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Detailed characterization of $\gamma\delta$T cells within the organs in mice: classification into three groups

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SUMMARY

$\gamma\delta$T cells are known to localize preferentially in the epithelial regions and the hepatic sinusoids, and exhibit highly restricted V gene usage depending on their location. In the present study, $\gamma\delta$T cells in mice were further characterized in terms of their expression of the interleukin-2 receptor beta-chain (IL-2Rβ), CD4 and CD8, and CD8α and β. This experiment was arranged to investigate whether $\gamma\delta$T cells have different properties depending on the organs and how $\gamma\delta$T cells are different from extrathymic $\alpha\beta$T cells, i.e. $\alpha\beta$T cells in the liver and intraepithelial lymphocytes in the intestine, in terms of the above phenotypes. Three-colour immunofluorescence tests using monoclonal antibodies revealed that $\gamma\delta$T cells can be classified into three groups: $\gamma\delta$T cells of the liver type are all IL-2Rβ^+, are comprised of double-negative (DN) CD8$^-$CD4$^-$ and single-positive CD8$^+$ (no CD4$^+$) cells, and express CD8$\alpha^+\beta^-$; $\gamma\delta$T cells of the thymus type are a mixture of IL-2Rβ^+ and IL-2Rβ^-, are mainly DN, and express CD8$\alpha^+\beta^+$ if they carry CD8 antigens; and $\gamma\delta$T cells of the intestine type are also IL-2Rβ^+ or IL-2Rβ^-, are all CD8$^+$, and express CD8$\alpha^+\beta^-$. $\gamma\delta$T cells in the spleen of normal mice are of the thymus type, while $\gamma\delta$T cells in the spleen of athymic nude mice seem to be of the liver type. All these properties of $\gamma\delta$T cells resemble those of extrathymic $\alpha\beta$T cells rather than regular $\alpha\beta$T cells of thymic origin. The present results reveal that $\gamma\delta$T cells and other extrathymic $\alpha\beta$T cells have many properties in common as primitive lymphocytes in phylogenetic development.

INTRODUCTION

T cells bearing T-cell receptor (TcR) $\gamma\delta$ are known to localize preferentially in the epithelial regions, including such sites as the skin, the reproductive organs, and the intestine.[1-4] These epithelial $\gamma\delta$T cells exhibit highly restricted V gene usage[5] and some of them were revealed recently to be generated extrathymically.[6-8] Similarly, $\gamma\delta$T cells with unique V gene usage exist in the hepatic sinusoids where extrathymic T-cell proliferation occurs after birth.[9,10] $\gamma\delta$T cells are also detected in the thymus and peripheral immune organs (i.e. the spleen, lymph nodes and blood) after birth, although their proportion is very small.[11] In the case of adult mice, the predominant V gene usage includes Vγ5/Vδ1 (the skin), Vγ6/Vδ1 (the reproductive organs), Vγ7/Vδ4 (the intestine), Vγ2/Vδ5 (the thymus, and the peripheral immune organs such as the spleen and the lymph nodes) and Vγ1, 2/Vδ6 (the liver).[1,10,11] Parallel with these studies, many phenotypic and functional studies on murine and human $\gamma\delta$T cells are ongoing.

Abbreviations: DN, double-negative; DP, double-positive; IEL, intraepithelial lymphocytes; IL-2Rβ, interleukin-2 receptor β-chain; LPL, lamina propria lymphocytes; MNC, mononuclear cells; TcR, T-cell receptor.

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In the course of our recent studies on extrathymic T cells seen in the liver of mice, it was demonstrated that such extrathymic $\alpha\beta$T cells have many properties distinct from those of regular $\alpha\beta$T cells of thymic origin.[12-17] For example, they have TcR$\alpha\beta$ (and CD3) of intermediate intensity, contain double-negative (DN) CD4$^-$8$^-$ cells, and constitutively express interleukin-2 receptor β-chain (IL-2Rβ) (similar to natural killer cells). It is speculated that extrathymic $\alpha\beta$T cells may be more primitive than regular $\alpha\beta$T cells in their phylogenetic development.[18] Moreover, comprehensive studies that have been conducted recently also reveal some unique properties of intraepithelial lymphocytes (IEL) as primitive lymphocytes (e.g. CD8$\alpha^+\beta^-$ of IEL).[19-22] In the present study, we also investigated $\gamma\delta$T cells within the organs in mice with respect to the expression levels of TcR$\gamma\delta$ (and CD3), DN phenotype, IL-2Rβ, $\alpha\alpha$ homodimer of CD8, and so on. Interestingly, $\gamma\delta$T cells can be classified into three groups according to the above-mentioned characteristics, which resemble those of IEL and hepatic $\alpha\beta$T cells. The present results might be very useful in determining the origins of $\gamma\delta$T-cell groups.

MATERIALS AND METHODS

Mice

B6-+/+ and B6-*nu*/*nu* mice, originally purchased from Jackson Laboratory Inc., Tokyo, Japan, were maintained under specific

pathogen-free conditions in the Animal Facility of Niigata University. These mice were used at the ages of 8–10 weeks (young age) and of approximately 40 weeks (old age).

Cell preparations

Hepatic mononuclear cells (MNC) were isolated by a previously described improved method.[23] Briefly, mice anaesthetized with ether were killed by total exsanguination by means of cardiac puncture. To obtain MNC, the liver was removed, pressed through a 200-gauge stainless steel mesh, and suspended in phosphate-buffered saline (PBS; 0·1 M, pH 7·2). After being washed once with PBS, MNC were isolated from hepatocytes and hepatocyte nuclei by Ficoll–Isopaque density (1·090) gradient centrifugation. The MNC collected from the interface were then suspended in Minimum Essential Medium (MEM) supplemented with 2% fetal calf serum (FCS). The preparations of hepatic MNC contained less than 4% Kupffer cells. Spleen cells were also collected by the Ficoll–Isopaque method, while thymocytes were obtained by forcing the thymus through a 200-gauge stainless steel mesh.

IEL and lamina propria lymphocytes (LPL) were collected from the intestine according to the method described in previous reports,[24,25] with some modifications. Briefly, the small intestine was removed and flushed with PBS to eliminate luminal contents. The mesentery and Peyer's patches were then resected. The intestine was opened longitudinally and cut into fragments 1–2 cm long. These fragments were incubated for 15 min in 20 ml Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing 5 mM EDTA, in a 37°-shaking water bath. The supernatant was collected and washed. This procedure for removing epithelium from gut fragments was performed twice. Pellets containing IEL were resuspended, passed through a glass-wool column to remove aggregates and dead cells, and washed twice before use. Gut fragments after removal of the epithelium, on the other hand, were incubated in 25 ml of PBS containing 2% FCS and 0·15 mg/ml collagenase for 75 min in 37°-shaking water bath. The supernatant and debris were collected and filtered through a stainless steel mesh, and LPL were then isolated from the debris by Ficoll-Isopaque density-gradient centrifugation. LPL were collected from the interface, and washed twice before use. Peyer's patch lymphocytes were obtained by forcing the patches, which were removed beforehand, through a 200-gauge stainless steel mesh.

Immunofluorescence tests

The surface phenotypes of cells were analysed by using monoclonal antibodies (mAb) in conjunction with the two- or three-colour immunofluorescence test.[17] The mAb used here included FITC-, phycoerythrin (PE)-, or biotin-conjugated reagents of anti-CD3 (145-2C11), anti-TcR$\alpha\beta$ (H57-597) and anti-TcR$\gamma\delta$ (GL3) (PharMingen Co., San Diego, CA). Biotin-conjugated anti-IL-2Rβ (TM-β1) mAb was kindly provided by Dr T. Tanaka at the Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan.[26] FITC- or biotin-conjugated reagents of anti-CD4 (L3T4), anti-CD8α (Lyt-2), and anti-CD8β (Lyt-3) were obtained from Becton Dickinson Co., Mountain View, CA. Biotin-conjugated reagents were developed with either PE- or Red 613-conjugated streptavidin (Caltag Laboratories, San Francisco, CA). The fluorescence-positive cells were analysed by a FACScan (Becton Dickinson Co.).

RESULTS

Distribution of $\alpha\beta$ and $\gamma\delta$T cells in various organs

When B6-+/+ and -*nu*/*nu* mice, aged 8–40 weeks, were analysed as to the proportion of $\alpha\beta$ and $\gamma\delta$T cells in various organs, including the liver, spleen, thymus and intestine, their distribution patterns were found to be very unique. The distribution patterns of $\alpha\beta$ and $\gamma\delta$T cells in various organs in a simple manner are represented in Fig. 1. More detailed analysis, including the individual variations of the phenotypes, are represented elsewhere.[17,23] IEL in the intestine and hepatic MNC of normal mice contained a considerably larger proportion of $\gamma\delta$T cells, while MNC in the spleen and thymus were comprised of only a small proportion of such cells (< 1%). This was also true in athymic nude mice, although the proportion of $\alpha\beta$T cells among IEL in these mice was quite low. As shown previously,[17] this was due to a relative increase in the proportion of natural killer (NK) cells.

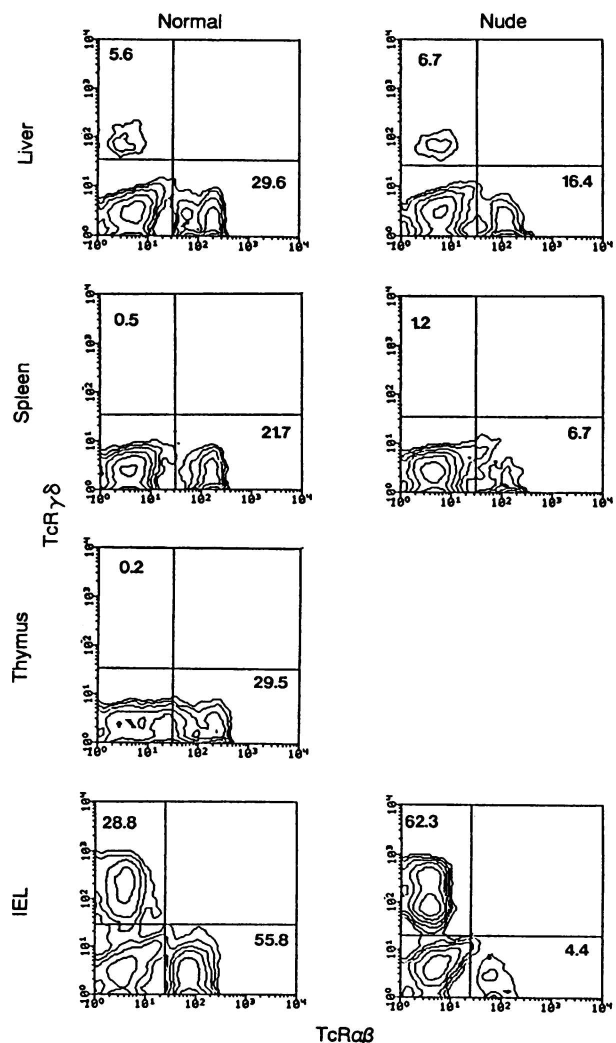

Figure 1. The distribution of $\alpha\beta$ and $\gamma\delta$T cells in various immune organs. One of the representative results is depicted. B6-+/+ and B6-*nu*/*nu* mice aged 10 weeks were used in this experiment.

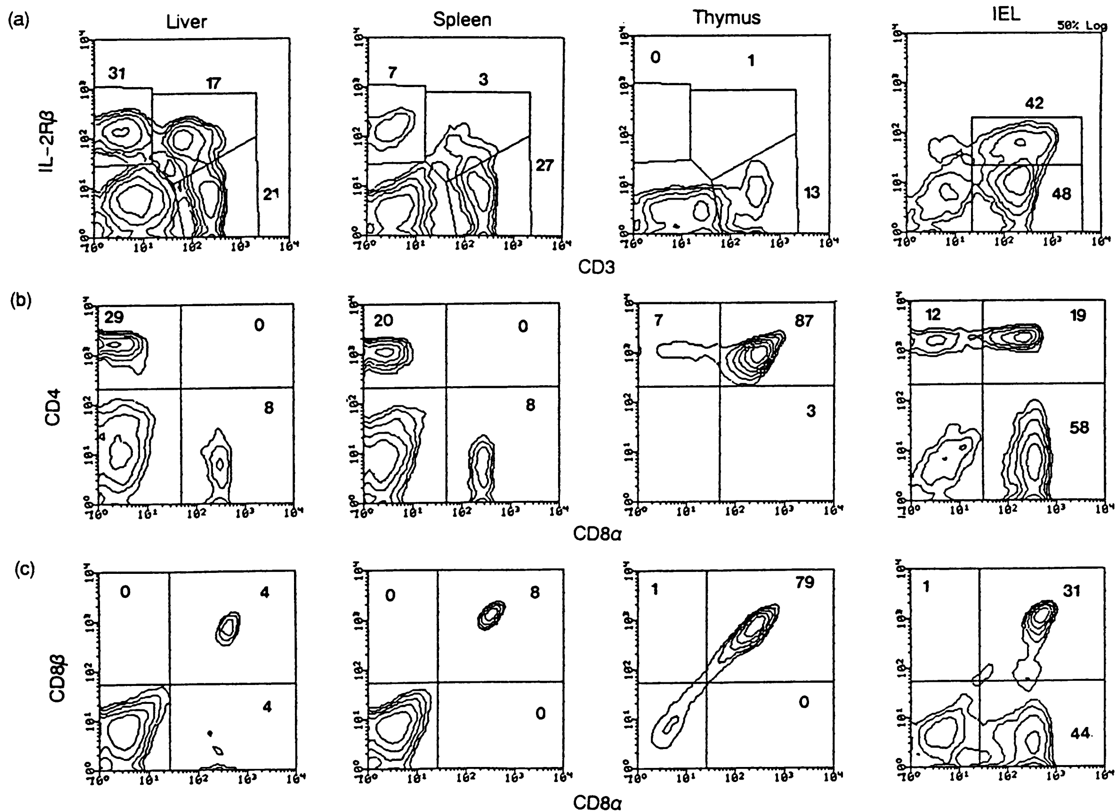

Figure 2. Phenotypic characterization of whole MNC in various organs of normal mice. (a) Two-colour staining for CD3 and IL-2Rβ. (b) Two-colour staining for CD4 and CD8. (c) Staining for CD8α and β. B6-+/+ mice aged 8 weeks were analysed. Representative results of three experiments are depicted.

Phenotypic characterization of whole MNC in various organs in normal mice

Before analysis of γδT cells, whole MNC in various organs were analysed by two-colour staining to determine the expression of CD3, IL-2Rβ, CD4 and CD8 (Fig. 2). When hepatic MNC were stained for CD3 and IL-2Rβ, four lymphocyte subsets were identified: $CD3^-$ IL-2$R\beta^-$ (mainly B cells), $CD3^-$ IL-2$R\beta^+$ (NK cells), CD3-intermediate$^+$ IL-2$R\beta^+$ (extrathymic T cells), and $CD3^+$ IL-2$R\beta^-$ (regular T cells of thymic origin). Here, almost all of $CD3^-$ IL2$R\beta^-$ cells expressed surface immunoglobulin (Ig), while all $CD3^-$ IL-2$R\beta^+$ cells were eliminated by the injection with asialo-GM1 antibody *in vivo*.[17,18] Although the staining patterns were almost the same in splenic lymphocytes, the proportions of NK and extrathymic T cells were very low. Thymocytes were all IL-2$R\beta^-$ and comprised of null, dull and bright TcR cells. As shown previously (and our unpublished observation), only intermediate TcR cells contained DN $CD4^-8^-$ cells and expressed $CD8\alpha^+\beta^-$. Since the majority of intermediate TcR cells in the liver are αβT cells, the above mentioned characteristics belong to intermediate TcR αβT cells. On the other hand, IEL displayed a very unique pattern, consisting of mainly $CD3^+$ IL-2$R\beta^+$ and $CD3^+$ IL-2$R\beta^-$ cells. The staining for CD4 and CD8α showed that DP cells were not present in the liver and spleen, although they comprised an extremely large proportion in the thymus and a significant proportion in the intestine. As already reported,[19] such DP cells in the intestine belong to αβT cells. Finally, in this section, the existence of $CD8\alpha^+\beta^-$ cells (possibly expressing a αα homodimer of CD8) was investigated among MNC in various organs. Although all $CD8^+$ cells in the spleen and thymus were $CD8\alpha^+\beta^+$, $CD8^+$ cells in the liver and intestine were comprised of $CD8\alpha^+\beta^-$ cells at a level of 50% or more. Since $CD8\alpha^+\beta^-$ cells as well as γδT cells seem to be more primitive T cells than other regular T cells, it is estimated that the liver and intestine are the sites where such primitive T cells preferentially resided.

The expression of IL-2Rβ on γδT cells in various organs

To directly analyse the phenotype of γδT cells, three-colour staining for CD3, TcRγδ and IL-2Rβ was performed. By gating TcRγδ$^+$ cells in the cell analyser, the expression of CD3 and IL-2Rβ on γδT cells was depicted (Fig. 3). In this experiment, normal young mice (8 weeks old), normal aged mice (40 weeks old) and athymic nude mice (8 weeks old) were examined in parallel. It was demonstrated that all hepatic γδT cells were IL-2$R\beta^+$ irrespective of the mice used. Since intermediate TcRαβ cells were IL-2$R\beta^+$ and bright TcRαβ cells were IL-2$R\beta^-$ in the liver, both γδT cells and intermediate TcRαβ cells were estimated to be primitive T cells expressing IL-2Rβ. On the other hand, γδT cells in the spleen, thymus and intestine were a mixture of IL-2$R\beta^+$ and IL-2$R\beta^-$ in normal young and old mice. In normal mice, there was a tendency that IEL γδT cells in young mice expressed IL-2Rβ but those in old mice lost IL-2Rβ. In the case of athymic nude mice, γδT cells in the spleen were IL-2$R\beta^+$, similar to those in the liver of these mice, while the

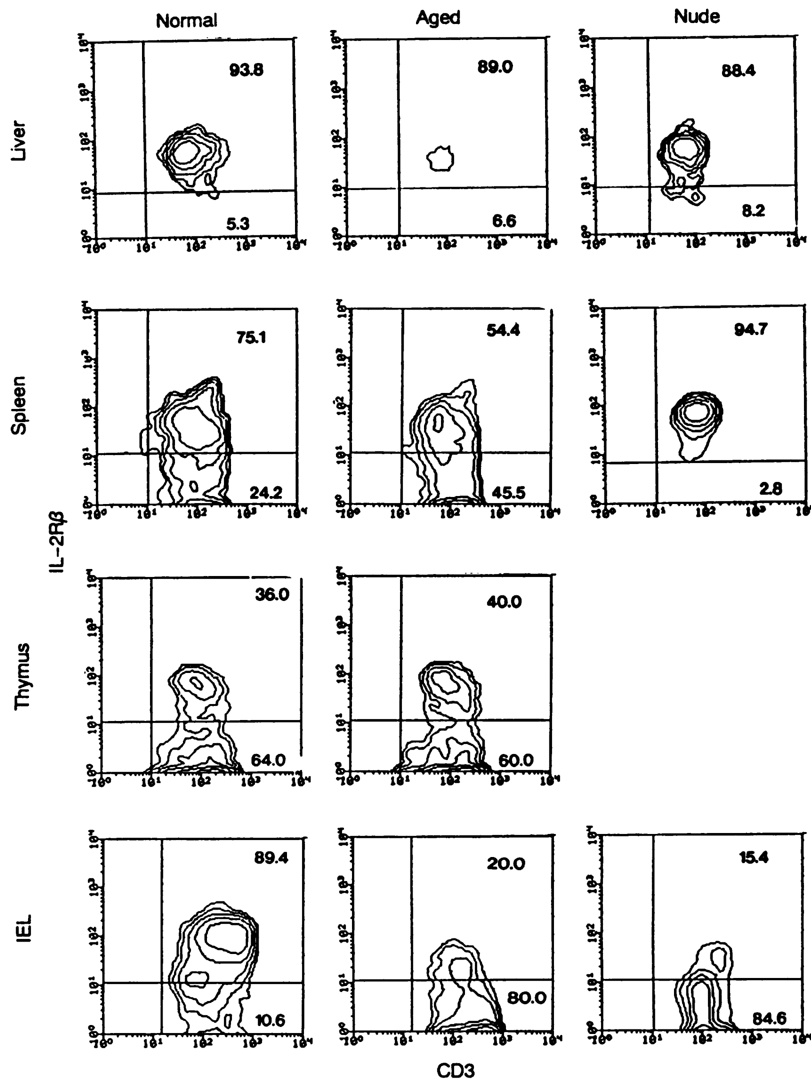

Figure 3. Expression of CD3 and IL-2Rβ on $\gamma\delta$T cells in various organs of normal and athymic nude mice. B6-+/+ mice at a young age (8 weeks) and an old age (40 weeks), and B6-*nu/nu* mice (8 weeks) were used. Three-colour staining for CD3, TcR$\gamma\delta$ and IL-2Rβ was performed. The staining pattern of CD3 and IL-2Rβ on $\gamma\delta$T cells is represented by the gating analysis of TcR$\gamma\delta$.

majority of $\gamma\delta$T cells in the intestine were IL-2Rβ^-. The gated analysis of IEL $\alpha\beta$T cells revealed that such T cells were mainly IL-2Rβ^- (data not shown).

The existence of DN CD4$^-$8$^-$ cells among $\gamma\delta$T cells in various organs

Three-colour staining for TcR$\gamma\delta$, CD4 and CD8 was performed and the staining pattern of CD4 and CD8α was depicted by the gating analysis of $\gamma\delta$T cells (Fig. 4). This analysis clearly showed the distribution of DN CD4$^-$8$^-$ cells as well as that of other phenotypes on $\gamma\delta$T cells. It was demonstrated that hepatic $\gamma\delta$T cells were a half-and-half mixture of DN CD4$^-$8$^-$ cells and CD8$^+$ cells irrespective of mice. In contrast, especially in normal mice, almost all $\gamma\delta$T cells, both in the spleen and thymus, were DN cells, while the majority of $\gamma\delta$T cells in the intestine were CD8$^+$. In case of athymic mice, the staining pattern of $\gamma\delta$T cells in the spleen resembled that of hepatic $\gamma\delta$T cells. However, $\gamma\delta$T cells in the intestine of athymic mice were only CD8$^+$ cells. The small proportion of DP cells in the thymus and intestine of all tested mice suggested that such DP cells mostly consisted of $\alpha\beta$T cells.

Identification of CD8$\alpha^+\beta^-$ cells among $\gamma\delta$T cells in various organs

To identify CD8$\alpha^+\beta^-$ cells among $\gamma\delta$T cells, three-colour staining for TcR$\gamma\delta$, CD8α and CD8β was performed. The expression of CD8α and CD8β on $\gamma\delta$T cells was depicted by the gating analysis of TcR$\gamma\delta^+$ cells (Fig. 5). Interestingly, it was revealed that all CD8$^+$ $\gamma\delta$T cells in the liver and intestine were CD8$\alpha^+\beta^-$ in normal young and old mice. On the other hand, the majority of CD8$^+$ $\gamma\delta$T cells in the spleen and thymus were CD8$\alpha^+\beta^+$. The liver CD8$^+$ $\gamma\delta$T cells in nude mice were unique, but the equivalent cells in the spleen were similar to those in aged mice. All IEL in the intestine of nude mice were CD8$\alpha^+\beta^-$, similar to the case in normal IEL.

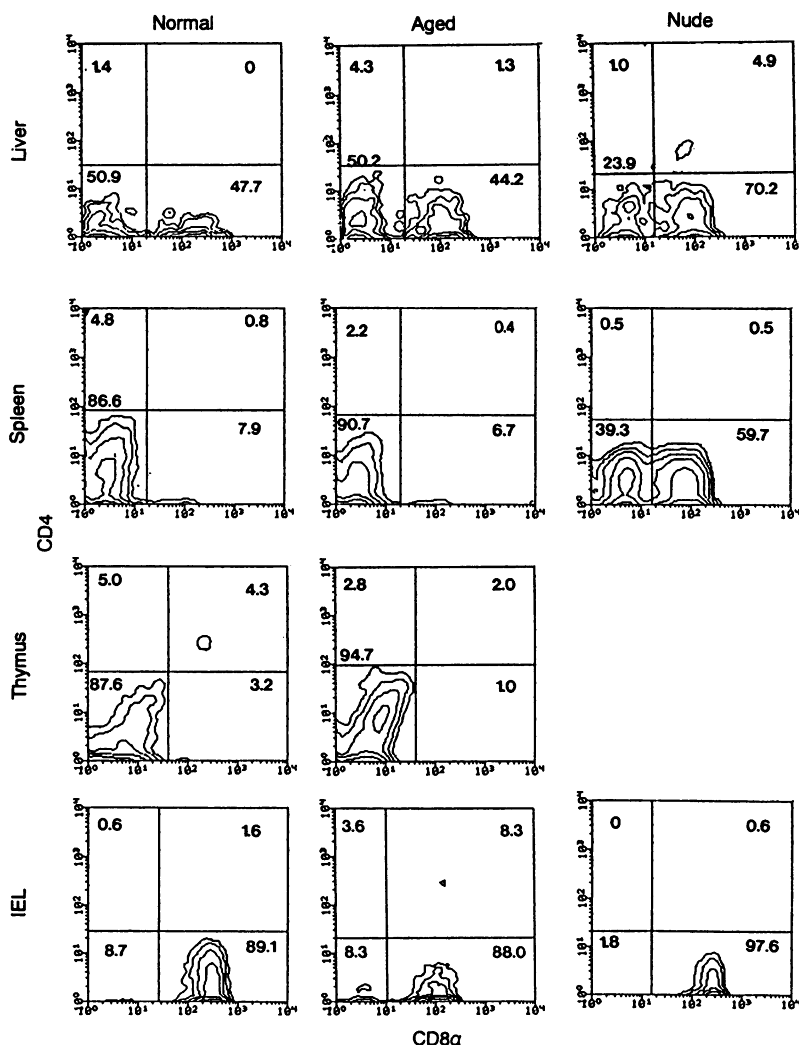

Figure 4. Expression of CD4 and CD8 antigens on $\gamma\delta$T cells in various organs of normal and athymic nude mice. B6-+/+ mice at a young age (8 weeks) and at an old age (40 weeks), and B6-*nu/nu* mice (8 weeks) were used. Three-colour staining for TcR$\gamma\delta$, CD4 and CD8 was performed. The staining patterns of CD4 and CD8 are represented by the gating analysis of TcR$\gamma\delta$.

A comparison of the light scatter among bright TcR$\alpha\beta$ cells, intermediate TcR$\alpha\beta$ cells, and $\gamma\delta$T cells

The light scatter analysis revealed that $\gamma\delta$T cells displayed a slightly larger light scatter than $\alpha\beta$T cells in every organ tested (Fig. 6). Among the $\alpha\beta$T cells, intermediate TcR cells showed a greater light scatter than did bright TcR cells, as shown in the liver. The smallest light scatter was seen in $\alpha\beta$T cells in the thymus, these cells possibly undergoing programmed cell death (i.e. apoptosis).

DISCUSSION

In the present study, it is demonstrated that $\gamma\delta$T cells are a unique T-cell population in terms of the expression of IL-2Rβ, and the composition of the DN CD4$^-$8$^-$ and CD8$\alpha^+\beta^-$ phenotypes. All of these phenotypes seem to be seen in more primitive lymphocytes than the regular T cells of thymic origin. All $\alpha\beta$T cells carrying TcR$\alpha\beta$ (and CD3) or bright intensity (i.e. bright TcR cells) appeared in the periphery of the thymus, lacked a population having IL-2Rβ (under resting conditions), the DN phenotype and CD8$\alpha^+\beta^-$.[17] On the other hand, all intermediate TcR cells seen in the liver (and other organs) were IL-2Rβ^+ and CD8$\alpha^+\beta^-$, and approximately one-third of such intermediate TcR cells were DN CD4$^-$8$^-$.[17,27] It is well-known that IL-2Rβ in constitutively expressed on NK cells under resting conditions.[28,29] All of the T cells seen in the liver and periphery of congenitally athymic nude mice are intermediate TcR cells, it being suggested that they are possibly of extrathymic origin.[17] Interestingly, the level of CD3 expression on $\gamma\delta$T cells was the same as the intermediate intensity of CD3 in hepatic intermediate TcR$\alpha\beta$ cells.[30] As shown in parallel, IEL $\alpha\beta$T cells were mainly IL-2Rβ^-, a mixture of CD8$^+$ and DP CD4$^+$8$^+$ cells and also a mixture of CD8$\alpha^+\beta^-$ and CD8$\alpha^+\beta^+$. Taken together, not only intermediate TcR$\alpha\beta$ cells and IEL $\alpha\beta$T cells, but also $\gamma\delta$T cells in various organs, are estimated to carry the primitive phenotype.

Concerning the expression of the above-mentioned phenotypes, $\gamma\delta$T cells in various organs can be classified into three

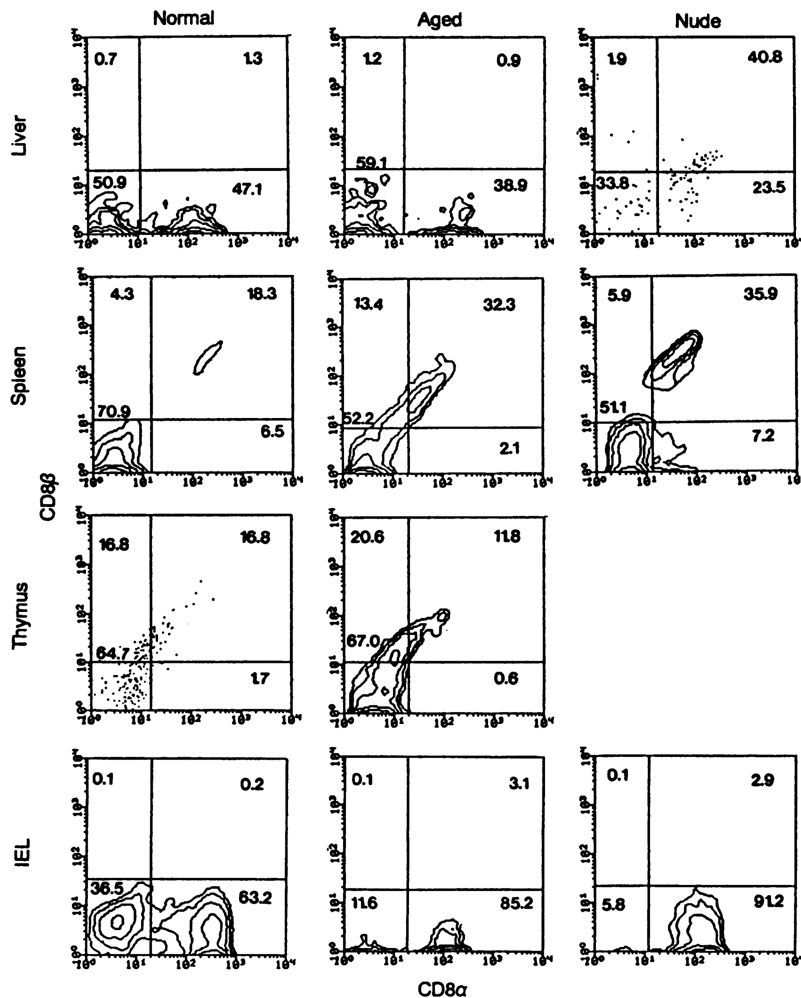

Figure 5. Expression of CD8α and β antigens on $\gamma\delta$T cells in various organs of normal and athymic nude mice. B6-+/+ mice at a young age (8 weeks) and at an old age (40 weeks), and B6-*nu*/*nu* mice (8 weeks) were used. Three-colour staining for TcR$\gamma\delta$, CD8α and CD8β were performed. The staining patterns of CD8α and β on $\gamma\delta$T cells are represented by the gating analysis of TcR$\gamma\delta$.

groups. In normal young and old mice, $\gamma\delta$T cells in the spleen and thymus appear to have many properties in common. Namely, both of them were a mixture of IL-2Rβ^+ and IL-2Rβ^-, almost all of them were DN CD4$^-$8$^-$, and some cells expressed CD8$\alpha^+\beta^+$ if they carried CD8 antigen. In sharp contrast, hepatic $\gamma\delta$T cells were all IL-2Rβ^+, were a mixture of DN and CD8$^+$, and expressed CD8$\alpha^+\beta^-$, whereas IEL $\gamma\delta$T cells were a mixture of IL-2Rβ^+ and IL-2Rβ^- (IL-2Rβ^+ was predominant at a young age), were all CD8$^+$, and all expressed CD8$\alpha^+\beta^-$. In a recent study, we observed that $\gamma\delta$T cells in the uterus resemble those in the intestine (our unpublished observation). Because of low cell yields from the skin, phenotypic analysis such as undertaken in this study could not be carried out. It is concluded that, at present, $\gamma\delta$T cells can be classified into three groups, namely, hepatic $\gamma\delta$T cells, thymic $\gamma\delta$T cells and IEL $\gamma\delta$T cells. Also, since thymectomy at a young age decreases the proportion of $\gamma\delta$T cells in the spleen significantly, some $\gamma\delta$T cells are of thymic origin (H. Watanabe and T. Abo, manuscript submitted for publication).

In the case of athymic nude mice, $\gamma\delta$T cells seen both in the liver and spleen showed the same phenotypes. They were all IL-2Rβ^+, consisted of DN and CD8$^+$, and were a mixture of CD8$\alpha^+\beta^-$ and CD8$\alpha^+\beta^+$. Except for the expression of CD8β^+ on some cells, all other phenotypes resembled those of hepatic $\gamma\delta$T cells in normal mice. To simplify all data produced in this study, $\gamma\delta$T cells are classified in Table 1. The data on $\alpha\beta$T cells, which were obtained in our previous studies[16–18] and in this study, are also listed for purposes of comparison. Although almost all intermediate TcR cells in the liver and IEL in the intestine were CD8β^-, and these T cells were of extrathymic origin, extrathymic T cells were not always CD8β^-. Some $\gamma\delta$T cells of athymic nude mice could express CD8β, despite their extrathymic origin. It is concluded that $\gamma\delta$T cells are more primitive lymphocytes than regular, bright TcR$\alpha\beta$ cells, and are rather similar to intermediate TcR$\alpha\beta$ cells (i.e. extrathymic T cells) in many respects and IEL $\alpha\beta$T cells in some respects. Moreover, both intermediate TcR cells, IEL $\alpha\beta$T cells and $\gamma\delta$T cells, share morphology similar to that of large granular lymphocytes.[9,31,32] The similarity of all these populations might be due to their early phylogenetic development.

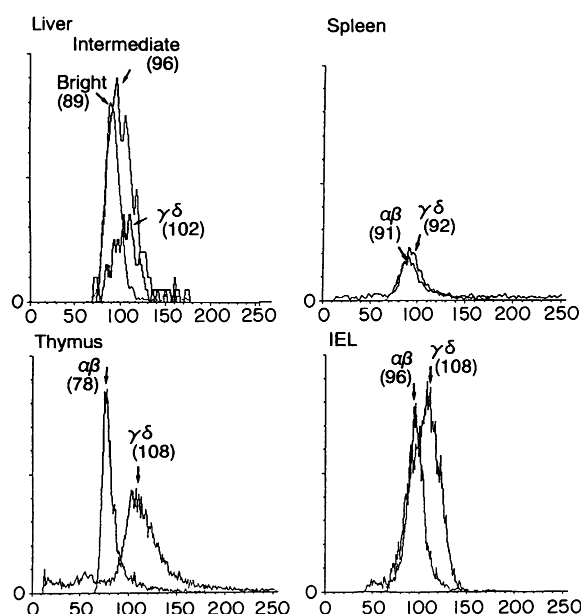

Figure 6. A comparison of the light scatter among $\alpha\beta$ and $\gamma\delta$T cells in various organs of normal mice. B6-+/+ mice aged 8 weeks were analysed. $\gamma\delta$T cells had a larger light scatter than $\alpha\beta$T cells in all organs tested. Numbers in parentheses indicate the mean fluorescence.

Table 1. Phenotypic characterization of $\alpha\beta$ and $\gamma\delta$T cells

Subject	Phenotype		
	IL-2Rβ	CD4 and CD8	CD8$\alpha\beta$
$\gamma\delta$T cells			
Liver type	+	DN and CD8	CD8$\alpha^+\beta^-$
Thymus type	+ and −	DN*	CD8$\alpha^+\beta^+$
IEL type	+ and −	CD8	CD8$\alpha^+\beta^-$
Nude type	+	DN and CD8	CD8$\alpha^+\beta^-$ and CD8$\alpha^+\beta^+$
$\alpha\beta$T cells			
Intermediate TcR cells	+	DN, CD4 and CD8	CD8$\alpha^+\beta^-$
IEL $\alpha\beta$T cells	+ and −	CD8, DP	CD8$\alpha^+\beta^-$
Bright TcR cells	−	CD4 and CD8	CD8$\alpha^+\beta^+$

* In this case, only a few CD8$^+$ cells, which had a CD8$\alpha^+\beta^+$ phenotype, were present.

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