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Early coupled up-regulation of interleukin-12 receptor beta-1 in CD8$^+$ central memory and effector T cells for better clinical outcomes in liver transplant recipients

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Summary

This study aimed to investigate the role of initial priming of interleukin (IL)-12 receptor beta-1 in CD8$^+$central memory T cells (initial IL-12RT$_{CM}$ priming) and CCR7-negative subsets (CNS) in effector cell expansion and clinical outcome after living donor liver transplantation (LDLT). One hundred and six patients who underwent LDLT were classified into the following three groups according to hierarchical clustering of CD8$^+$CD45 isoforms before LDLT: I, naive-dominant; II, effector memory-dominant; and III, effector-dominant. The pre-existing CD8$^+$effector cells (T$_E$) and activated immune status increased progressively from group I to group II to group III. Groups I, II and III received tacrolimus (Tac)/glucocorticoid (GC) regimens. Eighteen group III recipients received Tac/mycophenolate mofetil (MMF) and were defined as group IV. Initial IL-12RT$_{CM}$ priming was slightly, moderately and markedly decreased in droups I, II, and III, respectively. Initial priming of IL-12Rβ1 in CNS was decreased markedly in the three groups with marked decreases of T$_E$, perforin and interferon (IFN)-γ; all parameters were restored by up-regulation of IL-12Rβ1$^+$T$_{CM}$ through the self-renewal of T$_{CM}$. The lag time required until coupled up-regulation of IL-12Rβ1 of T$_{CM}$ and CNS to above baseline was 12, 20 and 32 days in groups I, II and III, respectively. Inferior clinical outcomes were associated with increasing lag time. In contrast, the initial priming of IL-12Rβ1 in T$_{CM}$ and CNS remained above baseline in group IV due to MMF-mediated increase of IL-12Rβ1. Early coupled up-regulation of T$_{CM}$ and CNS leads to efficient T$_E$ differentiation and optimal clinical outcomes.

Keywords: CD8$^+$ central memory T cells and CCR7$^-$ subsets, initial encounter with alloantigen, initial priming of IL-12Rβ1, living donor liver transplantation, Tac/GC or Tac/MMF regimen

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Introduction

In the transplant immunity field, many investigators always aim to choose an exact criterion to evaluate clinical outcomes of recipients prospectively. To date, there have been several studies regarding preoperative risk selection bias. For living donor liver transplantation (LDLT), pretransplant desensitization has been performed routinely for the selected recipients according to the degree of T lymphocyte cross-matching (LCM), human leucocyte antigen (HLA) mismatches or other immunogenetic tests. However, there is still no consensus on pretransplant desensitization, as shown in our studies [1]. Conversely, our previous study showed that CD8$^+$ pre-existing effector memory T cell (T$_{EM}$)-dominant recipients were related closely to hepatitis C virus infection, and that CD8$^+$ effector T cell (T$_E$)-dominant recipients?HPT$_E$-recipients?were associated with the highest frequencies of post-transplant complications and a much poorer probability of survival compared with the recipients with the lowest pre-existing T$_E$ (LPT$_E$-recipients) [2,3]. Such HPT$_E$-recipients comprised approximately 30% of all adult LDLT recipients in our hospital. Approximately 50% of these HPT$_E$ recipients had higher

rates of severe hepatitis C virus (HCV) infections than patients with hepatitis B virus (HBV) and non-viral infections [2,3]. Moreover, initial priming of interleukin (IL)-12 receptor beta-1^+ (Rβ1) cells was up-regulated in an early period after LDLT in uneventful recipients, whereas it was decreased in complicated recipients [4]. Consequently, it is of great importance to determine how accurate any of those measures are in predicting clinical outcome, the survival rates of recipients in particular.

In addition, the most commonly used immunosuppressive agents are generally administered without considering the pretransplant immune status of the recipients. It seems likely that the effects of various combined immunosuppressive regimens (ISR) would be influenced strongly by the presence of a high number of pre-existing T_E with activated immune status or by pretransplant sensitization of recipients.

Immediately after LDLT, the greater number of donor dendritic cells (DCs) [the most potent type of professional antigen-presenting cell (APC)] migrates to the secondary lymphoid organs and spleen at the sites where naive T cells (T_N) react with alloantigen-loaded APCs. In initiation of the alloimmune response, recipient T cells can recognize donor alloantigen through the direct pathway via native major histocompatibility complex (MHC) molecules expressed on graft-associated APCs [5]. The direct pathway predominates early post-transplantation. Recipient T cells that are primed through the direct pathway have the ability to engage the allograft directly and, thus, mediate effector function efficiently. Activated $CD8^+$ cytotoxic T lymphocytes (CTLs) specific for donor MHC class 1 can interact directly with the allograft through stimulation of allogeneic endothelial cells and their migration to secondary lymphoid organs [6] and cause allograft injury through prominent apoptosis, which culminates in the activation of caspases [7,8]. During those processes, the perforin/granzyme pathway participates to induce target cell death.

$CD8^+$ T cells must maintain the continuous production of T_E that provide anti-antigenic defence. A $CD8^+$ T cell clone is stimulated initially by the first encounter with alloantigen through the T cell receptor (TCR)/CD27 pathway in the secondary lymphoid tissue, leading to the processes of their self-renewing T_{CM} [9]. After expansion of the T_{CM} pool to achieve a high frequency of alloantigen-primed cells, signals initiate their differentiation to T_E that migrate to the periphery to eliminate alloantigens. Thus, alloantigen-activated $CD8^+$ T cells are established by the IL-2-independent generation of self-renewing T_{CM} [10,11].

IL-12 is produced by activated maturated DCs by CD40-dependent interaction with $CD4^+$ T cells. The immunological action of IL-12 has been suggested as follows: first, IL-12 acts directly on $CD8^+$ T_N to provide a third signal to support the $CD8^+$ T cell response to optimally activate differentiation into effector and memory cells [12–15]. Secondly, IL-12 up-regulates expression of both CD25 and IL-12Rβ1, providing positive cross-regulation of receptor expression [16]. Thirdly, IL-12 priming makes $CD8^+$ T cells less susceptible to cell death through anti-apoptotic effects [17]. Lastly, and more importantly in terms of IL-12R$\beta1^+$ T_{CM} up-regulation above the pretransplant levels, the IL-12R$\beta1^+$ cells of T_{CM} and T_E are regulated in a tightly coupled manner [4].

It remains poorly understood how initial priming of IL-12 regulates the generation of effector and memory $CD8^+$ T cells in response to the first encounter with alloantigen. In particular, the present study sought to clarify how the coupled initial priming of IL-12Rb1 in T_{CM} and CNS produces better post-transplant immune responses and clinical outcome despite a variety of primary diseases or a different pre-existing immune status of recipients. Additional aims were to clarify the mechanisms predictive of a poor clinical outcome, and to identify the optimal immunosuppressive drugs for HPT_E recipients.

Patients and materials

Patients

We performed standard LDLT [18] in 350 consecutive adult patients between December 2002 and July 2009 at Kyoto University Hospital. Of these patients, 190 recipients without immunological studies and 54 recipients undergoing ABO-incompatible LDLT were excluded. The remaining 106 recipients were classified into the following three groups according to a dendrogram of hierarchical clustering [19] by preoperative $CD8^+$CD45 isoform profiles: group I (naive-dominant); group II (effector memory-dominant); and group III (effector-dominant). All recipients in groups I, II and III were immunosuppressed with the Tac/GC regimen. Eighteen of the recipients in group III were immunosuppressed with the Tac/MMF regimen, and defined as group IV. Written informed consent was obtained from each recipient before starting the study, which was approved by the Ethics Committee of Kyoto University Hospital and conducted in accordance with the 1975 Declaration of Helsinki, as revised in 1996.

Immunosuppression

For all recipients, methylprednisolone (10 mg/kg) was administered just before the start of graft reperfusion [20]. The Tac/GC regimen was administered at 24 h after LDLT using our standard method [2]. We noticed the beneficial effect of a mycophenolate mofetil (MMF)-mediated increase in IL-121Rβ1 of $CD8^+$ T cell subsets 6

years ago. Thereafter, for group IV recipients, in addition to Tac administration, MMF (0·5–1 g every 12 h) was administered within 24 h after LDLT through the intestinal tube. Thereafter, these recipients were switched to oral MMF (0·5–0·75 g every 12 h).

Acute graft rejection, infection and tissue typing (HLA) were evaluated employing the methods reported previously [2]. Lymphocyte cross-matching (LCM) examinations (complement-dependent cytotoxicity and anti-human globulin assays) are performed routinely before LDLT [1]. The results of direct complement-dependent cytotoxicity assays were used in this study.

Flow cytometry

We examined peripheral blood mononuclear cells (PBMCs) from each recipient. Sample analyses were performed within 24 h after sampling in all cases. Blood that had a white blood cell count of less than 3000/mm^3 was excluded, because of incorrect measurements. For all recipients, the blood samples were taken and analysed every morning of post-operative day (POD) 0, 5, 8, 12, 20, 28, 32 and 40. Thereafter, the blood samples were taken every 7–10 days until discharge or hospital death. The PBMCs were stained with monoclonal antibodies, and cell immunostaining was performed using monoclonal antibodies, as reported previously [2]. The monoclonal antibodies used to immunostain the cell surface antigens were as follows: allophycocyanin (Coulter Immunotech, Miami, FL, USA) or PC-5 (Coulter Immunotech, Marseilles, France)-conjugated anti-CD4 or anti-CD8; fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO (Nichirei, Tokyo, Japan); TC-conjugated anti-CD45RA (Caltag Laboratories, Burlingame, CA, USA); phycoerythrin (PE)-conjugated anti-CD3 (Coulter Immunotech, Miami); FITC-conjugated anti-CD19 (Coulter Immunotech, Marseilles, France); PE-conjugated anti-human CCR7 (DakoCytomation, Kyoto, Japan); PE-conjugated anti-CD27 (Coulter Immunotech, Marseilles, France); and FITC-conjugated anti-CD28 (Nichirei, Tokyo, Japan).

Flow cytometric measurement of interferon (IFN)-γ and tumour necrosis factor (TNF)-α production was performed after previous stimulation, as described previously [2]. Intracellular perforin in CD8$^+$ cells was measured without previous stimulation according to a previously reported method [2].

The expression of IL-12 receptors was determined using R-PE-conjugated anti-IL-12Rβ1 and IL-12Rβ2 (BD Biosciences, San Diego, CA, USA). Flow cytometric levels of IL-12Rβ1$^+$ cells, perforin and IFN-γ in six CD8$^+$ T cell subsets were measured after the classification of CD8$^+$ T cells into three subsets, as reported previously [4]. Perforin-T_E* and IFN-γ-T_E* were expressed as the absolute proportion of perforin or IFN-γ specific for T_E within CD8$^+$ T cells (T_{CD8}).

Evaluation of post-transplant immune status

As a measure of post-transplant immune status, the proportion of variables immediately before LDLT was subtracted from the proportions at various time–points after LDLT, and expressed as the percentage difference [21].

Statistical analysis

To classify the recipients into three groups, we performed a hierarchical cluster analysis [19] using JMP 8 (SAS Institute Inc., Cary, NC, USA). Overall survival was defined as the interval from surgery until death from any cause. The survival curves were estimated using the Kaplan–Meier method and compared employing the log-rank test. Comparisons of continuous variables between groups were performed by applying Student's *t*-test and analysis of variance (ANOVA). Comparisons of proportions between groups were undertaken using Fisher's exact test or the χ^2 test. All statistical tests were two-tailed. Significance was defined as $P < 0{\cdot}05$.

Results

The subjects consisted of 106 recipients (40 HCV-infected, 20 HBV-infected and 46 non-viral-infected recipients) who had undergone standard LDLT. The changes in phenotypical and functional properties of CD8$^+$ T cell subsets seem to have been established by previous lifelong exposure to microbial or viral infection rather than by the ageing effect in each individual. Accordingly, we further clarified the fundamental mechanism underlying changes in phenotypical and functional features in CD8$^+$ T cell subsets before LDLT in four groups according to a dendrogram of hierarchical clustering by preoperative CD8$^+$CD45 isoform profiles

Hierarchical clustering by pretransplant CD8$^+$ CD45 isoform profiles

Table 1a shows the immunological characteristics of the recipients classified into the four groups before LDLT. The proportion of CD8$^+$ T cell subsets was expressed as a percentage of CD8$^+$ T cells, although T_{DP+} and T_{DP-} were not described. The T_N population was markedly larger in group I than in groups II, III and IV. The CD8$^+$ T cells in group II included the highest numbers of T_{EM} and T_{CM}, and those in groups III and IV included the highest numbers of T_E. In groups II, III and IV, the proportions of CD27$^-$CD28$^-$, IFN-γ, IL-12Rβ1 and perforin expression were markedly higher than in group I. In particular, a stepwise loss of CCR7, CD28 and CD27 associated with the progressive up-regulation of cytotoxic activity has been found to characterize CD8$^+$ T cell

Table 1a. Hierarchical clustering into the four groups

	I	II	III	IV
(*n*)	24	35	29	18
% T_N	56·76 ± 2·71	19·67 ± 2·00	21·46 ± 1·94	20·95 ± 2·74
% T_{CM}	6·15 ± 0·62	14·41 ± 1·25	4·78 ± 0·42	5·59 ± 0·78
% T_{EM}	6·55 ± 0·80	18·89 ± 1·34	6·69 ± 0·73	7·65 ± 1·43
% T_E	15·05 ± 1·40	24·08 ± 2·14	49·62 ± 2·49	48·49 ± 3·66
% $CD27^-CD28$	16·71 ± 2·42	44·24 ± 5·08	36·10 ± 8·67	61·18 ± 12·61
% IFN-γ	39·83 ± 3·78	63·75 ± 4·86	59·17 ± 3·58	60·73 ± 4·29
% IL-12Rβ1	35·14 ± 4·89	72·16 ± 5·22	70·64 ± 3·63	72·87 ± 4·79
% Perforin	15·85 ± 4·91	52·51 ± 6·32	62·45 ± 5·89	47·89 ± 8·17

Values are expressed as the mean ± standard error.

differentiation [22]. Thus, $CD8^+CD28^-CD27^-$ subsets can be characterized as those with the highest power effector activities. The number of $CD8^+CD28^-CD27^-$ subsets in group I was considerably lower compared with that in groups II, III or IV. Consequently, the effector activities of T_E in groups II, III and IV were all similar and markedly higher than those in group I. In addition, the pre-existing T_E levels within $CD8^+$ T cells were the lowest in group I, increased slightly in group II, but increased markedly in groups III and IV. Moreover, the percentage of pre-existing IL-12R$\beta1^+$ T_E increased progressively from 6·25% in group I to 22·56% in group II, to 42·12% in group III and to 42·75% in group IV. The same tendency was observed in the expression levels of $CD27^-CD28^-$, IFN-γ and perforin. As a result, the pre-existing immune status was activated only slightly in group I and moderately in group II, whereas it had already been activated fully in groups III and IV.

Table 1b shows the significance of the differences between the four groups in their proportions of the $CD8^+$ T cell subpopulation and their function. Significant differences were observed in the CD45 isoform proportions of T cells between the four groups. In particular, the proportion of T_E was significantly higher in groups III and IV than in groups I and II. The proportions of IFN-γ, IL-12Rβ1 and perforin were significantly higher in groups II, III and IV than in group I.

Next, clinical and immunological findings were analysed.

Pre-, peri- and post-transplant analyses

Table 2 shows the clinical characteristics of the four groups. Patient ages were significantly lower in group I than in groups II, III and IV. The viral infection rate was 60 (56·6%) of 106 recipients, including HCV, 40 of 106 (37·7%) and HBV, 20 of 106 (18·9%). The number of HCV-infected recipients was highest in group III, whereas the number of HBV-infected recipients was highest in group II. The numbers of other primary diseases were not significantly different among the four groups. The four groups did not differ significantly in terms of their clinical status according to the Model for End-Stage Liver Disease (MELD) score [23] and donor age. The number of recipients with more than three HLA-mismatched loci was highest in groups II and III. Regarding the risk of cytomegalovirus (CMV), the frequencies of donor-positive and recipient-negative status did not differ among the four groups. The frequencies of bacterial, cytomegaloviral and fungal infections were highest until discharge in group III. The incidence of severe sepsis

Table 1b. Result of *P*-values presenting differences among the four groups in the relative proportion of the $CD8^+$ T cell subpopulation

Variable	All *P**	I *versus* II *P*†	I *versus* III *P*†	I *versus* IV *P*†	II *versus* III *P*†	II *versus* V *P*†	III *versus* IV *P*†
% T_N	<0·0001	<0·0001	<0·0001	<0·0001	0·5470	0·7080	0·8869
% T_{CM}	<0·0001	<0·0001	0·3077	0·7090	<0·0001	<0·0001	0·5808
% T_{EM}	<0·0001	<0·0001	0·9326	0·5551	<0·0001	<0·0001	0·5922
% T_E	<0·0001	0·0070	<0·0001	<0·0001	<0·0001	<0·0001	0·7629
% $CD27^-CD28^-$	0·0005	0·0001	0·0119	0·0101	0·2894	0·3073	0·1435
% IFN-γ	0·0007	0·0002	0·0024	0·0007	0·4467	0·5983	0·7913
% IL-12Rβ1	<0·0001	<0·0001	<0·0001	<0·0001	0·8110	0·9113	0·7206
% Perforin	0·0301	0·0156	0·0036	0·0341	0·3179	0·6209	0·1544

**P*-values are based on analysis of variance (ANOVA).

†*P*-values are based on Student's *t*-test. $T_E = CD8^+$ effector T cells; $T_{EM} = CD8^+$effector memory T cells; T_{CM} = central memory; T_N = naive; IFN = interferon; IL = interleukin.

Table 2. Clinical analyses of the four groups

Group	I	II	III	*IV*	*P* (all)	*P* (III-versus IV)
Number of recipients	24	35	29	18		
Age	42 ± 12	56 ± 6	51 ± 14	54 ± 10	<0·0001*	0·3705*
Sex (male/female)	12/12	26/9	12/17	8/10	0·0372†	0·8363†
Primary disease	Numbers (%)				0·0521†	0·4196†
Viral hepatitis C	6 (25·0)	12 (34·3)	16 (55·2)	6 (33·3)		
Viral hepatitis B	2 (8·3)	13 (37·1)	3 (10·3)	2 (11·1)		
PSC	2 (8·3)	1 (2·9)	0 (0)	1 (5·6)		
Autoimmune hepatitis	2 (8·3)	1 (2·9)	1 (3·5)	0 (0)		
Fulminant hepatic failure	5 (20·8)	2 (5·7)	1 (3·5)	0 (0)		
PBC	3 (12·5)	4 (11·4)	2 (6·9)	4 (22·2)		
Biliary atresia	1 (4·2)	0 (0)	2 (6·9)	1 (5·6)		
Other	3 (12·5)	2 (5·7)	4 (13·8)	4 (22·2)		
MELD score	20 ± 12	17 ± 8	15 ± 7	21 ± 9	0·0827*	0·0195*
Donor age	42 ± 14	41 ± 14	40 ± 11	41 ± 12	0·7765*	0·4821*
HLA-mismatch numbers ≥3	7 (35·0) unclear: 4	17 (50·0) unclear: 1	20 (69·0)	6 (33·3)	0·0468†	0·0169†
LCM (positive/negative)	1/23	0/35	0/29	1/17	0·2833†	0·1621†
Risk of CMV [donor (+)/recipient (–)]	4 (20·0) unclear: 4	4 (12·1) unclear: 2	2 (7·4) unclear: 2	0 (0)	0·2120†	0·2375†
Infection						
Bacteria	9 (37·5)	13 (37·1)	23 (79·3)	4 (22·2)	0·0003†	0.0001†
CMV	9 (37·5)	6 (17·1)	16 (55·2)	1 (5·6)	0·0006†	0.0006†
Fungus	1 (4·2)	0 (0)	8 (27·6)	0 (0)	0·0004†	0.0144†
SS/MODS	2 (8·3)	1 (2·9)	7 (24·1)	0 (0)	0·0153†	0·0239†
Rejection						
ACR	6 (25·0)	4 (11·4)	5 (17·2)	3 (16·7)	0·6014†	0.9594†
Chronic rejection	1 (4·2)	0 (0)	2 (6·9)	0 (0)	0·3282†	0.2548†
Hospital deaths	1 (4·2)	3 (8·6)	8 (27·6)	0 (0)	0·0096†	0·0144†

Values are expressed as the mean ± standard deviation.

**P*-values based on analysis of variance

†*P*-values based on the χ^2 test. PSC = primary sclerosing cholangitis; PBC = primary biliary cirrhosis; MELD = model for end stage liver disease; HLA = human leucocyte antigen; LCM = lymphocyte cross-match; SS/MODS = severe sepsis leading to multiple organ dysfunction syndrome; ACR = acute cellular rejection; CMV = cytomegalovirus.

leading to multiple organ dysfunction syndrome (SS/MODS) was highest in group III, whereas no severe complications were noted in group IV. The frequencies of acute cellular rejection (ACR) and chronic rejection were not different among the four groups. Hospital death was defined as when recipients died within 3 months after LDLT. Group III recipients showed eight (27·6%), the highest number of hospital deaths. In contrast, no hospital deaths occurred in group IV.

Next, the changes in IL-12Rβ1 of CD8$^+$ T cell subsets after LDLT were examined.

Changes in IL-12Rβ1 of CD8$^+$ T cell subsets

Changes in IL-12Rβ1 of the CCR7-positive subsets (CPS: T_N, T_{CM} and T_{DP+}) and CCR7-negative subsets (CNS: T_E, T_{EM} and T_{DP-}) after LDLT in the four groups are shown in Fig. 1. The values of IL-12Rβ1 expression at POD 5 are referred to hereafter as the initial priming point, as the trough levels of Tac were increased to high levels on POD 5 after administration of the Tac/GC regimen [4] and the IL-2 expression of CD4$^+$ T_{CM} was decreased to the lowest values [4].

Recipients receiving the Tac/GC regimen

In the initial stage of group I, the initial priming of IL-12Rβ1$^+$ T_{CM} (initial IL-12RT_{CM} priming) was only slightly decreased with the first encounter with alloantigen, and then IL-12Rβ1 of the T_{CM} and T_{DP+} was increased markedly to above the pretransplant level during the post-operative period. The initial priming of IL-12Rβ1$^+$ T_N was decreased to slightly below the pretransplant levels. In contrast, the initial priming of IL-12Rβ1 of CNS was decreased markedly, but increased promptly to above baseline after POD 8, along with a marked increase in IL-12Rβ1$^+$ T_{CM}. The IL-12Rβ1$^+$ T_{CM} level was correlated significantly positively with IL-2Rβ1$^+$ T_E ($r = 0{\cdot}411$), IL-12Rβ1$^+$ T_{EM} ($r = 0{\cdot}687$) and IL-12Rβ1$^+$ T_{DP-} ($r = 0{\cdot}885$).

In the initial stage of group II, the initial IL-12RT_{CM} priming was decreased moderately along with IL-12Rβ1 of CNS, and then increased to around the pretransplant

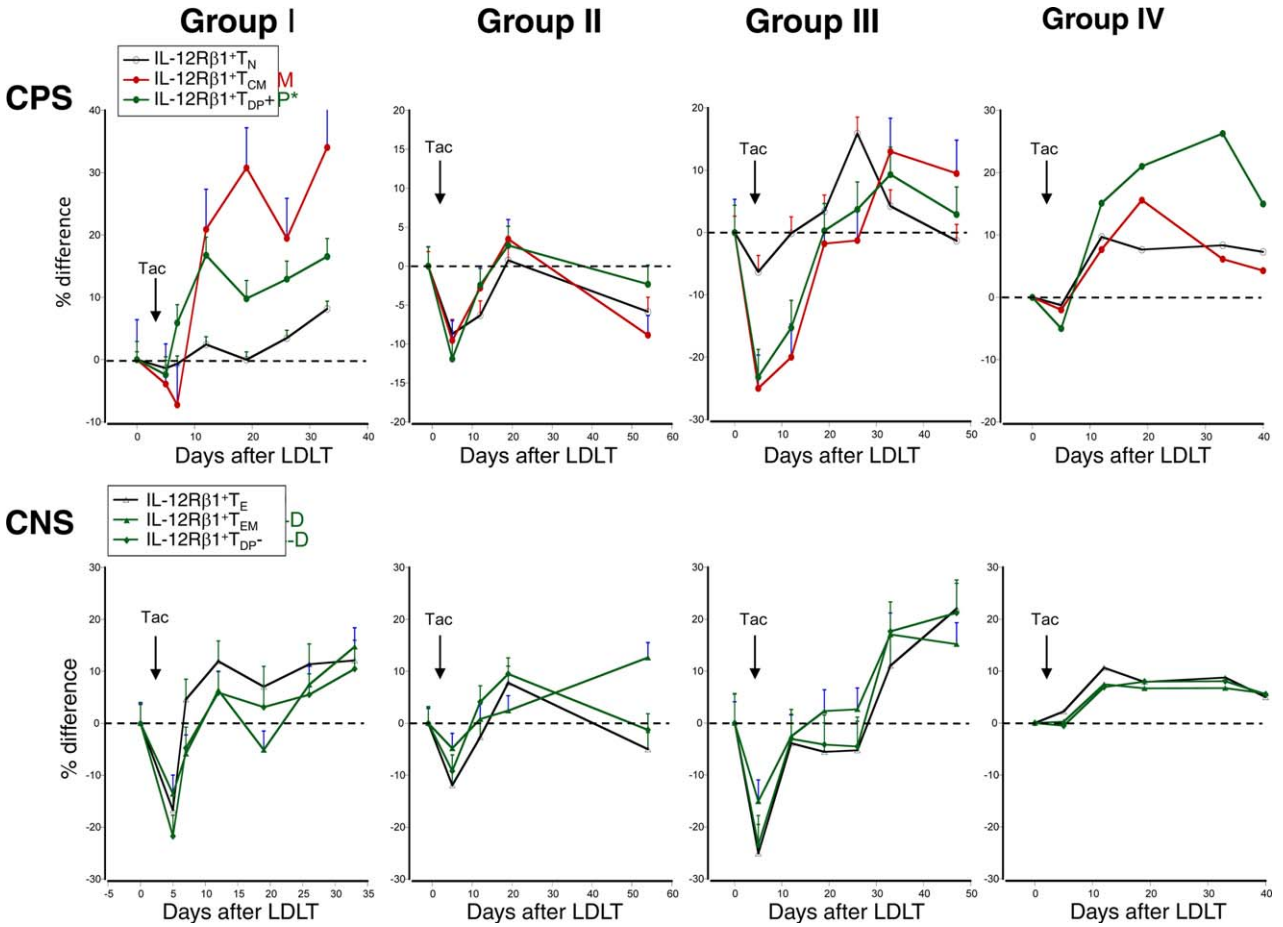

Fig. 1. Changes in the percentage difference in interleukin (IL)-12R$\beta 1^+$cells of CCR7-positive subset (CPS) and CCR7-negative subset (CNS) after living donor liver transplantation (LDLT) in the four groups. Follow-up studies were performed involving seven recipients from each group. The variable components were measured according to the method in Fig. 2. Tac = tacrolimus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

levels after POD 12, followed by decreases in CPS on POD 54. The IL-12R$\beta 1^+$ T_{CM} level was correlated significantly positively with IL-2R$\beta 1^+$ T_E ($r = 0{\cdot}877$), IL-12R$\beta 1^+$ T_{EM} ($r = 0{\cdot}741$) and IL-12R$\beta 1^+$ T_{DP-} ($r = 0{\cdot}581$).

In the initial stage of group III, the initial priming of IL-12β1 in T_{CM}, T_{DP+} and the CNS was decreased markedly during POD 5 to POD 12, and then returned to baseline after POD 18. The initial priming of IL-12R$\beta 1^+$ T_N was decreased slightly and then remained at baseline, although the level increased to approximately 15% above baseline on POD 25. The initial priming of IL-12R$\beta 1^+$ cells in T_E and T_{DP-} was decreased markedly to approximately −25% below baseline, and then increased to subpretransplant levels after POD 12, followed by an increase above baseline after POD 32. The initial priming of IL-12R$\beta 1^+$ T_{EM} was approximately −15%. IL-12R$\beta 1^+$ T_{CM} was correlated significantly positively with IL-12R$\beta 1^+$ T_E ($r = 0{\cdot}509$), IL12R$\beta 1^+$ T_{EM} ($r = 0{\cdot}623$), and IL-12R$\beta 1^+$ T_{DP-} ($r = 0{\cdot}627$).

Importantly, the lag time required until development of the coupled up-regulation of IL-12Rβ1 between T_{CM} and CNS was 12, 20 and 32 days in groups I, II and III, respectively. The decreased initial priming of IL-12Rβ1 of CNS was restored by a highly positive correlation with up-regulation of IL-12R$\beta 1^+$ $T_{CM.}$ In addition to antigenic and co-stimulatory signals, the IL-12 signal triggered a developmental programme by which $CD8^+$ T_N differentiated into effector and memory cells.

Recipients receiving the Tac/MMF regimen (group IV)

The initial priming of IL-12Rβ1 of T_{CM} and T_{DP+} was only slightly decreased below baseline, and then increased markedly to above baseline after POD 12. The initial priming of IL-12R$\beta 1^+$ T_N remained at baseline, and then increased to approximately 7% above baseline during the post-transplant period. The initial priming of IL-12Rβ1 of the CNS remained at baseline, and then increased

Table 3. Initial priming of interleukin (IL)-12Rβ1 in CD8$^+$ T cell subsets on post-operative day (POD) 5

	Group I	Group II	Group III	*P* I and II *versus* III	Group IV	*P* IV *versus* III
CPS						
IL-12Rβ1$^+$ T_N	−1·37 ± 0·48	−8·69 ± 3·49	−6·32 ± 3·52	0·7335†	−1·34 ± 1·37	0·9721†
IL-12Rβ1$^+$ T_{CM}	−3·90 ± 6·13	−9·52 ± 3·49	−27·01 ± 5·02	0·0767†	−2·88 ± 5·46	0·0428†
IL-12Rβ1$^+$ T_{DP+}	−2·43 ± 4·12	−11·90 ± 5·63	−23·16 ± 8·98	0·1969†	−4·67 ± 3·69	0·1467†
CNS						
IL-12Rβ1$^+$ T_E	−16·76 ± 7·23	−11·93 ± 8·30	−25·12 ± 6·58	0·2206†	−2·03 ± 6·45	0·0044†
IL-12Rβ1$^+$ T_{EM}	−13·60 ± 6·42	−4·83 ± 1·80	−15·06 ± 7·87	0·4068†	−1·57 ± 4·33	0·0557†
IL-12Rβ1$^+$ T_{DP-}	−21·73 ± 6·68	−9·19 ± 5·17	−23·48 ± 7·73	0·3302†	−2·30 ± 4·14	0·0084†
Lag time	12 days	20 days	32 days		0 days	

Values are expressed as the % difference (mean ± standard error).

**P*-values based on analysis of variance

†*P*-values based on the χ^2 test. Lag time: duration required for coupled development of interleukin (IL)-12Rβ1 of T_{CM} and the CCR7-negative subset (CNS) to above the pretransplant levels

slightly to 7% above baseline after POD 12. IL-2Rβ1$^+$ T_{CM} was correlated weakly positively with IL-12Rβ1$^+$ T_E ($r = 0{\cdot}568$), IL-12Rβ1$^+$ T_{EM} ($r = 0{\cdot}538$) and IL-12Rβ1$^+$ T_{DP-} ($r = 0{\cdot}442$).

The lag time in group IV was negligible.

Next, initial priming of IL-12β1 in CD8$^+$ T cell subsets on POD 5 was compared among four groups.

Initial priming of IL-12Rβ1 in CD8$^+$ T cell subsets on POD 5

The initial priming of IL-12Rβ1 in the CD8$^+$ T cell subsets of the four groups on POD 5 is shown in Table 3. In the recipients receiving the Tac/GC regimen, the initial priming of IL-12Rβ1$^+$ T_N was only slightly decreased. The initial IL-12RT_{CM} priming was decreased markedly progressively from group I to group II to group III. The initial priming of IL-12Rβ1$^+$ T_{DP+} was similar to that of IL-12Rβ1$^+$ T_{CM}. In contrast, the initial priming of IL-12Rβ1 of T_E, T_{EM} and T_{DP-} was decreased markedly in groups I and III, whereas that in group II was also decreased, but to a lesser extent. The lag time was 12 days in group I, 20 days in group II and 32 days in group III. In contrast, in group IV that received the Tac/MMF regimen, the initial priming of IL-12Rβ1 of CPS remained almost unchanged at baseline and the lag time was negligible.

Next, the effects of Tac/GC and Tac/MMF administration on CD8$^+$ T cells after LDLT were examined in a recipient that was followed longitudinally.

Changes in IL-12Rβ1, CD8$^+$ T cell subsets, perforin, IFN-γ and TNF-α in a group III recipient receiving the Tac/MMF regimen

The effects of Tac/GC and Tac/MMF administration on CD8$^+$ T cells after LDLT in a recipient that was followed longitudinally until post-operation day (POD) 150 are illustrated in Fig. 2. In this recipient, GC was administered only in the early period after LDLT and was then withdrawn.

Figure 2a–c shows the administered immunosuppressive agents and clinical course, as well as changes in the proportions and percentage differences of CD8$^+$ T cell subsets and IL-12Rβ1$^+$ cells in each subset of CD8$^+$ T cells in this recipient (a 56-year-old male) undergoing LDLT for HBV-related liver cirrhosis. As immunosuppression, Tac was administered to achieve a 10-ng/ml trough level until POD 20, and was then decreased to a 5-ng/ml trough level due to the onset of renal failure (Fig. 2a). MMF (0·5 g twice daily) was administered until POD 20, but was stopped from POD 21 to 29. MMF (a 50% dose, 0·25 g twice daily) was again administered after POD 30. A steroid bolus was administered for 3 days from POD 7, and then methylprednisolone was decreased gradually until POD 17. Prednisone (0·5 mg/kg) was administered after POD 102 until POD 138. Therefore, a prolonged period from POD 18 to 101 was steroid-free, similar to group IV. Sepsis (Se) occurred during the period between POD 8 and 16, and again between POD 32 and 34. SS/MODS developed around POD 28, with an increase in aspartate aminotransferase (AST) between POD 16 and 27. Biopsy-proven ACR was confirmed on POD 70. Microbial infection continued from POD 27 until 96 (Fig. 2b,i). T_E generation increased markedly to 77·4% on POD 17 at the time of MMF and Tac administration, decreased to 38·9% around POD 28 during the period of MMF withdrawal and Tac reduction, and then increased to near pretransplant levels (Fig. 2b,i). T_{EM} and T_{DP-} increased to approximately 20% from POD 28, and then remained slightly above pretransplant levels. The proportion of T_N, T_{CM} and T_{DP+} remained at the lowest level during the post-transplant period. These changes were depicted more clearly by the percentage difference (Fig. 2b,ii).

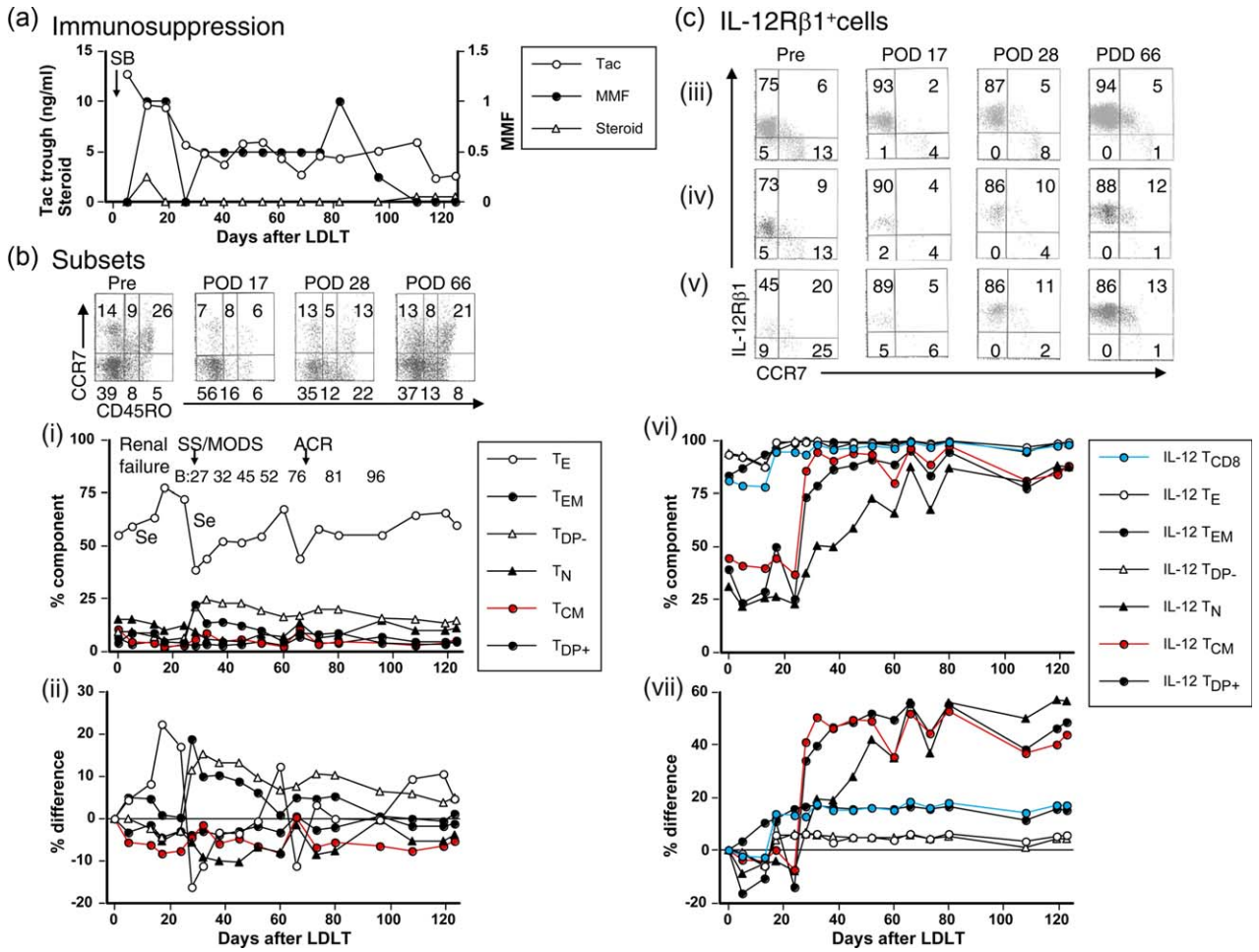

Fig. 2. Effect of immunosuppressive agents, flow cytometric assay of the changes in $CD8^+$ T cell subsets and their interleukin (IL)-12R$\beta1^+$ cells after living donor liver transplantation (LDLT) in a group IV recipient. (a) Effect of immunosuppressive agents. SB = steroid bolus. (b) Flow cytometric analysis of $CD8^+$ T cell subsets; proportion per $CD8^+$ T cells (b,i) and percentage difference (b,ii). For flow cytometry, the lymphocytes were stained with monoclonal antibodies to CD45RO and CCR7. The dot–plots show double-staining for $CD8^+$CCR7/CD45RO on gated lymphocytes, which identified six subsets of $CD8^+$ T cells: naive (T_N) ($CD45RO^-CCR7^+$), central memory (T_{CM}) ($CD45RO^+CCR7^+$), effector memory (T_{EM}) ($CD45RO^+CCR7^-$), effector T cells (T_E) ($CD45RO^-CCR7^-$), DP^+ T cells (T_{DP+}) ($CD45RORACCR7^+$) and DP^- T cells (T_{DP-}) ($CD45RORACCR7^-$), as reported7 previously [4]. Cells in six segments are presented as a ratio (%). (b,i) B27, bacterial infection on postoperative day (POD) 27 (*Pseudomonas aeruginosa*, bile); B32 = bacterial infection on POD 32 (*P. putida* and *Chryseobacterium*, bile); C29 = cytomegalovirus on POD 29; and Se = sepsis. (c) IL-12R$\beta1^+$cells. For flow cytometry, $CD8^+$ T cells were classified into three subsets based on triple-staining using antigen-presenting cell (APC)-conjugated anti-CD8 (Coulter Immunotech, Marseille, France), fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO (Coulter Immunotech, Marseille, France) and RD1-conjugated anti-CD45RA (Coulter clone 2H4-RD1; Beckman Coulter, Miami, FL, USA) for $CD8^+CD45RO^-$ cells (c,iii), $CD8^+CD45RO^+$ cells (c,iv) and $CD8^+CD45RO^{++}$ cells (c,v) in gated lymphocytes, as reported previously [4]. This figure shows the dot–plots in three different subsets presenting the IL-12R$\beta1$-expressing cells superimposed on double-staining for IL-12R$\beta1$ and CCR7 in gated $CD8^+CD45RO^-$ cells (c,iii), gated $CD8^+CD45RO^+$ cells (c,iv) and $CD8^+CD45RO^{++}$ cells (c,v). The percentage component (c,vi) and percentage difference of IL-12R$\beta1^+$cells (c,vii). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The changes in the percentage of IL-12R$\beta1^+$ cells in $CD8^+$ T cell subsets after LDLT are shown in Fig. 2c. IL-12R$\beta1^+$ cells in T_E, T_{EM} and T_{DP-} (CCR7-negative subsets, CNS) remained at the highest level of 90% during the post-transplant period (Fig. 2c,vi). In contrast, those in T_N, T_{CM} and T_{DP+} (CCR7-positive subsets, CPS) remained at the lowest level until POD 24, and then increased rapidly to the highest level in T_{CM} and T_{DP+} and later in T_N, as shown by the percentage difference (Fig. 2c,vii). More importantly, the time of marked up-regulation of IL-12R$\beta1^+$ cells of CPS was consistent with the starting point of the steroid-free protocol; this increase persisted for a prolonged period.

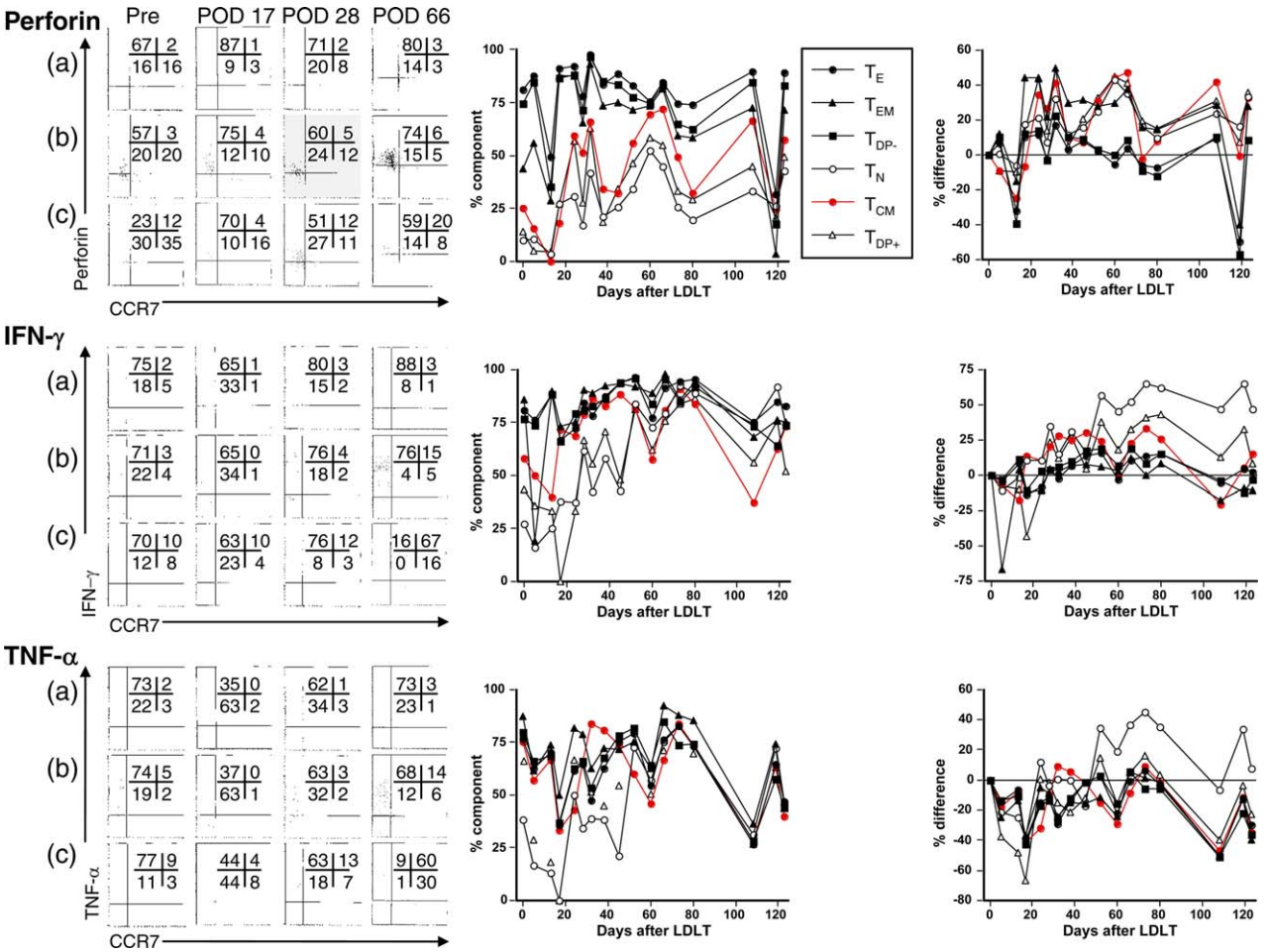

Fig. 3. Flow cytometric analysis of changes in the percentage proportion and percentage difference of perforin, interferon (IFN)-γ and tumour necrosis factor (TNF)-α in CD8$^+$ T cell subsets after living donor liver transplantation (LDLT) in the same recipient as that of Fig. 2. The dot-plots in three different subsets show the perforin-, IFN-γ- and TNF-α-expressing cells superimposed on double-staining of each of three variables and CCR7 in gated CD8$^+$CD45RO$^-$ cells (a), gated CD8$^+$CD45RO$^+$ cells (b) and CD8$^+$CD45RO^{++}cells (c). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Changes in the expression of perforin, IFN-γ and TNF-α

Flow cytometric analysis of changes in the proportions or percentage differences of perforin, IFN-γ and TNF-α in CD8$^+$ T cell subsets after LDLT in the same recipient as that shown in Fig. 2 are seen in Fig. 3. The proportion of perforin in CNS was expressed at the highest level and remained high, whereas that in CPS increased with a rise in IL-12Rβ1$^+$ cells in each of the CPS. Regarding IFN-γ expression, CNS showed the highest level and CPS showed an increase in IFN-γ, with an elevation in IL-12Rb1$^+$ cells in each subset. However, the TNF-α expression in T_N was increased greatly between POD 52 and 80, whereas the TNF-α level in other subsets remained near pretransplant levels in the period between POD 25 and 80 and then decreased. These findings indicate that perforin and IFN-γ expression was increased markedly along with the IL-12Rb1$^+$ cell increase, corresponding with the start of the steroid-free protocol.

Next, survival rate of the four groups was analysed by Kaplan–Meier survival curves.

Kaplan–Meier survival curves for the four recipient groups

The survival rate of the four recipient groups was analysed, although retrospective estimates have potential limitations. Figure 4 shows that the survival probability rate of groups I and II was approximately 90% beyond POD 2000. In contrast, the survival probability rate of group III decreased to approximately 50% beyond POD 2000, although most recipients died within the first 100 days. In contrast, the survival rate in group IV remained at 100% beyond POD 2000. However, recent follow-up studies indicated that, of all 38 recipients who received the Tac/MMF regimen, irrespective of pre-existing T_E levels, four died beyond POD 2500. A significant difference was seen between groups I, II and IV *versus* group III at

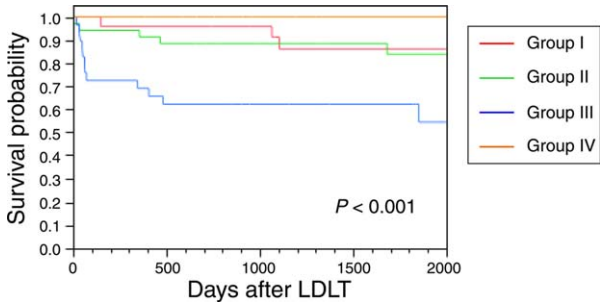

Fig. 4. Long-term survival rates in the four groups using Kaplan–Meier estimates. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

each time-point, when the entire follow-up period was compared by Kaplan–Meier analysis and the log-rank test. A significant ($P = 0{\cdot}001$) difference was also seen in the survival probability rate between groups I, II and IV *versus* III. No significant ($P = 0{\cdot}389$) difference was observed between groups I and II *versus* group IV; however, a significant ($P = 0{\cdot}0036$) difference was apparent between groups III and IV. Importantly, it is very likely that the survival probability of the four groups was related closely to the duration of the lag time.

Discussion

Role of initial priming of IL-12Rβ1 in T_{CM} and the CNS

HLA mismatches, lymphocyte cross-matches, MELD score and donor age were not different among the four groups, and did not relate significantly to survival rates and clinical outcomes. In contrast, the present study clearly showed that initial priming of $CD8^+$ IL-12Rβ1 immediately after LDLT plays a more crucial role in determination of better immune responses and clinical outcome.

The initial IL-12RT_{CM} priming by the first encounter with the largest numbers of alloantigens was decreased only slightly, moderately and markedly in groups I, II and III, respectively. The difference in programming of the initial priming of IL-12RT_{CM} in the three groups may be dependent on the extent of their pre-existing T_E numbers and activated immune status. In contrast, the initial priming of IL-12Rβ1 in CNS was down-regulated markedly in the three groups, but the lag time was prolonged progressively from groups I and II to group III. Moreover, the patients' post-transplant clinical outcomes worsened with increasing lag time. Conversely, in group IV, the early coupled initial priming of IL-12Rβ1 in T_{CM} and the CNS developed without a lag time. These results imply that an efficient effector function of CTLs is programmed during their first encounter with alloantigens and can develop in the absence of additional TCR signalling. This idea is consistent with accumulated data that the initial antigen encounter triggers a programme for autonomous clonal expansion and development into functional effector cells [17,24,25].

Post-transplant immune response after the Tac/GC regimen

The lag time was 12 days in group I and 20 days in group II. It seems likely that the capability of inducing the highest immune response was maintained, because of the lowest frequencies of post-transplant complications compared with group III. The immune status in group III recipients was characterized by the highest numbers of pre-existing T_E and immunologically full activation. In particular, the pre-existing IL-12Rβ1$^+$ T_{CM} was expressed at the highest levels. IL-2 production by $CD4^+$ helper T cells was inhibited strongly by administration of the Tac/GC regimen. Their initial priming of IL-12Rβ1 of T_{CM} and the CNS was down-regulated markedly for the longest period, along with marked decreases in the generation of $T_{E,}$ perforin and IFN-γ by CTLs. These results indicate a form of anergy characterized by marked down-regulation in lytic activities of CTLs. Conversely, it has been found that upon being fully activated by IL-12, CTLs develop activation-induced non-responsiveness (AINR), similar to split anergy that is characterized by an inability to produce IL-2, which supports continued expansion, as proposed by Mescher *et al.* [13]. The anergy status in group III appeared to be similar to AINR. Consequently, the AINR-like immune status in group III may have been induced by the marked down-regulation of both IL-12Rβ1 of the $CD8^+$ T cells and IL-2 production of $CD4^+$ helper T cells. It is most likely that the IL-12 produced by activated maturated DCs was strongly produced thereafter over time, along with IL-2 [16], following clearance of alloantigen. Those recipients had the highest susceptibility to post-transplant bacterial and viral infections, resulting in SS/MODS with the highest mortality rates.

Post-transplant immune responses after the Tac/MMF regimen

The initial priming of IL-12Rβ1 of T_{CM} and the CNS remained above baseline without a lag time, and was then followed by higher levels of IL-12Rβ1$^+T_{CM-}$. This result indicates that IL-2 production cannot be inhibited after administration of the Tac/MMF regimen, because IL-12 up-regulates expression of both CD25 and IL-12Rβ1 [16]. Moreover, the pre-existing highest T_E and the fully activated immune status were maintained during the post-transplant period.

Beneficial effects of Tac/MMF on clinical outcomes and survival rates after liver transplantation have been reported [26–28]. Although MMF exerts its effects through a multitude of mechanisms [29], in the present study the most significant benefits were the following

three events. First, the Tac/MMF regimen strongly up-regulated the initial priming of IL-12Rβ1 in CD8$^+$ T cell subsets. Secondly, the effect of the Tac/MMF regimen could not be affected by the pre-existing activated immune status of recipients. Thirdly, the enhanced coupled expression of IL-12Rβ1$^+$T$_N$ with IL-12Rβ1 in T$_{CM}$ and CNS resulted in efficient generation of the effector functions characteristic of activated CTLs.

Our findings provide an important insight into the mechanisms by which the early tight coupling of the initial priming of IL-12Rβ1 in T$_{CM}$ and CNS plays a crucial role in inducing the best effector cell expansion and their clinical outcomes. These results indicate that administration of the Tac/GC regimen should be avoided for HPT$_E$ recipients. Therefore, the addition of MMF to Tac-based immunosuppression leads to the best clinical outcomes.

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Disclosure

The authors declare that neither the submitted material nor portions thereof have been published previously or are under consideration for publication elsewhere. There are no conflicts of interest related to this manuscript. There are no financial and commercial conflicts of interest.

Author contributions

T. K., A. M., Y. F. and S. U. performed living donor liver transplantation for all recipients. S. U. and K. O. designed the research study. K. O. performed Doppler ultrasound examination. K. O. statistically analysed all data. S. Uemoto and K. O. wrote the paper.

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