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ORIGINAL ARTICLE

Liver sinusoidal endothelial cells contribute to alloreactive T-cell tolerance induced by portal venous injection of donor splenocytes

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Summary

We demonstrated that an indirect pathway of alloantigen presentation via liver sinusoidal endothelial cells (LSEC) is involved in alloreactive T-cell tolerance induced by portal venous injection (PI) of donor cells. Thirty million C57BL/6 (B6) splenocytes that were either untreated or treated with 30-Gy irradiation were injected via the portal vein into Balb/c mice. Host LSEC expressing major histocompatibility complex class II actively endocytosed the allogeneic naive splenocytes as well as irradiated splenocytes after PI. Using a transendothelial migration assay, it was demonstrated that host-type Balb/c $CD4^+$ T cells that transmigrated across LSEC that had captured irradiated B6 splenocytes were rendered tolerant to subsequent alloantigen presentation by host professional antigen-presenting cells. Consistently, PI of irradiated donor-type splenocytes led to remarkable prolongation of the survival of subsequently transplanted heart allografts. These results indicate that indirect antigen presentation by LSEC significantly contributes to alloreactive T-cell tolerance induced by PI of irradiated donor splenocytes.

Introduction

It has been postulated that the liver might be a site for induction of tolerance to exogenous major histocompatibility complex (MHC) class II-restricted antigens (food) entering the organ in large amounts via portal circulation from the gut [1,2]. Such a phenomenon has been used as a basis for a strategy designed to achieve the ultimate goal in transplantation, the induction of tolerance of T cells with allospecificity, i.e. portal venous injection (PI) of allogeneic cells has been reported to induce donor-specific tolerance across MHC and minor histocompatibility complex barriers. Various possible mechanisms underlying PI-induced tolerance have been proposed: clonal deletion [3–5], induction of anergy and generation of regulatory cells [5–9], and regulation of cytokine production [10,11]. However, the cellular basis of this immune tolerance is not fully understood.

Portal venous injection-induced tolerance must be a consequence of presentation of allogeneic antigens (Ag) to T cells in the liver. The ability to present exogenous Ag on MHC class I or II molecules is restricted to Kupffer cells and dendritic cells in the liver. The importance of antigen presentation by Kupffer cells in PI-induced tolerance is emphasized by the findings of prevention of antigen sequestration and prevention of tolerance following administration of gadolinium chloride (a rare earth metal that prevents Kupffer cell phagocytosis) [12,13]. Liver dendritic cells have abnormal antigen-presenting properties and low expression levels of co-stimulatory molecules, and they preferentially induce T-helper 2 (Th2) responses, suggesting that these cells mediate tolerogenicity [14,15]. In addition to Kupffer cells and liver dendritic cells, liver sinusoidal endothelial cells (LSEC), which constitute the lining or wall of the hepatic sinusoid, are also able to present Ag to T cells [16–18]. While a number of studies have demonstrated the importance of antigen

presentation by Kupffer cells and liver dendritic cells to PI-induced transplantation tolerance, the role of antigen presentation by LSEC in such immune tolerance has not been investigated. LSEC constitutively express all molecules necessary for antigen presentation (CD54, CD80, CD86, MHC class I and class II and CD40) and can function as antigen-presenting cells (APC) for $CD4^+$ and $CD8^+$ T cells [16]. It has recently been reported that LSEC 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 Ag [19,20]. This suggests that LSEC contribute to PI-induced tolerance to allogeneic Ag. In the present study, we demonstrated that LSEC actively endocytose allogeneic splenocytes injected via the portal vein and that alloreactive $CD4^+$ T cells are tolerized after contact with such LSEC that have taken up allogeneic cells.

Materials and methods

Mice

Balb/c (H-2^d), C57BL/6 (B6) (H-2^b) and C3H/He (C3H) (H-2^k) female mice (8–12-week old) were purchased from Clea Japan, Inc. (Osaka, Japan). All animals were maintained in a specific pathogen-free microisolator environment. Animal experiments were approved by the Institutional Review Board of Hiroshima University and conducted according to the guidelines of the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Portal venous injection of donor-type splenocytes

Splenocytes were prepared as a single cell suspension after lysis of erythrocytes with ammonium chloride/potassium solution. The splenocytes were either untreated or irradiated (30 Gy) before PI. Allogeneic splenocytes (30×10^6) in 0.5 ml of medium 199 (Sigma, St Louis, MO, USA) containing 1% HEPES buffer were injected through the superior mesenteric vein using a 30-gauge needle.

Heterotopic heart transplantation

Seven days after the PI, donor-type heart allografts were transplanted. Cervical heterotopic heart transplantation was performed using the cuff technique modified from a method described previously [21]. Briefly, the recipients were initially prepared before donor heart harvest to minimize graft ischemic time. The right external jugular vein and the right common carotid artery were dissected free, mobilized as far as possible, and fixed to the appropriate cuffs. The cuffs were composed of polyethylene tubes (2.5F; Portex Co. Ltd, London, UK), the diameters of which were adjusted by physical extension. The aorta and the main pulmonary artery of the harvested donor heart were drawn over the end of the common carotid artery and the external jugular vein for anastomoses. The ischemic time of the graft hearts was <30 min. The function of the grafts was monitored by daily inspection and palpation. Rejection was determined by the cessation of beating of the graft and was confirmed by histology.

Fluorescent cell labeling

Splenocytes were washed in phosphate-buffered saline (PBS) and labeled with PKH-26 (Sigma) for the experiment to detect LSEC endocytosis. PKH-26 labeling (2×10^{-6} M dye) was performed according to the manufacture's protocol.

For analysis of cell proliferation in indirect mixed lymphocyte reaction (MLR), splenocytes were adjusted to 5×10^7 cells/ml in PBS, and 5-(and -6)carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 5 μM. The cells were mixed gently and then incubated at 37 °C for 15 min in the dark. After incubation, the cells were immediately washed twice in cold PBS with 2% heat-inactivated fetal bovine serum (FBS).

Isolation of liver sinusoidal endothelial cells

Disaggregated liver cells were obtained from untreated Balb/c mice or Balb/c mice that had been treated with PI by the two-step collagenase perfusion method [22] and were centrifuged at $50 \times g$ for 1 min. The supernatant was centrifuged at $150 \times g$ for 5 min. The pellet was resuspended, and total cells obtained were stained with biotin-conjugated anti-CD105 (MJ7/18) (Bioscience, San Diego, CA, USA). This monoclonal antibody (mAb) binds to mouse LSEC. Cells were subsequently counterstained with streptavidin microbeads (Miltenyi Biotec, Auburn, CA, USA) and magnetically sorted using an autoMACS (Miltenyi Biotec). This sorting technique leads to a yield of $2–7 \times 10^6$ cells/body in the positive fraction. Positively sorted cells were cultured overnight and adherent cells were harvested. To analyze the purity of LSEC, aliquots of the sorted fractions were cultured in the presence of acetylated low-density lipoprotein (Ac-LDL)-Bodipy (final concentration of 15 μg/ml) (Molecular Probes) in a culture medium, DMEM containing 10% heat-inactivated FBS, 5 μM 2-mercaptoethanol, 1% HEPES buffer, and 100 IU/ml of penicillin–100 mg/ml streptomycin, on collagen I-coated 35-mm tissue culture dishes (Becton Dickinson Labware, Bedford, MA, USA) This fluorescence-labeled lipoprotein is exclusively taken

up by endothelial cells such as LSEC. After 12 h, the cells were additionally stained for the expression of CD11b as a marker for Kupffer cells.

Endocytosis of PKH-26-labeled cells by LSEC

Twelve hours after PI of either naive or irradiated B6 splenocytes (30×10^6) that had been labeled with PKH-26 into Balb/c mice, host LSEC were isolated as described above. Isolated LSEC were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse I-A/I-E (2G9) (BD Pharmingen, San Diego, CA, USA). The LSEC that had taken up the injected splenocytes could be identified as PKH-26-labeled cells by flow cytometric (FCM) analysis.

Preparation of stimulator cells for indirect MLR

Allogeneic B6 or third party C3H splenocytes (20×10^6) were injected intravenously into Balb/c mice. Seven days after injection, splenocytes obtained from the Balb/c mice were digested with ammonium chloride/potassium solution. The splenocytes were plated on 100-mm tissue culture dishes (Falcon 3003; Becton Dickinson Labware). After incubation for 2 h in a 5% CO_2 incubator, adherent cells were prepared as antigen-presenting stimulator cells for subsequent indirect MLR.

Transmigration across LSEC layer

Liver sinusoidal endothelial cells obtained from Balb/c mice 7 days after PI of naive or irradiated B6 splenocytes were placed on a fibronectin-coated Falcon biocoat cell culture insert polyethylene terephthalate (PET) filter of 3 μm in pore size, 12-well format (Becton Dickinson Labware) (4×10^6 cells/insert). After 24 h of culture, nonadhered cells were washed out from the PET filter membrane. CFSE-labeled Balb/c splenocytes (10×10^6 cells/filter) were overlaid and incubated for 24 h at 37 °C in a 5% CO_2 incubator (see Fig. 4a). Splenocytes that had transmigrated to the bottom of the wells of a 12-well plate were harvested and counted. These cells were used as responder cells in the subsequent indirect MLR.

Indirect CFSE–MLR assay

The transmigrated cells were used as responders after resuspension at 2×10^6 cells/ml in RPMI 1640 medium containing 15% controlled processed serum replacement (CPSR-3; Sigma), 5 μM 2-mercaptoethanol, 1% HEPES buffer, and 100 IU/ml of penicillin–100 μg/ml streptomycin. The stimulator cells were irradiated with 30 Gy. One $\times 10^6$ CFSE-labeled responder cells were cultured with 0.2×10^6 stimulator cells in a total volume of 1 ml of medium in a 48-well flat-bottom plate (Becton Dickinson Labware) at 37 °C in 5% CO_2 in the dark for 5 days.

Flow cytometric analysis

All analyses were performed using a FACSCalibur® cytometer (BD Bioscience, Mountain View, CA, USA). For phenotyping LSEC surface markers, LSEC were stained with the following mAbs: FITC-conjugated anti-mouse I-A/I-E (2G9), anti-CD40 (HM40-3), anti-CD80 (16-10A1) and anti-CD86 (GL1).

Proliferating T cells in the indirect MLR using CFSE-labeled lymphocytes were detected by a multi-parameter FCM setting as previously described [23–25]. The harvested cells were stained with phycoerythin (PE)-conjugated anti-mouse CD4 mAb (GK1.5) or CD8a mAb (53-6.7). T-cell proliferation (division) in each $CD4^+$ and $CD8^+$ T-cell fraction was visualized as serial halving of the fluorescence intensity of an intracellular fluorescent dye (CFSE). For all analyses, nonspecific Fcγ receptor binding of labeled Abs was blocked by CD16/32 (2.4G2). All mAbs were purchased from BD Pharmingen. Dead cells were excluded from the analysis by light-scatter and/or propidium iodide.

Statistical analysis

The results were statistically analyzed by Student's *t*-test of means or the log rank test when appropriate. $P > 0.05$ was considered to be statistically significant.

Results

PI of irradiated allogeneic splenocytes led to significant prolongation of the survival of subsequently grafted allogeneic hearts

To determine whether tolerance or hyporesponsiveness to fully allogeneic organ grafts could be achieved by PI of allogeneic cells, Balb/c mice were treated with PI of allogeneic B6 splenocytes (30×10^6 cells) that were either untreated or irradiated (30 Gy), and B6 heart allografts were subsequently transplanted 7 days after PI. Survival curves of the grafted hearts are shown in Fig. 1. The median survival times of B6 heart allografts were 8 days in the untreated Balb/c recipients ($n = 5$), 15 days in the Balb/c recipients with PI of untreated B6 splenocytes ($n = 6$), and more than 100 days in the Balb/c recipients with PI of irradiated B6 splenocytes ($n = 5$). Thus, modification of allogeneic splenocytes with irradiation enhanced the prolonging effect of PI of those cells on the survival of subsequently transplanted heart allografts.

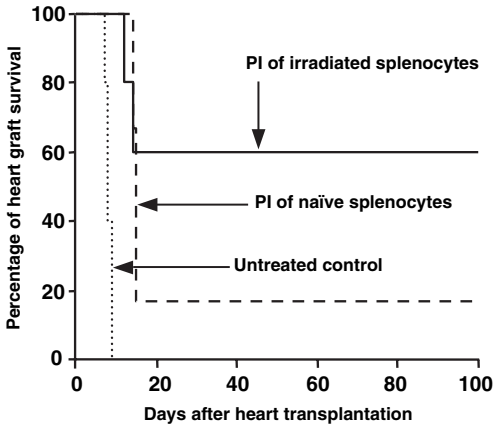


Figure 1 Portal venous injection (PI) of irradiated allogeneic splenocytes led to significant prolongation of the survival of subsequently grafted allogeneic hearts. Thirty million B6 splenocytes that were either naive or irradiated (30 Gy) were injected via the portal vein into Balb/c mice. Seven days later, B6 heart allografts were heterotopically transplanted into the Balb/c mice. Five untreated Balb/c mouse recipients were used as controls, six Balb/c mouse recipients were treated with PI of naive B6 splenocytes, and five Balb/c mouse recipients were treated with PI of irradiated B6 splenocytes. Survival curves of the grafted hearts are shown. $P < 0.005$, untreated control Balb/c mice versus Balb/c mice treated with PI of naive B6 splenocytes. $P < 0.05$, untreated control Balb/c mice versus Balb/c mice treated with PI of irradiated B6 splenocytes. $P < 0.001$, Balb/c mice treated with PI of naive B6 splenocytes versus Balb/c mice treated with PI of irradiated B6 splenocytes.

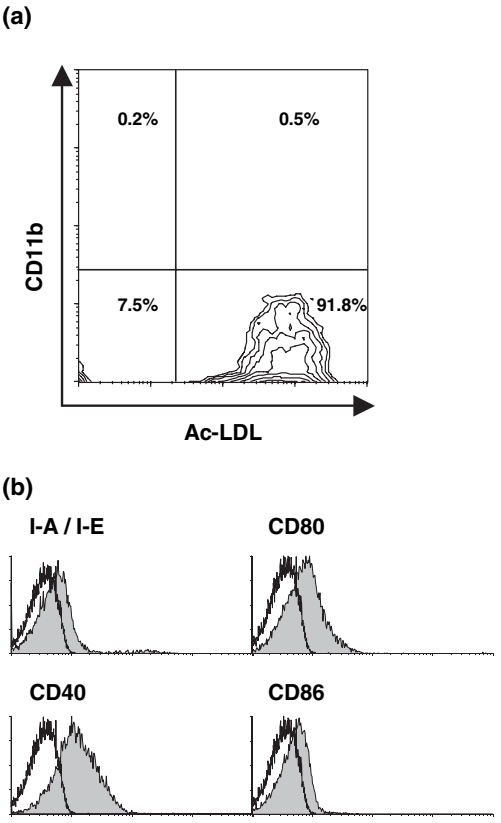


Figure 2 LSEC constitutively express all molecules necessary for antigen presentation (MHC class II, CD80, CD86, and CD40). Total liver cells from untreated Balb/c mice were stained with anti-CD105 mAb and sorted by MACS. (a) The positive fraction was cultured overnight in the presence of acetylated, Bodipy-labeled low-density lipoprotein (Ac-LDL). After 12 h, the cells were additionally stained for the expression of CD11b as a marker for Kupffer cells. (b) For phenotyping, LSEC were stained with FITC-conjugated anti-I-A/I-E, anti-CD40, anti-CD80, and CD86. A gate was set on vital cells, and 10 000 counts were acquired. Open histograms represent negative control staining isotype-matched Abs. FCM profiles shown are representative of three independent experiments.

LSEC constitutively express all molecules necessary for antigen presentation (MHC class II, CD80, CD86, and CD40)

It has recently been reported that LSEC function as APCs in the context of both MHC class I and class II restriction with the resulting development of specific T-cell tolerance to both exogenous and endogenous soluble Ag [19,20]. This suggests that LSEC contribute to PI-induced tolerance to allogeneic Ag. To determine the role of LSEC in PI-induced transplantation tolerance, we first isolated LSEC from Balb/c mice. Results of immunohistochemical studies have shown that LSEC were positive for the expression of CD105 molecules (endoglin) but that the endothelia of central veins or other vessels in the mouse liver were not (T. Onoe, D. Tokita and H. Ohdan, unpublished data), indicating that liver endothelial cells other than LSEC do not express CD105 molecules or that they express CD105 molecules at much lower levels than LSEC. Therefore, $CD105^+$ cells were positively selected by MACS to isolate LSEC from liver constituent cells. The results of endocytosis assays using (Ac-LDL)-Bodipy demonstrated that positive sorted cells contained between 90% and 95% LSEC (Fig. 2a). To characterize the phenotype of naive LSEC from untreated Balb/c mice, $CD11b^-$ cells that had taken up Ac-LDL-Bodipy were selected by gating and were examined for their expression of surface molecules necessary for the efficient presentation of Ag to T cells. As shown in Fig. 2b, LSEC were MHC class II^+, $CD40^+$, $CD80^+$ and $CD86^+$ phenotypes, consistent with those of APC.

LSEC actively endocytosed allogeneic splenocytes injected via the portal vein

Although it has been reported that LSEC show huge endocytic capacity for many ligands, including glycoproteins, components of the extracellular matrix, immune

complexes, transferrin and ceruloplasmin, it is not clear whether LSEC take up (endocytose) allogeneic splenocytes after PI. To address this issue, we isolated LSEC from Balb/c mice that had been treated with PI of either naive or irradiated allogeneic B6 splenocytes. Prior to PI, B6 splenocytes were labeled with PKH-26 so that LSEC that had taken up the injected splenocytes could be identified as $CD105^+$ cells that have PKH-26 labeling. At 12 h after PI, approximately 20% of $CD105^+$ cells had taken up PKH-26-labeled splenocytes that were either naive or irradiated (Fig. 3). There was no statistically significant difference between either proportions or intensities of PKH-26 labeling in $CD105^+$ cells from the mice that received naive splenocytes and those that received irradiated splenocytes. The kinetics of LSEC uptake of naive and irradiated allogeneic splenocytes were also not different (data not shown). Thus, LSEC actively endocytosed allogeneic naive splenocytes as well as irradiated splenocytes after PI. Notably, $CD105^+$ cells capturing allogeneic splenocytes highly expressed MHC class II, suggesting their capacity for antigen presentation to $CD4^+$ T cells.

Induction of nonresponsiveness of T cells to alloantigens by transmigration across LSEC that had captured allogeneic splenocytes

The various functions of APC (professional myeloid APC), such as uptake, processing and presentation of Ag, are separated in time and space. APC take up Ag in the periphery and then mature during their migration into lymphatic tissue, where they encounter T cells in a specialized microenvironment. The LSEC, which has been described as a new type of APC that is organ-resident, executes all three salient features of an APC at one time and induces immune tolerance in naive T cells [17,18]. It is possible that LSEC process the captured allogeneic splenocytes and subsequently present allo-Ag to naive T cells. Through antigen presentation by the LSEC, alloreactive T cells might be tolerized. We examined the effect of antigen presentation by LSEC to naive T cells on the responsiveness of those T cells to subsequent antigen presentation by professional APCs. Balb/c mouse splenocytes first underwent transmigration across the LSEC from Balb/c mice that had been treated with PI of either naive or irradiated allogeneic B6 splenocytes, enabling direct interaction between T cells and LSEC (Fig. 4a). The number of transmigrated T cells across LSEC from mice that had received PI of either naive or irradiated allogeneic splenocytes was markedly greater than that of transmigrated T cells across LSEC from untreated mice (Fig. 4b).

Such transmigrated Balb/c splenocytes, which were predominantly $CD4^+$ T cells, were subsequently stimulated with splenic APCs from Balb/c mice that had been stimulated with intravenous injection of the splenocytes from either donor-type B6 or third-party C3H mice. A remarkable proliferative response of $CD4^+$ T cells that

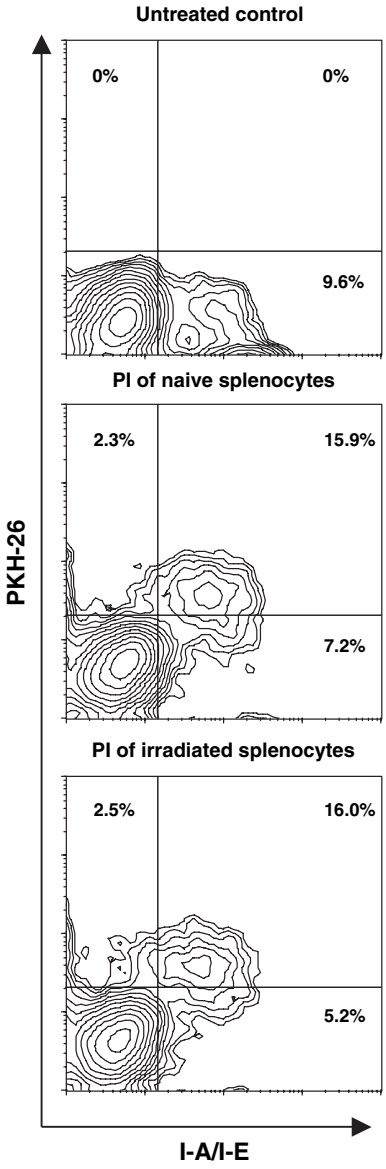


Figure 3 LSEC actively endocytosed allogeneic splenocytes injected via the portal vein. Prior to PI, B6 splenocytes were labeled with PKH-26. Twelve hours after PI of either naive or irradiated B6 splenocytes into Balb/c mice, host LSEC were isolated by autoMACS using biotin-conjugated anti-CD105 mAb + streptavidin microbeads. The positive fraction was cultured overnight to eliminate nonadherent cells. Isolated LSEC were stained with FITC-conjugated anti-I-A/I-E. The LSEC that had taken up the injected splenocytes could be identified as PKH-26-labeled cells by flow cytometric (FCM) analysis. FCM study using anti-CD105 mAb revealed that the inocula of splenocytes prepared from B6 mice did not include $CD105^+$ cells (<0.5%) (data not shown). FCM profiles shown are representative of three independent experiments.

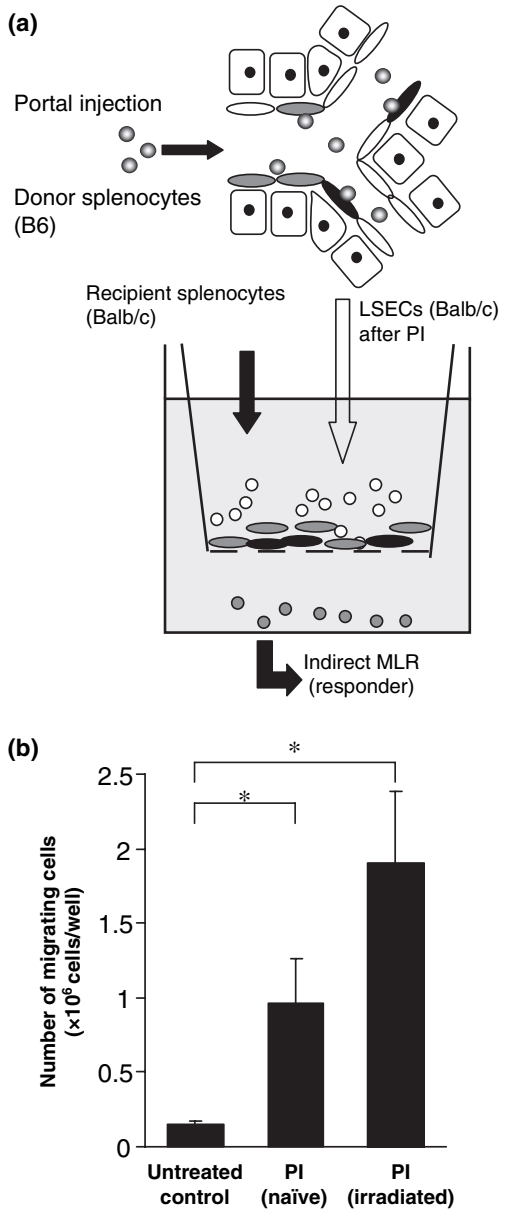


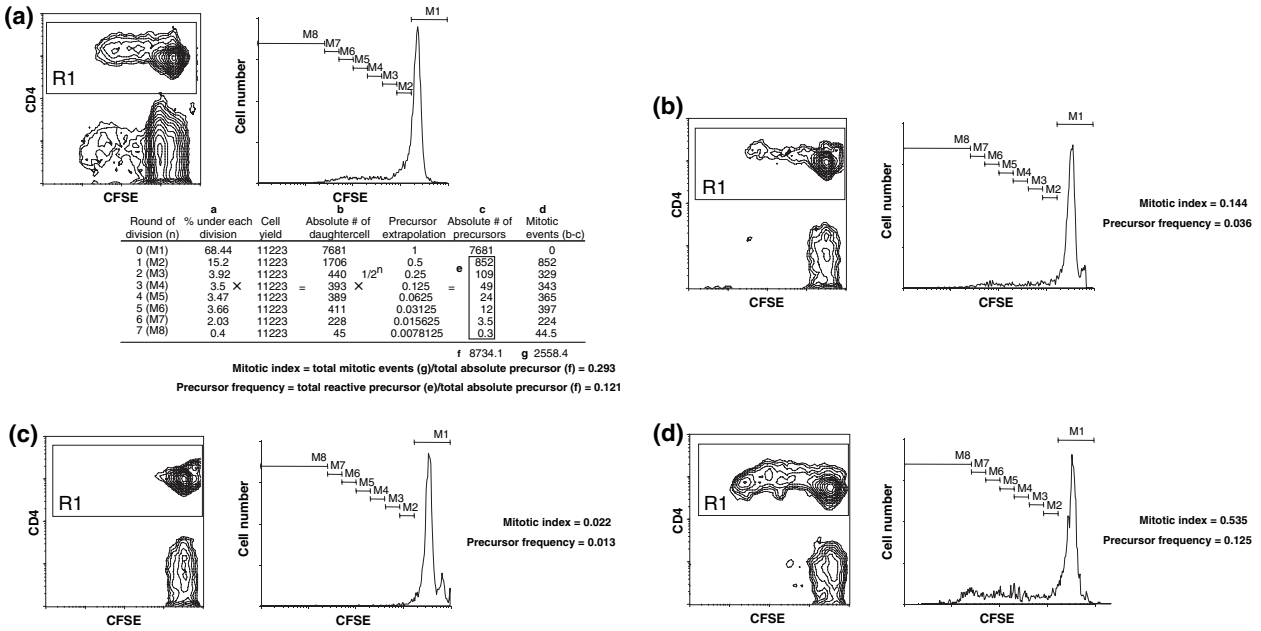
Figure 4 T-cell-LSEC layer transmigration assay. (a) System of transendothelial migration assay. LSEC were isolated from Balb/c mice that were either untreated or treated with PI of naive or irradiated B6 splenocytes 7 days after the PI by autoMACS. The isolated LSEC were placed on a fibronectin-coated Falcon biocoat cell culture insert polyethylene terephthalate (PET) filter (4×10^6 cells/insert well). After 24 h of culture, CFSE-labeled Balb/c splenocytes (1×10^7 cells/filter) were overlaid and incubated for 24 h. Splenocytes transmigrating to the bottom of the wells were harvested. The cells were used as responder cells of indirect MLR. (b) The number of T cells that transmigrated across LSEC from mice having received PI of either naive or irradiated allogeneic splenocytes was significantly greater than that of T cells that transmigrated across LSEC from untreated mice. The results shown are mean values of three independent experiments. Average values ± SEM for individual groups are shown (*$P < 0.05$).

had transmigrated across the LSEC from untreated Balb/c mice to Balb/c APCs pulsed with B6 splenocytes was observed. However, the $CD4^+$ T cells that had transmigrated across the LSEC from mice that had been treated with PI of irradiated B6 splenocytes lacked a proliferative response to Balb/c APCs pulsed with B6 splenocytes but a normal response to Balb/c APCs pulsed with C3H splenocytes (Fig. 5). Thus, T cells that transmigrated across antigen-presenting LSEC that had captured irradiated allogeneic splenocytes were rendered tolerant to alloantigens in an antigen-specific manner.

Discussion

It is generally accepted that there are two pathways of allorecognition, direct antigen presentation and indirect antigen presentation that together contribute to allograft rejection. The direct pathway results from the recognition of foreign major histocompatibility molecules, intact, on the surfaces of donor cells. Indirect allorecognition occurs when donor histocompatibility molecules are internalized, processed, and presented as peptides by host APCs. Little is known about the actual contributions of direct allorecognition and indirect allorecognition to the physiology of allograft rejection *in vivo*. There is accumulating evidence, however, that both direct alloreactivity and indirect alloreactivity represent an essential component of the allograft rejection process [26,27]. Transplantation studies using $CD4^+$ and $CD8^+$ T-cell-deficient mice or $CD4^+$ and $CD8^+$ T-cell-depleting mAbs have revealed that $CD4^+$ T cells, not $CD8^+$ T cells, are essential for the rejection of skin, heart and islets allografts [28,29]. These studies, along with others using MHC class I and class II-deficient donors, suggest that indirect antigen presentation is an important pathway mediating MHC class I- and class II-disparate allograft rejection [30]. Therefore, any strategies designed to achieve the ultimate goal in transplantation, i.e. the induction of tolerance, will need to take into account both pathways. PI of allogeneic cells has been reported to induce donor-specific tolerance across MHC and minor histocompatibility complex barriers. Although various possible mechanisms underlying PI-induced tolerance, including clonal deletion [3–5], induction of anergy and generation of regulatory cells [5–9], regulation of cytokine production [10,11], and promotion of microchimerism [31], have been postulated, the cellular basis of this immune tolerance is not fully understood. The difficulty in determining the precise mechanisms may be the result of a difference between tolerizing mechanisms of T cells with direct allospecificity and T cells with indirect allospecificity.

Single treatment with PI of allogeneic cells induces persistent donor-specific tolerance across multiple minor



Round of division (n)	a % under each division	Cell yield	b Absolute # of daughtercell		Precursor extrapolation		c Absolute # of precursors	d Mitotic events (b-c)
0 (M1)	68.44	11223	7681		1		7681	0
1 (M2)	15.2	11223	1706		0.5	e	852	852
2 (M3)	3.92	11223	440	$1/2^n$	0.25		109	329
3 (M4)	3.5 ×	11223	= 393 ×		0.125	=	49	343
4 (M5)	3.47	11223	389		0.0625		24	365
5 (M6)	3.66	11223	411		0.03125		12	397
6 (M7)	2.03	11223	228		0.015625		3.5	224
7 (M8)	0.4	11223	45		0.0078125		0.3	44.5
							f 8734.1	g 2558.4

Mitotic index = total mitotic events (g)/total absolute precursor (f) = 0.293

Precursor frequency = total reactive precursor (e)/total absolute precursor (f) = 0.121

Figure 5 T cells that transmigrated across the LSEC that had captured irradiated allogeneic splenocytes were rendered tolerant to alloantigens. Balb/c mouse splenocytes first underwent transmigration across the LSEC from Balb/c mice that were either untreated or treated with PI of naive or irradiated B6 splenocytes, as shown in Fig. 4. The transmigrating cells, which were predominantly $CD4^+$ T cells, were labeled with CFSE and then subsequently stimulated *in vitro* with irradiated splenic APCs from Balb/c mice that were pulsed with intravenous injection of donor-type B6 or third-party C3H splenocytes (indirect MLR). After the indirect MLR, harvested lymphocytes were stained with PE-conjugated anti-CD4 mAb. Then, by FCM analysis, T-cell proliferation (division) was visualized as serial halving of the fluorescence intensity of an intracellular fluorescent dye (CFSE). Balb/c splenocytes that transmigrated across the LSEC from Balb/c mice that were either untreated (a) or treated with PI of naive (b) or irradiated B6 splenocytes (c) were subsequently stimulated with splenic APCs pulsed with donor-type B6 splenocytes. (d) Balb/c splenocytes that transmigrated across the LSEC from Balb/c mice that were treated with PI of irradiated B6 splenocytes were subsequently stimulated with splenic APCs pulsed with third-party C3H splenocytes. FCM contour plot profiles shown are representative of three independent experiments. CFSE fluorescence histograms of $CD4^+$ T cells show their proliferative history in response to indirect allostimulation. $CD4^+$ T cells were selected by gating and analyzed for CFSE fluorescence. Divisions of alloreactive T cells, which were identified and determined by their CFSE intensities, were labeled from 0 to *n* as division round. The limit of detection is seven or eight division cycles caused by compression of peaks as the CFSE intensity approaches autofluorescent levels. Thus, divisions beyond six cycles are indistinguishable and are collectively referred to as division 7+. The methods used for quantitative analysis of precursor frequencies and mitotic indexes in the responses to indirect stimulator cells are shown. From the experimentally determined values of percentages of total cell events in each division *n* (a) and total number of living cell events at the time of harvest, the total number of daughter cells that underwent division *n* times was calculated (b). The total number of original or precursor cells required to have generated these daughter cells was extrapolated by dividing the number of daughter cells at *n* divisions by 2^n (c). The absolute number of mitotic events was determined by subtracting the number of division precursors from the number of daughter cells generated by each precursor population (d). (e) Absolute number of precursor cells in each division (reactive precursors); (f) sum of the precursors; (g) sum of mitotic events.

histocompatibility and MHC class I incompatible barriers [5,32]. Such tolerance could be induced by the injection of not only allogeneic living cells but also noncellular Ag [19,20], raising the possibility that this strategy efficiently induces tolerance among T cells with indirect allospecificity. In contrast, other treatments such as administration of immunosuppressants [8,33–35] and co-stimulatory blockades [36], in addition to PI of donor cells, are usually required to induce donor-specific tolerance across MHC class II incompatible barriers, suggesting that the tolerizing effects of PI of donor cells might be more efficient among T cells with indirect allospecificity than T cells with direct allospecificity.

In tolerizing T cells with indirect allospecificity, host APCs, which internalize, process and present donor histocompatibility molecules, should play a significant role. The LSEC has been described as a new type of APC that induces immune tolerance in naive T cells [17,18]. LSEC are capable of even stimulating naive $CD4^+$ T cells. However, following priming by Ag-presenting LSEC, $CD4^+$ T cells fail to subsequently differentiate towards a Th1 phenotype but, instead, become regulatory T cells, expressing IL-4 and IL-10 upon restimulation [20,37]. LSEC also have the capacity to present exogenous Ag on MHC class I molecules to $CD8^+$ T cells, a process termed cross-presentation [19]. Previously believed to occur exclusively in

myeloid APC, cross-presentation in LSEC occurs with extraordinary efficiency, requiring only minute amounts of Ag and occurring within 60–120 min after exposure to Ag. Stimulation of naive $CD8^+$ T cells by LSEC results first in the proliferation of T cells and the release of cytokines but finally leads to Ag-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. These findings suggest that LSEC contribute to PI-induced tolerance of T cells with indirect allospecificity.

In the present study, we demonstrated that host LSEC actively endocytose allogeneic splenocytes injected via the portal vein. Host LSEC capturing allogeneic splenocytes highly expressed MHC class II. It is likely that a subset of MHC class II-rich LSEC were either selectively endocytic or expressed a high level of MHC class II because of the processing of captured allogeneic cells. In either case, it is possible that the LSEC process captured allogeneic splenocytes and subsequently present alloantigens to naive $CD4^+$ T cells through the interaction between MHC class II and T-cell receptors. Such antigen presentation by LSEC might induce tolerance among $CD4^+$ T cells with indirect allospecificity. Passenger leukocytes are forced into contact with LSEC because of the small diameters of the sinusoids. The cumulative surface area of LSEC is very large, and hepatic microcirculatory parameters allow for frequent contact between LSEC and passenger leukocytes. Given the large volume of blood passing daily through the liver, it seems probable that LSEC are ideally positioned within the liver to establish peripheral immune tolerance [4,17]. We carried out a T-cell transendothelial migration assay to mimic such anatomical features of the interaction between LSEC and T cells. In this system, $CD4^+$ T cells that received stimuli from LSEC adhered to a PET filter exclusively transmigrated across the LSEC layer. PI of either naive or irradiated allogeneic splenocytes prior to harvesting LSEC resulted in a significant increase in T-cell transmigration, suggesting that presentation of alloantigens by LSEC that had captured allogeneic splenocytes to $CD4^+$ T cells took place during their period of direct contact. The responsiveness of $CD4^+$ T cells that had transmigrated across LSEC to the syngeneic splenic APCs pulsed with allogeneic splenocytes was reduced but remained detectable when the LSEC had captured naive allogeneic splenocytes (Fig. 5). In contrast, such responsiveness of $CD4^+$ T cells was completely abrogated when the LSEC had captured irradiated allogeneic splenocytes, indicating tolerance of T cells to the responsible alloantigens. This finding is consistent with the results presented in Fig. 1 showing remarkable prolongation of survival of heart allografts by prior PI of irradiated donor-type splenocytes. At present, it is not clear why PI of irradiated allogeneic splenocytes more efficiently tolerized T cells with indirect allospecificity than did PI of naive allogeneic splenocytes. As the activities of LSEC uptake of naive and irradiated allogeneic splenocytes were comparable, the difference between the tolerizing effects of naive and irradiated allogeneic splenocytes might be the result of a difference in either processing or presentation of alloantigens by LSEC. It is possible that LSEC obtain tolerogeneic activity toward alloreactive T cells when they endocytose allogeneic cells undergoing apoptosis (induced by irradiation) but do not obtain so much activity when they endocytose living allogeneic cells. This possibility is currently under investigation.

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