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Self-reactive forbidden clones are confined to pathways of intermediate T-cell receptor cell differentiation even under immunosuppressive conditions

T. MORODA,*† Y. KAWACHI,† T. IIAI,† A. TSUKAHARA,† S. SUZUKI,† T. TADA,† H. WATANABE,* K. HATAKEYAMA† & T. ABO* **Department of Immunology and the †First Department of Surgery, Niigata University School of Medicine, Niigata, Japan*

SUMMARY

It is believed that self-reactive forbidden T-cell clones are generated by 'failure' of the pathway of T-cell differentiation in the thymus, if it is disturbed. We examined how such forbidden clones are generated under immunosuppressive conditions. Mice were treated with an injection of deoxyspergualin, FK506, or cycloporin A. From day 3, the number of cells yielded by various organs decreased. Because of the resistance of intermediate (int) T-cell receptor (TCR) cells (i.e. TCR^{int} cells), they became more prominent in proportion than TCR^{high} cells. TCR^{high} cells are conventional T cells generated through the mainstream in the thymus, whereas TCR^{int} cells are primordial T cells generated by the extrathymic pathway or an alternative intrathymic pathway. Similar to untreated mice, forbidden $V\beta3^+$ and $V\beta11^+$ clones in C3H/He (Mls-1^b2^a) mice were confined to TCR^{int} cells after treatment; there was no leakage of forbidden clones into TCR^{high} cells in the thymus and periphery. In parallel with the increase in the proportion of TCR^{int} cells, the proportion of forbidden clones also increased under immunosuppressive states, especially in the liver. Liver mononuclear cells isolated from treated mice still had the potential to mediate autologous killing. The present results suggest that the generation of self-reactive clones is highly restricted to the pathways of TCR^{int} cell differentiation even under immunosuppressive conditions.

INTRODUCTION

Immunosuppressive drugs, including deoxyspergualin (DSG),[1] FK506 and cyclosporin A (CsA), exert their suppression at the immature stage of T-cell differentiation or at the mature stage at functional levels.[1–3] Although these drugs are used as immunosuppressants in organ transplantation, they themselves sometimes induce autoimmune-like diseases.[4–6] It was believed that these drugs disturb the pathway of T-cell differentiation in the thymus and that some self-reactive forbidden clones appear in the peripheral organs due to failure of the pathway.

Recent studies have revealed that T cells with intermediate (int) levels of T-cell receptor (TCR) (i.e. TCR^{int} cells), which are generated in the liver, contain self-reactive forbidden clones in terms of both phenotype and function.[7–9] A similar population of TCR^{int} cells (or $NK1{\cdot}1^+$ T cells) generated by an alternative intrathymic pathway is also known to exist in the thymus.[10–17] Since these pathways were found to produce consistently functional self-reactive forbidden clones,[7,18] we examined how forbidden clones were generated by the extrathymic pathway, by an alternative pathway in the thymus, and by the major intrathymic pathway, when immunosuppressive drugs were used. Irrespective of the use of immunosuppressive drugs, forbidden clones, which were estimated based on anti-$V\beta$ monoclonal antibodies (mAb) and the Mls system,[19–21] were confined to TCR^{int} cells in the liver, spleen and thymus. Moreover, liver mononuclear cells (MNC), which contained the highest proportion of TCR^{int} cells, were found to mediate autologous killing against syngeneic thymocytes. These results suggest that self-reactive T-cell clones are only generated through the extrathymic pathway in the liver and an alternative pathway in the thymus.

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Abbreviations: CsA, cyclosporin A; DSG, deoxyspergualin; int, intermediate; IL-2Rβ, IL-2 receptor β-chain; MNC, mononuclear cells.

Correspondence: Dr T. Abo, Department of Immunology, Niigata University School of Medicine, Asahimachi 1, Niigata 951, Japan.

MATERIALS AND METHODS

Mice

C3H/He mice at 8 weeks of age were used. These mice were originally obtained from Charles River Japan (Kanagawa, Japan). All mice were fed under specific pathogen-free conditions.

Immunosuppressive drugs

DSG (Nihon Kayaku Co., Tokyo, Japan), FK506 (Fujisawa Pharmacential Co., Tokyo, Japan) and CsA (Sandoz Seiyaku Co., Tokyo, Japan) were used in this study. In a preliminary study, sublethal doses of these drugs were determined: DSG,

10 mg/kg/mouse (intraperitoneally; i.p.); FK506, 5 mg/kg/mouse (subcutaneously; s.c.); and CsA, 20 mg/kg/mouse (s.c.).

Cell preparation

Hepatic MNC were isolated by an improved method as described elsewhere.[22] To obtain MNC, the liver was removed, pressed through a 200-gauge stainless steel mesh, and then suspended in phosphate-buffered saline (PBS) (pH 7·2). MNC were isolated by Percoll gradient (35% Percoll containing 100 IU/ml heparin) centrifugation.

Spleen cells were collected by Ficoll–Isopaque gradient (1·090 g/ml),[8] whereas thymocytes were obtained by forcing the thymus through a 200-gauge steel mesh.

Immunofluorescence tests

The surface phenotypes of cells were identified by using mAb in conjunction with either a two- or three-colour immunofluorescence test.[7] The mAb used included fluorescein isothiocyanate- (FITC), phycoerythrin- (PE) or biotin-conjugated reagents of anti-CD3 (145–2C11) anti-CD4 (L3T4), anti-CD8 (Lyt-2) and anti-IL-2 receptor β-chain (IL-2Rβ) (TM-β1) (PharMingen, San Diego, CA). All biotin-conjugated reagents of anti-Vβ mAb against Vβ2, 3, 8 (8·1+8·2) and 11 were obtained from PharMingen. Each population of Vβ^+ cells was identified by three-colour staining for CD3 (FITC), IL-2Rβ (PE) and corresponding Vβ (Red 613). Biotin-conjugated reagents were developed with either PE or Red 613-conjugated streptavidin (Becton Dickinson, Mountain View, CA). The fluorescence-positive cells were analysed with a FACScan (Becton Dickinson).

Cytotoxicity assay

The activity of autologous killing against syngeneic thymocytes by MNC in the liver was examined by means of a specific ^{51}Cr-release assay.[7] ^{51}Cr-labelled thymocytes (5×10^4 cells) were incubated with effector cells at the indicated ratios for 4 hr. Almost all of this killing of thymocytes (Fas$^+$) was mediated by TCR^{int} cells with Fas ligand. Namely, Fas$^-$ thymocytes in B6-*lpr/lpr* mice were not killed by Fas ligand$^+$ TCR^{int} cells of B6 mice.

Statistical analysis

The significance of differences was estimated by the Student's *t*-test.

RESULTS

Effects of immunosuppressive drugs on various pathways of T-cell differentiation

An immunosuppression by a single administration of each drug was investigated in C3H/He mice. Since the maximum depletion of the cell number was seen at days 3–5, the numbers of cells on day 5 are depicted (Fig. 1). Although the numbers of cells in the liver and spleen decreased ($P<0·05$, except CsA in the spleen), the most prominent effect was seen in the thymus ($P<0·01$).

Phenotypic characterization

To determine which subsets of lymphocytes were affected by the drugs, two-colour staining for CD3 and IL-2Rβ was carried out. As shown previously,[7–9] natural killer (NK) cells and extrathymic T cells constitutively express IL-2Rβ. In this regard, these populations could be identified as IL-2Rβ^+ CD3$^-$ cells and IL-2Rβ^+ CD3^{int} cells, respectively. In a recent study, we confirmed that purified IL-2Rβ^+ CD3$^-$ cells eventually mediated NK activity against YAC-1 target.[23]

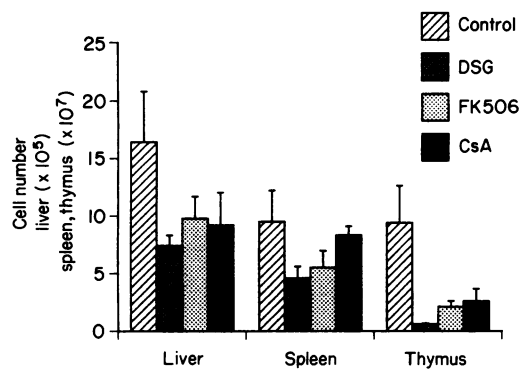

Figure 1. Immmunosuppressive effects of DSG, FK506 and CsA on the number of cells yielded by the liver, spleen and thymus. A single injection of DSG, FK506 or CsA was administered into mice and these mice were killed on day 5. Five mice were used for each drug and the mean + 1 SD were calculated. The immunosuppressive effects were prominent in the liver and spleen (*P0·05, except CsA in the spleen) and in the thymus (all P0·01).*

The results of two-colour staining on day 5 are shown in Fig. 2a. Despite the prominent decrease in the cell numbers, the staining patterns in the liver and spleen in DSG-treated mice were not significantly changed. However, the staining pattern of thymocytes in DSG-treated mice showed an increase in the proportions of IL-2Rβ^- CD3^{high} and IL-2Rβ^+ CD3^{int} cells. In other words, the proportions of CD3$^-$ and CD3^{dull} immature thymocytes decreased in the case of DSG.

When attention was focused on FK506 and CsA, a unique change in the staining pattern was observed. The proportion of IL-2Rβ^+ CD3^{int} cells increased while the proportion of IL-2Rβ^- CD3^{high} cells decreased in the liver. In the case of CsA, IL-2Rβ^+ CD3$^-$ NK cells increased prominently in the liver. Although the change in the spleen was minimal, that in the thymus was prominent for both drugs. The proportion of IL-2Rβ^+ CD3^{int} cells became prominent, whereas the proportion of IL-2Rβ^- CD3^{high} cells decreased profoundly. It is speculated that the maturation of CD3^{dull} to CD3^{high} cells was arrested by FK506 and CsA.

Two-colour staining for CD4 and CD8 was then carried out (Fig. 2b). Reflecting the above results, double-positive (DP) CD4$^+$ CD8$^+$ cells decreased (from 80% to 20%) in mice treated with DSP but not at all in mice treated with FK506 and CsA. With these drugs, although DP CD4$^+$ CD8$^+$ cells were abundant, single-positive CD4$^+$ and CD8$^+$ cells were absent. In other words, all the immunosuppressive drugs arrested the maturation of T cells in the mainstream of T-cell differentiation, but the effective stage during maturation was different depending on the drug used.

Restriction of self-reactive clones to CD3^{int} cells even after treatment with immunosuppressants

Experiments were then conducted to determine how forbidden clones are distributed among various T-cell subsets after

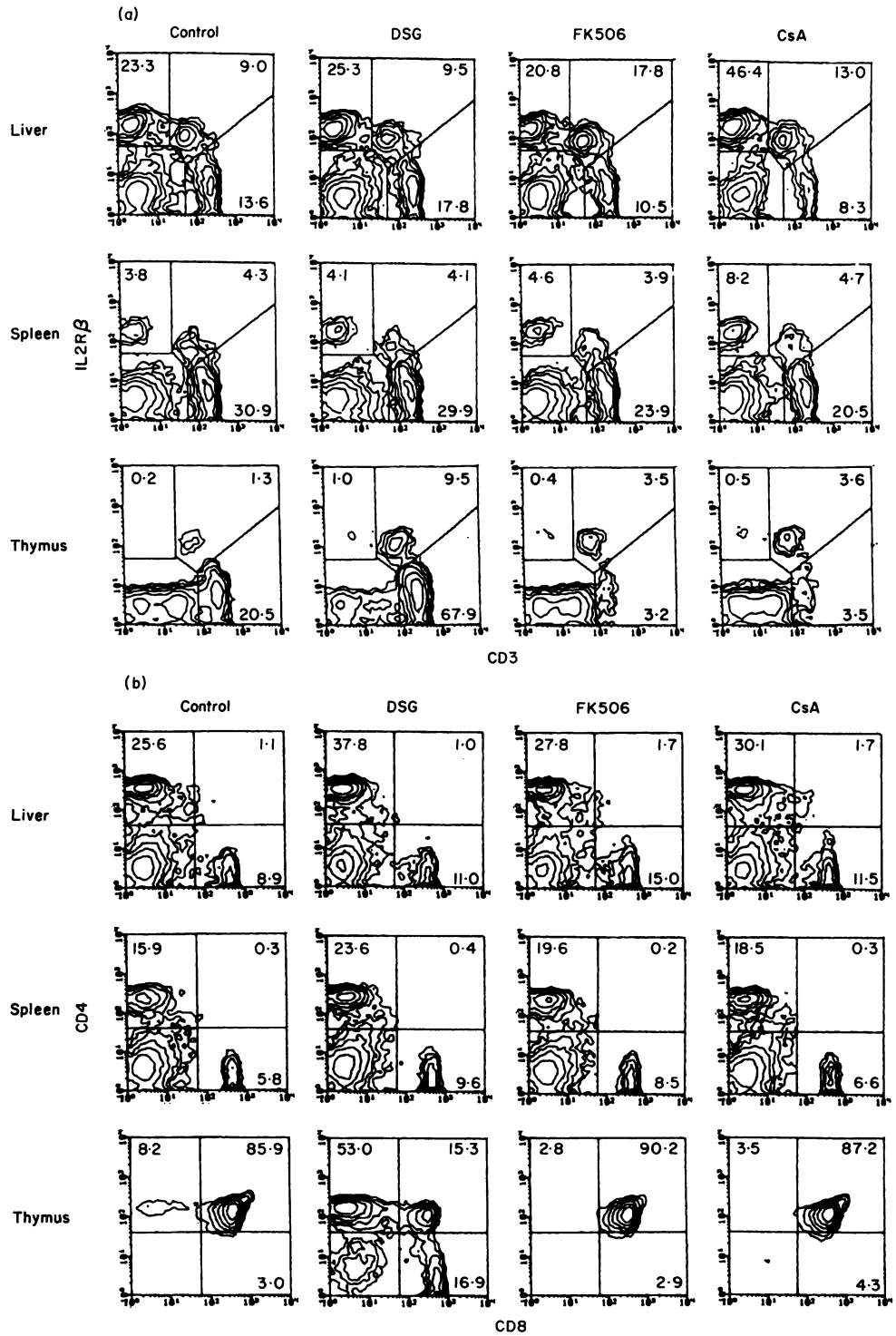

Figure 2. Increase in the proportion of $CD3^{int}$ cells in the liver and thymus of mice treated with various immunosuppressive drugs. (a) Two-colour staining for CD3 and IL-2Rβ. (b) Two-colour staining for CD4 and CD8. A prominent increase in the proportion of IL-2Rβ^+ $CD3^{int}$ cells was induced, especially in the liver of mice treated with FK506 and CsA and in the thymus of mice treated with any of the immunosuppressants. The proportion of DP $CD4^+$ $CD8^+$ cells decreased prominently in the case of DSG. Numbers in the figure represent the percentages of fluorescence-positive cells in the corresponding areas. Representative results of three experiments are depicted.

treatment with immunosuppressive drugs (Figs 3–5). MNC were isolated from treated mice on day 5. Three-colour staining for CD3, IL-2Rβ and each Vβ was carried out. In C3H/He mice (Mls-1^{b}2^{a}), Vβ2^{+} and Vβ8^{+} cells are non-forbidden clones while Vβ3^{+} and Vβ11^{+} cells are self-reactive forbidden clones. In the case of liver and spleen (Figs 3 and 4), Vβ2^{+} and Vβ8^{+} cells were distributed to all T-cell fractions. In contrast, forbidden clones, Vβ3^{+} and Vβ11^{+}, were confined to CD3^{int} cells, irrespective of treatment. In some cases, the proportion of forbidden clones increased more in treated mice than in control mice.

Attention was then focused on the thymus (Fig. 5). Since the expression level of TCR as well as of CD3 is very low at the stage of CD3^{dull} cells in the thymus, the proportions of non-forbidden clones, Vβ2^{+} and Vβ8^{+}, were also very low, irrespective of treatment. However, the proportions of Vβ2^{+} and Vβ8^{+} cells became elevated at the CD3^{high} stage. The CD3^{int} cells also contained these non-forbidden clones.

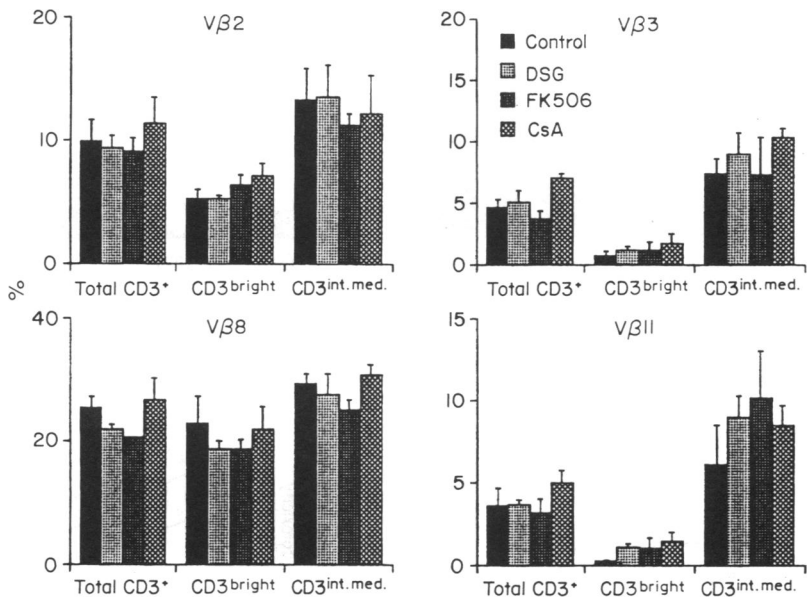

Figure 3. Distribution of self-reactive clones among T-cell subsets in the liver before and after treatment with immunosuppressive drugs. Mice were examined on day 5 after the treatment. Three-colour staining of MNC for CD3, IL-2Rβ, and each Vβ was carried out. To determine the proportion of each type of Vβ^{+} cell, gated analysis of either TCR$^{high(bright)}$ cells or TCR^{int} cells was performed. In C3H/He mice (Mls-1^{b}2^{a}), Vβ2^{+} and Vβ8^{+} (Vβ 8·1+8·2) cells were found to be non-forbidden clones while Vβ3^{+} and Vβ11^{+} cells were self-reactive forbidden clones. The mean+1 SD of four separated experiments are represented.

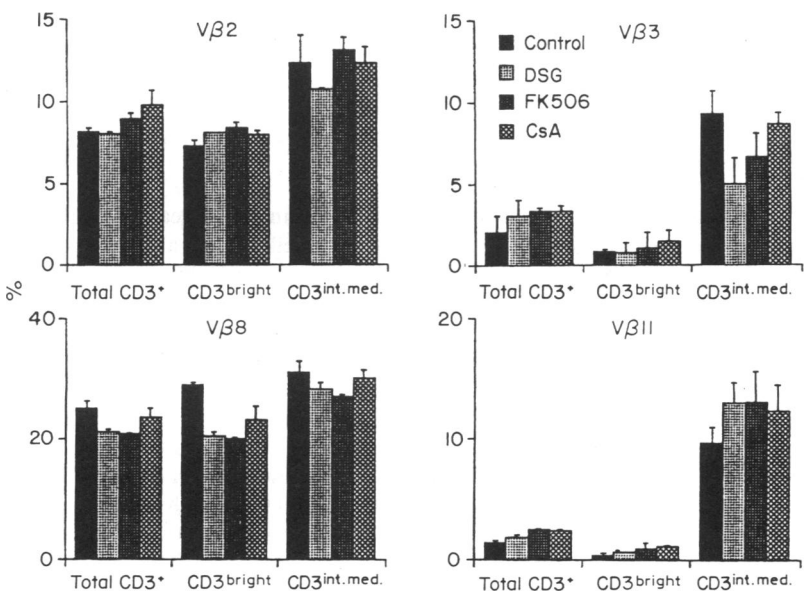

Figure 4. Distribution of self-reactive clones among T-cell subsets in the spleen before and after treatment with immunosuppressive drugs. Further details as for Fig. 3.

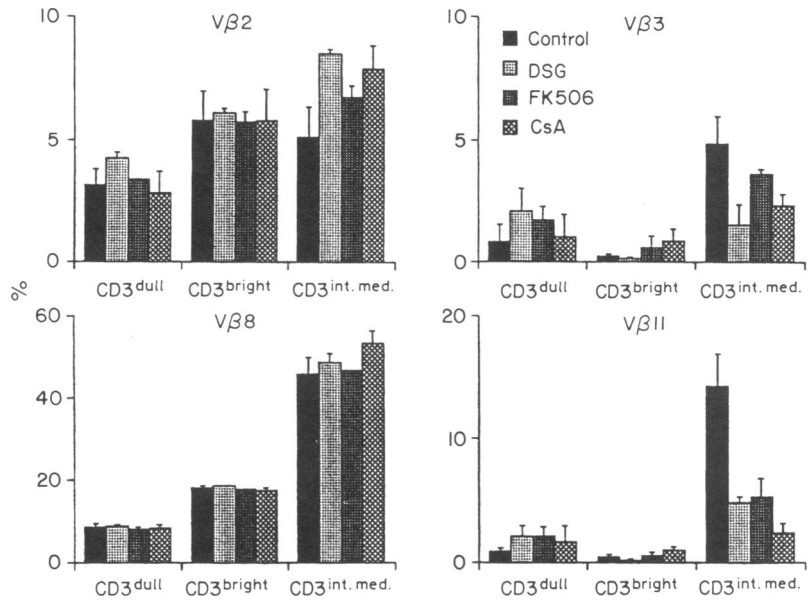

Figure 5. Distribution of self-reactive clones among T-cell subsets in the thymus before and after treatment with immunosuppressive drugs. Further details as for Fig. 3.

The situation of forbidden clones in the thymus was, however, quite different (Fig. 5, right). A low proportion of forbidden clones, $V\beta3^+$ and $V\beta11^+$, appeared in the $CD3^{dull}$ fraction. However, such forbidden clones were eliminated at the stage of $CD3^{high}$ cells. This was true in both control mice and treated mice. In contrast, forbidden clones, $V\beta3^+$ and $V\beta11^+$, were mainly found in the $CD3^{int}$ cell fraction. The only difference in forbidden clones in $CD3^{int}$ cells between the liver and thymus was that the levels increased in the liver while those in the thymus decreased after treatment with any drug.

Increase in the activity of autologous killing by liver MNC after treatment

Since treatment consistently increased the proportion of TCR^{int} cells in the liver, whether liver MNC in such treated mice had an elevated level of autologous killing was examined (Fig. 6). The activity of autologous killing against syngeneic thymocytes increased significantly in mice treated with FK506, CsA and DSG ($P<0{\cdot}05$). Cell separation experiments to show that autologous killing against syngeneic thymocytes is mediated by $CD3^{int}$ cells in the liver, but not by NK cells or $CD3^{high}$ cells, are represented elsewhere (manuscript submitted for publication).

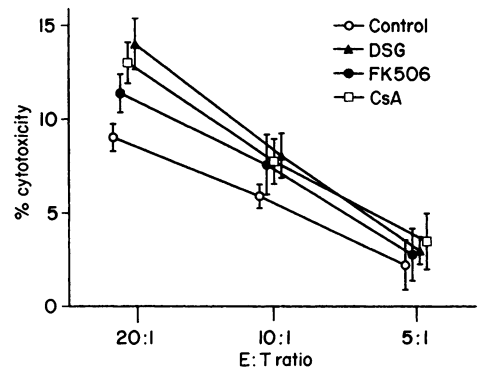

Figure 6. Increase in the activity of autologous killing by liver MNC in mice treated with FK506, CsA or DSG. MNC were isolated from the liver of mice after treatment (on day 5) or without treatment. Liver MNC were cultured at the indicated effector : target (E : T) ratios (0·2 ml/well) for 4 hr. Liver MNC from treated mice showed higher levels of activity of autologous killing than those from control mice. The mean + 1 SD of triplicate assays are depicted.

DISCUSSION

It has been thought that self-reactive clones, which appear in the peripheral immune organs after treatment with immunosuppressive drugs, are generated by 'failure' through the major intrathymic pathway of T-cell differentiation. Since we have previously reported that extrathymically generated T cells always contain a significant proportion of self-reactive forbidden clones,[7,8] we investigated how forbidden clones were distributed among various T-cell subsets. We have demonstrated that, irrespective of the administration of immunosuppressants, self-reactive forbidden clones are confined to $CD3^{int}$ cells, which are generated by the extrathymic pathway in the liver and by an alternative pathway in the thymus. There was no leakage of forbidden clones into $CD3^{high}$ cells in the thymus and periphery.

We used three immunosuppressive drugs: DSG, FK506 and CsA. These drugs induced thymic atrophy and resulted in a decrease in the number of splenic and liver MNC. When attention was focused on the major pathway in the thymus, DSG showed a very unique pattern of suppression. Many $CD3^-$ and $CD3^{dull}$ thymocytes with immature phenotypes were exhaused and a relative increase in the proportions of $CD3^{high}$ cells (and $CD3^{int}$ cells) was induced. On the other hand, FK506 and CsA suppressed the maturation of $CD3^{dull}$ cells to $CD3^{high}$ cells, and $CD3^{high}$ cells were exhausted.

We, as well as other investigators, have focused attention

not on only $CD3^{int}$ cells in the liver but also on $CD3^{int}$ cells in the thymus.[10–17] $CD3^{int}$ cells correspond to $NK1{\cdot}1^+$ T cells and these $NK1{\cdot}1^+$ T cells are generated through an alternative pathway in the thymus. In the present study, forbidden clones were confined to $CD3^{int}$ cells in the liver, spleen and thymus. Despite their being present at different sites, these $CD3^{int}$ cells might be categorized as a similar lineage of T cells in terms of their selection for TCR repertoire.[7,18] In addition, they share many other properties as primordial T cells, e.g. the morphology of large agranular lymphocytes, $CD44^+$ L-selectin$^-$ and IL-2R$\alpha^-\beta^+$. In sharp contrast, $CD3^{high}$ cells are small lymphocytes, $CD44^-$ L-selectin$^+$ and IL-2R$\alpha^-\beta^-$, under resting conditions.[24] After activation, these $CD3^{high}$ cells become large lymphoblasts, having the phenotype of $CD44^+$ L-selectin$^+$ and IL-2R$\alpha^+\beta^+$ (high affinity IL-2R).[25] $CD3^{int}$ cells were not able to acquire IL-2Rα even after stimulation (they remained intermediate affinity IL-2R). Moreover, one-third of $CD3^{int}$ cells were double-negative $CD4^-$ $CD8^-$ cells but $CD3^{high}$ cells do not include such cells.

Reflecting the existence of self-reactive forbidden clones in the liver, liver MNC always showed the highest activity of autologous killing.[7] Even after the treatment, this activity was not suppressed but was elevated. This phenomenon is important for understanding the aetiology of the onset of autoimmune-like syngeneic graft-versus-host diease (GVHD).[26] In addition to a relative predominance of TCR^{int} cell differentiation under immunosuppressive states, this pathway becomes active under certain conditions. Such a phenomenon has been seen with ageing,[27–30] under conditions of intracellular infection,[31, 32] and during pregnancy.[33]

There is the question of why the pathways of TCR^{int} cell differentiation are sometimes more predominant than that of TCR^{high} cell differentiation (i.e. mainstream T-cell differentiation in the thymus) under immunosuppressive conditions. It seems that there are two reasons: (1) TCR^{int} cell differentiation is resistant to the immunosuppressive states, and (2) the pathways of TCR^{int} cell differentiation are very quick for the production of TCR^{int} cells. When immunosuppressive states become free, only TCR^{int} cells appear in the liver and periphery (i.e. only 3 days are needed to produce them by this pathway). On the other hand, the pathway of TCR^{high} cell differentiation requires at least 2 weeks to produce TCR^{high} cells from the precusor in the bone marrow.[8,34]

In our most recent study, we demonstrated that hepatic T cells express high levels of Fas mRNA and undergo apoptosis.[27] However, because of the lower level of TCR-CD3 complex on $CD3^{int}$ cells, the level of apoptotic stimuli affecting $CD3^{int}$ cells might be lower than those affecting $CD3^{high}$ cells. In this regard, forbidden clones may remain in the extrathymic pathway and the alternative intrathymic pathway.

When mice were treated with immunosuppressive drugs, the proportion of forbidden clones always increased in the liver while that in the thymus decreased. This phenomenon is quite similar to that seen with ageing.[7] In the case of ageing, the number of $CD3^{high}$ cells derived from the thymus decreased in the liver and other peripheral organs. At that time, the proportion of forbidden clones in the liver increased, whereas that in the thymus decreased. It is conceivable that thymic microenvironments more eliminate effeciently such forbidden clones than hepatic microenvironments do.

Thus far, the distribution of forbidden clones has been investigated in normal mice,[7,9] mice with autoimmune-like chronic GVHD mice,[8] and mice with syngeneic GVHD.[26] Including the mice treated with immunosuppressive drugs in this study, forbidden clones are always confined to IL-2Rβ^+ $CD3^{int}$ cells. IL-2Rβ^- $CD3^{high}$ cells in the thymus and periphery never comprise such forbidden clones. Therefore, it has been revealed that the generation of self-reactive forbidden clones is highly restricted to the event of TCR^{int} cell differentiation in adult mice, even under immunosuppressive conditions. This concept might be extremely important for understanding the mechanisms involved in self-tissue damage by T cells.

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