EXTRATHYMIC DIFFERENTIATION OF T LYMPHOCYTES AND ITS BIOLOGICAL FUNCTION

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REVIEW

Abstract	2
Introduction	2
The Sites Where and the Periods When Extrathymic T Cell Differentiation Occurs	3
Characterization of IEL in the Intestine and Thy-1 ⁺ Dendritic Epidermal Cells (DEC) in the Skin	4
Extrathymic Generation of T Cells in the Liver	6
Characterization of Extrathymic T Cells Generated in the Liver	6
Further Characterization of T Cells in GALT	11
Isolation of Hepatic MNC and Identification of Intermediate TCR Cells in Mice	14
Predominant Appearance of Self-Reactive T Cell Clones in the Liver	14
Possible Reciprocal Regulation of Extrathymic and Intrathymic Pathways of T Cell Differentiation	16
Factors to Regulate the Reciprocal Response	16
Recognition Molecules of Extrathymic T Cells	17
Homing Receptors of Extrathymic T Cells	18
Extrathymic T Cells in Nude, Thymectomized and Scid Mice	19
Cancer and Extrathymic T Cells	21
Activation of Extrathymic T Cell Differentiation during Hepatocyte Regeneration after a Partial Hepatectomy	22
Intimate Association of Extrathymic T Cells and Bacterial Infection	22
Successive Activations of Either Extrathymic or Intrathymic T Cells in the Liver after Viral and Parasite Infections	23
Autoimmune Diseases and Extrathymic T Cells	23
Transplantation and Extrathymic T Cells	24
Immediate Hypersensitivity and the Extrathymic Pathway of T Cell Differentiation	25
A Possible Mechanism Underlying the 'Breaking of Self-Tolerance' in the Thymus	25
The Development of Autoimmune-Like Lesions with Age	27
Microenvironments of the Hepatic Sinusoids	27
Primitive CD5 ⁺ B Cells Reside in the Liver of Autoimmune NZB/W F ₁ Mice	27

Phylogeny of Extrathymic T Cells	28
Extrathymic T Cell Differentiation in Humans	33
Conclusion	33
References	34

ABSTRACT

Extrathymic pathways of T cell differentiation exist in the liver and intestine of humans and animals. Although such extrathymic pathways are relatively minor at young ages, they become predominant at older ages. The extrathymic differentiation pathway occurring in the hepatic sinusoids is also activated under conditions of bacterial infection, malignancies and autoimmune diseases. During the most activated state, these extrathymic T cells even appear in the periphery. In contrast to regular T cells differentiated in the thymus, these extrathymic T cells comprise a large population of self-reactive forbidden T cell clones and have unique properties. The generation of forbidden T cell clones in the liver might be due to the lack of a double-positive $CD4^+8^+$ stage for negative selection seen in the thymus. Therefore, the activation of the extrathymic pathway might be beneficial for the surveillance of atypical cells generated *in vivo* by virtue of their autoreactivity. However, the overstimulation might be responsible for the onset of certain autoimmune diseases. Since the extrathymic pathways appear phylogenetically earlier than the thymic pathway in animals, these pathways might comprise fundamental immune systems before and after thymic development. Cumulative evidence indicates that extrathymic T cells may also be associated with other immunological phenomena, including immediate hypersensitivity and organ transplantation.

INTRODUCTION

It is well established that T lymphocytes differentiate in the thymus to form the repertoires of mature T cells (33, 70, 121, 130). Here, they undergo positive or negative T cell clone selections in a double-positive (DP) $CD4^+8^+$ stage, differentiate into single-positive $CD4^+$ or $CD8^+$ cells, and then migrate to the periphery. On the other hand, it is suspected that an extrathymic pathway of T cell differentiation exists since unique T cells are detected in the periphery not only in congenitally athymic nude mice, but also in aged humans and animals bearing an involuted thymus (68, 101, 140). Nevertheless, there is so far no definite consensus as to where

such extrathymic T cells are generated. In a series of recent studies on humans and mice (2, 97, 98, 113, 114, 116), we have demonstrated that after birth, the liver is a major site for extrathymic T cell differentiation (Fig. 1). These extrathymic T cells differentiate in the hepatic sinusoids and sometimes migrate to the periphery. Extrathymic T cells generated in the liver have T cell receptors (TCR) of intermediate intensity and consist of double-negative (DN) $CD4^-8^-$ cells and single-positive $CD4^+$ or $CD8^+$ cells. The most striking difference between regular T cells and extrathymic T cells is that after maturation, the formers do not contain self-reactive forbidden T cell clones, whereas the latter contain a large proportion of such clones (2, 109). This is presumed to be due to the lack of a clonal deletion system in the liver. In this regard, T cells of thymic origin recognize foreign antigens in the context of self-major histocompatibility complex (MHC) antigens, whereas extrathymic T cells may recognize atypical cells generated *in vivo*. Therefore, these systems may represent a dichotomy of immune functions.

Abbreviations: DP, double-positive; TCR, T cell receptors; DN, double-negative; MHC, major histocompatibility complex; IEL, intraepithelial lymphocytes; SPF, specific pathogen-free; MNC, mononuclear cells; DEC, dendritic epidermal cells; mAb, monoclonal antibody; Mls, minor lymphocyte stimulatory; CTL, cytotoxic T lymphocytes

Fig. 1 Scheme of the intrathymic and extrathymic pathways of T cell differentiation in the thymus and liver

Further to these studies, it was reported that both intraepithelial lymphocytes (IEL) in the intestine (13, 43, 109) and T cells in the omentum of the peritoneal cavity (7, 62) also undergo extrathymic T cell differentiation *in situ*. In contrast to extrathymic T cells in the liver, these extrathymic T cells, especially in the intestine, do not appear in the periphery and probably work in the organ where they were generated. However, all of these extrathymic T cells share a common characteristic; they become predominant under conditions of thymic involution or thymic atrophy (e.g., aging and bacterial infection). The extrathymic and intrathymic pathways therefore seem to be reciprocally regulated by yet undetermined factors. The object of this article is to introduce extrathymic pathways of T cell differentiation and to elucidate their biological functions.

The Sites Where and the Periods When Extrathymic T Cell Differentiation Occurs

The major sites where extrathymic T cell differentiation occurs have so far been reported to be the liver (2, 97, 98, 113, 114, 116), intestine (13, 43, 109) and peritoneal cavity (7, 62). Both $\alpha\beta$ and $\gamma\delta$ T cells were demonstrated to be generated in the hepatic sinusoids and the intraepithelial region of the intestine. In a recent study, we observed that extrathymic T cells with unique properties were also present in the splenic red pulp and the thymic medulla. It is still uncertain whether all of these

extrathymic T were generated originally *in situ* or come from other sites (e.g., from the liver).

In vertebrates following thymic development, these pathways of extrathymic T cell differentiation are extremely minor not only during the fetal stage, but also just after birth. During these periods of life, only the intrathymic T cell differentiation primarily occurs in the thymus. However, at adolescence there is a significant increase in the number of extrathymic $\alpha\beta$ and $\gamma\delta$ T cells in the liver (97) and intestine (32). This typical activation of the extrathymic pathways is represented in a case of mice (Fig. 2) (R. Okuyama, S. Seki, T. Abo, K. Sugiura, T. Ohteki, A. Kusumi, K. Ohmori and K. Kumagai, Age-dependent increase of extrathymic $\alpha\beta$ T cells in the liver and their appearance in the periphery of older mice; submitted). Here, male BALB/c mice were fed, either conventionally or under specific pathogen-free (SPF) conditions; and the numbers of mononuclear cells (MNC) obtained from various organs were enumerated. Approximately 50% of hepatic MNC were T cells and at least one-third of such T cells was of extrathymic origin; whereas, almost all of MNC in the thymus (T cells, >95%), spleen (T cells, ~40%) and lymph nodes (inguinal lymph nodes were used here) (T cells, ~70%) were comprised of T cells of thymic origin, as shown later. Two weeks after birth, hepatic MNC became prevalent and the number was increased as a function of age. The increase was more prominent in mice under conventional feeding conditions than under SPF conditions. In contrast, the num-

Fig. 2 Variation of the number of MNC in various organs of mice as a function of age. BALB/c mice fed under SPF and conventional feeding conditions were examined (data from Okuyama *et al.*, in preparation: *vide supra*).

ber of thymocytes decreased with age, showing a drastic decline at 10 weeks of age. Again, this decline was more significant in mice under conventional feeding conditions. Changes in the numbers of MNC in the spleen and lymph nodes were minimal. It is postulated that extrathymic pathways of T cell differentiation might be physiologically activated as a function of age and that this activation occurs when the intrathymic pathway is inactivated.

Characterization of IEL in the Intestine and Thy-1⁺ Dendritic Epidermal Cells (DEC) in the Skin

To prevent infection from various microorganisms and dietary antigens in the alimentary tract, the intestine consists of gut-associated lymphoid tissues (GALT), including IEL, and lymphocytes of lamina propria, Peyer's patches and regional lymph nodes (Fig. 3) (21, 22). Among these, IEL are known to be comprised of both extrathymic and

Fig. 3 Immune system of the intestine. T, T cells; B, B cells; m, macrophages

Table 1 *T Cell Subsets of IEL in the Intestine of Mice*

Mice	Intrathymic T cells	Extrathymic T cells
Young, normal	$\alpha\beta$ T cells with CD8 $_{\alpha\beta}$	$\gamma\delta$ T cells with CD8 $_{\alpha\alpha}$
Old, normal (>20 weeks)	$\alpha\beta$ T cells with CD8 $_{\alpha\beta}$ ↓	$\alpha\beta$ T cells with CD8 $_{\alpha\alpha}$ ↑ ^a $\gamma\delta$ T cells with CD8 $_{\alpha\alpha}$
Young, nude	—	$\gamma\delta$ T cells with CD8 $_{\alpha\alpha}$
Old, nude (>20 weeks)	—	$\alpha\beta$ T cells with CD8 $_{\alpha\alpha}$ ↑ ^a $\gamma\delta$ T cells with CD8 $_{\alpha\alpha}$

^aThese cells have bright TCR but contain a significant proportion of DP CD4⁺8⁺ cells (up to 15%).

intrathymic T cells (32). On the other hand, the lymphoid tissues other than IEL are mainly, if not completely, comprised T cells of thymic origin. In addition, these tissues also contain a considerable proportion of B cells which play an important role in the production and secretion of IgA in the alimentary tract (88, 91).

In an early study using thymectomized mice, approximately half of the IEL were demonstrated to be of extrathymic origin and these extrathymic T cells increased with age (32). In recent studies, analyses of the distribution of $\alpha\beta$ and $\gamma\delta$ T cells among IEL were reported (25, 26, 43, 44, 81, 109, 134). Summarized data of murine studies are presented in Table 1. In normal young mice aged 6 to 10 weeks, $\alpha\beta$ and $\gamma\delta$ T cells were present in the intestinal epithelium with a ratio of approximately 1:1 (43, 103). These $\alpha\beta$ and $\gamma\delta$ T cells preferentially express CD8 antigens on the cell surface. Here, $\alpha\beta$ T cells are of thymic origin and have CD8 $_{\alpha\beta}$, whereas $\gamma\delta$ T cells are of extrathymic origin and have CD8 $_{\alpha\alpha}$. In other words, regular T cells that differentiated in the thymus express a CD8 heterodimer, α and β , however, extrathymic $\gamma\delta$ T cells express a CD8 homodimer, α and α . In mice aged over 30 weeks, $\alpha\beta$ T cells of thymic origin gradually decreased, whereas the $\alpha\beta$ T cells of extrathymic origin having CD8 $_{\alpha\alpha}$ increased. This age-associated increase of $\alpha\beta$ T cells (including $\gamma\delta$ T cells) was also observed in athymic nude mice (25). Irrespective of the age of these nude mice, thymic $\alpha\beta$ and $\gamma\delta$ T cells were not detected.

It has been shown that $\gamma\delta$ T cells in the intestine preferentially exhibit the V γ 7/V δ 1 combination of V gene segments (10, 73, 128). In a recent study using the polymerase chain reaction (PCR) method (43), the extrathymic generation of IEL was demonstrated by the presence of RAG-1 (rearrangement activation gene).

Since IEL do not display a phagocytic function against bacteria, they may exert cytotoxic functions against both bacterial-infected cells and atypical cells generated *in vivo* (including damaged or regenerating epithelial cells) in the intestine. Iwanaga T. *et al.* (Niigata University School of Medicine, Niigata) recently observed in a study using guinea pigs that IEL attack old epithelial cells in the intestine which are subsequently processed by macrophages (personal communication). It was also reported that in mice IEL acquire specific or non-specific (non-MHC restricted) cytotoxicity after activation (44, 77) and that activated T cells of extrathymic origin acquire a morphology similar to large granular lymphocytes (LGL) (115).

Thy-1⁺ DEC are unique T lymphocytes in the skin of mice, mainly consist of $\gamma\delta$ T cells (51, 73, 127). Thy-1⁺ DEC are found in mice but not in humans. These $\gamma\delta$ T cells seen in the skin of mice preferentially exhibited the V γ 5/V δ 1. Since $\gamma\delta$ T cells with the V γ 5/V δ 1 are found transiently in the thymus during the early fetal stage, these $\gamma\delta$ T cells are considered to be of thymic origin. In addition, it was reported that $\gamma\delta$ T cells with the V γ 5/V δ 1 appear in the skin of nude mice after thymus transplantation (50). In the fetal thymus, $\gamma\delta$ T cells appear earlier than $\alpha\beta$ T cells (e.g., 14 days after gestation in mice) (49). However, $\alpha\beta$ T cells then appear and begin to increase, whereas $\gamma\delta$ T cells decrease to undetectable levels (<1%) in the thymus at birth. It was proposed that perhaps $\gamma\delta$ T cells migrate from the thymus to the periphery (e.g., skin and reproductive organs) at fetal stage and then expand by antigenic stimulation in the periphery after birth (73). On the other hand, $\gamma\delta$ T cells with V γ 1/V δ 6 become prevalent and increase in the skin of old mice. Since the most common V gene allele combination of $\gamma\delta$ T cells generated in the liver is the V γ 1/V δ 6 (97), it is possible that the

aforementioned $\gamma\delta$ T cells are of extrathymic origin (e.g., hepatic origin).

Extrathymic Generation of T Cells in the Liver

Although extrathymic generation of T cells occurs in several places in the body, the extrathymic pathway in the liver might be especially important. The extrathymic T cells in the intestine appear to only work locally the original site where they were generated. However, the extrathymic T cells generated in the liver not only work in the liver but also migrate, during the most activated states, to work in the periphery, including the circulation, spleen, lymph nodes, etc. Such activation of the hepatic pathway has been widely observed during aging (97, Okuyama *et al.*, in preparation; *vide supra*), and under conditions of bacterial stimulation (2), malignancies (113, 116) and autoimmune diseases (98, 114).

The discovery of the extrathymic pathway of T cell differentiation was initiated by studying natural killer (NK) cells and $\gamma\delta$ T cells. NK cells are known to contain several properties of both those of myelomonocytes and T lymphocytes (1, 75). Most notably, a significant proportion of NK cells simultaneously express the myelomonocyte antigens, Mac-1 (CDw 18) and CD56, and the T cell antigens, CD2 and CD3. Moreover, NK cells are phagocytic against certain bacteria (3). Since NK cells are not significantly present in the thymus, it was believed that they were generated extrathymically. Interestingly, it has subsequently been demonstrated that NK cells are in fact generated in the hepatic sinusoids, yet preferentially reside in the liver (63, 137). These results therefore facilitated us to investigate whether or not extrathymic T cells are also differentiated in the liver of humans and mice.

In addition, several investigators showed that $\alpha\beta$ T cells could be induced from their early precursors in *in vitro* cultures (107). We previously reported that $\gamma\delta$ T cells were also induced and activated from their precursors by heat-killed bacteria in humans (4). Surprisingly, the induction and activation of such $\gamma\delta$ T cells were reproduced only when a medium supplemented with 20% or more (e.g., >50%) autologous (or allogeneic) sera was used (115). This phenomenon further prompted us to consider the possibility that extrathymic T cells are differentiated in the serum-rich area such as the hepatic sinusoids.

We consequently found that unique $\gamma\delta$ T cells

with a lymphoblastic morphology were present in the hepatic sinusoids of humans and mice, especially with malignancies (113). We further observed that in the periphery of autoimmune MRL-*lpr/lpr* mice, the abnormally expanding $\alpha\beta$ T cells with a DN CD4⁻8⁻ phenotype were generated in the liver (98). It has been demonstrated that both extrathymic $\alpha\beta$ and $\gamma\delta$ T cells in the liver reside in the hepatic sinusoids, adhering to the endothelial cells and Kupffer cells (82). Here, the endothelial cells and Kupffer cells may play an important role in providing the hepatic microenvironments for supporting the generation of extrathymic T cells. When such extrathymic T cells increase in the liver, for instance, when mice are injected with bacteria; the increase is notable by both light and electron microscopy. The accumulation of hepatic MNC is represented here by light microscopy (Fig. 4).

It is well documented that the liver, during a fetal stage, is one of the major hematopoietic organs in mammals (66). This hematopoiesis occurs in the parenchymal space (i.e., Disse's space), but not in the sinusoidal lumen (Fig. 5a). Here, many progenitors of erythroid, myelomonocytic and lymphocytic origins are seen between the parenchymal hepatocytes in mice during the fetal stage. This hematopoiesis, however, ceases within 1 week following birth. On the other hand, extrathymic T cell generation begins as early as 2 weeks after birth. These T cells are present only in the sinusoidal lumen, firmly adhering to the Kupffer cells and sinusoidal endothelial cells. In the most activated state, these extrathymic T cells occupy a large space of the sinusoidal lumen as shown in Fig. 5b (autoimmune MRL-*lpr/lpr* mice). It should be emphasized that the hepatic sites between fetal hematopoiesis and extrathymic T cell generation are in completely different locations.

Characterization of Extrathymic T Cells Generated in the Liver

Extrathymic T cells generated in the liver have properties that are distinguishable from regular T cells of thymic origin. A significant proportion (up to 10%) of hepatic MNC show lymphoblastic morphology and display a large light scatter in FACS analysis (82, 97, 98, 113, 136). It is conceivable that some T cells in the liver may proliferate *in situ*. In fact, it has been confirmed by the results that hepatic MNC have not only an ability to proliferate spontaneously in an *in vitro* culture for more than 18 h after cell isolation (97, 98, 113), but also an ability

Fig. 4 Light microscopy of the liver. The liver in normal mice (a) and mice injected with 10^8 heat-killed *Propionibacterium acnes* 4 days after injection (b) ($\times 400$). Note the sinusoidal dilatation and accumulation of MNC in the sinusoids of mice injected with bacteria (data from reference 2).

a

b

Fig. 5, a and b

Fig. 6 Distribution patterns of T cell subsets in the liver, periphery (e.g., spleen) and thymus of mice. T cells are classified into $\alpha\beta$ and $\gamma\delta$ T cells with either DN (double-negative), SP (single-positive) or DP (double-positive) cells.

to undergo DNA synthesis *in vivo* by means of bromodeoxyuridine (BrdU)-injection experiment (Ohmori K., Niigata University School of Medicine, manuscript in preparation).

Phenotypic analysis revealed that in young mice, hepatic MNC consist of both $\alpha\beta$ and $\gamma\delta$ T cells (approximately 4:1) (Fig. 6). In addition, almost all of the $\gamma\delta$ T cells were of extrathymic origin, whereas the $\alpha\beta$ T cells were a mixture of both extrathymic and thymic origins. It has been shown that a considerable proportion of $\alpha\beta$ and $\gamma\delta$ T cells in the liver contain DN CD4⁻8⁻ cells as well as single-positive CD4⁺ or CD8⁺ cells (109). This distribution pattern is significant when compared with that of T cells in the periphery (e.g., spleen). In the periphery, almost all of the T cells are $\alpha\beta$ T cells with a single-positive phenotype. Although DN T cells are also detected in the thymus, they are transient precursors undergoing maturation into T cells. In

addition, they do not appear in the periphery. However, since both hepatic DN T cells and single-positive T cells can grow in an *in vitro* culture in the presence of IL-2 without their phenotypic switching (our unpublished observation), these DN T cells seem to be at an independent stage (i.e., one of the mature T cells) of maturation. It is well established that thymocytes comprise a large proportion (70–80%) of the T cells with DP CD4⁺8⁺ phenotype (33, 70, 121, 130). However, T cells with a DP phenotype are never present in the liver of mice (and humans) even under activated conditions (2). The $\gamma\delta$ T cells generated in the liver also display a unique set of the V gene (i.e., V γ 1/V δ 6), although they have a potential to produce the various sets of V gene under certain conditions (97, 116).

The fact that TCR of the $\alpha\beta$ T cells in the liver show a two-peak pattern of $\alpha\beta$ TCR (and CD3) expression (i.e., intermediate TCR cells and bright TCR cells) in terms of immunofluorescence staining intensity (Fig. 7) is particularly interesting (2). As already demonstrated, the TCR of $\alpha\beta$ T cells in the thymus also show the two-peak pattern of TCR (and CD3) expression (dull TCR cells and bright TCR cells). Dull $\alpha\beta$ TCR cells in the thymus are immature T cells with a DP phenotype before positive or negative selection, whereas bright $\alpha\beta$ TCR cells are mature T cells with a single-positive CD4⁺ or CD8⁺ phenotype after selection (33, 70, 121, 130). These mature T cells undergo clonal deletion of self-reactive forbidden clones and then migrate to the periphery. The relatively dull $\alpha\beta$ TCR cells

Fig. 5 A comparison of the sites between hematopoiesis during the fetal stage (a) and extrathymic T cell differentiation after birth (b). Electron micrographs of fetal liver (BALB/c mice, 18 days after gestation) and liver of autoimmune MRL-*lpr/lpr* mice after the onset of disease ($\times 3,200$). Hematopoiesis is present in the parenchymal area (Disse's space), whereas extrathymic T cell differentiation occurs in the sinusoids. Extrathymic T cells are not present in the fetal sinusoids during these periods. (Data provided by Dr Hiromi Iwanaga and Toshihiko Iwanaga, Niigata University School of Medicine).

Fig. 7 A comparison of the FACS profiles of MNC from various organs performed by a single-color immunofluorescence test using anti- $\alpha\beta$ TCR or anti-V β 8 mAbs. MNC from the thymus and liver displayed a two-peak pattern of TCR staining, whereas those from the spleen, lymph nodes and blood did the single-peak pattern (data from reference 2).

A. Liver

B. Thymus

Fig. 8 A direct comparison of the $\alpha\beta$ TCR (and CD3) staining patterns and the two-color staining of CD4 and CD8 between hepatic MNC and thymocytes. Relatively dull TCR in the liver was estimated as 'intermediate TCR'. In contrast to thymocytes, hepatic MNC did not comprise DP CD4⁺8⁺ cells (data from reference 136).

seen in the liver was termed 'intermediate TCR cells' because their peak is in the intermediate position between those of the dull and bright TCR in thymocytes (Fig. 8) (12). We then concluded that intermediate $\alpha\beta$ TCR cells are truly of extrathymic origin, since congenitally athymic nude mice and thymectomized mice (2 months after thymectomy) only have intermediate $\alpha\beta$ TCR cells in the liver (S. Seki, T. Abo, K. Sugiura, T. Ohteki, K. Hirokawa, M. Utsuyama and K. Kumagai, $\alpha\beta$ T cells with intermediate intensity of T cell receptors in the liver may be of extrathymic origin, submitted). Bright $\alpha\beta$ TCR cells in the liver of normal mice may be cells that migrated from the thymus. Only intermediate TCR cells comprise the DN cells (up to 40% of intermediate $\alpha\beta$ TCR cells) in the liver (Fig. 9). In the periphery of young mice, including the spleen, lymph nodes and peripheral blood, bright TCR cells of thymic origin are present (see Fig. 7). In older mice, a significant proportion of intermediate $\alpha\beta$ TCR cells appear even in the periphery.

Therefore, not all of the T cells seen in the periphery of older mice are always long-lived T lymphocytes of thymic origin as previously believed by many investigators.

Further Characterization of T Cells in GALT

With respect to the intensity of TCR, the IEL in the intestine are known to have the unique intensity in both mice (87) and rats (30). Despite the extrathymic differentiation of IEL, they generally display an intensity comparable to the bright TCR cells in the thymus and periphery. Moreover, among such bright $\alpha\beta$ TCR cells, there are DP CD4⁺8⁺ cells (Fig. 10). These results signify the possibility that the IEL undergo a unique system of extrathymic T cell differentiation. As already mentioned, the figure clearly showed that only IEL in GALT consisted of a large proportion of $\gamma\delta$ T cells (41%) and of DP CD4⁺8⁺ cells (33%). In contrast, lymphocytes from lamina propria and Peyer's

A. Liver **B. Spleen**

Fig. 9 Only intermediate TCR cells in the liver were consisted of DN CD4⁻8⁻ cells. Hepatic MNC and splenic lymphocytes were stained by three-color immunofluorescence using anti-CD4 (FITC), anti-CD8 (Phycoerythrin), and anti-CD3 (biotin) mAbs. Biotin-conjugated reagent was developed with Streptavidin-Red 613. The gating analysis of each area on CD3 staining was performed. Only intermediate TCR cells in the liver, but not bright TCR cells in the liver and spleen, were consisted of DN CD4⁻8⁻ cells.

Fig. 10 A comparison of T cell marker expression among IEL, lamina propria lymphocytes (LPL) and lymphocytes of Peyer's patches in the intestine. C3H/He mice aged 8 weeks were used.

patches lacked such T cells but contained a large proportion of B cells.

It was reported that ruminant animals, such as sheep and cattle have a large number of extrathymic (DP phenotype) and $\gamma\delta$ T cells in the intestine as well as the circulation (55). Since these animals have a highly developed intestine to digest plant cellulose and harbor an abundant of bacterial flora, the extrathymic $\alpha\beta$ and $\gamma\delta$ T cells generated in the intestine are always in the most activated state. In these animals, the extrathymic T cells generated in the intestine may even migrate to the periphery.

Isolation of Hepatic MNC and Identification of Intermediate TCR Cells in Mice

We do not recommend to use the collagenase perfusion method, for the isolation of hepatic MNC (136). After total bleeding or after perfusion of the liver with 5 ml of PBS (0.1 M, pH 7.2) in anesthetized mice, the liver is removed, cut into small pieces, and forced through the 200-gauge stainless steel mesh. After washing once with PBS, hepatic MNC were isolated by the Ficoll-Isopaque gradient (1.090) centrifugation method. The cells were then suspended in the medium supplemented with se-

rum (5 to 10%) to avoid cell damage. Approximately 0.5 to 1.5×10^6 hepatic MNC could be obtained from one young mouse. This method did not yield a significant proportion of phagocytic cells (i.e., Kupffer cells) since they trapped with the cell debris. More than 95% of the hepatic MNC were lymphoid cells, and the cell yield ranged from 5 to 15% (average 10%) (136). Therefore, normal young mice (8 to 10 weeks old) have approximately 1×10^7 lymphoid cells in the liver. This number increased with age (see Fig. 2). Ten million of lymphoid cells present in the liver seemed to be substantial since a similar number was also present in the spleen of young mouse (~ 5 weeks old) fed under SPF conditions. When the collagenase perfusion method was performed before the gradient centrifugation in other studies, more than 60% of the yielded cells were Kupffer cells. In addition, we noticed that perfusion with 5 ml of PBS did not significantly reduce the number of cells (less than 20%) when compared to the yield obtained after total bleeding (136). In other words, more than 80% of the isolated hepatic MNC were residential lymphocytes in the liver. In conclusion, it was possible for us to obtain residential hepatic MNC immediately after total bleeding, without collagenase perfusion or PBS perfusion. Furthermore, the distribution patterns of intermediate TCR cells, bright TCR cells, B cells and NK cells in hepatic MNC were not significantly different between the PBS perfusion and total bleeding methods.

At present, the most sensitive method to identify intermediate TCR cells (i.e., extrathymic T cells in the liver) in mice is a two-color immunofluorescence analysis using anti-CD3 (or anti- $\alpha\beta$ TCR) mAb (FITC-conjugated) and anti-IL-2 receptor (R) β chain (biotin-conjugated) mAb (a study in collaboration with Tanaka T. and Miyasaka M., Tokyo Metropolitan Institute of Medical Science, Tokyo). The results are depicted in Fig. 11. There are two large populations of IL-2R β^+ cells in the liver and other organs in normal mice (Fig. 11, right column), one is the CD3 $^-$ NK cells and the other is the TCR cells with a CD3 of intermediate intensity. By using this sensitive method, both NK and intermediate TCR cells were easily detectable even in organs other than the liver. If anti-asialo GM $_1$ antibody was injected *in vivo*, almost all of the NK cells disappeared within 3 days following the injection (Fig. 11, left column). This treatment did not affect the intermediate TCR cells which were present in all of the tested organs. NK and intermediate TCR cells share similar properties in terms of their

IL-2R β expression, non-MHC restricted cytotoxicity and hepatic generation. They may therefore be more primitive lymphocytes than the bright TCR cells of thymic origin. We have recently demonstrated that both NK and intermediate $\alpha\beta$ TCR cells are more resistant to total body irradiation (7 Gy) than bright TCR cells, since almost all of the bright $\alpha\beta$ TCR cells disappeared within 2 days after irradiation (unpublished data).

Predominant Appearance of Self-Reactive T Cell Clones in the Liver

When the extrathymic pathway of T cell differentiation in the liver is activated in mice during aging or under conditions of bacterial stimulation, malignancies and autoimmune diseases, a considerable proportion of self-reactive forbidden T cell oligoclones appears in the liver. Such self-reactive clones can be identified by a series of anti-V β mAbs (2). For example, anti-V β 6 mAb reacts with Mls-1 a , whereas anti-V β 3 reacts with Mls-2 a . Since AKR/J mice have Mls-1 a antigens, V β 6 $^+$ T cell oligoclones are deleted by negative selection in the thymus which results in few V β 6 $^+$ T cells ($<1.0\%$) in the periphery. Also, since C3H/He mice have Mls-2 a antigens, V β 3 $^+$ T cell clones are deleted in the thymus which results in few V β 3 $^+$ T cells in the periphery. Despite these situations, after bacterial stimulation a considerable proportion of forbidden T cell clones is induced in the liver of these mice (2). In their most activated states, these forbidden clones migrate, to some extent, to the periphery. These forbidden clones were actually further demonstrated to be self-reactive in an autologous mixed lymphocyte culture.

In recent studies, V β 8 $^+$ T cell oligoclones were reported to be responsible for the onset of certain autoimmune diseases. The autoreactivity and alloreactivity of V β 8 $^+$ cells were also reported (76, 117). V β 8 $^+$ cell preponderance among the $\alpha\beta$ T cells most notably was observed in autoimmune MRL-

Fig. 11 FACS profiles of MNC for various organs performed by a two-color immunofluorescence test using anti-CD3 and anti-IL-2R β mAbs. Normal C3H/He mice are compared to mice injected with anti-asialo GM $_1$ antibody (3 days after the injection). CD3 $^-$ IL-2R β^+ NK cells and CD3 $^+$ IL-2R β^+ intermediate TCR cells are encompassed by squares. The numbers in the figures indicate the percentages of positive cells.

Fig. 11

lpr/lpr mice (96, 108), diabetic NOD mice (12), and mice with experimental autoimmune encephalomyelitis (EAE) (132, 141). Moreover, an injection of anti- $V_{\beta}8^{+}$ mAb into these mice prevented the onset of such diseases. Approximately 30% of the $V_{\beta}8^{+}$ T cells among total $\alpha\beta$ T cells reside in the various peripheral immune organs in normal mice (78, 125). However, more than 50% of the $V_{\beta}8^{+}$ cells make up the hepatic MNC of normal mice (114, Seki *et al.*, in preparation: *vide supra*). If this enumeration is gated to the intermediate $\alpha\beta$ TCR cell fraction in FACS analysis, the percentage of $V_{\beta}8^{+}$ T cells comprises up to 70%. It can be therefore concluded that, in addition to forbidden T cell clones, self-reactive $V_{\beta}8^{+}$ T cell clones are preferentially generated in the liver. The generation of forbidden T cell clones might be due to the lack of the clonal deletion system in the liver, whereas the preponderance of $V_{\beta}8^{+}$ cells might be caused by the existence of some positive selection mechanism in the liver.

Possible Reciprocal Regulation of Extrathymic and Intrathymic Pathways of T Cell Differentiation

When an extrathymic T cell differentiation in the liver is activated in mice, an intrathymic T cell differentiation seems to be reciprocally inactivated. As previously mentioned, such conditions include aging as well as bacterial infection, malignancies and autoimmune diseases. If heat-killed *Propionibacterium (P.) acnes* (10^8 /mouse) were intraperitoneally injected into mice, 3 days after injection a prominent two or three-fold increase in the number of hepatic MNC was induced (2). At the same time, profound thymic atrophy (more than 95%) was observed. Here, the increase in the number of hepatic MNC was due to the selective increase of intermediate TCR cells, whereas the decrease in the number of thymocytes was mainly due to the accelerated death of the DP $CD4^{+}8^{+}$ cells. In addition, in the *in vitro* culture, the increase in the spontaneous cell proliferation of hepatic MNC preceded the increase in the number of hepatic MNC, and the decrease in the spontaneous cell proliferation of thymocytes preceded the decrease in the number of thymocytes. When bacterial injections were repeated every 3 days, thymocytes were not obtainable from the atrophic thymus, yet many hepatic MNC, containing a large proportion of intermediate TCR cells (up to 70%), were obtained 2 weeks after the initial injection. It is possible that

this phenomenon reflects a reciprocal regulation of the extrathymic and intrathymic pathways of T cell differentiation. In the case of aging, this reciprocal response progresses very slowly; but in the cases of malignancies and autoimmune diseases, the response progresses more quickly. When we injected heat-killed bacteria or mitomycin C-treated syngeneic tumor cells into mice, the reciprocal response transiently occurred and the normal state was regained several days later (116). These results were also observed when we injected curable numbers of live bacteria and tumor cells. On the other hand, when we injected incurable numbers of live bacteria and tumor cells, irreversible changes occurred, including thymic atrophy, which resulted in death. Nevertheless, the liver and thymus are dynamic primary immune organs for the extrathymic and intrathymic differentiation of T cells, respectively.

In contrast to the so far described phenomena, the stimulations of mice with foreign antigens (e.g., xenogeneic proteins) and viruses, resulted in the activation of intrathymic T cell differentiation (Watanabe H. and Abo T., manuscript in preparation). For example, a considerable proportion of bright TCR cells in the periphery, yet none of the intermediate $\alpha\beta$ TcR cells, acquired IL-2R. These responses reflect the fact that foreign antigens induce CTL of thymic origin, but not intermediate $\alpha\beta$ TCR cells of extrathymic origin.

Factors to Regulate the Reciprocal Response

In subsequent studies, we have demonstrated that a single *in vivo* injection of either lymphotoxin (TNF_{β}) or estrogen simultaneously induced the activation of extrathymic T cell differentiation in the liver and the inactivation of intrathymic T cell differentiation (99). Similar effects, yet less effective, were induced by TNF_{α} and IL-1. IFN_{α} and IFN_{δ} did not affect significantly T cell differentiation when injected into mice. Summarized data are presented in Fig. 12. Since effect of lymphotoxin (10^4 unit/mouse, intraperitoneal injection) was transient, but that of estrogen (1 mg/mouse, subcutaneous injection) was considerably long-lasting, it is possible that lymphotoxin and estrogen might be physiological factors that regulate extrathymic and intrathymic T cell differentiation. It is well-known that lymphotoxin induces apoptotic death (due to DNA fragmentation) in certain tumors (28, 42). Since it is well established that the process of clonal deletion of forbidden T cell clones is attributed to their apoptosis, i.e., programmed death

Fig. 12 A comparison of the number of MNC from the liver and thymus of mice treated with various cytokines or hormones. Mice were examined 3 to 7 days after injection. Lymphotoxin and estrogen administrations could induce the activation of hepatic MNC and, instead, induce thymic atrophy. Hydrocortisone administration induced simultaneously the decrease in the numbers of the liver and thymus.

during the DP stage (9, 58), the similar process may be responsible for the accelerated apoptotic death of DP CD4⁺8⁺ thymocytes. Even an injection of estrogen seems to accelerate the same process as seen in that of lymphotoxin. A large dose of irradiation (10 Gy/mouse) or hydrocortisone (5 mg/mouse) simultaneously suppresses both the extrathymic and intrathymic pathways and results in a decrease in the numbers of hepatic MNC and thymocytes as early as 2 days after treatment (99). It is therefore conceivable that the reciprocal responses seen in mice injected with bacteria and mitomycin C-treated tumor cells might be mediated by cytokines such as TNF β , TNF α and IL-1 rather than the stress-associated hormones, glucocorticoids.

Finally, we introduce an interesting phenomenon in which mice treated with an ovariectomy do not display a reduction in the size of their thymus with aging (11). Ovariectomized mice may have higher serum levels of luteinizing hormone (LH) and luteinizing hormone releasing hormone (LHRH). Hence, it is possible that such upper hormones to stimulate estrogen production may activate the intrathymic pathway of T cell differentia-

tion while simultaneously inactivating the extrathymic pathway.

Recognition Molecules of Extrathymic T Cells

After birth, a considerable proportion of $\gamma\delta$ T cells in the circulation and other areas appears to be of extrathymic origin. Many investigators established $\gamma\delta$ T cell lines or clones in *in vitro* cultures from murine and human systems. Such cultured $\gamma\delta$ T cells were demonstrated to be reactive with heat shock proteins (HSP) (48, 64), mycobacterial antigens (e.g., PPD) (45, 65, 94) and autologous class I (and class II) MHC antigens (15, 16, 31, 106, 133). HSP are widely distributed in bacteria, yeasts, parasites and eukaryocytes, and their structures are conserved throughout different type of organisms (89, 95). HSP are produced in large amounts by eukaryocytes which suffer from stress and bacterial infection. It is therefore conceivable that $\gamma\delta$ T cells are responsible for the surveillance of atypical cells, or damaged cells, that are generated *in vivo*. In addition to classical class I and class II MHC antigens which display a great degree of polymorphic diversity depending on individuals, less polymorphic

Table 2 *The Expression of Homing Receptors on T Cell Subsets in Normal Mice*

Organ	Subset	Homing receptor				
		CD44	Mel-14	ICAM-1	LFA-1	CD2
Liver	Intermediate TCR cells	++	—	+	++	+
	Bright TCR cells	—	+	—	+	+
Periphery	Bright TCR cells	—	+	—	+	+
Thymus	Dull TCR cells	—	+	—	+	+
	Bright TCR cells	—	+	—	+	+
Intestine	IEL	+	—	+	+	—

Similar homing receptors are expressed between hepatic intermediate TCR cells and IEL. The data provided from Ohteki T., Tohoku University School of Dentistry.

(or monomorphic) class I MHC antigens are present. Interestingly, many established $\gamma\delta$ T cell clones were demonstrated to recognize such class I MHC antigens, including CD1 (31, 106), Qa-1 (133) and TL antigens (15, 16) (i.e., TLA complex antigens). As described later, extrathymic T cells may be phylogenetically generated earlier than regular T cells of thymic origin. In this regard, extrathymic $\gamma\delta$ T cells may recognize either the monomorphic class I MHC antigens which develop even phylogenetically earlier, or recognize certain self-antigens in the context of these monomorphic MHC antigens. Monomorphic class II MHC antigens were also reported in a recent study (67). Similarly, certain extrathymic $\alpha\beta$ T cell clones with the DN CD4⁺8⁺ phenotype were reported to recognize HSP. It is well established that CD4 and CD8 antigens are accessory molecules which support cellular interaction by binding with classical class II and class I MHC antigens, respectively. Some extrathymic $\alpha\beta$ and $\gamma\delta$ T cells acquire CD4 and CD8 antigens without thymic microenvironments, especially in the liver (Seki *et al.*, in preparation: *vide supra*). These $\alpha\beta$ and $\gamma\delta$ T cells may recognize class I and II MHC antigens or their altered antigens. On the other hand, there are other populations of extrathymic $\alpha\beta$ and $\gamma\delta$ T cells, especially prevalent in the liver, which have DN CD4⁺8⁺ phenotype (98, 114). It is postulated that such T cells may recognize the monomorphic MHC antigens (e.g., TL antigens). It was reported that, abnormal T cells appear in the thymus and periphery of TL-transgenic mice (93). These abnormal cells were determined to be $\alpha\beta$ and $\gamma\delta$ T cells with the DN phenotype (our unpublished data). Similarly, many strains of autoimmune mice (e.g., MRL-*lpr/lpr*, C3H/He-*gld/gld*, NOD, NZB/W F₁, and BXSB mice) show increased proportions of DN, but not

single-positive, T cells in the liver (82). Perhaps some yet undetermined self-antigens in the context of monomorphic MHC antigens are recognized by such DN $\alpha\beta$ T cells in these autoimmune mice.

In recent studies (56, 138), TL antigens were also reported to be expressed on the cell surface of intestinal epithelia in mice. Such TL antigens might be the molecules that give the stimulations to the IEL, one of the extrathymic T cells.

Homing Receptors of Extrathymic T Cells

As previously mentioned, T cells in particular regions display unique V _{γ} and V _{δ} gene segments. For example, in mice the Thy-1⁺ DEC have the V γ 5/V δ 1, the $\gamma\delta$ T cells in female reproductive organs have the V γ 6/V δ 1, and the IEL in the intestine display the V γ 7/V δ 1 (73). Bonneville *et al.* (17) produced transgenic mice containing specific alleles of the V gene to examine whether or not T cells migrate to particular organs. However, since a specific homing was not observed, $\gamma\delta$ TCR themselves are not considered to be homing receptors.

We recently demonstrated that in the liver both $\alpha\beta$ and $\gamma\delta$ T cells with TCR of intermediate intensity have a unique pattern of homing receptors (Ohteki T., Tohoku University School of Dentistry, Sendai). For instance, intermediate TCR cells in the liver were CD44 (Pgp-1)⁺⁺, Mel-14⁺, ICAM-1⁺, LFA-1⁺⁺ and CD2⁺ (Table 2). In contrast, bright TCR cells in the periphery and thymus were CD44⁺, Mel-14⁺, ICAM-1⁺, LFA-1⁺ and CD2⁺. IEL in the intestine also expressed a unique pattern. Furthermore, it is interesting to note that extrathymic T cells expressed CD44, but lack Mel-14; whereas regular T cells differentiated in the thymus inversely expressed Mel-14, but lacked CD44.

Details concerning the structures and possible functions of Mel-14 (39) and CD44 antigens (27) were reported. If hepatic MNC or splenic lymphocytes were radio-labelled with ^{51}Cr and then intravenously injected into mice, 2 days after the injection, more than 60% of the hepatic MNC, yet only 10% of the splenic lymphocytes, migrate to the liver (our unpublished observation). This result further supports the fact that hepatic MNC may have unique homing receptors on their cell surface. Since regular T cells acquired CD44 and lost Mel-14 after activation (e.g., mitogenic stimulation), it is conceivable that these activated T cells (i.e., bright TCR cells), like intermediate TCR cells with CD44^+ Mel-14 $^-$ phenotype migrate to the hepatic sinusoids. In fact, we observe such bright TCR cells in the liver. Namely, we demonstrate that bright TCR cells isolated from the liver have more potent cytotoxic activity against immunized allogeneic lymphocytes when compared with bright TCR cells from the spleen and lymph nodes.

Extrathymic T Cells in Nude, Thymectomized and Scid Mice

BALB/c-*nu/nu* mice congenitally lack the thymus but are known to have a small number of T cells in the peripheral lymphoid organs, spleen and lymph nodes (61, 74, 79). They express Thy-1 and CD3 antigens in terms of immunofluorescence staining and have a relatively dull intensity of $\alpha\beta$ and $\gamma\delta$ TCR (35, 68, 74, 80, 108). In previous studies, many investigators considered that these cells were progenitor T cells that had not undergone thymic education. However, it is now believed that these T cells are extrathymic T cells generated in the liver. Both $\alpha\beta$ and $\gamma\delta$ T cells both in the liver and periphery of nude mice are determined to have an intermediate TCR (and CD3) intensity similar to the intensity seen in the liver of normal mice (T. Ohteki, T. Abo, S. Seki, K. Sugiura, A. Kusumi, K. Ohmori, H. Watanabe and K. Kumagai, Extrathymic generation of $\alpha\beta$ and $\gamma\delta$ T cells occurs in the liver of athymic nude mice; submitted, and Seki *et al.*, in preparation: *vide supra*). In addition, these $\alpha\beta$ T cells comprised a considerable proportion of the self-reactive forbidden T cell clones which were estimated by a series of anti- V_β mAbs (35, 60, 74, 80, 108). It was also reported that T cells in the periphery of nude mice have a relatively low responsiveness to T cell mitogens (e.g., phytohemagglutinin and concanavalin A) (38, 104). Extrathymic intermediate TCR cells isolated from the liver in

normal mice also display the same low responsiveness to these T cell mitogens. More importantly, many of the T cells in the hepatic sinusoids of nude mice not only displayed lymphoblastic morphology, but also underwent DNA synthesis *in vivo* (Ohteki *et al.*, in preparation: *vide supra*). The DNA synthesis was observed by the *in vivo* BrdU injection method in conjunction with BrdU-anti-BrdU immunohistochemical staining. On the other hand, almost all of $\alpha\beta$ and $\gamma\delta$ T cells in the periphery of nude mice display a morphology similar to small resting lymphocytes and did not significantly undergo DNA synthesis *in vivo*. It is therefore possible that T cells are generated in the liver and then migrate to the periphery. We also observed that the number of extrathymic intermediate TCR cells in the liver and periphery increased with age in nude mice (97, Ohteki *et al.*, Seki *et al.*, in preparation: *vide supra*).

Normal young mice aged 4 weeks were thymectomized and systematically characterized by the staining pattern of T cells (Fig. 13) (Seki *et al.*, in preparation: *vide supra*). Here, normal control mice contained both intermediate and bright TCR cells in the liver, but only bright TCR cells in the periphery. After a thymectomy, the staining peaks of the bright $\alpha\beta$ TCR cells, in both the liver and periphery, gradually became small. Two months after a thymectomy the peak of bright $\alpha\beta$ TCR cells in the liver disappeared. It is conceivable that even after birth, the bright TCR cells are continuously supplied from the thymus. It was also noted that after the thymectomy, the lymph nodes and spleen keep bright TCR cells longer than does the liver after thymectomy. However, 6 months after the thymectomy intermediate TCR cells began to appear and become predominant even in the periphery. This replacement occurred more rapidly if the thymectomy was performed at earlier age. It was also reported that neonatally thymectomized mice have a considerable proportion of self-reactive forbidden T cell clones in the periphery (7, 64, 122). Since a considerable proportion of intermediate TCR cells appear in the periphery of normal older mice without a thymectomy, all of peripheral T cells are therefore not long-lived T cells of thymic origin.

Scid (severe combined immunodeficiency) mice are known to have a congenital deficiency of both T and B lymphocytes (20). Interestingly, we could not detect any lymphocytes in the liver, thymus and periphery of these mice (Fig. 14) (136). On the other hand, nude mice have only intermediate

Fig. 13 Only intermediate TCR cells may be of extrathymic origin. BALB/c-(+/+) mice were thymectomized at the age of 4 weeks. These mice were examined 30 and 60 days after thymectomy (Tx); BALB/c-(nu/nu) mice aged 8 weeks were also examined.

TCR cells. Although extrathymic and intrathymic pathways of T cell differentiation occur independently in the corresponding organs, perhaps the earlier progenitor cells of both pathways share common stem cells in the bone marrow.

Cancer and Extrathymic T Cells

It is widely known that malignancies in patients and animals cause atrophy of the thymus and the degree of atrophy depends on the progress of tumor development (5, 14, 29, 129). In a recent study, it

Fig. 14 FACS profiles of MNC in nude and scid mice

was proposed that atrophy of the thymus is induced by certain cytokines (e.g., TNF and GM-CSF) rather than the stress-associated hormones, glucocorticoids (36). In view of these findings, we injected syngeneic tumor cells (1×10^7 cells, intraperitoneally) into mice and examined the activation levels of T cell differentiation in both the thymus and liver (116). As expected, a profound thymic atrophy (more than 95%) was induced several days after the injection. However, since there was an increase in the numbers of hepatic MNC and intermediate TCR cells, it was demonstrated that extrathymic pathway of T cell differentiation in the

liver was reciprocally activated during the same period. Even in human autopsy cases, we demonstrated that hepatic T cells in the subjects with malignancies were in an activated state (113). As previously mentioned, an injection of hydrocortisone (5 mg/mouse), not only induced thymic atrophy (more than 90%), but also simultaneously reduced the number of hepatic MNC. It is therefore conceivable that factors other than glucocorticoids, such as lymphotoxin (TNF_β) and other monokines (TNF_α and IL-1) as previously discussed, might be responsible for the effect of injecting syngeneic tumor cells into mice.

The phenomenon described above seems to demonstrate, that extrathymic T cells rather than regular T cells (e.g., CTL) differentiated in the thymus, might be directly associated with tumor immunity. This is further supported by the fact that athymic nude mice do not reject allogeneic transplants and show a low incidence of spontaneous malignancy generation. It is presumed that extrathymic T cells generated in the liver, by virtue of their autoreactivity, play an important role in the surveillance of malignant cells *in vivo*. Such *in vivo* hepatic T cell function and their specificity are under investigation in murine and human systems. In a preliminary study, we have demonstrated after injecting syngeneic tumor cells (2×10^6 cells, intraperitoneally) into mice, intermediate TCR cells in the liver, and to some extent in the spleen, were specifically activated. Interestingly, a larger number of normal lymphocytes or syngeneic mitogen-stimulated lymphoblasts (10^8 cells, intraperitoneally) did not induce such activation. Whereas, an injection of allogeneic tumor cells (2×10^6 cells, intraperitoneally) only activated bright TCR cells in the liver and spleen. These results suggest that the immune system has an ability to distinguish foreign cells and own cells (i.e., syngeneic tumors, syngeneic normal cells and allogeneic tumors), and respond differently to them, probably by recognizing their MHC antigens (plus tumor-specific antigens) on the cell surface.

Activation of Extrathymic T Cell Differentiation during Hepatocyte Regeneration after a Partial Hepatectomy

After a partial hepatectomy (two-thirds of the liver) in mice, intensive hepatocyte regeneration occurs; and within 10 days following the treatment, the normal size of the liver is regained. It is known that activated T cells appear in the spleen during hepatocyte regeneration (34, 100, 105, 112). To examine the nature of such activated T cells, we thoroughly analyzed MNC isolated from the liver, spleen and thymus of these mice (Satoh Y., Niigata University School of Medicine, Niigata). We observed that after the partial hepatectomy, the number of MNC in the liver increased, whereas the number of thymocytes decreased. The increased number of hepatic MNC was mainly due to the increase in the number of intermediate $\alpha\beta$ TCR cells, whereas the decreased number of thymocytes was due to the decrease in the number of DP $\alpha\beta$ T cells in the thymus. A significant proportion of the activated T

cells in the spleen was also the same intermediate $\alpha\beta$ TCR cells seen in the liver. This phenomenon is quite similar to that seen in mice with malignancies. It is conceivable that the rapid regeneration of normal hepatocytes may be inactivated by intermediate TCR cells. Since extrathymic T cells preferentially reside in the intestinal epithelial region (i.e., IEL), the skin (i.e., Thy-1⁺ DEC) and the liver, they may play an important role in regulating normal cell regeneration. In other words, extrathymic T cells may recognize not only malignant cells, but also rapidly dividing normal cells.

Intimate Association of Extrathymic T Cells and Bacterial Infection

Extrathymic T cell population with intermediate TCR intensity is comprised of a considerably large proportion of self-reactive clones, and induced by bacterial stimulations, including *P. acnes* and *Escherichia (E.) coli* (2). Although the bacterial induction was performed with an intraperitoneal injection of 10^8 heat-killed bacteria, similar results were obtained by using 10^4 – 10^5 live bacteria (our unpublished data). In other words, bacterial infections efficiently activate the extrathymic pathway of T cell differentiation. It is possible that the induction of self-reactive T cell clones may be beneficial for the surveillance of bacterially infected cells. However, overstimulation of such T cell clones may be responsible for the onset of certain autoimmune diseases. In fact, it is well-known that anamnestic bacterial infections precede the onset of autoimmune disease.

Listeria monocytogenes are a class of intracellular pathogens which preferentially infect and grow in Kupffer cells of the hepatic sinusoids. We recently demonstrated in mice that the intraperitoneal infection of only 10^3 live *Listeria* effectively activated extrathymic T cell differentiation. Three to seven days after the infection, an increased proportion (and absolute number) of intermediate $\alpha\beta$ TCR cells was seen in the liver. Interestingly, after day 7, the level of intermediate $\alpha\beta$ TCR cells returned to its normal level, whereas the level of bright TCR cells of thymic origin increased. The infected mice became healthy around day 10. It is postulated that during the initiation of infection, extrathymic T cells attack the bacterially infected cells (e.g., infected Kupffer cells), whereas regular T cells of thymic origin interact subsequently with the bacteria disseminated from the infected cells. We feel that both pathways of T cell differentiation

might be successively activated in an appropriate fashion and work in collaboration with each other during the course of bacterial infection.

Successive Activations of Either Extrathymic or Intrathymic T Cells in the Liver after Viral and Parasite Infections

Both viral and parasite antigens are typical foreign antigens in animals. Indeed, these infections were demonstrated to activate regular T cells of thymic origin, which resulted in the increase of bright TCR cells in the liver and periphery of mice (our unpublished observations). However, during these experiments, we encountered interesting phenomena. First, when the extrathymic pathway of T cell differentiation was activated beforehand in mice by some agents described so far, viral infections (e.g., influenza virus) often became immortal. It is possible that atypically generated cells in which viral replication occurred, were effectively eliminated by the activated extrathymic T cells at the initiation period of viral infection. On the other hand, during the chronic course of parasite infection, the extrathymic T cells were activated especially when the parasites made up a granuloma formation. It is also conceivable that in such a case, the granuloma may be recognized as atypical cells generated *in vivo* by extrathymic T cells. These results indicate that immune responses after infections vary uniquely, depending on the natures of infected agents. Again, we feel that both pathways of T cell differentiation are under a subtle regulation.

Autoimmune Diseases and Extrathymic T Cells

MRL-*lpr/lpr* mice develop a severe autoimmune disease that resembles SLE and rheumatoid arthritis in humans (8, 92, 118). The predominant immunological abnormality in these mice is the development of peripheral lymphadenopathy which is due to the increase in the number of $\alpha\beta$ T cells with the DN CD4⁻8⁻ phenotype. Since there was no evidence for increased proliferation of such T cells in the thymus, bone marrow and periphery, the location where these abnormal T cells are generated was still unknown. Some investigators reported that a thymectomy sometimes reduced the diseased state in autoimmune MRL-*lpr/lpr* mice (119, 126, 131). Therefore, many studies of autoimmune MRL-*lpr/lpr* mice have focused on the intrathymic differentiation of T cells. However, it was reported that when a normal thymus was trans-

planted into thymectomized MRL-*lpr/lpr* mice, lymphadenopathy still occurred (131). Furthermore, it has been observed that a chronic injection of certain immune activators into thymectomized MRL-*lpr/lpr* mice also caused lymphadenopathy (119). Recently, it has also been reported that forbidden T cell oligoclonal populations are normally deleted in the thymus of MRL-*lpr/lpr* mice (71, 120). These findings raise the possibility that although the thymus in MRL-*lpr/lpr* mice may play significant supporting roles in the activation of lymphadenopathy and autoimmune disease, the thymus itself is not necessarily abnormal (98). We have demonstrated that abnormal $\alpha\beta$ T cells with the DN phenotype are generated in the liver (98, 114). In addition, just before and after the onset of disease, there was a significant increase in the proliferation of $\alpha\beta$ T cells in the hepatic sinusoids. Radioisotope injection experiment revealed that such T cells were generated in the liver and then migrated to the periphery. Although the T cells that migrated to the periphery had already lost the cytotoxicity, the cells remaining in the liver were highly cytotoxic. It is therefore essential to examine the extrathymic pathway of T cell differentiation in the liver for studies on autoimmune diseases. In addition, DN $\alpha\beta$ T cells are also known to mediate helper functions of autoantibody production for B cells (23, 139). In earlier studies, several investigators reported that abnormal T cells in the periphery of autoimmune mice were predominantly V β 8⁺ T cell clones (96, 108) with relatively dull TCR intensity (24, 71, 86). These properties correspond well to those of the extrathymic T cells in the liver as already described. C3H/HeJ-*gld/gld* mice, like MRL-*lpr/lpr* mice, develop autoimmune disease and lymphadenopathy in the periphery. Abnormal proliferation of the DN $\alpha\beta$ T cells was also observed in the liver of these mice (82). BXSB, NOD and NZB/W F₁ mice, however, are known to be autoimmune models without peripheral lymphadenopathy. In this study, a significant proliferation of the DN $\alpha\beta$ T cells was also observed in the liver of these mice, although the magnitudes were not as high as those in MRL-*lpr/lpr* and C3H/HeJ-*gld/gld* mice (Fig. 15). Therefore, the expansion of extrathymic DN $\alpha\beta$ T cells in the liver is a common feature of autoimmune mice. In humans, abnormal DN $\alpha\beta$ T cells were also observed in the peripheral blood of patients with SLE (118). Here, the effect of estrogen on the activation of extrathymic T cell differentiation might be responsible for a predominant occurrence of autoimmune diseases in females (99).

* indicates a statistical significance ($p < 0.01$) analyzed by Student t-test.

Fig. 15 The appearance of DN T cells in the liver of various autoimmune mice

The actual autoimmune diseases and the male to female ratios in humans and animals are listed elsewhere (6).

Transplantation and Extrathymic T Cells

It is well established that allogeneic skin grafts are rejected by $CD8^+$ T cells (i.e., CTL) of thymic origin in murine systems. Here, $CD4^+$ T cells initiate the generation of CTL. However, under specific transplant conditions, extrathymic T cells seem to be responsible for allogeneic skin graft rejection. For instance, when athymic nude mice are transplanted with an allograft, they are unable to promptly reject it. However, it was shown that when these mice were intravenously injected with $CD4^+$ T cells from the same background mice, they rejected the allograft (41). Interestingly, it was demonstrated that the effector cells were of nude mouse origin. Therefore, extrathymic T cells might become direct effectors when aided by regular $CD4^+$ T cells.

We recently observed that in mice transplanted with allografts, intermediate TCR cells are slightly activated in the liver (Iiai T., Niigata University School of Medicine, Niigata, unpublished data).

Perhaps yet undetermined antigens (e.g., monomorphic MHC antigens) are recognized by extrathymic T cells during allograft transplantation. In this regard, an allogeneic liver transplantation experiment in rats may contain a clue to resolve the above question. These rats sometimes failed to reject an allograft, and in this case, experienced profound thymic atrophy with proliferation of extrathymic T cells of recipient (and donor) origin in the liver (Kobayashi E. and Kamata N., National Children's Medical Center, Tokyo). It is possible that extrathymic T cells might mediate a regulatory function (e.g., suppressor function) against T cells of thymic origin.

When F_1 ($B6 \times bm12$) hybrid mice were injected with parental B6 splenic $CD4^+$ T cells of class II MHC-disparate, they suffered from a graft-versus-host (GVH) reaction and displayed autoimmune-like symptoms (111). Such mice also showed an increased number of intermediate $\alpha\beta$ TCR cells in the liver, accompanied with profound thymic atrophy (Ikarashi Y. *et al.*, The University of Tokyo, Tokyo, manuscript in preparation). We postulate that first, the donor $CD4^+$ T cells may recognize the recipient class II MHC antigens and then, the recipient extrathymic $\alpha\beta$ T cells may be activated

to destroy these damaged autologous cells. It is interesting to note that the intermediate TCR cells proliferating in the liver of recipient F_1 mice consist of mainly $CD4^+$ T cells. Perhaps these processes result in the onset of autoimmune-like disease. In any case, extrathymic T cells generated in the liver seem to be intimately associated with the allogeneic transplantation.

Immediate Hypersensitivity and the Extrathymic Pathway of T Cell Differentiation

Immediate Type I hypersensitivity, (allergy), includes atopic dermatitis and allergic asthma in children, and allergic reaction to different types of pollens in adults. Although a complex series of events are associated with these reaction, the production of IgE against corresponding antigens is an important fundamental process. To effectively produce IgE against a small amount of such antigens, an appropriate helper function is required from $CD4^+$ T cells of thymic origin (103). Genetic backgrounds (including a genetic background of hyperfunction of the thymus) might be responsible for the high incidence of hypersensitivity. When we injected the extracts of parasites or mites into mice in preliminary studies, only bright TCR cells that were differentiated in the thymus were activated in terms of an increased expression of IL-2R. On the other hand, intermediate TCR cells in the liver were noticeably inactivated. This experimental result further supports the notion as stated above. Namely, foreign antigens are processed by T cells of thymic origin in the context of self-MHC antigens.

We therefore propose the working hypothesis that since the extrathymic and intrathymic pathways are always reciprocally regulated, preactivation of the extrathymic pathway might reduce the incidence of immediate hypersensitivity. As described earlier, extrathymic $\alpha\beta$ T cells may, by virtue of their autoreactivity, also have a regulatory function against regular T cells. There are presently several procedures to induce this reciprocal regulation, including bacterial stimulation (even oral administration) and lymphotoxin injection.

Before and following World War II, a number of children in Japan had sinusitis with purulent nasal discharges, most of which have a fate to be swallowed. During this period, the incidence of atopic dermatitis and allergic asthma was very low in Japanese children. This situation might be explained by the present belief that activation of the extrathymic T cell differentiation pathway can be induced by

the oral administration of bacteria via the alimentary tract. On the other hand, the intrathymic pathway, which is the source of helper T cells for IgE production, might be reduced. As shown in a murine study (see Fig. 2), mice under conventional feeding conditions, but not under SPF conditions, showed an activated state of the extrathymic T cell differentiation. It is therefore conceivable that bacterial flora in the intestine is also an important source of stimuli for the extrathymic pathway in the liver. Since Japanese children (and adults) today may not have appropriate bacterial flora due to nutritional improvements and the frequent administration of antibiotics, this might result in a decrease of activation of the extrathymic pathway of T cell differentiation. Perhaps this explains why many Japanese children (and adults) are suffering from immediate hypersensitivity these days.

A Possible Mechanism Underlying the 'Breaking of Self-Tolerance' in the Thymus

When thymocytes from young mice were stained by a two-color immunofluorescence test using anti-CD3 and anti-IL-2R β mAbs, a small population of intermediate TCR cells (mainly $\alpha\beta$ T cells) was detectable in the FACS profile (see Fig. 11). However, in mice with an involuted or atrophic thymus, due to either aging or bacterial stimulation, the relative proportion of such intermediate TCR cells increased. Atrophic thymus comprises mainly the medullary region. It is therefore conceivable that intermediate $\alpha\beta$ TCR cells primarily reside in the medulla of the thymus which subsequently becomes prominent after atrophy of the cortex region. Both the hepatic sinusoids and the thymic medulla have intermediate $\alpha\beta$ TCR cells with extrathymic T cell properties (e.g., containing self-reactive T cell clones and the predominance of $V\beta 8^+$ T cell clones). It is presently still unknown whether intermediate $\alpha\beta$ T cells in the thymus are generated *in situ* or supplied from other organs (e.g., the liver). In either case, under induced thymic atrophy, thymocytes contain a large proportion of self-reactive forbidden clones in the medulla. This phenomenon seems to have been previously referred to the 'breaking of self-tolerance' in the thymus. Thymic atrophy induced by the GVH reaction seems to be the case (37). In earlier studies, it was revealed that the epithelia in the thymic medulla are of endodermal origin, but those in the thymic cortex, which bear a representative set of self-antigens and therefore educate T cells, are of

Fig. 16 Effect of X-ray irradiation and the appearance of intermediate TCR cells in the thymus. Intermediate and bright TCR cells are encompassed and the numbers of the figures indicate the percentages of positive cells. NK cells appeared in the thymus after irradiation are indicated by arrowheads.

ectodermal origin. Hence, we prefer to consider that the medullary region of the thymus developed phylogenetically earlier than the cortex region. If this is correct, the thymic medulla, like the hepatic sinusoids, is comprised of primitive intermediate $\alpha\beta$ TCR cells.

In addition to the conditions described above, X-

ray irradiation (7 Gy/mouse), hydrocortisone injection (5 mg/mouse), and anti-CD3 mAb treatment *in vivo* (100 μ g/mouse) can selectively eliminate bright TCR cells in the thymus (including dull TCR cells). The FACS profiles of MNC obtained from irradiated mice are depicted in Fig. 16. The MNC were stained with a two-color immunofluo-

rescence test using anti-CD3 and anti-IL-2R β mAbs. Since most intermediate TCR cells are not sensitive to irradiation, they remained in the various organs (indicated by an arrow). This was even true in the thymus, where the thymic cortex was profoundly reduced and the medullary region became prominent. The intermediate TCR cells that remained in the thymic medulla, like the extrathymic T cells in the liver, were comprised of a significant proportion of autoreactive forbidden T cell clones. Furthermore, the T cells recovered after the treatment were mainly generated from intermediate TCR cells in the liver. The predominance of intermediate TCR cells in various organs continued for several days thereafter.

After atrophy of the thymic cortex, not only intermediate $\alpha\beta$ TCR cells but also NK cells (indicated by arrowheads), became predominant (see Fig. 16). It is conceivable that the thymic medulla, even under a normal condition, contains both populations. Many investigators have established clonal lines of autoreactive T cells and NK cells from the thymus (19, 84, 110, 135). We believed that these clones originated in the thymic medulla. Furthermore, we have discovered that it is easier to use hepatic MNC for the establishment of such clones. It has been reported that $\gamma\delta$ T cell clones and lines established from the adult thymus, like $\alpha\beta$ T cells in the liver, have the same V gene segment, V γ 1/V δ 6 (69, 83). Since red pulp in the spleen is mostly comprised of extrathymic T cells with intermediate TCR (18, and our unpublished data), it is possible that the sinusoidal structure in splenic red pulp is also the site for extrathymic T cell differentiation.

The Development of Autoimmune-Like Lesions with Age

Despite a decline in immunologic activities in aging humans and animals, organ-specific or multi-organ autoimmune-like lesions are known to develop (54, 59). The inflammatory lesions occur in the salivary glands, kidneys, lungs, pancreas and liver. The cells which infiltrate in the lesions are mainly lymphocytes, most notably CD4 $^{+}$ T cells. In accordance with the results so far described, the infiltrating cells might be intermediate TCR cells of extrathymic origin. In fact, we recently demonstrated that in mice aged more than 2 years, not only the liver, but also the periphery, including the spleen, lymph nodes and thymus, were comprised of a large proportion of intermediate TCR cells

(>60%) (Okuyama *et al.*, in preparation: *vide supra*). As shown in Fig. 1, the number of hepatic MNC increased with age which resulted in a predominant absolute number of intermediate TCR cells in old mice. Although we are currently investigating this phenomenon, we postulate that these extrathymic T cells migrate to the autoimmune-like lesions and by virtue of their autoreactivity, mediate cytotoxicity.

Microenvironments of the Hepatic Sinusoids

Intermediate TCR cells apparently proliferate in the hepatic sinusoids. They display a lymphoblastic morphology and a large light scatter in FACS analysis. Moreover, it was reported that null cells depleted of Thy-1 $^{+}$ cells, which were originally obtained from the liver of MRL-*lpr/lpr* mice, were able to acquire TCR in an *in vitro* culture (98). It is conceivable that in order for the intermediate TCR cells of extrathymic origin to obtain their unique properties as intermediate TCR cells of extrathymic origin, the unique microenvironments are required to educate their progenitors. Since intermediate TCR cells always adhere to either sinusoidal endothelial cells or Kupffer cells in the liver, they may facilitate the homing of the progenitors and instigate stroma function. The unique properties of hepatic endothelial cells (e.g., myeloid antigen expression and phagocytic function) and Kupffer cells were reported (90). These cells may have some of the unique stroma cell functions, that have been observed in the thymic epithelial and dendritic cells, and in the stroma cells of the bone marrow. Nevertheless, the responsible cytokines remain to be determined. We have recently induced hepatic cirrhosis in mice by injecting with CCl $_4$ (Iiai T. *et al.*, unpublished observation). Surprisingly, the mice with advanced cirrhosis did not produce intermediate TCR cells in the liver. This result further elucidates that the appropriate microenvironments are essential for extrathymic T cell differentiation in the liver.

Primitive CD5 $^{+}$ B Cells Reside in the Liver of Autoimmune NZB/W F $_1$ Mice

We have so far described the presence of intermediate TCR and NK cells in the liver of mice. These cells seem to be primitive lymphocytes that exist in a phylogenetically intermediate position between monocytes and regular T cells. A series of recent studies demonstrate that CD5 $^{+}$ B cells reside in the

Fig. 17 Evolution of myeloid and lymphoid cells. Cells differentiated in the hepatic sinusoids are underlined.

liver of autoimmune NZB/W F₁ mice (Ohteki T., Tohoku University School of Dentistry, Sendai). CD5⁺ B cells (i.e., Lyt-1⁺ B cells in mice and Leu-1⁺ B cells in humans) are considered to be of a discrete lineage from conventional CD5⁻ B cells (46, 47, 57). CD5⁺ B cells are involved in the production of autoantibodies and are, therefore, possibly responsible for the onset of autoimmune diseases. They appear in both autoimmune-prone NZB/W F₁ mice and patients with autoimmune diseases. Conventional CD5⁻ B cells are replenished by bone marrow progenitors and distributed to many peripheral immune organs (e.g., the spleen and lymph nodes) where they constitute the major population of B cells (52, 53). On the other hand, CD5⁺ B cells normally constitute only 1 to 3% of the lymphocytes in such peripheral lymphoid organs. These cells uniquely reside in the peritoneal cavity, constituting 10 to 40% of the total peritoneal exudate cells (40 to 80% of the B cells) (52, 57). They also appear relatively independent of bone marrow supply and characterized by self-renewal. CD5⁺ B cells begin to appear in the liver just before the onset of disease in NZB/W F₁ mice and increase as a function of age. The phenotypic features of hepatic CD5⁺ B cells were the same as those of known CD5⁺ B (dull-CD5, bright-IgM and dull-B220 expression) cells in the peritoneal cavity and

spleen. However, in an *in vitro* culture, hepatic CD5⁺ B cells were unable to spontaneously produce IgM- and IgG-autoantibodies against and double-stranded DNA. On the other hand, CD5⁺ B cells in the peritoneal cavity produced IgM-, but not IgG-autoantibodies, whereas splenic CD5⁺ B cells were able to produce both IgM- and IgG-autoantibodies. It is postulated that CD5⁺ B cells in the liver may be the most primitive subset of the CD5⁺ B cell population. By incorporating the data, a scheme of various myeloid and lymphoid cell developments can be depicted (Fig. 17). From a stem line of macrophages, typical phagocytes develop as granulocytes, whereas a lineage of lymphoid cells diverts to give rise to a large number of subsets including NK cells, T cells and B cells. Earlier subsets of these lymphoid cells might be NK cells, extrathymic αβ and γδ T cells and CD5⁺ B cells.

Phylogeny of Extrathymic T Cells

To determine the phylogeny of the extrathymic pathway of T cell differentiation, we examined the livers of various animals at stages before and after thymic development. The microscopic observation revealed that the livers of mollusks (e.g., shell fishes and cuttlefishes) and arthropods (e.g., crab and shrimp) contain a large number of lymphoid cells

Fig. 18, A, B and C

Fig. 18, D, E and F

Fig. 18, G, H and I

Fig. 19 Scheme of primitive lymphocyte differentiation in the liver. A, animal with an open vessel system; B, animal with closed vessel system

in the sinusoidal regions (Fig. 18). In addition, we were able to isolate such lymphoid cells by Ficoll-Isopaque gradient (1.090 in density) centrifugation. Interestingly, hepatic lymphocytes in these animals, like in humans and mice, comprise a considerable proportion (up to 10%) of lymphoblasts. It is probable that this morphology reflects lymphocyte proliferation *in situ*. Namely, before thymic development, animals make lymphocytes in the hepatic sinusoids which become a source of lymphocytes in the body cavity (coelom). In addition, since their open vessel system directly connects the hepatic sinusoids with the body cavity (Fig. 19A), it is easy to understand why the epithelia in the peritoneal cavity and sinusoidal endothelial cells in the liver are of the same origin. A recent study has demonstrated that the omentum also supports

extrathymic development of T cells in mice (7). It was further reported that during the fetal stage, the omentum contains precursors of CD5⁺ B cells (123). It is therefore reasonable that there is a preponderance of CD5⁺ B cells in both the peritoneal cavity and liver. In acelomates, the coelom cavity, in which many organs of mesoderm origin are present, is not developed. With the development of the coelom, coelomates develop the hepatic sinusoids of the immune system. Perhaps the phylogenetic development of this immune system is a means to survey the various organs of mesoderm origin (and others) in the coelom, including, but not limited to, the urinary, genital and neural systems. After the development of a closed vessel system (Fig. 19B), hepatic lymphocytes only migrate to the circulation.

The lampreys represent a modern derivative of the most primitive vertebrate line. Their thymus consists of protothymi on the gills (40). A microscopic observation of such protothymi displayed that it is primarily comprised of a considerably large medullary region. In addition, a large number of lymphocytes were depicted in the hepatic sinusoids (see Fig. 18, G-I). The lymphocytes isolated by gradient centrifugation contained cells of a lymphoblastic morphology. However, such lymphoblastic cells were not detected in the samples obtained from the blood and spleen.

Finally, we have to emphasize that after thymic development, animals do not produce a large num-

Fig. 18 Histology of the liver and cell smears of hepatic lymphoid cells from various animals. A: Cell smear of hepatic MNC in mice (May-Grünwald and Giemsa staining, $\times 960$). B: Cell smear of splenic cells in mice (May-Grünwald and Giemsa staining). C: The liver of crab. D: The liver of crab [hematoxylin-eosin (H-E) staining, $\times 320$]. E: The liver of crab (H-E staining, $\times 640$). F: Cell smear of hepatic MNC in crab (May-Grünwald and Giemsa staining, $\times 960$). G: The liver of lamprey (H-E staining, $\times 320$). H: The liver of lamprey (H-E staining, $\times 640$). I: Cell smear of hepatic MNC in lamprey (May-Grünwald and Giemsa staining, $\times 960$)

ber of lymphocytes in the liver until the thymus is either involuted due to aging or atrophied during the diseased states as described thus far. In the fetal and neonatal stages of mice, hematopoiesis is very active in the parenchymal area of the liver, but the extrathymic differentiation of T cells is not detected in the sinusoidal area. It is not until 2 or 3 weeks that extrathymic differentiation begins to occur, although on a small scale, in the sinusoids of mice.

Extrathymic T Cell Differentiation in Humans

Humans also contain the extrathymic $\alpha\beta$ and $\gamma\delta$ T cells that have been so thoroughly characterized in mice. In humans, the two major subsets of $\gamma\delta$ T cells are either V γ 9-C γ 1/V δ 2 or C γ 2/V δ 1 (72, 85, 124). Since BB3 and Ti γ A mAbs react with V δ 2 and V γ 9, respectively, these mAbs can be used to identify the former subset. On the other hand, the latter subset can be identified with δ -TCS1 mAb, since they react with V δ 1. Three-fourths of the $\gamma\delta$ T cells in the peripheral blood belong to the former subset; whereas one-fourth of the $\gamma\delta$ T cells in the blood, and all of the $\gamma\delta$ T cells in the thymus, belong to the latter subset. Interestingly, only $\gamma\delta$ T

cells with V γ 9-C γ 1/V δ 2, like the extrathymic $\gamma\delta$ T cells in the liver of mice (97), show an age-associated increase, especially at adolescence (102). Therefore, the former subset may be of extrathymic origin and the latter subset may be of thymic origin. We further observed that extrathymic $\gamma\delta$ T cells were predominant in the liver of patients with malignancies (113). In a recent study, we have demonstrated that intermediate $\alpha\beta$ TCR cells were present in the liver and periphery of aged people (Okuyama R. and Seki S., Tohoku University School of Medicine, Sendai, manuscript in preparation). However, the characterization of such human intermediate $\alpha\beta$ TCR cells is still under investigation.

CONCLUSION

Before and after thymic development in animals, extrathymic pathway of T cell differentiation in the liver (as well as intestine) seems to be the most primitive lymphocyte differentiation system. After thymic development, this extrathymic pathway plays a relatively minor role, especially during the fetal and neonatal stages. However, thereafter the

Fig. 20 Function of extrathymic (or primitive) T cells

extrathymic pathway is gradually activated by aging, and under conditions of bacterial infection, malignancies and autoimmune diseases. Since there is not a DP CD4⁺8⁺ stage for negative selection of forbidden T cell clones seen in the thymus, the hepatic pathway mainly produces self-reactive forbidden T cell clones. This production of extrathymic T cells might be beneficial for the surveillance of atypical cells generated *in vivo* under the conditions mentioned above (Fig. 20). However, overstimulation of extrathymic production may be responsible for the onset of certain autoimmune diseases. On the other hand, regular T cells differentiated in the thymus interact with foreign antigens in the context of self-MHC antigens. Extrathymic T cells have TCR (and CD3) of intermediate intensity (i.e., intermediate TCR cells), and consist of DN CD4⁺8⁻ cells and single-positive CD4⁺ or CD8⁺ cells. In addition, they have a unique set of homing receptors. In the most activated state, differentiation extrathymic T cells migrate from the liver to the periphery. In the periphery, they reside in unique areas of the spleen (i.e., red pulp) and thymus (i.e., medulla). In order to elucidate the mechanism of 'breaking self-tolerance' in the thymus and the immunology of incurable diseases, including malignancies and autoimmune diseases, further studies involving the extrathymic pathway of T cell differentiation will have to be conducted.

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