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ORIGINAL ARTICLE

Liver sinusoidal endothelial cells have a capacity for inducing nonresponsiveness of T cells across major histocompatibility complex barriers

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allografts, liver nonparenchymal, liver sinusoidal endothelial cells, liver transplantation, tolerance.

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Summary

Livers transplanted across major histocompatibility complex (MHC) barriers in mice are normally accepted without recipient immune suppression. To identify the cell type that contributes to induction of such a tolerance state, we established an allogeneic mixed hepatic constituent cell-lymphocyte reaction (MHLR) assay. Hepatic constituent cells were isolated from C57BL/6 (B6) and Balb/c mice as stimulators, and splenocytes were isolated from B6 mice as responders. Irradiated hepatic constituent cells were co-cultured with fluorescent dye (CFSE)-labeled B6 splenocytes. In the allogeneic MHLR using either whole hepatic constituent cells or parenchymal hepatocytes as stimulators, a lack of T-cell proliferation was observed. Only when $CD105^+$ cells, which are exclusively liver sinusoidal endothelial cells (LSECs), were depleted from hepatic constituent cell stimulators, the MHLR resulted in marked proliferation of both allo-reactive $CD4^+$ and $CD8^+$ T cells. These results indicate that $CD105^+$ LSECs have the capacity to induce nonresponsiveness of T cells across MHC barriers.

Introduction

Liver allografts are extraordinarily tolerogenic, and stable grafts can be maintained without immunosuppression in some species [1–4]. Furthermore, the presence of a liver allograft can suppress both the rejection of other solid tissue grafts from the same donor [5,6] and graft-versus-host disease against host alloantigens that are expressed on a liver allograft [7]. Elucidation of the immune mechanism underlying the acceptance of liver allografts in animal models should lead to the establishment of novel means to promote acceptance of transplant organs. Although various possible mechanisms underlying the acceptance of liver allografts, including action of soluble class I antigens [8–10], clonal deletion [11–13], induction of anergy, generation of regulatory cells [14–16], and microchimerism established by liver-derived leukocytes [17–20], have been proposed, the cellular basis of this immune tolerance is not fully understood.

It is generally accepted that the immunogenecity of solid-organ allografts is due to the presence of antigen-presenting cells (APCs) expressing major histocompatibility complex (MHC) class II within the grafts. MHC molecules on these cells are directly recognized by host $CD4^+$ T cells, which become activated and produce various cytokines that initiate a cascade of alloimmune events. Liver dendritic cells have abnormal APC properties and low expression of co-stimulatory molecules, and they preferentially induce T helper 2 (Th2) responses, suggesting dendritic cell-mediated tolerogenicity [21,22]. In addition to liver dendritic cells, liver sinusoidal endothelial cells (LSECs), which constitute the lining or wall of the hepatic sinusoid, are also able to present antigens to T cells [23]. While a number of studies have demonstrated the importance of antigen presentation by liver dendritic cells to liver allograft tolerance, the role of antigen presentation by LSECs in such immune tolerance has not been investigated. LSECs constitutively express all

molecules necessary for antigen presentation (MHC class I, class II CD54, CD80, CD86 and CD40) and can function as APCs for $CD4^+$ and $CD8^+$ T cells [24]. It has recently been reported that LSECs may function as APCs in the context of both MHC-I and MHC-II restriction with the resulting development of specific T-cell tolerance to both exogenous and endogenous soluble antigens [25,26]. In the present study, we investigated the immunogenicity of LSECs in mouse liver allografts, which are normally accepted without recipient immune suppression across MHC barriers, by the use of a mixed hepatic constituent cell-lymphocyte reaction (MHLR) assay.

Materials and methods

Mice

Female C57BL/6 (B6, H-2^b) and Balb/cA Jcl (Balb/c, H-2^d) mice of 8–12 weeks of age in the experiments were used. All animals were purchased from Clea Japan, Inc. (Osaka, Japan) and maintained in a specific pathogen-free microisolator environment. All animal experiments were performed according to the guidelines set by the National Institutes of Health (NIH publication 86-23, revised 1985).

Isolation of hepatic cells and preparation of fractions

Fractions of hepatic constituent cells, parenchymal cells (PCs) (hepatocytes), CD105-positive nonparenchymal cells (NPCs) (LSECs), and CD105-negative NPCs (LSEC-depleted NPCs) were prepared following the procedure schematically shown in Fig. 1, modified as described previously [27]. Disaggregated hepatic constituent cells were obtained from mice by the collagenase perfusion technique, modified as previously described [28]. In brief, livers were removed after inferior vena cava perfusion with Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY, USA) containing 0.02% ethylene glycol-bis (β-aniroethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Sigma, St Louis, MO, USA) and HBSS-containing 0.05% collagenase (Wako, Osaka, Japan). The livers were digested, and disaggregated whole hepatic constituent cells were centrifuged three times at 50 *g* for a minute each time. The pellet and supernatant were used for the preparation of hepatocytes and NPCs, respectively. A fraction containing hepatocytes was isolated as a pellet obtained by centrifuging the former through 45% Percoll (Sigma) at 50 *g* for 24 min. The latter was centrifuged at 150 *g* for 5 min, and the pellet was suspended and similarly centrifuged twice at 150 *g*. This pellet was suspended and centrifuged at 50 *g* for 2 min to eliminate contaminative hepatocytes, and a fraction of NPCs was obtained as a supernatant. Antimouse CD105 monoclonal antibody (mAb; MJ7/18) (eBioscience, San Diego, CA, USA) was used as a marker of endothelial cells. This mAb binds to CD105 molecules (endoglin) expressed specifically on mouse endothelial cells, including LSECs [29]. After NPCs had been stained by biotin-conjugated antimouse CD105 Ab and streptavidin-conjugated microbeads (Militenyi Biotec, Bergish Gladbach, Germany), NPCs were depleted of LSECs by magnetic cell sorting system using auto MACS® (Militenyi Biotec) following the manufacturer's instructions, and then $CD105^+$ NPCs and $CD105^-$ NPCs were obtained. Similarly, $CD105^-$ hepatic constituent cells (LSEC-depleted hepatic constituent cells) were obtained by the same method. After depletion, the purity was always <5% of $CD105^+$ cells by negative selection and more than 90% of $CD105^+$ cells by positive selection.

Preparation of responder cells and CFSE labeling

After harvest of spleens from B6 mice and erythrocyte lysis with ammonium chloride/potassium solution, the splenocytes were suspended in phosphate-buffered saline (PBS) (Nissui, Tokyo, Japan). A final volume of 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Inc., Eugene, OR, USA) was added, and the cells were gently mixed and incubated for 15 min at 37 °C in a CO_2 incubator protected from light. Labeling of cells was stopped by adding cold PBS with 2% fetal bovine serum (FBS; Sanko, Tokyo, Japan), and the cells were then washed and resuspended in mixed lymphocyte reaction (MLR) medium, i.e. RPMI culture medium containing 15% controlled process serum replacement-type 3 (Sigma), 5 μM 2-mercaptoethanol (Katayama, Osaka, Japan), 1% HEPES buffer (Gibco), and 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco).

Preparation of stimulator cells

Fractions of whole hepatic cells, hepatocytes, $CD105^+$ NPCs (LSECs), $CD105^-$ NPCs (LSEC-depleted NPCs), and $CD105^-$ hepatic constituent cells (LSEC-depleted hepatic constituent cells) were prepared from C57BL/6J and Balb/c mice as described above. After each fraction had been irradiated with 30 Gy, the cells were resuspended in culture medium as stimulator cells.

In vitro CFSE-MHLR

The stimulator and responder cells in the MLR medium were adjusted to 4×10^6 cells/ml and 0.8×10^6 cells/ml of medium, respectively, and co-cultured in a total volume of 2 ml of medium in 24-well flat-bottom plates (BD Labware, Franklin Lakes, NJ, USA) at 37 °C in a 5% CO_2 incubator in the dark for 5 days.

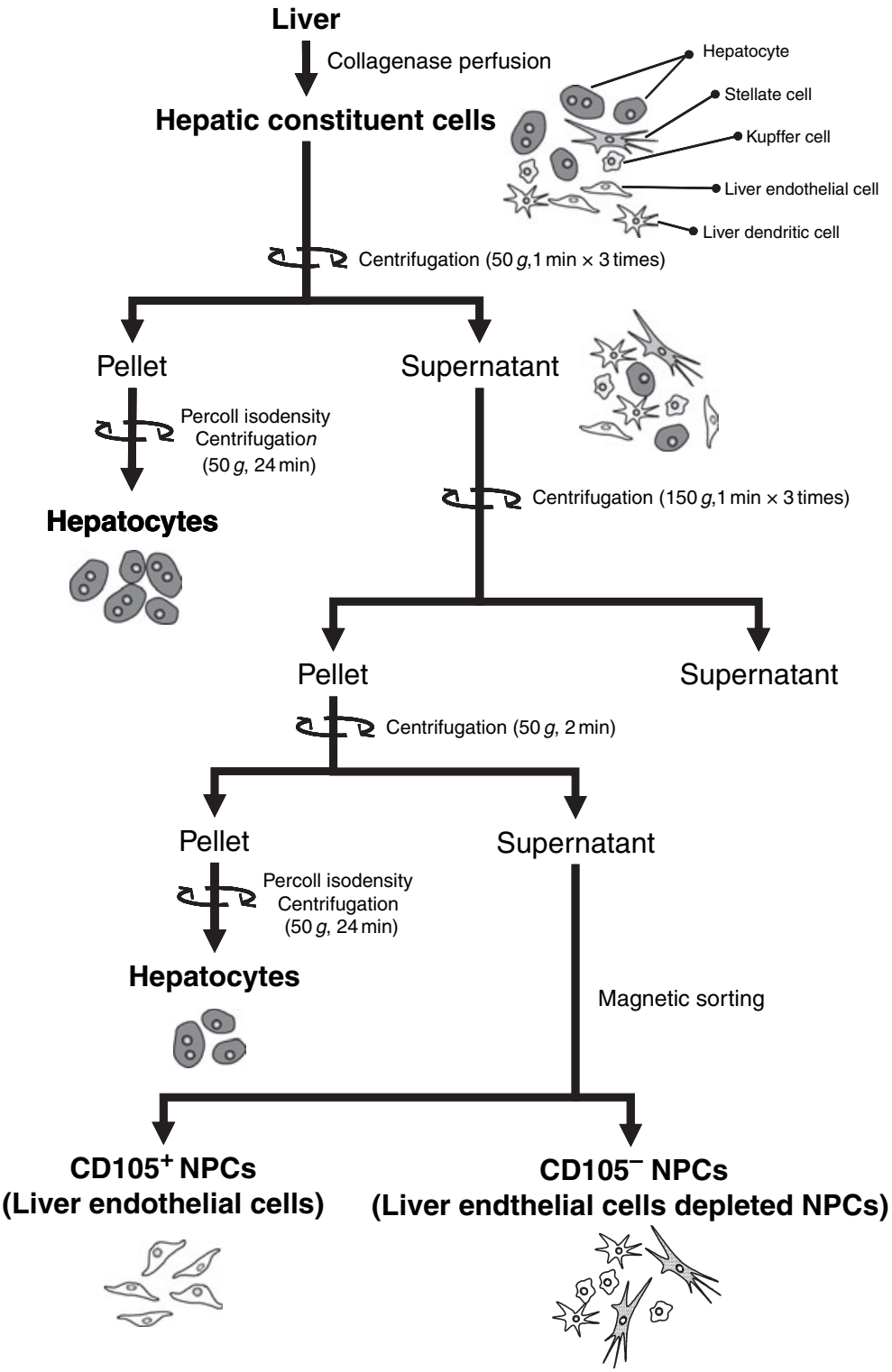

Figure 1 Schematic illustration of the methods used to prepare fractions of hepatic constituent cells, hepatocytes, $CD105^+$ nonparenchymal cells (NPCs), and $CD105^-$ NPCs. The symbols represent hepatocytes, stellate cells, Kupffer cells, liver sinusoidal endothelial cells and liver dendritic cells, as indicated. Each fraction was subjected to fluorescence-activated cell sorter (FACS) analysis to determine purity as shown in Fig. 3.

Flow cytometric analyses

Proliferation of MLR-cultured cells was analyzed by multiparameter flow-cytometry (FCM) as follows. All analyses were performed on a FACScan® cytometer (Becton Dickinson, Mountain View, CA, USA). The harvested cells (at least 1.5×10^5 cells/sample) were stained with phycoerythin (PE)-conjugated antimouse CD4 mAb (GK1.5) or CD8a mAb (53–6.7). For all analyses, nonspecific Fcγ-receptor binding of labeled Abs was blocked by rat antimouse CD16/32 mAb (2.4G2). All mAbs were purchased from PharMingen (San Diego, CA, USA). Dead cells were excluded from the analysis by forward-scatter and propidium iodide (PI) staining.

Results

Isolation of hepatic constituent cells

The liver comprises a diversity of constituent cells with unique features that might be conducive to induction of tolerance. Investigation of the immunogenicity and tolerogenicity of individual hepatic constituent cells should provide clues for elucidation of the mechanisms for induction of a state of tolerance after liver allografting. For this purpose, we isolated a PC fraction and a NPC fraction by the 2-step collagenase perfusion method schematically shown in Fig. 1. The purity of these fractions was determined by morphology and FCM (Figs 2 and 3). As PCs (hepatocytes) and NPCs differ in granularity and autofluorescence, FCM analyses of parameters of right-angle scatter (SSC) and fluorescence 2 (FL2) could be used to distinguish them. PC hepatocytes, which are characterized by their high degrees of granularity and autofluorescence, were identified in the region 1 (R1) (Fig. 3). NPCs, which are agranular and less autofluorescent, were identified in the R2 and R3 (Fig. 3). The purities of the fractions of PCs and NPCs were found to be approximately 90% and 85–95%, respectively.

The LSECs were isolated from the NPC fraction. Results of immunohistochemical studies showed that LSECs express CD105 molecules in the mouse liver but not in endothelia of central veins or other vessels (data not shown). Therefore, $CD105^+$ cells were positively or negatively selected by MACS to isolate or deplete LSECs from hepatic constituent cells. Positive sorted cells contained between 90 and 95% LSECs (Figs 2a and 3c).

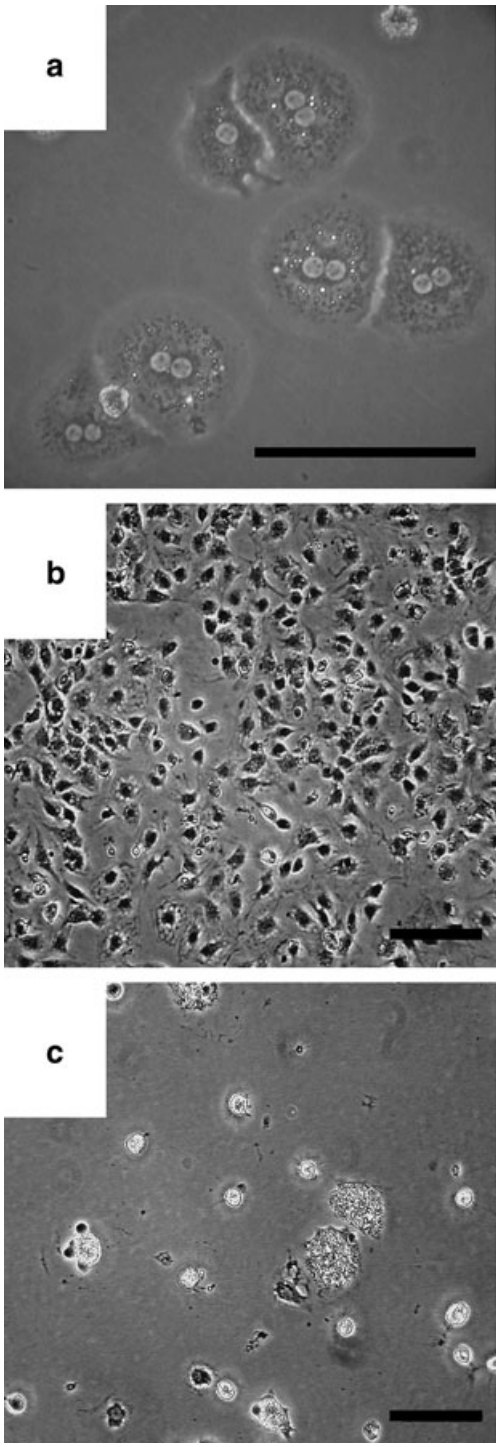

Figure 2 Phase contrast images of hepatocytes, $CD105^+$ nonparenchymal cells (NPCs) and $CD105^-$ NPCs separated from whole liver constituent cells, as shown in Fig. 1. (a) Hepatocytes with binuclear or mononuclear, which are characterized by their high degrees of granularity and autofluorescece. (b) $CD105^+$ NPCs, which are characterized by their polygonal shape and cobblestone appearance. The cells were cultured on collagen-coated dishes to confirm morphologic character. (c) $CD105^-$ NPCs, which consist of morphologically various cells. By the trypan blue exclusion test, viabilities of hepatocytes, $CD105^+$ NPCs and $CD105^-$ NPCs were consistently >95%, >90% and >85%, respectively. Bar indicates 100 μm.

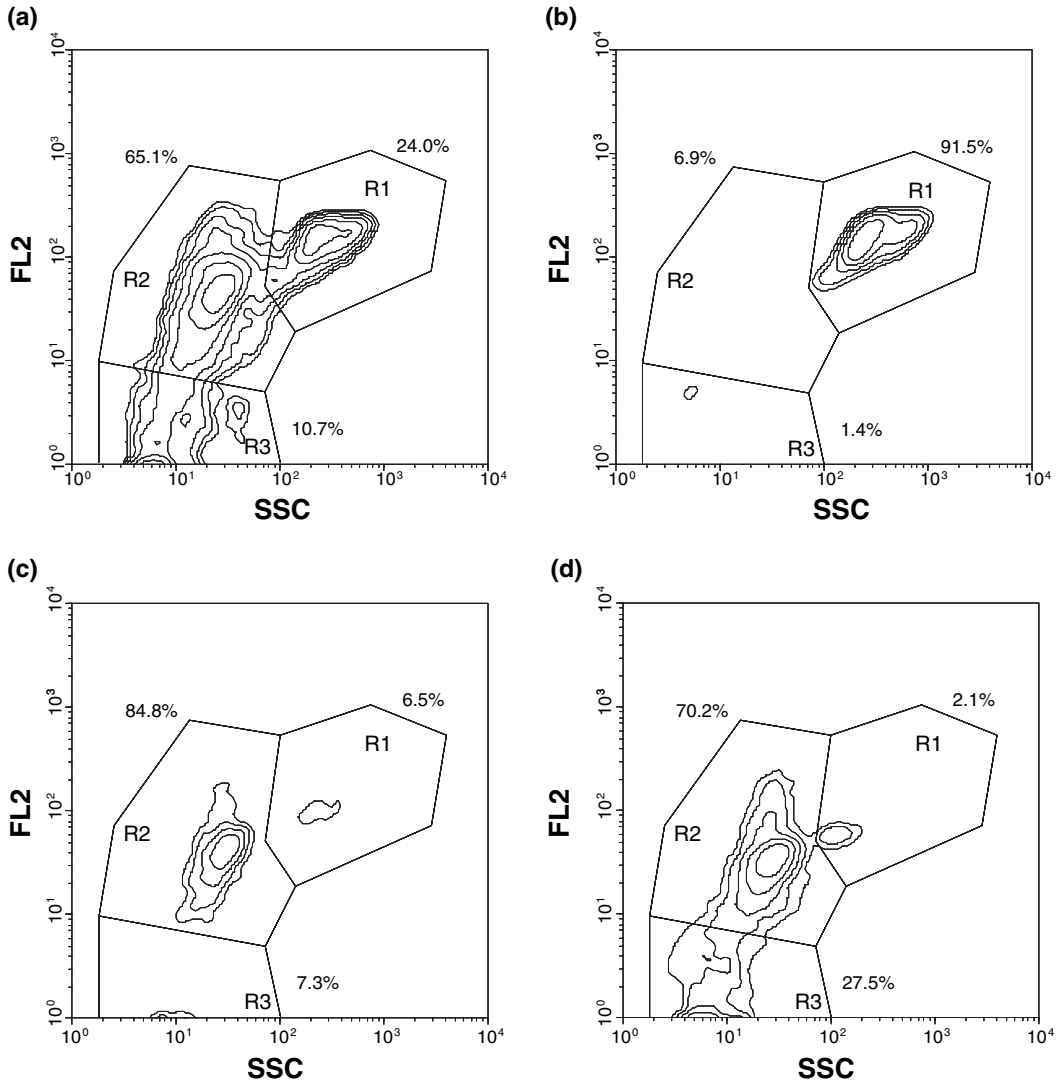

Figure 3 Fractions of hepatocytes, $CD105^+$ nonparenchymal cells (NPCs) (liver sinusoidal endothelial cells, LSECs) and $CD105^-$ NPCs (LSEC-depleted NPCs) were enriched, as illustrated in Fig. 1. Fractions separated from whole hepatic constituent cells were analyzed by FCM using parameters of right-side scatter (SSC) (log scale) and fluorescence 2 (FL2) to evaluate granularity and autofluorescence. Dead cells were excluded from analyses by forward-scatter and propidium iodide staining. Three different populations were distinguished among the (a) whole hepatic constituent cells: parenchymal cells (i.e. hepatocytes), liver mesenchymal cells (i.e. stellate cells and liver endothelial cells), and liver-resident haematopoietic cells (i.e. Kupffer cells and liver dendritic cells) were identified in region 1 (R1) (SSC-high, FL2-high), R2 (SCC-low, FL2-medium) and R3 (SCC-low, FL2-low), respectively. Hepatocytes (parenchymal cells) (in R1) are characterized by their high degrees of granularity and autofluorescence, as previously reported [27]. Liver endothelial cells and stellate cells (mesenchymal cells) (in R2) are characterized by their low degrees of granularity and relatively high degrees of autofluorescence. Kupffer cells and liver dendritic cells (liver-resident haematopoietic cells) (in R3) are characterized by their low degrees of granularity and autofluorescence. Purities of (b) hepatocytes, (c) $CD105^+$ NPCs, and (d) $CD105^-$ NPCs were 91.5%, 84.8% and 97.7%, respectively. FCM profiles shown are representative of three independent experiments.

Only the $CD105^-$ NPC fraction induced proliferation of $CD4^+$ and $CD8^+$ alloreactive T cells

Isolated fractions of hepatic constituent cells were subjected to MHLR assays to examine their capacity to stimulate allogeneic T cells. In the present study, FCM analysis of lymphocyte proliferation in response to allogeneic stimulation by serial halving of the fluorescence intensity of the intracellular fluorescent dye, CFSE, was used in the MHLR assay. This method was employed to avoid the use of titrated thymidine, which is possibly incorporated by stimulator hepatic constituent cells that are relatively resistant to irradiation. CFSE stably stains intracellular proteins without toxicity, and

the fluorescence of each stained cell segregates equally to daughter cells upon cell division, resulting in sequential halving of cellular fluorescence intensity with each successive generation [30]. When analyzed by FCM, this sequential halving of fluorescence is visualized and can be used to track lymphocyte division in populations of proliferating cells. This, then, allows phenotypic analysis of proliferating cells by multicolor FCM analysis, as previously reported [31].

In the syngeneic MHLR using whole hepatic constituent cells as stimulators, T-cell proliferation was not seen as expected (Fig. 4a). Notably, even in the allogeneic MHLR using whole hepatic constituent cells as stimulators, no proliferation of either $CD4^+$ or $CD8^+$ T cells was observed at all (Fig. 4b). When a nearly pure population of parenchymal hepatocytes as stimulators was used, only limited proliferation of T cells was seen in the allogeneic MHLR, indicating their poor ability as alloantigen presenting stimulators (Fig. 4b). In contrast, when $CD105^+$ NPCs (LSECs) were depleted from NPC stimulators by magnetic cell sorting, the MHLR resulted in marked proliferation of both alloreactive $CD4^+$ and $CD8^+$ T cells, demonstrating that an immunogenic population that could promote alloimmune responses was included in this population (Fig. 5b). A pure population of $CD105^+$ NPCs (LESCs) did not induce proliferation of either alloreactive $CD4^+$ or $CD8^+$ T cells (Fig. 5b).

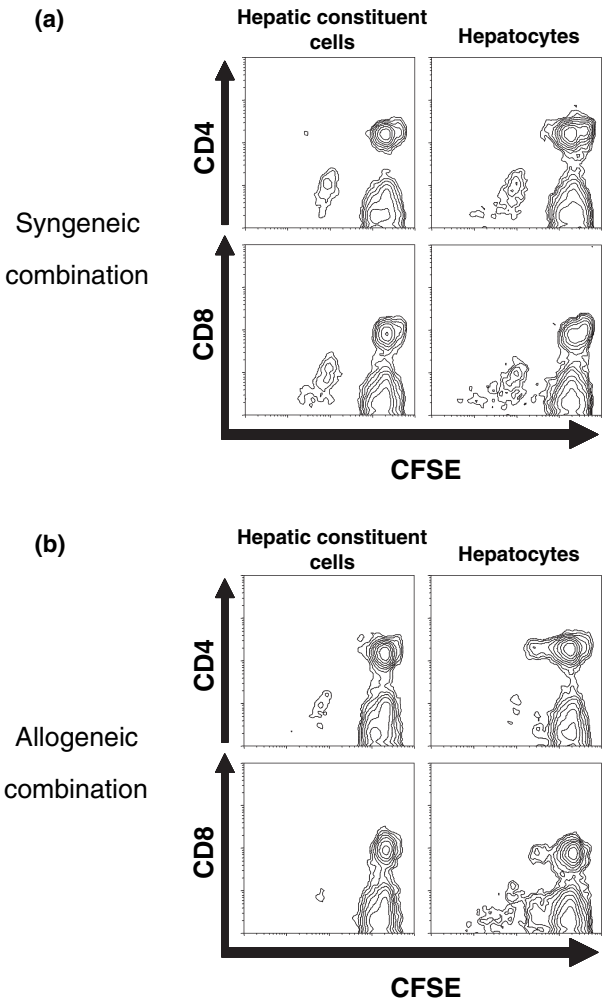

Figure 4 Whole hepatic constituent cells and hepatocytes did not promote proliferation of either $CD4^+$ or $CD8^+$ alloreactive T cells. Irradiated whole hepatic constituent cells or purified hepatocytes that were prepared from B6 and Balb/c mice, as shown in Fig. 1, were co-cultured with fluorescent dye (CFSE)-labeled responder splenocytes from B6 mice (mixed hepatic constituent cell-lymphocyte reaction, MHLR) in a syngeneic combination (a) and an allogeneic combination (b). After the MHLR, harvested lymphocytes were stained with either phycoerythin (PE)-conjugated anti-CD4 monoclonal antibody (mAb) or anti-CD8 mAb. Then, by FCM analysis, T-cell proliferation (division) was visualized as serial halving of the fluorescence intensity of an intracellular fluorescent dye (CFSE). Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining. FCM profiles shown are representative of three independent experiments.

$CD105^+$ LSECs have the capacity for inducing nonresponsiveness of alloreactive T cells

Poor proliferation of T cells in response to the allogeneic purified hepatocytes might be explained by the lack of MHC class II molecules on hepatocytes [32]. The complete absence of T-cell proliferation in response to the allogeneic whole hepatic constituent cells, even which included a strong immunogeneic NPC fraction ($CD105^-$), raises the possibility that whole hepatic constituent cells include a tolerogenic cell population. To determine whether $CD105^+$ LSECs have such tolerogeneic activity toward allogeneic T cells, $CD105^+$ LSEC-depleted hepatic constituent cells were subjected to an allogeneic MHLR assay. Only in the absence of the $CD105^+$ LSECs, hepatic constituent cells induced remarkable proliferation of both alloreactive $CD4^+$ and $CD8^+$ T cells (Fig. 6b), indicating that $CD105^+$ LSECs have a tolerogenic property.

Discussion

It is generally accepted that there are two pathways of allorecognition, direct antigen presentation and indirect antigen presentation, which together contribute to allograft rejection. The direct pathway results from the recognition of foreign MHC molecules on the surfaces of donor cells. Indirect allorecognition occurs when donor histocompatibility molecules are internalized, processed, and presented as peptides by host APCs. In order to determine the mechanism for the acceptance of liver allografts, both pathways should be taken into account. In

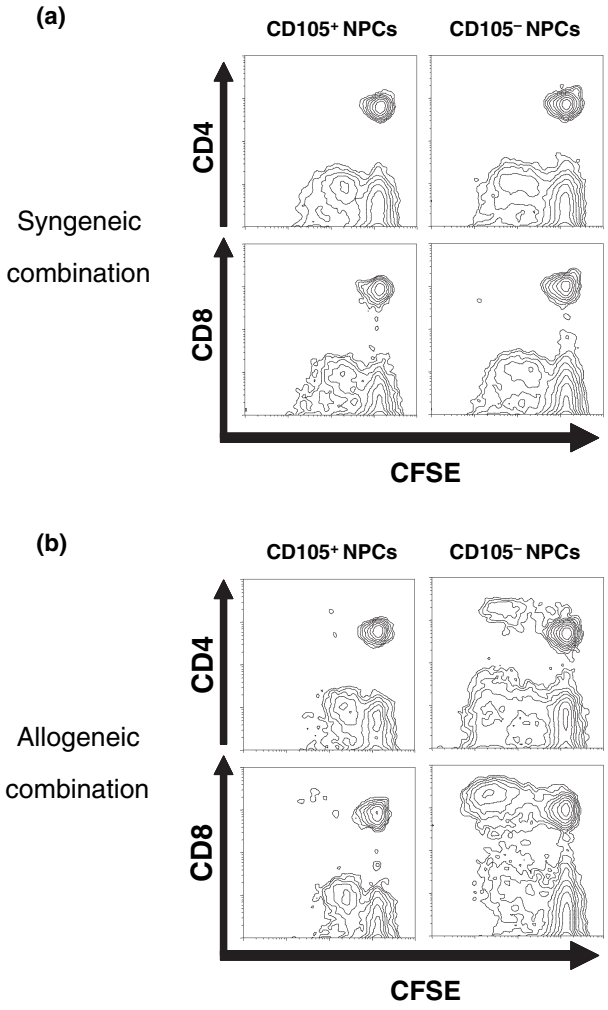

Figure 5 $CD105^-$ nonparenchymal cells (NPCs) (liver sinusoidal endothelial cells, LSEC-depleted NPCs) induced marked proliferation of both $CD4^+$ and $CD8^+$ alloreactive T cells, but $CD105^+$ NPCs (LSECs) did not. Irradiated $CD105^+$ NPCs or $CD105^-$ NPCs, which were prepared from B6 and Balb/c mice, as shown in Fig. 1, were co-cultured with fluorescent dye (CFSE)-labeled responder splenocytes from B6 mice (mixed hepatic constituent cell-lymphocyte reaction, MHLR) in a syngeneic combination (a) and an allogeneic combination (b). After the MHLR, harvested lymphocytes were stained with either phycoerythin (PE)-conjugated anti-CD4 monoclonal antibody (mAb) or anti-CD8 mAb. Then, by FCM analysis, T-cell proliferation (division) was visualized as serial halving of the fluorescence intensity of CFSE. Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining. FCM profiles shown are representative of three independent experiments.

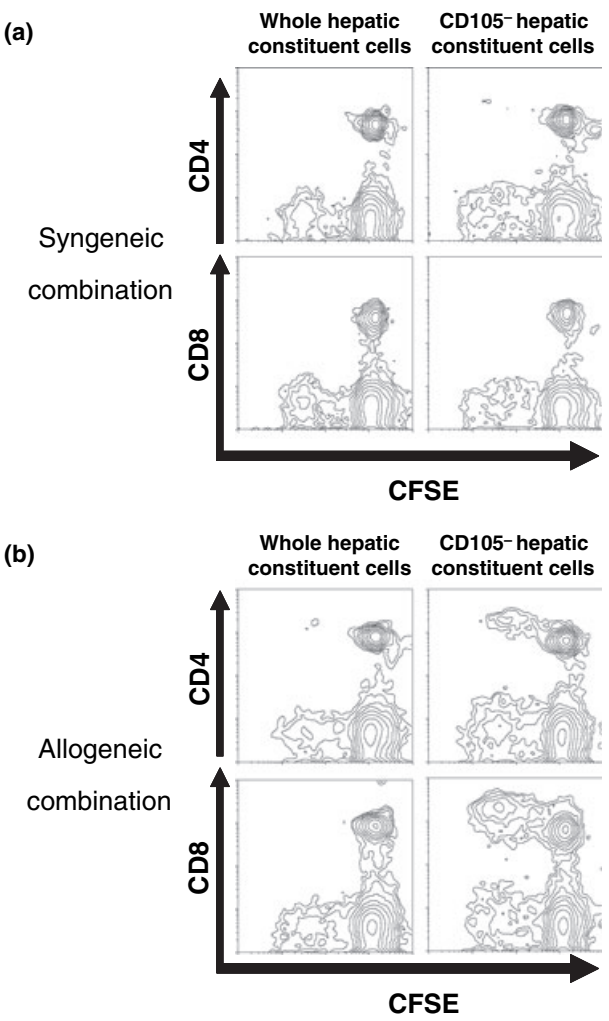

Figure 6 Depletion of $CD105^+$ cells from whole hepatic constituent cells facilitated proliferation of alloreactive T cells. Whole hepatic constituent cells were stained with biotin-conjugated anti-CD105 monoclonal antibodies (mAbs) and streptavidin microbeads and then magnetically sorted to deplete $CD105^+$ liver sinusoidal endothelial cells (LSECs). Either irradiated whole hepatic constituent cells or $CD105^-$ hepatic constituent cells were co-cultured with co-cultured with fluorescent dye (CFSE)-labeled responder splenocytes (mixed hepatic constituent cell-lymphocyte reaction, MHLR) in a syngeneic combination (a) and an allogeneic combination (b). After the MHLR, harvested lymphocytes were stained with either phycoerythin (PE)-conjugated anti-CD4 mAb or anti-CD8 mAb. Then, by FCM analysis, T-cell proliferation (division) was visualized as serial halving of the fluorescence intensity of CFSE. Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining. FCM profiles shown are representative of three independent experiments.

our MHLR assay, T-cell proliferation was induced in response to allogeneic hepatic constituent cells predominantly through the direct pathway, as results obtained by using of purified T cells as responders for the allogeneic MHLR were the same as those obtained from the allogeneic MHLR using unfractionated splenocyte responders (data not shown). Therefore, the MHLR assay might be a useful tool to investigate the mechanism for tolerizing T cells with direct allospecificity after transplantation of liver allografts.

The APCs expressing MHC class II molecules in liver allografts should play a significant role in tolerizing T cells with direct allospecificity. The LSEC has been described as a new type of APC that induces immune tolerance in naive T cells in the context of both MHC-I and MHC-II restriction [25,26,33,34]. LSECs are capable of even stimulating naive $CD4^+$ T cells. However, following priming by antigen-presenting LSECs, $CD4^+$ T cells fail subsequently to differentiate toward a Th1 phenotype but, instead, become regulatory T cells, expressing interleukin (IL)-4 and IL-10 upon re-stimulation [25]. LSECs also have the capacity to present exogenous antigens on MHC class I molecules to $CD8^+$ T cells, a process termed cross-presentation [26]. Previously believed to occur exclusively in myeloid APCs, cross-presentation in LSECs occurs with extraordinary efficiency, requiring only minute amounts of an antigen and occurring within 60–120 min after exposure to the antigen. Stimulation of naive $CD8^+$ T cells by LSECs results first in the proliferation of T cells and the release of cytokines but finally leads to antigen-specific tolerance, as demonstrated by a loss of cytokine expression at the same time as the failure of $CD8^+$ T cells to develop into cytotoxic effector T cells. Despite such progress in elucidation of immune functions of LSECs to autologus T cells, it has not been determined whether LSECs tolerize allogeneic T cells through the direct pathway. We here for the first time demonstrated that LSECs have the capacity to induce nonresponsiveness of alloreactive T cells across MHC barriers.

In view of the anatomical features of the liver, circulating host leukocytes are forced into contact with LSECs in the liver allograft owing to the small diameters of the sinusoids. The cumulative surface area of LSECs is very large, and hepatic microcirculatory parameters allow for frequent contact between LSECs and circulating host leukocytes. Given the large volume of blood passing daily through the liver, it seems probable that LSECs are ideally positioned within the liver allografts to establish immune tolerance. At present, it is not clear how LSECs in the liver allograft tolerized host T cells with direct specificity. Since LSECs resemble immature dendritic cells (DCs) but are resistant to maturation, the LSECs might have insufficient expression of co-stimulatory molecules, resulting in their incapability to generate T-cell immunity. This possibility is currently under investigation.

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