Liver Sinusoidal Endothelial Cells Tolerize T Cells across MHC Barriers in Mice

Takashi Onoe, Hideki Ohdan, Daisuke Tokita, Masayuki Shishida, Yuka Tanaka, Hidetaka Hara, Wendy Zhou, Kohei Ishiyama, Hiroshi Mitsuta, Kentaro Ide and Toshimasa Asahara

*J Immunol* 2005; 175:139-146; doi: 10.4049/jimmunol.175.1.139
http://www.jimmunol.org/content/175/1/139

---

**References**
This article cites 54 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/175/1/139.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Liver Sinusoidal Endothelial Cells Tolerize T Cells across MHC Barriers in Mice

Takashi Onoe, Hideki Ohdan, Daisuke Tokita, Masayuki Shishida, Yuka Tanaka, Hidetaka Hara, Wendy Zhou, Kohei Ishiyama, Hiroshi Mitsuta, Kentaro Ide, and Toshimasa Asahara

Although livers transplanted across MHC barriers in mice are normally accepted without recipient immune suppression, the underlying mechanisms remain to be clarified. To investigate the cell type that contributes to induction of such a tolerance state, we established a mixed hepatic constituent cell-lymphocyte reaction (MHLR) assay. Irradiated C57BL/6 (B6) or BALB/c mouse hepatic constituent cells (HCs) and CFSE-labeled B6 splenocytes were cocultured. In allogeneic MHLR, whole HCs did not promote T cell proliferation. When liver sinusoidal endothelial cells (LSECs) were depleted from HC stimulators, allogeneic MHLR resulted in marked proliferation of reactive CD4+ and CD8+ T cells. To test the tolerizing capacity of the LSECs toward alloreactive T cells, B6 splenocytes that had transmigrated through monolayers of B6, BALB/c, or SJL/j LSECs were restimulated with irradiated BALB/c spleenocytes. Nonresponsiveness of T cells that had transmigrated through allogeneic BALB/c LSECs and marked proliferation of T cells transmigrated through syngeneic B6 or third-party SJL/j LSECs were observed after restimulation. Transmigration across the Fas ligand-deficient BALB/c LSECs failed to render CD4+ T cells tolerant. Thus, we demonstrate that Fas ligand expressed on naive LSECs can impart tolerogenic potential upon alloantigen recognition via the direct pathway. This presents a novel relevant mechanism of liver allograft tolerance. In conclusion, LSECs are capable of regulating a polyclonal population of T cells with direct allospecificity, and the Fas/Fas ligand pathway is involved in such LSEC-mediated T cell regulation. *The Journal of Immunology, 2005, 175: 139–146.*

Liver allografts are extraordinarily tolerogenic, and stable grafts can be maintained without immunosuppression in some species (1–3). In addition, the presence of a liver allograft can suppress the rejection of other solid tissue grafts from the same donor (4, 5).

The high capacity of the transplanted liver to establish tolerance in an allogeneic host has been attributed to the unique features and architecture of hepatic constituent cells (HCs). The abundant release of allogeneic (donor-type) MHC class I molecules from the liver into the blood (6), the emigration of mobile and surviving passenger lymphocytes and phagocytes from the donor liver (thereby creating microchimerism with a high zone tolerance effect) (7), the regulatory properties of the prevalent NKT cell subset in the liver (8), and the termination of immune responses by intrathoracic entrapment and deletion of activated T cells by resident APCs (9) are some of the multiple factors that are likely to be involved in the tolerogenicity of liver allografts. However, the details of each mechanism remain to be elucidated.

It is generally accepted that the immunogenicity of solid organ allografts is due to the presence of APCs expressing MHC class II within the grafts (10). MHC molecules on these cells are directly recognized by the host CD4+ T cells, which become activated and produce various cytokines that initiate a cascade of alloimmune events. Liver dendritic cells (LDCs) have abnormal APC properties and a low expression of costimulatory molecules, and they preferentially induce Th2 responses, suggesting a dendritic cell-mediated tolerogenicity (11, 12). In contrast, Kupffer cells (KCs), which are also resident APCs in the liver, participate in rejection, rather than tolerance, induction (13).

In addition to LDCs and KCs, liver sinusoidal endothelial cells (LSECs), which constitute the lining of the hepatic sinusoid, are also able to present soluble exogenous Ags to T cells with transgenic T cell receptors (14–17). Although a number of studies have demonstrated the importance of Ag presentation by LSECs for liver allograft tolerance, the role of direct alloantigen presentation by LSECs in such tolerance has not been extensively investigated.

In the present study we investigated the tolerogenicity of LSECs in mice in which liver allografts are normally accepted without recipient immune suppression across MHC barriers. Through the use of a mixed hepatic constituent cell-lymphocyte reaction (MHLR) assay and a transendothelial migration assay, we have demonstrated a novel and surprising effect of LSECs, i.e., naive allogeneic LSECs selectively render reactive CD4+ and CD8+ T cells tolerant at least in part via the Fas/Fas ligand (FasL) pathway. This result provides the first demonstration that LSECs are capable of regulating a polyclonal population of T cells with certain specificity through direct Ag recognition.

---

Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Received for publication December 17, 2004. Accepted for publication April 19, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by a Grant-in-Aid for the Creation of Innovations through the Business-Academic-Public Sector Cooperation of Japan and by the Uehara Memorial Foundation.

2 Address correspondence and reprint requests to Dr. Hideki Ohdan, Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-Ku, 734-8551 Hiroshima, Japan. E-mail address: hohdan@hiroshima-u.ac.jp

3 Abbreviations used in this paper: HC, hepatic constituent cell; 7-AAD, 7-aminoactinomycin D; Ac-LDL, acetylated low density lipoprotein; BM, bone marrow; FasL, Fas ligand; FCM, flow cytometry; KC, Kupffer cell; LDC, liver dendritic cell; LNPC, liver nonparenchymal cell; LSEC, liver sinusoidal endothelial cell; MHLR, mixed hepatic constituent cell-lymphocyte reaction; PI, propidium iodide; SA, streptavidin; PD-L1, programmed death ligand-1.
Materials and Methods

Animals

Eight- to 12-wk-old female wild-type C57BL/6 (B6, H-2 b), BALB/c (BALB/c, H-2 d), and SJL/Jorllco (SJL/j, H-2 s) mice were purchased from The Jackson Laboratory. Age-matched (8- to 12-wk-old) mice were used for the experiments. All animals were maintained under pathogen-free conditions and in compliance with national and institutional ethics committee.

Immunohistochemistry

To detect CD105 expressed on LSECs, we fixed murine liver cryosections with formaldehyde and preincubated them with normal rabbit serum to block nonspecific binding. Sections were incubated with anti-CD105-biotin mAb for 1 h at room temperature. Biotinylated mAbs were visualized by a standard avidin-biotin-alkaline phosphatase method.

Isolation of LSECs, whole HCs, and LSEC-depleted HCs

Whole HCs were isolated using the collagenase perfusion technique, as previously described (18, 19). Liver nonparenchymal cells (LNPCs) containing LSECs and KCs were separated from whole HCs by low speed centrifugation, as previously described (18). LNPCs were pre-incubated with FeγR-blocking mAb (2.4G2; BD Pharmingen) and incubated with anti-CD105-biotin mAb (M17/18; ebioscience) and streptavidin (SA) microparticles (Miltenyi Biotech). The LSECs were then isolated from LNPCs by positive selection using auto MACs (Miltenyi Biotec). The purity of LSECs was confirmed by phase contrast microscopy and flow cytometry (FCM) after culture for 12 h in collagen I-coated dishes with Bodipy-labeled acetylated low density lipoprotein (Ac-LDL). LSEC-depleted HCs were obtained directly from whole HCs by negative selection using anti-CD105-biotin mAb, as described above. The obtained whole HCs, LSECs, and LSEC-depleted HCs were used as stimulators in the MHLR.

Construction of bone marrow chimeras

Bone marrow (BM) transplants were performed from B6 mice to BALB/c mice, as previously described (20). In brief, BALB/c recipients were lethally irradiated (13 Gy) and injected with T cell-depleted BM cells (10 × 10^6 cells/mouse) from B6 mice. T cell depletion was performed by MACS using biotin-conjugated anti-mouse CD4 and CD8 mAbs and streptavidin-conjugated microbeads (Miltenyi Biotec). BM chimeras were used at least 90 days after the BM transplant.

In vitro MHLR

For MHLR using a [3H]thymidine technique, fractions of whole HCs and LSEC-depleted HCs were prepared from B6 and BALB/c mice as described above. After each fraction had been irradiated (30 Gy) for use as stimulator cells, 4 × 10^6 naive splenocytes isolated from B6 mice were cocultured with 10^5 whole HCs or LSEC-depleted HCs. Cells were incubated in 24-well, flat-bottom plates (BD Labware) in culture medium at 37°C with 5% CO_2 for 5 days, including a final 12-h pulse with [3H]thymidine (10 μCi/well). Cells were harvested, and the amount of [3H]thymidine was measured using a scintillation counter.

For MHLR using a CFSE-labeling technique (CFSE-MHLR), naive splenocytes from B6 mice were labeled with 5 μM CFSE (Molecular Probes), as previously described (21), and resuspended in culture medium as responder cells. Fractions of whole HCs, LSEC-depleted HCs, and isolated LSECs were prepared from B6, BALB/c, and BM chimeric mice. After each fraction had been irradiated (30 Gy) for use as stimulator cells, the stimulator (0.8 × 10^6 cells) and responder (4 × 10^6 cells) cells were cocultured in 24-well, flat-bottom plates at 37°C in a 5% CO_2 incubator in the dark for 5 days. In some experiments isolated LSECs were returned to the LSEC-depleted MHLR culture wells using culture inserts that contained polycarbonate filters of 0.45 μm pore size (Kurabou) to separate LSECs from MHLR. Control inserts contained an equivalent volume of medium without isolated LSECs.

FCM analyses

The following reagents were used for surface staining: anti-CD4-PE (GK1.5), anti-CD8-PE (53-6.7), anti-CD11b-PE (M1/70), anti-CD11c-PE (HL-60/ 

mAbs were purchased from eBioscience. All analyses were performed on a FACSCalibur (BD Biosciences). Non-specific FcγR blocking of labeled mAbs was blocked by 2.4G2. Dead cells were excluded from the analysis by forward scatter and propidium iodide (PI). To detect T cell apoptosis in some experiments, annexin V-allophycocyanin and 7-aminooctacyclin D (7-AAD) (BD Pharmingen) stainings were performed in accordance with the manufacturer’s instructions.

Quantification of T cell proliferation

Stimulation indexes as alloreactivities of responder CD4+ and CD8+ T cells were quantified by their CFSE fluorescence intensities, modified as previously described (22, 23). On CFSE fluorescence histograms, CD4+ and CD8+ T cells were selected by gating and were analyzed for CFSE fluorescence. Theoretically, the CFSE fluorescence intensity of cells that have divided once shows half the value of CFSE fluorescence intensity of non-divided cells. According to this theory, the number of divisions of allogeneic T cells could be mathematically determined by the logarithm of CFSE intensities on the basis of the peak at the extreme right (the peak of undivided cells). The limit of detection is eight division cycles caused by the compression of peaks as the CFSE intensity approaches autofluorescence levels. Thus, the numbers of divisions beyond seven cycles are indistinguishable and are collectively referred to as division 7+. A single cell dividing n times will generate 2^n daughter cells. Using this mathematical relationship, the number of division precursors was extrapolated from the number of daughter cells of each division, and mitotic events were calculated in each CD4+ and CD8+ T cell subset. Using these values, mitotic indexes were calculated by dividing the total mitotic events by the total precursors. Stimulation indexes of allogeneic combinations were calculated by dividing the mitotic indexes of allogeneic combination by those of the control syngeneic combination.

Transendothelial migration assay

The transmigration experiments were conducted as follows. LSECs (1 × 10^6 cells/200 μl) were grown on culture inserts containing polycarbonate filters, with a pore size of 8 μm (Costar), precoated with 100 μl of recombinant human fibronectin (50 μg/ml; Sigma-Aldrich), and left overnight to form a monolayer. Monolayers were rinsed, and the inserts were transferred into wells of 24-well plates (800 μl/well). CFSE-labeled, nonadherent lymphocytes (10 × 10^6 cells/200 μl) from B6 mice were added to each well and were left for 12 h to migrate through the monolayer. The migrated lymphocytes were subsequently collected from the lower chambers. Control inserts contained an equivalent number of lymphocytes. These transmigrated B6 lymphocytes were cocultured with irradiated (30 Gy) BALB/c splenocytes in a total volume of 2 ml of medium in 24-well, flat-bottom plates (BD Biosciences) for 5 days (subsequent MLR).

Statistical analysis

Statistical analysis among experimental groups was performed by ANOVA, and Tukey’s test was used to compare individual groups. A value of p < 0.05 was considered statistically significant.

Results

Naive LSECs constitutively express all molecules necessary for Ag presentation, but do not induce proliferation of allogeneic T cells

To characterize the phenotype of naive LSECs in mice, we first developed a method to isolate LSECs. As observed by immuno-histochemical studies, LSECs highly express CD105 molecules (endoglin), which have been used as a marker for vessel endothelia (24, 25), compared with the endothelia of central veins or other vessels in the liver (Fig. 1A). Therefore, CD105- cells were positively selected by MACS to isolate LSECs from the LNPC fraction of disaggregated HCs. The mean yield of CD105- sorted cells from a mouse liver was 3.4 × 10^6 ± 8.5 × 10^6 cells (n = 10). The appearance of CD105- sorted cells was consistent with that of LSECs (Fig. 1B). To analyze the purity of LSECs, we cultured aliquots of the sorted fractions in the presence of Ac-LDL-Bodipy (this fluorescence-labeled lipoprotein is exclusively taken up by endothelial cells, such as LSECs). The CD105- sorted cells always contained >95% of CD11b+ cells that had taken up Ac-LDL-Bodipy, which represented the LSECs, and were not contaminated with CD45 (leukocyte common Ag)-positive cells.
LSECs exclusively express CD105 molecules and all molecules necessary for Ag presentation. A, CD105 molecule expression on LSECs in normal murine livers (original magnification: left, ×100; middle, ×200; right, ×400). Cryosections of normal murine liver were immunostained with anti-CD105 mAb (red). In normal liver tissue, LSECs express CD105 molecules, whereas endothelia of central veins do not. CV, central vein. B, Phase contrast image of CD105+ LNPCs separated from whole HCs by MACS. CD105+ LNPCs were cultured on collagen-coated dishes to confirm morphological character. The cells are characterized by their polygonal shape and cobbledstone appearance. Scale bar indicates 100 μm. C, Representative FCM results of sorted CD105+ LNPCs. Sorted CD105+ LNPCs were stained for the expression of CD45 as a marker for hemopoietic cells (right panel), were cultured and analyzed for purity in the presence of Ac-LDL-Bodipy (this fluorescence-labeled lipoprotein is exclusively taken up by endothelial cells such as LSECs), and were stained for the expression of CD11b as a marker for Kupffer cells (left panel). The percentages shown are of total sorted CD105+ LNPCs. The FCM profiles shown are representative of two independent experiments. D, Freshly isolated LSECs constitutively express all molecules necessary for Ag presentation and FasL.

LSECs inhibit proliferation, whereas hepatic BM-derived cells promote proliferation of alloreactive T cells

The inability of LSECs to induce proliferation of allogenetic T cells might be merely due to their poor alloantigen-presenting capacity or might be a consequence of their tolerogenic capacity. To address these possibilities, we established an MHLR assay in which irradiated HCs and CFSE-labeled splenocytes were cocultured. In the syngeneic combination (B6 vs B6), T cell proliferation was undetectable, as expected (Fig. 3, A and E). Even in the fully allogeneic combination (BALB/c vs B6), the MHLR assay revealed minimal proliferation of both allogeneic CD4+ and CD8+ T cells (Fig. 3, B and E). When LSECs were depleted from whole HC stimulators, the MHLR assay resulted in a marked proliferation of both alloreactive CD4+ and CD8+ T cells (Fig. 3, F and E), indicating that LSECs might have the capacity to inhibit the proliferation of alloreactive T cells. Consistent results were observed in an MHLR assay using the [3H]thymidine incorporation technique, although this conventional method did not allow phenotypic analysis of proliferating T cells and was less sensitive than the CFSE-MHLR (26) (Fig. 3C). These results also indicate that an immunogen population promoting allogeneic responses should be included in the LSEC-depleted HC fraction. To investigate this issue, we created BM chimeras by lethally irradiating BALB/c mice (H-2b) and reconstituting them with BM from B6 mice (H-2b). Thus, we refer to BALB/c mice receiving B6 BM as BALB/c(B6). The chimeric mouse livers expressed H-2d on tissue parenchyma and endothelial cells and expressed H-2b on hemopoietic resident cells. Even in the case where LSECs were depleted from whole HCs, the MHLR assay, using BALB/c(B6) HCs as stimulators and B6 splenocytes as responders, resulted in limited proliferation of both alloreactive CD4+ and CD8+ T cells (Fig. 3, D).
LSECs inhibit the proliferation of alloreactive T cells, and cell-cell contact is necessary for their inhibitory effects. Stimulation indexes of alloreactive T cells are shown. CFSE-labeled splenocytes from B6 mice were cocultured with irradiated LSEC-depleted HCs from BALB/c mice in the absence (control; □) or the presence of increasing numbers of LSECs from BALB/c mice (○ and ●). Isolated LSECs were returned to allocigenic LSEC-depleted MHLR with (●; n = 5) or without (○; n = 5) cell contact. The mean ± SEM of five independent experiments are shown. *p < 0.05 vs control.

HCs from B6 (A) and BALB/c (B) as stimulators are shown. C, Mean proliferation ([3H]thymidine incorporation) of splenocytes isolated from B6 in MHLR are shown. Whole HCs (n = 4) from B6 (syngeneic) mice and whole HCs (n = 4) or LSEC-depleted HCs (n = 4) from BALB/c (alloigenic) mice as stimulators were used. [3H]Thymidine incorporation of splenocytes from B6 mice was estimated with a scintillation counter as described in Materials and Methods. The mean ± SEM of four independent experiments are shown. **p < 0.01 vs all groups. D, Representative FCM results of the MHLR using CFSE-labeled splenocytes from B6 mice as responders and irradiated whole HCs or LSEC-depleted HCs from BALB/c(B6) BM chimeric mice as stimulators are shown. LSECs were depleted from whole HCs by magnetic cell sorting, as described in Materials and Methods. E, Stimulation indexes of alloreactive T cells in MHLR are shown. Whole HCs (□; n = 8) or LSEC-depleted HCs (●; n = 8) from B6, BALB/c, or BALB/c(B6) BM chimeric mice as stimulators were used. Stimulation indexes of alloreactive T cells were estimated by FCM analysis of CFSE intensity, as described in Materials and Methods. The mean ± SEM of eight independent experiments are shown. **p < 0.01 vs all groups.
we returned the isolated LSECs to the LSEC-depleted MHLR culture wells with or without cell-cell contact using a Transwell culture system. When cell-cell contact occurred, LSECs inhibited the proliferation of alloreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, whereas the physical separation of LSECs from the responder and stimulator cells in the MHLR abrogated the LSEC-induced inhibitory effects on alloreactive T cell proliferation (Fig. 4). Even in the presence of cell-cell contact, when MHC class II on the isolated LSECs were blocked with anti-I-A/I-E mAb before the MHLR culture, LSEC-induced inhibitory effects on alloreactive T cell proliferation were also abrogated (data not shown). These results indicate that the alloreactive T cells are tolerized via direct recognition of MHC class II expressed on LSECs. Interestingly, the enhanced proliferation of both CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells was observed in the contact-independent condition compared with LSEC-depleted conditions (Fig. 4). It has been previously reported that LSECs have the capacity to produce proinflammatory cytokines and chemokines, including IL-1, IL-6, TNF-α, and interferon-γ inducible protein-10 (27–29), which can promote T cell activation/proliferation. Such soluble mediators that can pass through the Transwell membrane may enhance the proliferation of alloreactive T cells in the absence of contact-dependent, LSEC-induced inhibition.

**LSECs induced apoptosis of alloreactive T cells in the allogeneic MHLR**

Several groups have reported that T cells undergo apoptosis in liver allografts (30–32). Based on this fact and the FasL expression on LSECs (Fig. 1D), we assumed that LSECs inhibited alloreactive T cell proliferation via apoptosis. To investigate this possibility, the cells harvested after allogeneic MHLR culture, using whole HC stimulators, including LSECs, were stained with annexin V to detect apoptotic cells along with 7-AAD to exclude necrotic cells (33, 34). Four-color FCM revealed that the proliferation of 7-AAD-negative alloreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells was detectable (Fig. 5). These proliferating T cells during the early stage of cell division were bound to annexin V with a higher frequency compared with the alloreactive T cells proliferating in response to LSEC-depleted HCs (Fig. 5, A and B). These findings are consistent with a model in which the apoptosis of alloreactive T cells activated in response to LSECs is a critical event in the induction of liver allograft tolerance.

In the FCM shown in Fig. 3, demonstrating that LSECs inhibit the proliferation of alloreactive T cells, PI was used for the exclusion of nonviable cells. Only PI-negative viable cells were subjected to additional analyses, so that proliferating apoptotic cells as well as necrotic cells would be excluded from the analyses. Hence, proliferation was not observed in Fig. 3.

**Fas/FasL signal plays a significant role in specific tolerance among alloreactive T cells induced by transmigration across naive allogeneic LSECs**

Blood passes through the liver in a meshwork of sinusoids formed by LSECs. Circulating leukocytes are forced into frequent contact with LSECs due to the small diameter (7–12 μm) of the sinusoids (35). To investigate the possibility that the sinusoidal architecture promotes the immunomodulatory activity of LSECs toward alloreactive T cells, we performed a T cell transendothelial migration assay to mimic the anatomical features of the interaction between LSECs and T cells (Fig. 6A). Nonadherent B6 lymphocytes that had transmigrated through a monolayer (with pores 8 μm in diameter) of B6, BALB/c, or SJL/J LSECs were subsequently stimulated with irradiated BALB/c splenocytes. Nonresponsiveness of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells transmigrated through allogeneic BALB/c LSECs and marked proliferation of those T cells transmigrated through syngeneic B6 and third-party SJL/J LSECs were observed upon the subsequent stimulation (Fig. 6, B and C). The transmigration of B6 T cells (in particular, CD4<sup>+</sup> T cells) across
the FasL-deficient BALB/c (BALB/c-gld) LSECs failed to induce such nonresponsiveness on subsequent stimulation with the irradiated BALB/c splenocytes, including professional APCs. Thus, T cells that transmigrated across the allogeneic LSECs were rendered tolerant to alloantigens at least in part via the Fas/FasL pathways.

Discussion

Although the immunomodulatory activity of LSECs is currently noticed, even its phenotypic property remains controversial. The conflicting results regarding the expression of the molecules necessary for Ag presentation (CD80, CD86, CD40, and MHC classes I and II) on LSECs have been reported (36, 37). This may be attributed to the difference in the methods of preparation of LSECs in the previous studies, i.e., contamination with other cells or degeneration of surface molecules by excessive enzymatic digestion, or in vitro subculture for purity possibly affects their phenotypic profile results. Because the specific surface marker for LSECs remains to be defined, previous methods of LSEC isolation do not directly confirm the presence of LSECs with regard to their anatomical distribution (16, 37, 38). Instead of the previously reported methods, i.e., counterflow elutriation (16, 38) or negative selection using mAbs against molecules that are not expressed on LSECs (37), we used positive selection of CD105/H11001 cells using MACS to isolate LSECs. CD105 is known to be predominantly expressed on endothelial cells (25, 39–41), and its promoter is strongly and selectively active in endothelial cells (42, 43). Elevated levels of CD105 expression were detected on microvascular endothelium and on vascular endothelial cells in regenerating tissue undergoing active angiogenesis (39, 40, 44). Similarly, LSECs, which have an exceptional capacity for angiogenesis, highly expressed CD105 compared with endothelia of other vessels in the livers, as observed in the present immunohistochemical studies (Fig. 1A). We cannot rule out the possibility that the CD105+/H11001 cell fraction isolated from livers contaminates with primitive hemopoietic cells, which has been also shown to express CD105 Ags (45). However, we confirmed complete absence of contamination with CD45+ hemopoietic cells, which might have the capacity for Ag presentation, in the isolated CD105+ cells, indicating the absence of hemopoietic APCs (Fig. 1C). The freshly isolated CD105+ cells expressed MHC class II, CD40, CD80, and CD86, reflecting their potential role as APCs (Fig. 1D). A symmetrical histogram with a single peak obtained in the analysis of the expression of such molecules on isolated CD105+ cells proved minimal contamination with cells other than LSECs.

It has been reported that naive CD4+ T cells primed by Ag-presenting LSECs (MHC class II-restricted presentation of soluble Ags) fail to differentiate toward the effector Th1 cells, but express high levels of immune-suppressive mediators (IL-4 and IL-10) (16). It has also been reported that the stimulation of naive CD8+ T cells by LSECs presenting exogenous Ags (cross-presentation) results first in the proliferation of T cells and the release of cytokines and finally leads to Ag-specific tolerance, as demonstrated by a loss of cytokine expression simultaneously with the failure of migrated lymphocytes to coculture with irradiated (30 Gy) splenocytes from BALB/c mice in subsequent MLR. B, Representative FCM results of CFSE-labeled CD4+ and CD8+ T cell division in the subsequent MLR are shown. LSECs from B6, BALB/c, BALB/c-gld, or SJL/j mice were used to form a monolayer. C, Stimulation indexes of alloreactive T cells in the subsequent MLR using transmigrated B6 lymphocytes as responder and irradiated BALB/c splenocytes as stimulator are shown. The mean ± SEM of four independent experiments are shown. *, p < 0.05; **, p < 0.01.
CD8⁺ T cells to develop into cytotoxic effector T cells. 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 class I and II restriction (17, 36, 38, 46). However, such immune regulatory effects of LSECs have been observed only in a model in which the interaction of soluble exogenous Ags and respective transgenic T cell receptors takes place; thus, the capacity of LSECs to regulate a polyclonal population of nontransgenic T cells with allelogeneic specificity remained to be elucidated. In addition, despite the progress in elucidation of immune functions of LSECs to autologous T cells, the manner in which host T cells respond to allelogeneic LSECs in liver allografts has not been determined. In the present study, LSECs failed to induce the proliferation of both allelogeneic CD4⁺ and CD8⁺ T cells (Fig. 2). The inability of LSECs to induce the proliferation of allelogeneic T cells was not merely due to their poor Ag-presenting capacity, but was a consequence of their tolerogenic capacity, as proven by results showing that the presence of LSECs in an allelogeneic MHLR assay led to inhibition of alloreactive T cells, and T cells transmigrated across the allelogeneic LSECs were rendered tolerant to subsequent stimulation with alloantigens. In the presence of LSECs, the cytokine analyses of the allelogeneic MHLR supernatants revealed a remarkable reduction of IL-2 and IFN-γ, rather than the increase in IL-4 and IL-10 (data not shown), suggesting that the tolerogenicity of LSECs toward alloreactive T cells is not predominantly due to the release of such immune-suppressive cytokines. As shown in Fig. 4, the proliferation of allelogeneic CD4⁺ and CD8⁺ T cells was inhibited when in contact with LSECs, whereas the physical separation of LSECs using a dual chamber Transwell culture system abrogated the LSEC-induced inhibitory effects on allelogeneic T cell proliferation. The inhibition of CD4⁺ T cells in contact with LSECs was less significant compared with that of CD8⁺ T cells. The inhibition of allelogeneic CD4⁺ to the same degree as that of CD8⁺ T cells apparently requires a greater number of LSECs. Such a difference between allelogeneic CD4⁺ and CD8⁺ T cells in susceptibility to inhibition when in contact with LSECs might be caused by the differences in their LSEC-induced inhibition mechanisms. Using FasL-deficient mice, we have demonstrated that the deficiency of FasL on LSECs significantly abrogated their tolerogenicity toward CD4⁺ T cells, but only partially toward CD8⁺ T cells (Fig. 6, B and C), suggesting that FasL on LSECs is more important for the inhibition of CD4⁺ T cells than that of CD8⁺ T cells. Consistently, it has been reported that FasL engagement inhibits CD4⁺ T cell proliferation, cell cycle progression, and IL-2 secretion, but CD8⁺ T cells are inherently resistant to the FasL-mediated regulation (47, 48). Mechanisms other than the Fas/FasL pathway would also be involved in the LSEC-induced tolerance of allelogeneic CD8⁺ T cells. It has been recently reported that LSECs constitutively expressed programmed death ligand-1 (PD-L1) (the ligand for the immunoinhibitory receptor PD-1) and inhibited the proliferation of effector T cells expressing PD-1 (49). There is a possibility that the PD-1/PD-L1 pathway also plays an important role in the LSEC-induced tolerance of allelogeneic CD8⁺ T cells. Additional studies are required to address this possibility.

Nevertheless, our results indicating the critical role of FasL on LSECs in liver allograft-induced T cell tolerance are consistent with two previously reported findings: 1) that extensive apoptosis of infiltrating cells was observed in the portal areas of liver allografts (30); and 2) that liver allografts from FasL-deficient mice were eventually rejected, whereas those from wild-type mice were spontaneously accepted (50). FasL-mediated tolerization by LSECs resembles the previously reported model in which FasL present in nonlymphoid tissue deletes reactive lymphoid cells during viral infections and is responsible for protecting immune-privileged sites from cellular immune-mediated damage (51, 52). The constitutive expression of FasL on LSECs might be induced by the unique liver microenvironment, i.e., endotoxin, as a physiological constituent of portal venous blood, induces release of the anti-inflammatory mediators from various liver resident hemopoietic cells (i.e., intrahepatic macrophage, dendritic, NK, and NKT cells), which, in turn, may promote FasL on naive LSECs (53). Such a hypothesis is consistent with the earlier findings of hepatic transplantation tolerance being due to a radioresponsive population that include intrahepatic hemopoietic cells (54). Elucidation of the immune mechanism underlying tolerogenicity of LSECs might lead to the establishment of novel means to promote acceptance even of transplant of organs other than liver.

Acknowledgments
We thank Drs. Hirotaka Tashiro, Makoto Ochi, and Yasuhiro Fudaba for their helpful comments.

Disclosures
The authors have no financial conflict of interest.

References


