (SLE). These mice also show severe lymphadenopathy and splenomegaly, comprising abnormal DN $CD4^-8^-$ $\alpha\beta$ T cells, after onset of the disease.[57,58] It was demonstrated that these DN $\alpha\beta$ T cells correspond to the intermediate TCR cells in our studies.[5,9,11] In these earlier studies, we pointed out that the major site for the proliferation of such intermediate TCR cells is the liver. Subsequently, we observed that such proliferation occurred at multiple sites in these mice, including the thymus, spleen, lymph nodes, etc. (T. Iiai and T. Abo, manuscript submitted for publication). Some data supporting this are presented in Fig. 14. Apparently before onset of the disease (5 wk of age), intermediate TCR cells (i.e., CD3-intermediate$^+$ IL-2Rβ^+) cells became prominent in the liver and, to some extent, in the spleen. At this stage, such an abnormality was not yet obvious in the thymus. However, intermediate TCR cells, some of which had a relatively low intensity of IL-2Rβ, increased throughout the organs tested, except for IEL in the intestine (15 wk old mice). This was more striking in mice at the age of 25 wk. We confirmed that these abnormal intermediate TCR cells localized in the medulla of the thymus and the red pulp of the spleen. In other words, the expansion of intermediate TCR cells occurs at multiple sites in the body. At all of these sites, a small but significant proportion of intermediate TCR cells are primarily present, even in normal mice.[29,59]

Subsequent studies revealed that a similar expansion of intermediate TCR cells was more or less evoked in mice with chronic GVH disease, which showed autoimmune-like diseases (Y. Ikarashi et al., manuscript submitted for publication).

In earlier studies in humans[60] and our recent experiments in mice (Kimura et al., in preparation), profound thymic atrophy was observed in the late trimester of pregnancy. Interestingly, an intensive activation of intermediate TCR cells was found to be induced in the organs of pregnant mice. Such a reciprocal response of intra- and extrathymic T-cell differentiation might, at least in part, be mediated by a hormonal regulation such as that by estrogen. We recently demonstrated that estrogen, but not glucocorticoids, progesterone and prolactin, was able to produce a similar reciprocal response in the two T-cell differentiation pathways in mice.[12]

It is known that placetal trophoblasts express non-classical (or monomorphic) MHC (e.g., HLA-G in human) but lack the expression of classical (or polymorphic) MHC (e.g., HLA-A, B, C).[19,35,61,70,75] In this regard, extrathymic T cells and NK cells might be important in protecting the mother from invasion of the growing fetal tissues by utilizing the process of recognition of such non-classical MHC or in a non-MHC restricted manner. Indeed, it has been reported that primitive lymphocytes such as $\gamma\delta$ T cells, $CD56^+$ T cells, and NK cells increase in number at the site of the decidua.[61-66] Instead, a decrease in regular T cells of thymic origin due to thymic atrophy in the

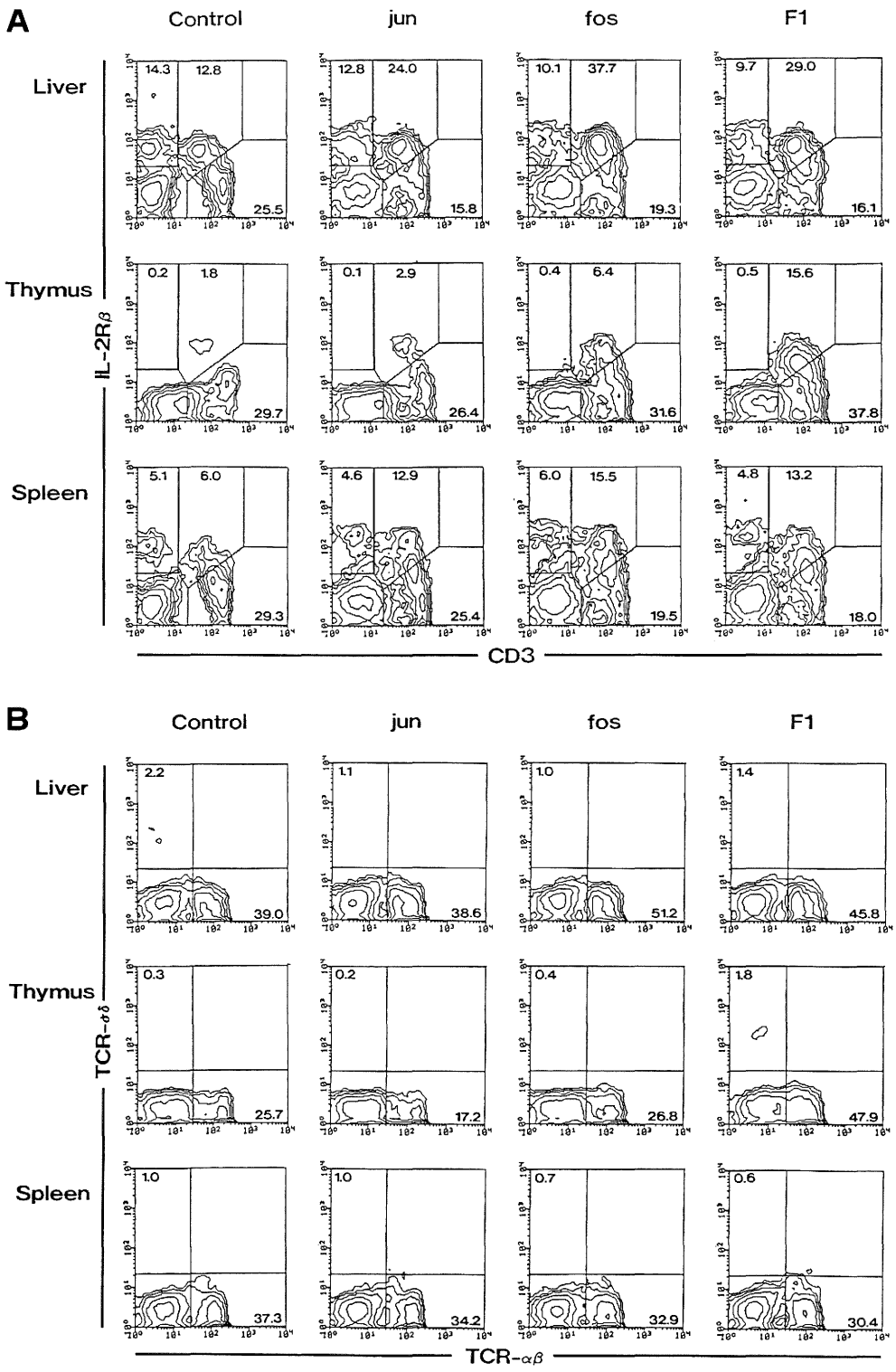

Fig. 12. Activation of intermediate TCR cells in mice transgened with *jun* or *fos*, and F_1 (*jun* × *fos*) mice. **A.** Two-color staining for CD3 and IL-2Rβ, **B.** Two-color staining for TCR-$\alpha\beta$ and -$\gamma\delta$. These oncogene-transgenic mice showed the increased level of intermediate TCR-$\alpha\beta$ cells.

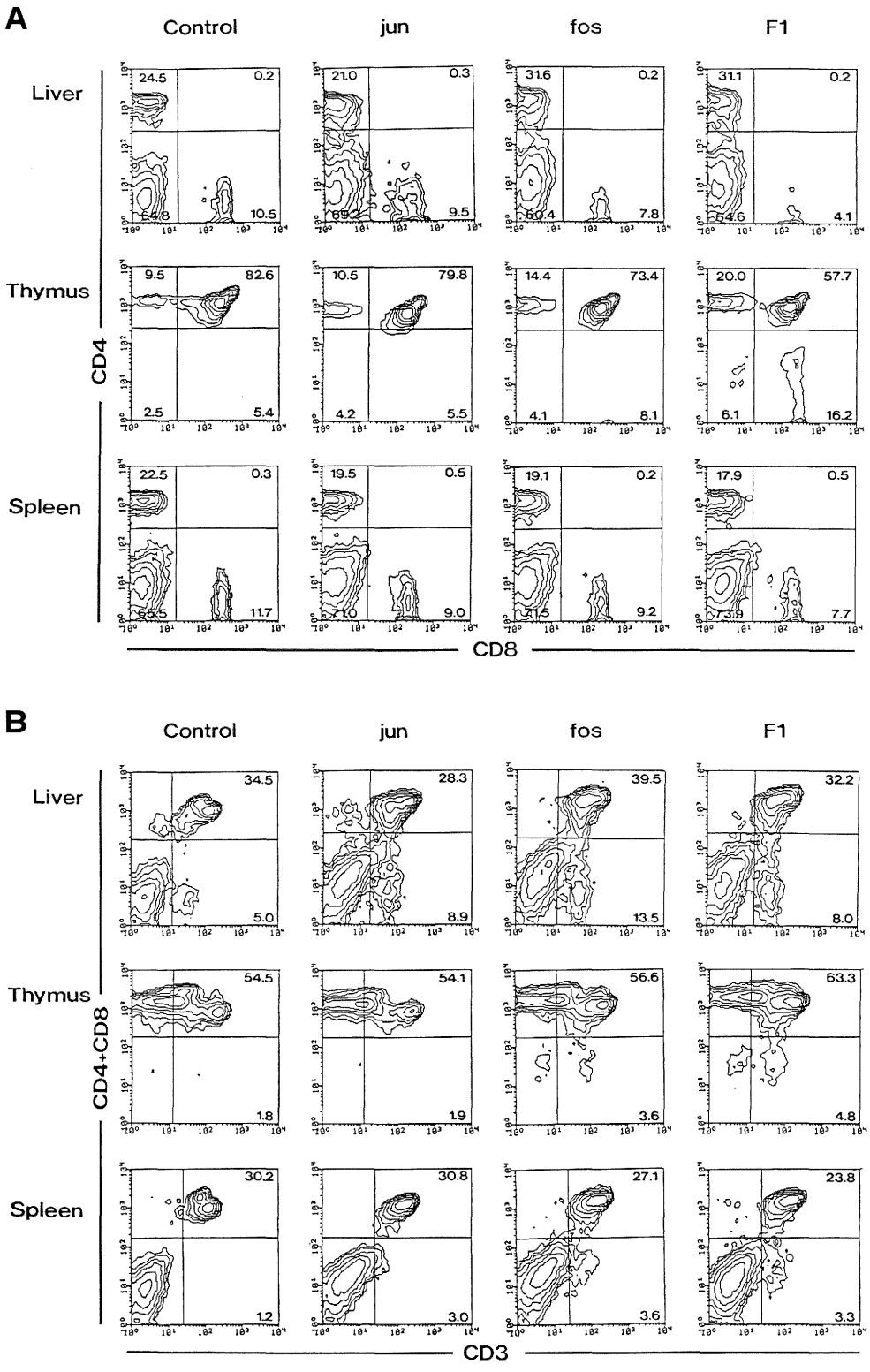

Fig. 13. Analysis of the expression of CD4 and CD8 antigens in *jun*-mice, *fos*-mice and F_1 (*jun* × *fos*) mice. **A.** Two-color staining for CD4 and CD8, **B.** Two-color staining for CD3 and a mixture of CD4 and CD8.

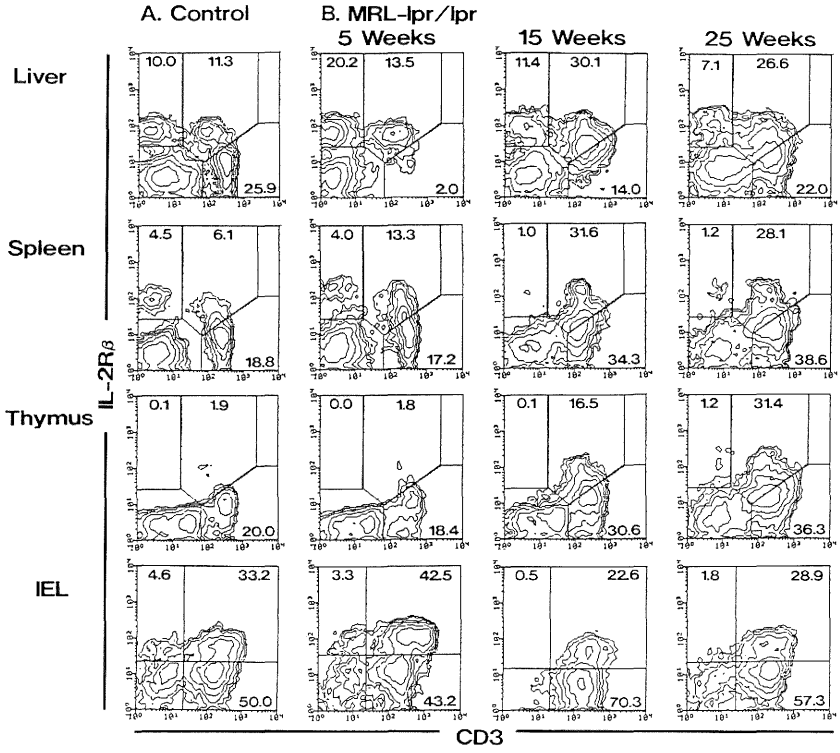

Fig. 14. Expansion of intermediate TCR cells not only in the liver but also in the thymus in MRL-lpr/lpr mice. Two-color staining for CD3 and IL-2Rβ reveals that intermediate TCR cells with IL-2Rβ of slightly lower density expand in the liver, thymus and spleen. IEL in the intestine are almost intact.

mother may be essential for the continuation of pregnancy to avoid the rejection of the fetus which carries the polymorphic MHC of paternal origin.

The primitive immune system, which may contribute to the continuation of pregnancy, is also related to several phenomena seen in females, such as the predominance of autoimmune diseases, a longer lifespan, and a lower incidence of malignancies.

VII. Hypothesis as to why primtive pathways of T-cell differentiation occur at multiple sites

One question of interest is why intermediate TCR cells exist in the above-mentioned organs, including the liver, spleen and thymus. It can be speculated that primitive T cells may be first generated at the intraepithelial sites which include cells of endodermal origin (Fig. 15). Such sites are the intraepithelial sites both in the gills and intestine. To improve respiratory function, the ectodermal clefts are developed and the gill holes are produced. In parallel, such ectodermal clefts wrap a diffuse protothymus (i.e., possibly the thymic medulla), resulting in the formation of a complete thymus consisting of both the cortex and medulla.[67] Reflecting these phenomena, the thymic medulla still contains cells with properties similar to those in the hepatic sinusoids and intestine. Similarly, the splenic red pulp, which also develops from the alimentary tract, carries primitive T cells. Since intermediate TCR cells are present and may differentiate *in situ* even in the thymus, the currently used term, i.e., "extrathymic pathways of T-cell differentiation", should be changed to "primitive pathways of T-cell differentiation".

In this regard, we propose a schema of T-cell differentiation in the thymus that includes the regular pathway occurring mainly in the cortex region and the primitive pathway occurring in the medullary region (Fig. 16). The understanding and acceptance of this schema are extremely important, because the primitive pathway in the thymic medulla sometimes becomes more predominant than the regular pathway in the cortex under conditions of aging, malignancies, bacterial infections, autoimmune diseases and chronic GVH disease. Since the primitive pathways often comprise self-reactive forbidden T-cell clones

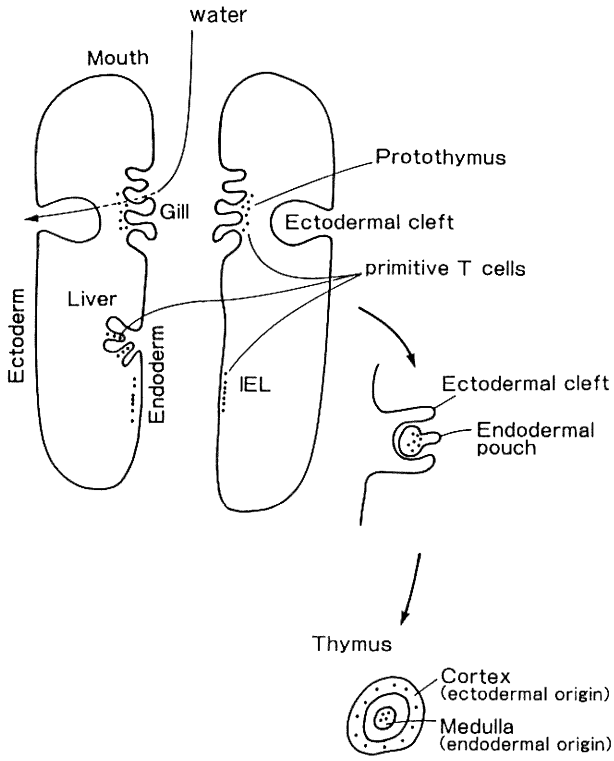

Fig. 15. A schema representing why the thymic medulla contains primitive T cells as well as regular T cells.

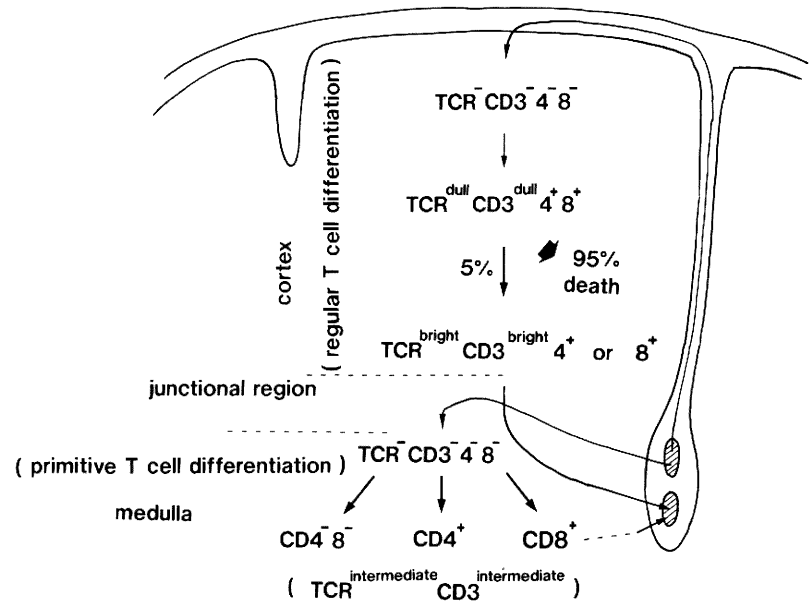

Fig. 16. Two distinct T-cell differentiation pathways in the thymus. The regular, intrathymic pathway occurs in the cortex and the primitive pathway occurs in the medulla. The T cells in the latter pathway have the phenotypes similar to those of T cells in the hepatic pathway.

under the above conditions, such T-cell differentiation in the thymus may be misinterpreted as a "breakdown of self-tolerance". Thus, care should be taken when the population of DP $CD4^+8^+$ cells in the thymus decreases (e.g., $< 70\%$). In this case, the primitive pathway may be reciprocally activated in the entire body, including the thymic medulla, the hepatic sinusoids and others.

VIII. Possible proliferation sites of effector T cells in organ specific autoimmune diseases

Many organ-specific autoimmune diseases are known to be evoked in animals by injections with organ-specific tissue antigens in conjunction with the complete Freund's adjuvant. Such animals include rats with experimental autoimmune pericarditis (EAM) injected with cardiac myosin,[68,69] and rats with experimental autoimmune encephalomyelitis (EAE) injected with myelin basic protein.[70,71]

During the course of these experiments, we found that proliferation of extrathymic T cells in these rats might possibly occur in the cavity near the diseased lesions. In the case of EAM,[72] it was the pericardial cavity (Fig. 17), while it was the spinal cavity (i.e., subarachnoid space) in the case of EAE (M. Tsuchida et al., manuscript submitted for publication). In this figure, there are many activated lymphoblasts with well-developed microvilli attached to the outer layer of the heart (Fig. 17A). On the other hand, the internal surface of the heart was completely intact (Fig. 17B). Until the present, many investigators have believed that effector T cells come from the circulation, as indicated by the results of cell transfer experiments using cell lines derived from the spleen and lymph nodes in diseased mice. However, we propose the possibility that a major site for the proliferation of effector T cells might be a cavity in close contact with the diseased lesions. Interestingly, proliferating T cells are always comprised of DN $CD4^-8^-$ cells (up to one-third of them), and only $CD4^+$ T cells among them invade the diseased tissue directly through the outer surface of the organs in the above-mentioned autoimmune rats.

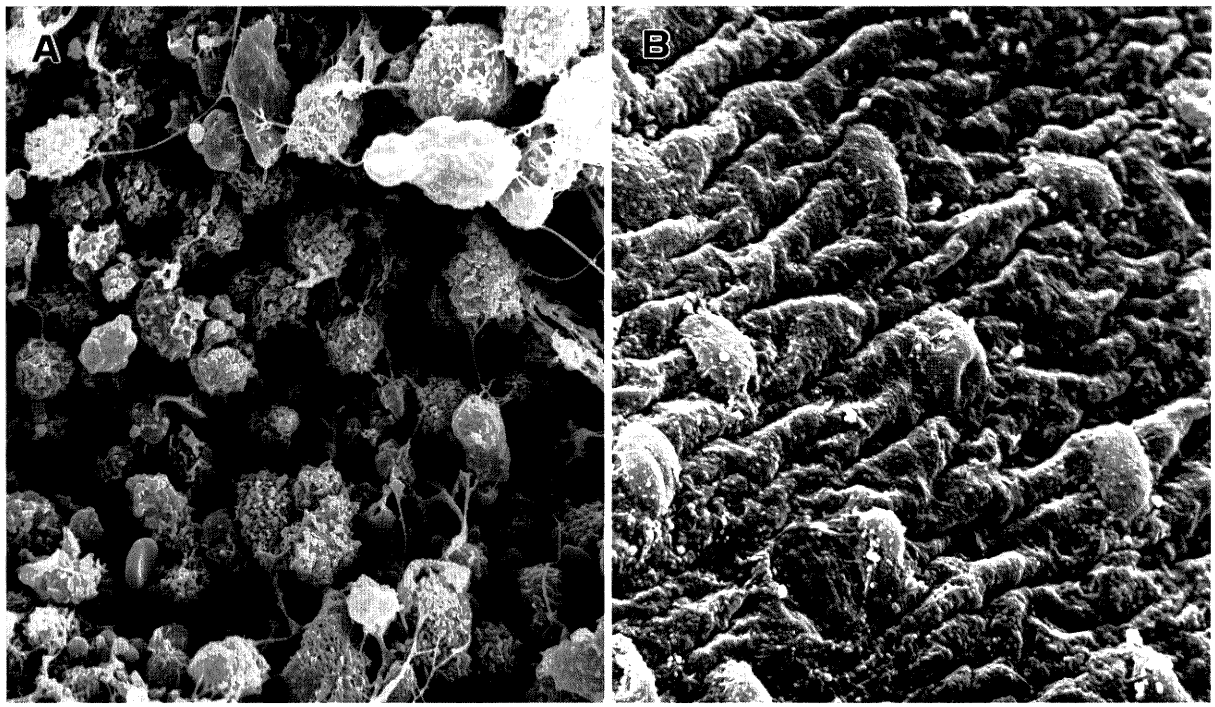

Fig. 17. Possible sites where extrathymic T cells proliferate in rats with organ-specific autoimmune diseases. **A.** The outer surface of the heart (×3,500), **B.** The inner surface of the heart (×3,500). Rats were injected with human cardiac myosin/complete Freund's adjuvant to elicite experimental autoimmune myocarditis (EAM). Activated lymphoblasts with well-developed microville, which contain a considerable proportion of DN $CD4^-8^-$ T cells, attach to the outer surface of the heart. It is presumed that such activated T cells directly invade the heart tissue.

IX. Conclusion

In the present communication, we propose the possibility that primitive T cells with extrathymic properties differentiate or proliferate at multiple sites in the living body. We term them primitive T cells rather than extrathymic T cells, since they are present even in the medulla of the thymus. It is speculated that primitive T cells may be generated before thymic development in phylogeny. Even after the development of the thymus, primitive T cells seem to play pivotal roles in aging and under conditions of bacterial infections, malignancies and pregnancy. However, the overstimulation of such primitive pathways of T-cell differentiation might be intimately associated with certain autoimmune states evoked by genetic traits, experimental autoimmune procedures, and chronic GVH disease.

Although mainly the primitive T cells were here introduced, $CD5^+$ B cells, which are known to produce autoantibodies such as anti-DNA antibody and others,[73-75] preferentially coexist with them in the liver as well as in the omentum.[76,77] It is therefore conceivable that primitive T and B cells might be still present at the sites (e.g., the intestinal epithelium, hepatic sinusoids and decidua in the pregnant uterus) where primitive (i.e., monomorphic MHC and others) MHC antigens are expressed in higher invertebrates. We feel that without understanding the primitive pathways of T-cell differentiation, many of the phenomena underlying various immune responses can not be properly understood.

Acknowledgments. This work was supported by a Grant-in-Aid for Scientific Research and Cancer Research from the Ministry of Education, Science and Culture, and a grant from the Medical Research Foundation of Niigata University, Japan.

The major contributors to this study are our colleagues, T. Iiai, K. Ohtsuka, M. Kimura, K. Sato, M. Ogawa, H. Hirahara, H. Hanawa, M. Tsuchida, S. Hashimoto and others, at the Niigata University School of Medicine, Niigata, Japan. The authors wish to thank Ms. H. Karasawa and Mrs. M. Watanabe for preparation of the manuscript.

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