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Relative Contribution of Direct and Indirect Allorecognition in Developing Tolerance After Liver Transplantation

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The interaction of donor passenger leukocytes and host leukocytes in recipient secondary lymphoid tissues during the early posttransplantation period is crucial in directing host immune reactions toward allograft rejection or acceptance. Responsible T cell clones could be activated through the direct and indirect pathways of allorecognition. We examined the role of the indirect pathway in liver transplantation (LT) tolerance by depleting host antigen-presenting cells (APC) with phagocytic activity [e.g., cluster domain (CD)68$^+$/CD163$^+$ macrophages, CD11c$^+$ dendritic cells (DC)] using liposome-encapsulating clodronate (LP-CL). After Lewis rat cell or liver graft transplantation, Brown Norway (BN) rat recipients pretreated with LP-CL showed a significantly reduced type 1 helper T cell cytokine up-regulation than control-LP-treated recipients. In the LT model, LP-CL treatment and host APC depletion abrogated hepatic tolerance; Lewis liver grafts in LP-CL-treated-BN recipients developed mild allograft rejection, failed to maintain donor major histocompatibility complex (MHC) class II$^+$ leukocytes, and developed chronic rejection in challenged donor heart allografts, while control-LP-treated BN recipients maintained tolerance status and donor MHC class II$^+$ hepatic leukocytes. Furthermore, in the BN to Lewis LT model, LP-CL recipient treatment abrogated spontaneous hepatic allograft acceptance, and graft survival rate was reduced to 43% from 100% in the control-LP group. In conclusion, the study suggests that host cells with phagocytic activity could play significant roles in developing LT tolerance. *Liver Transpl 14:346–357, 2008.* © 2008 AASLD.

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Bone marrow–derived "passenger" leukocytes in solid organs migrate to recipient secondary lymphoid tissues after transplantation, and their subsequent interactions with recipient leukocytes during the early posttransplantation period appear to be crucial in directing host immune reactions toward allograft rejection and/or graft acceptance.[1-7] An essential role of host secondary lymphoid tissues in providing a microenvironment for the interaction of host T cells and migrating donor leukocytes has been substantiated in splenectomized *aly/aly* (alymphoplastic) mice that lack secondary lymphoid tissues and fail to reject cardiac allografts.[8] In host secondary lymphoid organs, responsible host T cell clones could be activated by

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Abbreviations: APC, antigen-presenting cells; BN, Brown Norway rat; CD, cluster domain; CFSE, carboxyfluorescein succinimidyl ester; CIITA, class II transactivator; DC, dendritic cells; FITC, fluorescein isothiocyanate; GMCSF, granulocyte-macrophage colony-stimulating factor; HTx, heart transplantation; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LEW, Lewis rat; LP-CL, liposome clodronate; LT, liver transplantation; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; mRNA, messenger RNA; OLTx, orthotopic liver transplantation; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RPMI, Roswell Park Memorial Institute; RT, reverse transcription; TAC, tacrolimus; Th1, type 1 helper T cell.

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donor and recipient antigen presenting cells (APC) through 2 distinct pathways of allorecognition; in the direct pathway, recipient T cells recognize intact donor major histocompatibility complex (MHC) molecules complexed with peptide on the surface of migrating donor APC, while, in the indirect pathway, recipient T cells recognize donor-derived allopeptides processed and presented in the context of recipient MHC on host APC.[9,10] Historically, the direct pathway has been considered to play a primary role in initiating alloimmune responses during the early posttransplantation period and lead to acute rejection.[1,2,11] In contrast, the indirect pathway has been shown to play roles in initiating cluster domain (CD)4^+ T cell responses and mediating chronic rejection during a later phase of transplantation, when donor MHC class II^+ passenger leukocytes disappear from organ allografts. Recently, there has been increasing interest in a prominent role of the indirect pathway in initiating alloimmune responses during the early phase of organ transplantation.[8,12-14] However, the relative contributions of the direct and indirect pathways during the early acute phase of organ transplantation in initiating graft rejection and tolerance remain largely unclear.

In vivo administration of liposome clodronate (LP-CL) is known to selectively and efficiently deplete mononuclear phagocytic cells without affecting other leukocyte populations.[15-17] After ingestion via endocytosis, liposomes encapsulating clodronate are disrupted by lysosomal phospholipases, release clodronate within the cell, and lead to cell death. The technique has been widely used to investigate the in vivo role of phagocytic cells, such as macrophages and dendritic cells (DC), in defined biological processes, including infection, autoimmunity, graft-versus-host disease, and transplantation.[18-24]

We have previously shown that liver transplantation (LT) associates with prompt migration of a large number of graft leukocytes into host lymphoid tissues.[25,26] However, it remains undetermined how migrating donor leukocytes present alloantigens to host T cells and initiate immune reactions. In this study, we attempted to paralyze the indirect pathway of allorecognition by eliminating host APC with phagocytic activity using the LP-CL method. Using animals pretreated with LP-CL as recipients, the primary purpose of the study was to evaluate in vivo roles of host phagocytes and indirect pathway of allorecognition after LT in initiating acute rejection and tolerance.

MATERIALS AND METHODS

Animals

Inbred male Lewis (LEW, $RT1^l$), Brown Norway (BN, $RT1^n$), and ACI ($RT1^a$) rats weighing 200 to 250 g were obtained from Harlan Sprague Dawley (Indianapolis, IN) and maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh with a standard diet and water ad libitum. All procedures in this study were performed according to the guidelines of the Council of Animal Care at the University of Pittsburgh within the guidelines of National Institutes of Health and the Public Health Service Policy of the humane use and care of laboratory animals.

Monoclonal Antibodies (mAbs)

Monoclonal antibodies (mAbs) used in this study included purified or fluorescent-conjugated 8A2 (CD11c), ED1 (CD68), ED2 (CD163), R7.3 [αβ T cell receptor], OX33 (CD45RA; B cell), OX35 (CD4), OX8 (CD8α), OX6 (MHC class II) and anti-rat immunoglobulin (Ig)M (B cell) (BD PharMingen, San Diego, CA or Serotec, Oxford, UK). mAb L21-6 (mouse IgG_1) that binds to MHC class II antigens of LEW, but not BN, was used to identify donor MHC class II^+ cells.[27] Biotinylated rat anti-mouse IgG, or Cy3 or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch laboratories, Inc., West Grove, PA) were used as secondary antibodies. Isotype-matched irrelevant mAbs were used as negative controls.

Organ Transplantation

Orthotopic liver transplantation (OLTx) with arterial reconstruction and heterotopic heart transplantation (HTx) into the abdomen were carried out as previously described.[26] Liver graft rejection was defined as recipient animal survival. Heart graft survival was monitored by transabdominal palpation, and rejection was defined as the cessation of heartbeat.

Immunosuppression

Tacrolimus (TAC; Astellas Pharma Inc, Tokyo, Japan) in normal saline was injected intramuscularly on days 0-6, 13, and 20 after OLTx at a dose of 1.0 mg/kg/day. This dose of TAC has been shown to induce long-term rat organ allograft survival.[26,28]

Preparation of LP-CL and Recipient Treatment

LP-CL was prepared as previously described with minor modifications.[15-17,29] Briefly, lipids composed of 86 mg egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) and 9 mg cholesterol (Sigma-Aldrich, St. Louis, MO) were dissolved in 10 mL chloroform. The organic solvent was removed under reduced pressure in a rotary evaporator, and resulting lipid film was desiccated and dispersed in 10 mL phosphate buffered saline (PBS) containing 2.5 g clodronate (Sigma-Aldrich). The liposome was rapidly frozen in liquid nitrogen and slowly thawed at 4°C. The freeze-thaw step was repeated 3 times to increase the entrapment efficacy of clodronate in the liposome.[29] LP-CL was purified by 3 centrifugation washes at 10,000*g* for 15 minutes, and resuspended in 4 mL sterilized PBS. For the control, liposome without clodronate was prepared. Control-LP or LP-CL (4.0 mL/kg, relevant to 100 mg/kg clodronate) was intravenously injected 2 times to BN recipients via the penal vein on 10 and 7 days before OLTx.

Leukocyte Isolation and Purification

Single-cell suspensions were obtained by injecting Roswell Park Memorial Institute (RPMI) medium into excised cervical and mesenteric lymph nodes and spleens and following filtration through nylon mesh. After red blood cell lysis with NH_4Cl buffer, T cells were positively isolated using anti-rat pan-T cell microbeads (OX52) and LS columns (Miltenyi Biotec, Germany) with purity of 85 to 90%. The non-T cell population, which contained 50 to 55% of MHC class II^+ ($OX6^+$) and 60 to 65% of B ($OX33^+$) cells, was also collected.

Carboxyfluorescein Succinimidyl Ester (CFSE) Labeling

Carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) was used as described for cell labeling, with some modifications. Typically, single-cell suspensions (1×10^7/mL) were incubated with 5 μM CFSE for 30 minutes at room temperature. Labeled cells were washed 3 times with RPMI medium with 20% vol/vol fetal calf serum to eliminate unconjugated CFSE, and then resuspended in PBS for injection.

Experimental Design

Organ Transplantation

To examine the influence of LP-CL-induced host phagocyte depletion on allograft survival, LEW liver grafts were transplanted into Control-LP- or LP-CL-treated BN recipients with or without TAC immunosuppression. Animals were followed for 200 days or until allograft rejection. At 100 days, to determine the status of allograft acceptance, LEW heart grafts were transplanted to surviving liver recipients with a follow-up of additional 100 days. At the time of sacrifice, graft tissue samples were fixed in 10% formalin for routine histopathology, in OCT compound and frozen in liquid nitrogen cooled isopentane for immunohistochemistry, and snap-frozen for messenger RNA (mRNA) isolation. The host spleen was obtained for flow cytometry.

Cell Migration

The influence of LP-CL-induced host phagocyte depletion on early cell migration pattern was studied by infusing LEW CFSE-labeled T cell or non-T cell populations (2×10^8 cells per animal) into BN recipients treated with Control-LP or LP-CL. Animals were sacrificed at 24 or 72 hours after cell injection for immunohistochemical analyses of cell migration and polymerase chain reaction (PCR) for cytokine mRNA levels. No immunosuppressive drug was used.

Allogenic cell migration in host spleen was also studied by in vivo real-time confocal fluorescent imaging. Naive BN animals received intravenous injection of 0.5 mL of 0.02 μm-diameter microspheres containing red fluorescent dye (Fluo-Spheres; Molecular Probes) to detect macrophages or DC by phagocytosis. Then, unfractionated LEW CFSE-labeled splenocytes were injected into recipients 30 minutes after microsphere injection. Migration pattern of injected cells in the spleen was sequentially visualized by confocal fluorescent imaging as described before.[30] At 30 minutes, 4 hours, and 24 hours after cell injection, animals were anesthetized and a small laparotomy was performed. The spleen was mobilized and examined with fluorescent confocal microscopy. The imaging probe tip was positioned in contact with the surface of the spleen. Laser excitation utilized the combined 488 nm/568 nm spectral lines of a 50-mW air-cooled krypton-argon laser. Migrated $CFSE^+$ cells into the spleen were visualized as green using a 505-nm long-pass filter for detection (Chroma Technology, Brattlebor, VT). Emitted fluorescence from red microspheres contained within macrophages or DC by phagocytosis was detected using a 585-nm long-pass filter (Chroma Technology).

Routine Histopathology

Tissue samples were fixed in 10% buffer formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. In the liver allografts, particular attention was directed to the presence or absence of bile duct damage and/or loss, bile ductular proliferation, subendothelial mononuclear infiltration in the portal or central veins, and severity of portal tract inflammation.[31,32]

Immunohistochemistry

Frozen samples were cut into 6-μm sections onto gelatin-coated slides and stained with immunoperoxidase or immunofluorescent methods. The avidin-biotin-peroxidase immune complex was visualized with 3-amino-9-ethylcarbazole (AEC; ScyTek Laboratories, Logan, UT) and counterstained with hematoxylin. For immunofluorescent staining, after blocking with 10% (vol/vol) normal goat serum in super block (ScyTek Laboratories), the sections were incubated with each primary antibody, and then exposed to fluorescent-conjugated secondary antibody. Hoechst dye (bisBenzimide, Sigma, 1 μg/100 mL) was used for 30 seconds to stain nuclear DNA. The sections were washed and coverslipped with Gelvatol, a water-soluble mounting media (23 g polyvinyl alcohol, 50 mL glycerol, 0.01% sodium azide in 100 mL PBS), and visualized with an Olympus BX51 epifluorescence microscope and digitized with an Olympus color video camera (Olympus, Center Valley, PA). Positively-stained cells were counted in 4 randomly selected high power fields (×400) per section.

SYBR Green Real-Time Reverse Transcription (RT)-PCR

mRNA for interleukin (IL)-2, IL-10, interferon (IFN)-γ, and glyceraldehyde-3-phosphate dehydrogenase were quantified by SYBR Green real-time reverse transcription (RT)-PCR as described before.[33] After removal of potentially contaminating DNA with DNase I, 1 μg of total RNA from each sample was used for RT with an oligonucleotide dT and a Superscript II (all from Life Technologies, Grand Island, NY). The PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) using previously described primers.[34]

Thermal cycling conditions were 10 minutes at 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute on an ABI-Prism 7000 Sequence Detection System (PE Applied Biosystems). Each gene expression was normalized with glyceraldehyde-3-phosphate dehydrogenase mRNA content.

Flow Cytometry

Lineages of splenocytes from experimental animals were analyzed with fluorescent-conjugated mAbs R7.3-FITC, OX8-phycoerythrin, OX39-FITC, OX33-FITC, ED1-FITC, ED2-FITC, and CD11c-FITC. The samples were fixed in paraformaldehyde and analyzed on a Coulter Elite ESP (Coulter, Miami, FL). Isotype-matched nonspecific antibodies were used for controls.

Mixed Leukocyte Reaction (MLR)

One-way mixed leukocyte reaction (MLR) was conducted using unfractionated cervical lymph node lymphocytes or spleen cells obtained from experimental animals. RPMI 1640 supplemented with 25 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid buffer, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, penicillin (50 U/mL), streptomycin (50 μg/mL), NG-monomethyl-L-arginine-HOAc (NMA; $Cyclo_3$pss Biochemical, State Lake, UT) and 10% heat-inactivated normal rat serum was used as culture medium. For MLR, responder cells (1.75×10^5 cells/well) and irradiated (20 Gy) stimulator cells (3×10^5 cells/well) were placed in round-bottom 96-well plates. Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C for 4 days. Then 1 μCi of ^{3}H-thymidine was added to each well 16 hours before the termination of the cultures. Cultures were harvested and ^{3}H-thymidine incorporation was determined in a liquid scintillation counter.

Statistical Analysis

The differences of graft survivals among groups were analyzed using the log-rank test. Other data were expressed as mean ± standard deviation, and statistical analysis was performed with the Student t test or analysis of variance. A probability level of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of LP-CL on Phagocytic Macrophages and Immature DC in the Spleen

The depleting efficacy of LP-CL was examined at 7 days after the second LP-CL injection using immunofluorescent staining with mAb ED1, ED2, and CD11c. In the spleen of naive and Control-LP-treated animals, abundant ED1$^+$ and ED2$^+$ macrophages were seen (Fig. 1A). CD11c expression also was seen in the red pulp, splenic marginal zone, and splenic periarteriolar lymphoid sheath (T cell area). LP-CL treatment successfully reduced the number of ED1, ED2, and CD11c expressing macrophages and DC. The numbers of positively stained cells were reduced to 7 to 22% (ED1, 22%; ED2, 7%; CD11c, 15%) of those in Control-LP and naive animals. By 30 days after the second injection, ED1, ED2, and CD11c positive cells were repopulated with normal frequencies and distributions (data not shown). The result indicated temporary reduction of macrophages and CD11c$^+$ DC in the spleen with LP-CL.

When we examined other secondary lymphoid organs (cervical and mesenteric lymph node, and thymus), the frequencies of ED1$^+$, ED2$^+$, and CD11c$^+$ cells were similar between naive, Control-LP-treated, and LP-CL-treated animals (Supplementary Fig. S1). The result is consistent with previous studies demonstrating that intravenously administered LP-CL targets phagocytes in the liver and spleen without affecting those in the lymph nodes because liposomes are not able to cross capillary walls and other vascular barriers (e.g., blood brain barrier).[16,17]

Flow cytometric analysis of splenocytes showed that in LP-CL-treated animals, ED1$^+$, ED2$^+$, and CD11c$^+$ cells significantly decreased, while R7.3$^+$, CD4$^+$, CD8$^+$, and OX33$^+$ B cell populations marginally increased when compared with those of Control-LP-treated or naive animals (Supplementary Fig. S2). Results suggested that the elimination of ED1$^+$, ED2$^+$, and CD11c$^+$ DC resulted in a relative percentage increase of other populations in flow cytometry (Supplementary Fig. S2). Further analysis of T cell function in LP-CL-treated animals in MLR revealed that splenocytes from LP-CL-treated animals had normal proliferative capability against allogenic stimulator cells (Fig. 1B).

LP-CL Recipient Treatment Did Not Alter Allogenic Cell Migration Patterns

To examine the effects of LP-CL treatment and phagocytic cell depletion on the early migration patterns of allogenic passenger leukocytes, we first adopted an in vivo confocal fluorescent imaging system and conducted real-time tracking of infused allogenic CFSE$^+$ cells in the spleen. Intravenously infused allogenic cells were identified in the imaging field of the host spleen immediately after infusion. Initially, the majority of CFSE$^+$ cells appeared in the visual field but quickly disappeared from the field without lodging, suggesting that CFSE$^+$ cells were in the splenic circulation. Nevertheless, a few CFSE$^+$ cells started to lodge into the splenic environment soon after infusion (Fig. 2A). Once infused CFSE$^+$ cells were lodged, they appeared to be fixed and were not relocated. The number of lodged cells gradually increased during the first 24 hours. The results obtained from real-time tracking suggest that infused allogenic cells embed at permanent positions in the spleen without repositioning.

Subsequently, migration patterns and immune responses were studied at 24 to 72 hours after injection of CFSE-labeled T cell or non-T cell populations (2×10^8 cells per animal) into BN recipients treated with Control-LP or LP-CL without immunosuppression. At 24 hours after infusion, infused T cells were found in splenic T cell areas, while non-T populations lodged in splenic B cell follicles and red pulp, regardless of CL-LP or Control LP treatment (Fig. 2B), indicating that migration patterns

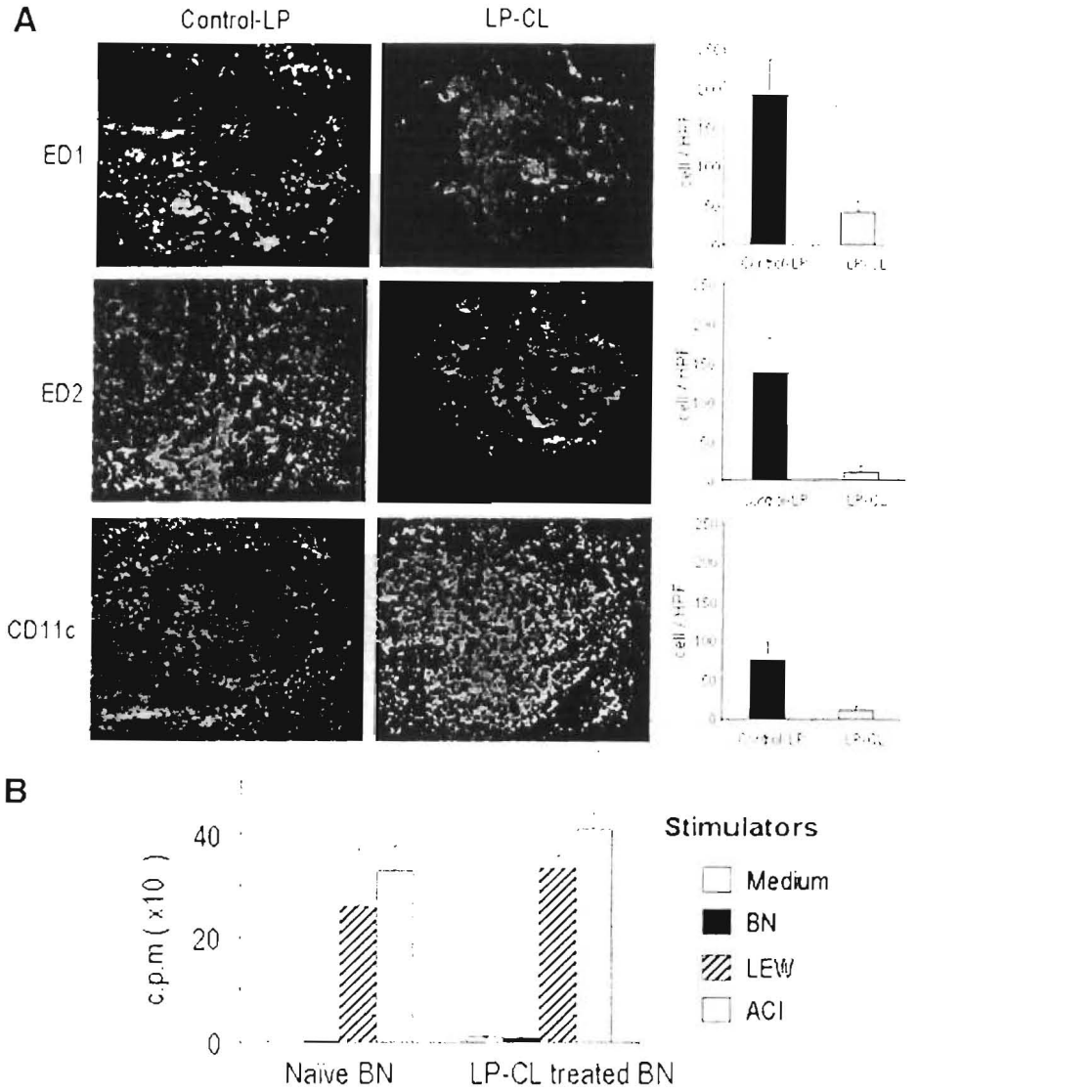

Figure 1. Efficacy of in vivo LP-CL treatment. (A) LP-CL or Control-LP was injected into BN animals at −10 and −7 days of analysis at a dose of 4.0 mL/kg (relevant to 100 mg/kg clodronate). The spleen sections were obtained at 7 days after the last liposome injection and stained with mAb for CD68 (ED1), CD163 (ED2), or CD11c with Cy3-conjugated goat anti-mouse IgG as secondary antibody (Ab) (red) to determine the efficacy to deplete phagocytes. The nucleus was stained with Hoechst dye (blue). Control-LP-treated animals showed normal distribution/frequency of ED1, ED2, and CD11c$^+$ cells as seen in naive animals. LP-CL-treated animals demonstrated successful elimination of host APC; ED1$^+$ and ED2$^+$ macrophages and CD11c$^+$ DC normally found in the splenic red pulp, marginal zone, and periarteriolar lymphoid sheaths (PALS) disappeared after treatment. The numbers of ED1$^+$, ED2$^+$, or CD11c$^+$ cells/high power field (HPF: ×400) in the spleen of Control-LP- or LP-CL-treated recipients (n = 3 each) were counted and shown in bar graphs. Original magnification; ×400. (B) MLR of splenocytes obtained from LP-CL-treated animals. Splenocytes of LP-CL-treated animals (n = 3) at 7 days after last liposome injection showed intact proliferative capacity against allogenic stimulator cells in MLR.

were not altered by LP-CL treatment. Most injected CFSE$^+$ cells disappeared by 72 hours of injection in non-immunosuppressed recipients (data not shown).

LP-CL Recipient Treatment Reduced IFN-γ Up-Regulation After Allogenic Cell Injection

Type 1 helper T cell (Th1) type immune response was analyzed in the spleen at 24 and 72 hours after allogenic cell injection to determine the effects of host phagocyte depletion. mRNA levels for IFN-γ dramatically increased in animals treated with Control-LP, and the non-T cell population induced stronger IFN-γ mRNA up-regulation than the T cell population (Fig. 2C). In contrast, animals treated with LP-CL showed significantly lower IFN-γ mRNA increases after T or non-T cell injection. The result suggests the considerable impact of depletion of host phagocytic cells with LP-CL treatment on early cytokine responses. There was no significant difference in IL-2 mRNA levels among groups. mRNA levels for IFN-γ were reduced to the baseline levels by 72 hours after injection.

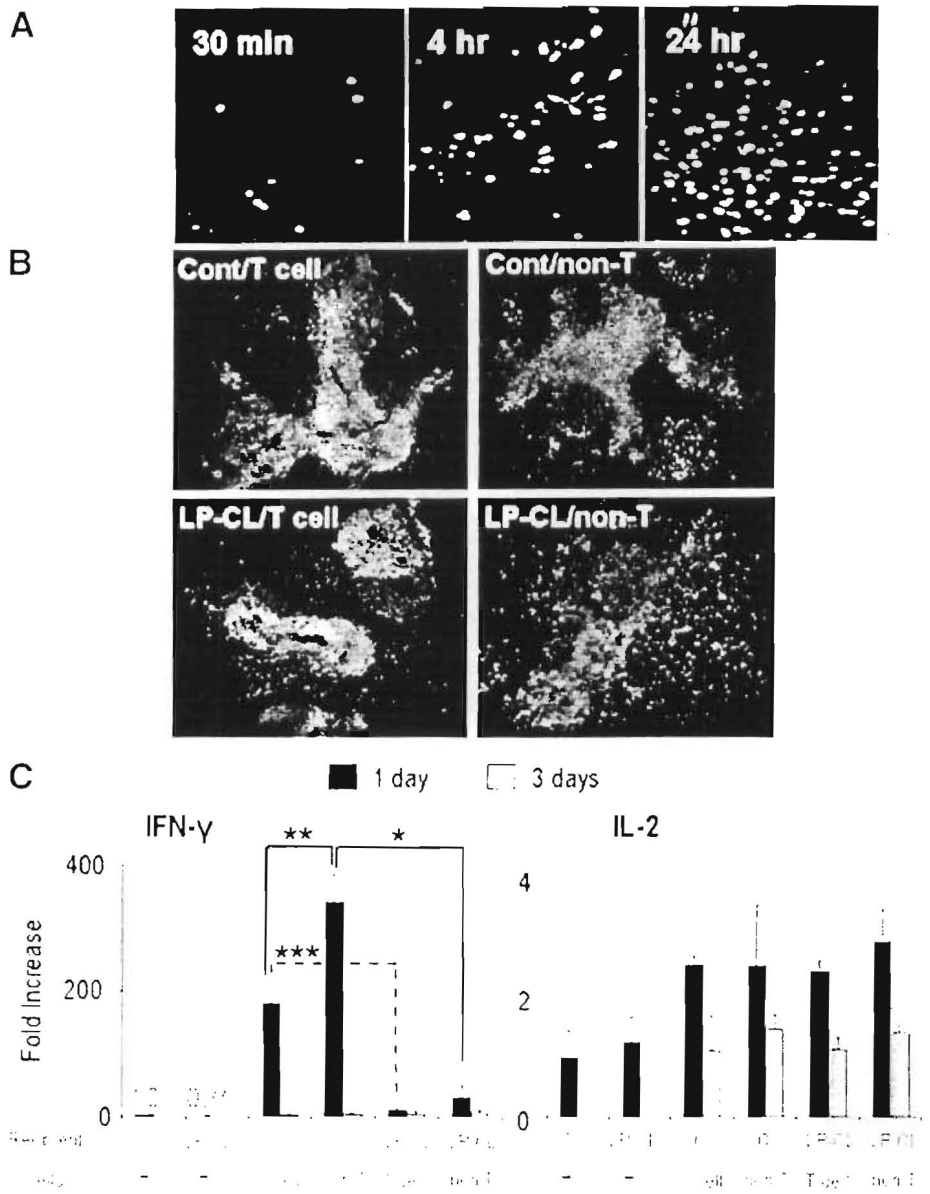

Figure 2. **Real-time visualization and migration pattern of infused CFSE$^+$ allogenic cell and subsequent Th1 cytokine mRNA up-regulation in the spleen. (A) Sequential real-time tracking images of infused CFSE labeled LEW splenocytes were captured in host BN spleen using in vivo confocal fluorescent microscopy. Infused allogenic cells immediately migrated to host spleen and promptly localized at stable positions. The number of lodged CFSE$^+$ cells increased with time (original magnification: approximately ×400). (B) Migration patterns of CFSE-labeled LEW T cells or non-T cells (2×10^8 cells per animal) were analyzed in the spleen of recipients treated with Control-LP or LP-CL (40 mL/kg at −10 and −7 days). The spleen samples were stained with phycoerythrin (PE)-conjugated R7.3 mAb to mark the T cell area [periarteriolar lymphoid sheaths (PALS), red]. At 1 day, infused CFSE$^+$ (green) T cells were found in the T cell area, while non-T cells lodged in B cell follicles and red pulps in control recipients. In LP-CL recipients, the migration patterns were not altered, and allogenic T cells and non-T cells were found in T cell area and B cell follicles/red pulps, respectively (original magnification: ×100). (C) mRNA levels of cytokines in the host spleen. Splenic Th1 type immune response was examined by real time RT-PCR at 1 and 3 days after cell infusion into BN recipients treated with Control-LP (C) or LP-CL (n = 3-4 in each group and each time point). Values were shown as fold increase versus normal spleen. *P < 0.01; **P < 0.05; ***P < 0.001.**

LP-CL Recipient Treatment Augmented Anti-Donor Alloimmune Responses After LT

Host phagocytic cell elimination could modify allosensitization, and in vivo influence of host LP-CL treatment was examined by conducting allogenic LT into LP-LC-treated recipients. Without immunosuppression, LEW liver allograft survival in Control-LP-treated BN recipients was 23.5 days (median, n = 4). In LP-CL-treated BN recipients, LEW liver graft survival was slightly shorter, with a median of 17.5 days (n = 4); however, no statistical difference was observed. When recipients

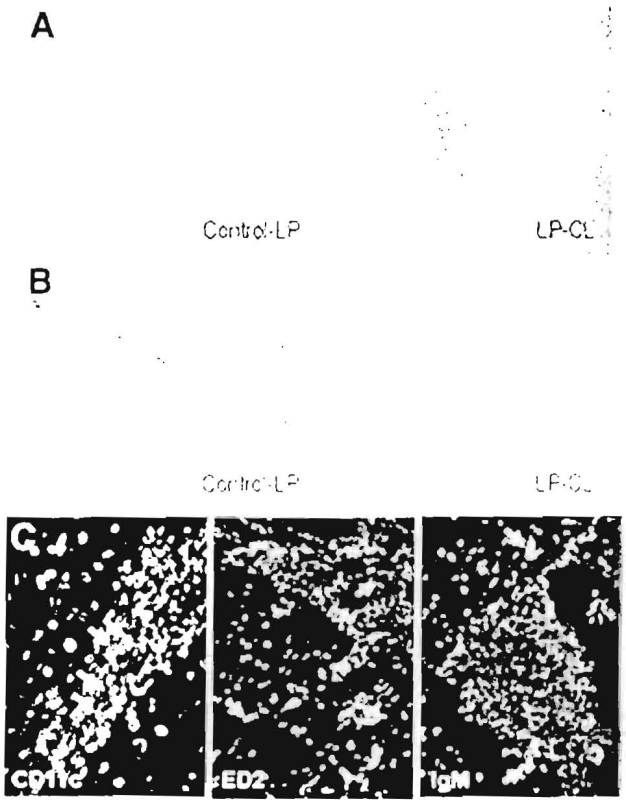

Figure 3. **Histopathology and characterization of donor-derived MHC class II$^+$ cells in liver allograft at 100 days after OLTx. At 100 days after OLTx, liver allografts were obtained from Control-LP- or LP-CL-treated BN recipients and examined by routine histopathology and immunohistochemistry with donor MHC class II mAb (L21-6). (A) Hematoxylin and eosin (H&E)-stained section showed that liver grafts in control (left) were free from chronic rejection (CR). In the LP-CL group (right), the liver allografts were essentially normal, but mild lymphocytic infiltration in the portal triad was more prominent (original magnification; ×200). (B) L21-6$^+$ donor MHC class II$^+$ cells (brown) were found in the portal triad of control liver graft (left), while they totally disappeared from liver grafts in LP-CL-treated recipients (right). Biliary epithelial cells in the LP-CL group were positive for L21-6 (original magnification: ×200). (C) Further characterization of donor MHC class II$^+$ cells by double immunofluorescence using Cy3-conjugated L21-6 (red) demonstrated that the majority of L21-6$^+$ cells were CD11c (green) positive DC. There were few L21-6$^+$/ED2$^+$ and L21-6$^+$/IgM$^+$ cells (original magnification: ×600). Representative images of 4 experiments.**

were treated with a short course of TAC immunosuppression (1.0 mg/kg on days 0-6, 13, and 20), liver allograft survival was prolonged to >100 days in both Control-LP- and LP-CL-treated recipients (n = 8 for each). Histopathological analysis of liver allografts at 100 days revealed that liver allografts in the Control-LP group were free from chronic rejection (Fig. 3A, left), as previously reported in naive BN recipients.[26] Liver allografts in the LP-CL group at 100 days also showed essentially normal hepatic architecture; however, lymphocytic infiltration in the portal triad was more evident than in the Control-LP group (Fig. 3A, right).

A remarkable difference between the Control-LP and LP-CL groups, however, was noticed in the frequency of donor MHC class II$^+$ (L21-6$^+$) cells maintained in the liver allografts (Fig. 3B). At 100 days, liver allografts in Control-LP group showed abundant L21-6$^+$ cells in the portal triad with a similar distribution pattern and frequency seen in naive LEW liver (Fig. 3B, left); this finding was consistent with our previous study using naive BN as recipients.[35] Further analysis using double immunofluorescence confirmed that the majority of L21-6$^+$ cells persisting in liver allografts were CD11c$^+$ DC (Fig. 3C, left) with few ED2$^+$ Kupffer cells (Fig. 3C, middle) and IgM$^+$ B cells (Fig. 3C, right). In contrast, almost all L21-6$^+$ cells disappeared from liver allografts in LP-CL-treated recipients at 100 days. Instead, the biliary epithelial cells in liver allografts became L21-6$^+$, indicating the up-regulation of MHC class II on graft bile ducts due to alloimmune reactions (Fig. 3B, right).

Donor MHC Class II Disappeared Early After LT in LP-CL-Treated Recipients

To investigate whether the difference in the number of L21-6$^+$ cells in the liver allografts took place during the early posttransplantation period, liver grafts and host spleens were analyzed 1 and 7 days after transplantation. In liver allografts in LP-CL-treated recipients, almost all L21-6$^+$ cells disappeared by 7 days after OLTx, while donor MHC class II$^+$ cells were found in the portal triad of liver grafts in Control-LP-treated recipients at 7 days after OLTx (Fig. 4A).

Likewise, in the host spleen, at 1 day after OLTx, L21-6$^+$ cells were detected in the red pulp, periarteriolar lymphoid sheath, and marginal zone in both groups (Fig. 4A); however, by 7 days, L21-6$^+$ cells in LP-CL-treated recipients mostly disappeared from the spleen. Significantly more donor MHC class II$^+$ cells remained in the spleen of control recipients (Fig. 4B).

Early Cytokine Up-Regulation Was Inhibited in LP-CL-Treated Recipients

Splenic mRNA levels for IFN-γ and IL-2 increased in Control-LP-treated recipients at 1 day after OLTx under TAC, while LP-CL-treated recipients showed a significant reduction of IFN-γ and IL-2 up-regulation (Fig. 5). There was no difference in splenic mRNA expression of IL-10. By 7 days, mRNA levels decreased to near baseline levels in both groups of recipients.

Donor Strain Challenge HTx Into Liver Allograft Recipients

Although both groups of liver allografts survived >100 days, there was an apparent difference in the presence of donor MHC class II$^+$ (L21-6$^+$) cells in liver allografts due to LP-CL treatment. To determine if the disappearance of donor MHC class II$^+$ cells caused further changes in recipient anti-donor immune responses, we challenged BN recipients bearing LEW liver allograft with LEW donor heart grafts at 100 days after OLTx. Heart grafts in both the Control-LP (n = 3) and LP-CL

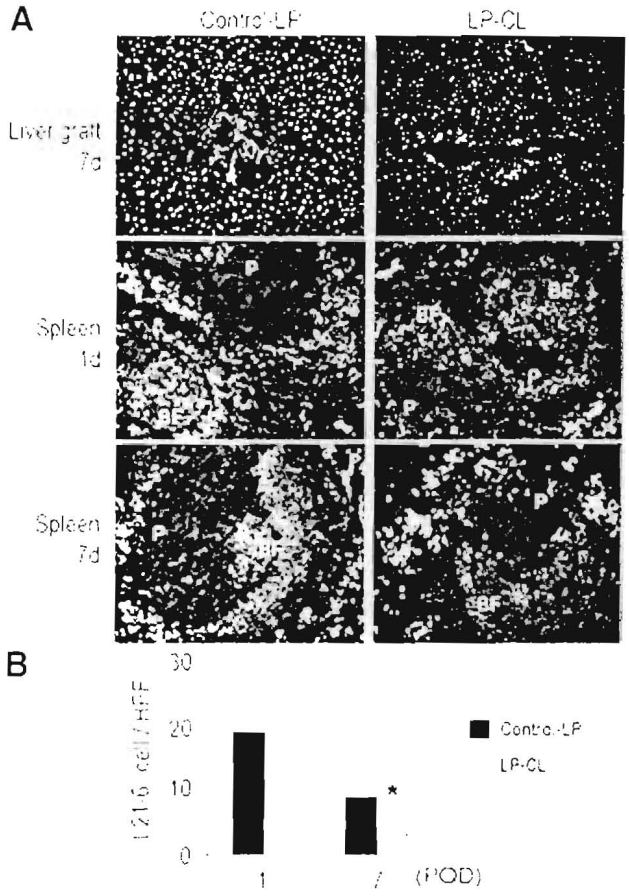

Figure 4. Donor MHC class II$^+$ cell migration early after OLTx. (A) Upper: At 7 days after OLTx under TAC, L21-6$^+$ cells (donor MHC class II$^+$ DC) were found in the portal triad of control liver grafts. In contrast, L21-6$^+$ cells disappeared from liver grafts of LP-CL-treated recipients at 7 days. Immunofluorescent staining with Cy3-conjugated L21-6 (red) (original magnification: ×200). Middle and lower: In host spleen, at 1 day after OLTx, L21-6$^+$ cells were detected in the red pulp, periarteriolar lymphoid sheath (PALS), and marginal zone in both Control-LP and LP-CL-treated recipients. However, at 7 days after OLTx, donor L21-6$^+$ cells in LP-CL-treated recipients mostly disappeared from the spleen, while significant numbers of L21-6$^+$ cells remained in the spleen of control recipients. Double immunofluorescence using Cy3-conjugated L21-6 (red) and FITC-conjugated anti-rat IgM (green) with nuclear Hoechst dye (Blue). BF, B cell follicle (IgM$^+$); MZ, marginal zone; P, periarteriolar lymphoid sheath (PALS). (B) The number of donor L21-6$^+$ cells/high power field (HPF) in Control-LP- or LP-CL-treated recipient spleen at 1 and 7 days after OLTx (n = 4 in each time point). *P < 0.05, Control-LP versus LP-CL.

(n = 4) groups survived for >100 days. However, heart grafts in the Control-LP group appeared to beat more vigorously than those in the LP-CL group. Histopathological analysis revealed that cardiac challenge allografts in the Control-LP group were essentially normal (Fig. 6A, left), whereas those in LP-CL group had moderate endocardial, pericardial, interstitial, and minimal periarterial mononuclear aggregation (Fig. 6A, right). Furthermore, L21-6 stain of challenge heart grafts in the Control-LP group showed homogeneous distribution of spindle-shaped interstitial donor MHC class II$^+$ cells through the myocardium with a frequency of 18.6 ± 5.7 cells/high power field, as seen in naive heart (Fig. 6B, left). On the other hand, no definite L21-6$^+$ interstitial cells were found in challenge heart grafts in the LP-CL group. In this group, L21-6 staining was seen on the vascular endothelial cells, indicating MHC class II up-regulation due to rejection (Fig. 6B, right).

MLR was conducted at different time points before and after challenge HTx. At 100 days after OLTx (before challenge HTx), lymphocytes from Control-LP-treated recipients demonstrated donor-specific hyporesponsiveness. LP-CL-treated recipients showed significantly higher proliferative responses to donor strain stimulator cells compared with control recipients, although anti-donor proliferation was low compared to responses to third party stimulator cells. Both groups of recipients showed lower anti-donor (LEW) proliferation when compared to that of naive BN (Fig. 7). After donor strain challenge heart grafting, in vitro anti-donor reactivity was further enhanced in recipients in the LP-CL group. At 100 days after challenge HTx (200 days after OLTx), although recipients in the Control-LP group maintained donor-specific hyporeactivity, LP-CL-treated recipients showed significantly ($P < 0.05$) increased proliferation to donor antigens compared to Control-LP-treated recipients.

LP-CL Treatment Abrogated Spontaneous Acceptance of Liver Allografts

To further assess the effects of host phagocyte depletion on in vivo LT outcomes, we next applied recipient LP-CL pretreatment (4 mL/kg on days −7 and −10) in the model of spontaneous liver allograft acceptance. When Control-LP-pretreated LEW recipients received BN liver allografts, all liver grafts were accepted without immunosuppression, and recipients survived for >100 days. In contrast, with BN liver grafts transplanted into LP-CL-treated recipients, 4 of 7 (57%) died within 32 days

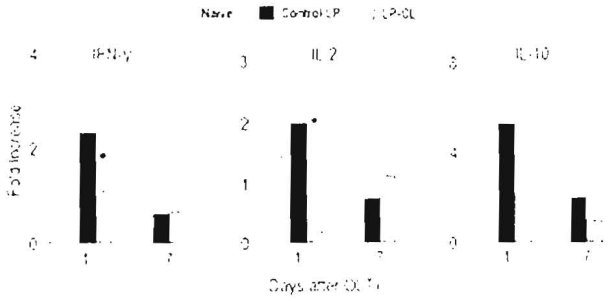

Figure 5. mRNA levels for cytokines in the spleen by real-time RT-PCR. Spleen samples were obtained from control (n = 4) and LP-CL recipients (n = 4) at 1 and 7 days after OLTx under TAC immunosuppression. At 1 day after OLTx, mRNA levels for IFN-γ and IL-2 were significantly lower in LP-CL recipients than in control recipients. However, IL-10 mRNA levels were not altered. By day 7 after OLTx, IFN-γ and IL-2 mRNA levels returned to the baseline values. Values were shown as fold increase compared to normal spleen (open bar). *P < 0.05, Control-LP versus LP-CL.

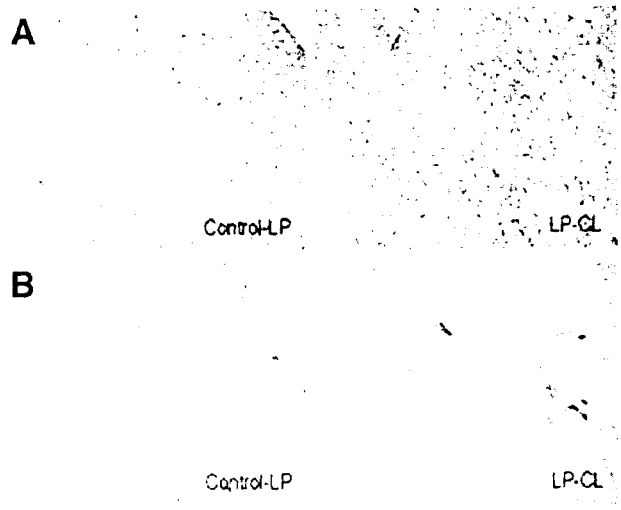

Figure 6. **Histopathology and donor MHC class II$^+$ cells in challenge heart allografts. At 100 days after OLTx in Control-LP- or LP-CL-treated BN animals, LEW challenge heart grafts were placed. After following additional 100 days, heart allografts were obtained for routine histopathology and donor MHC class II$^+$ stain. (A) Hematoxylin and eosin (H&E)-stained sections of cardiac challenge allografts in Control-LP-treated recipients showed essentially normal architecture. In contrast, challenge heart grafts in LP-CL-treated recipients had moderate endocardial, pericardial, interstitial, and minimal periarterial mononuclear aggregation (original magnification: ×200). (B) In challenge heart allografts from Control-LP-treated recipients, L21-6$^+$ spindle-shaped interstitial cells (brown) were easily found [18.6 ± 5.7 cells/high power field (HPF)]. No definite L21-6$^+$ cells were found in challenge heart grafts of LP-CL-treated recipients. Instead, endothelium of the coronary artery showed L21-6 positivity (MHC class II up-regulation) (original magnification ×200). Representative images of 4 experiments.**

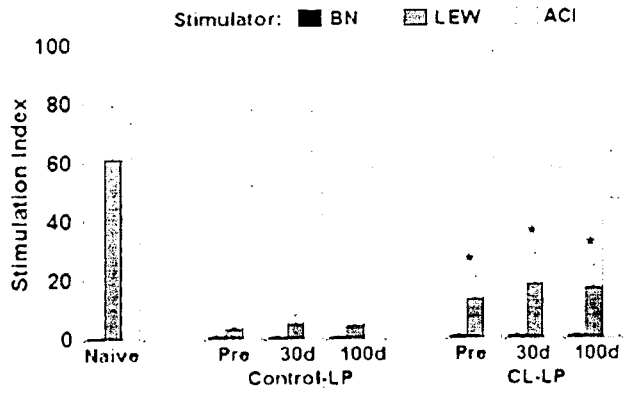

Figure 7. **One-way MLR after OLTx and subsequent challenge HTx. At 100 days after OLTx and just before challenge HTx, lymphocytes from Control-LP- and LP-CL-treated recipients demonstrated donor-specific hyporesponsiveness; however, LP-CL-treated recipients showed significantly higher anti-donor proliferative responses compared with Control-LP-treated recipients. Anti-donor proliferative reactivity increased in recipients in the LP-CL group after donor strain challenge HTx. At 100 days after HTx (200 days after OLTx), Control-LP-treated recipients maintained donor-specific hyporeactivity, and LP-CL-treated recipients showed significantly increased proliferation to donor antigens compared to Control-LP-treated recipients (*$P < 0.05$). n = 3-4 for each group.**

(Fig. 8). The result indicated the beneficial roles of host phagocytes in inducing liver allograft acceptance in this model.

DISCUSSION

This study demonstrated the influence of host splenic phagocyte depletion with LP-CL on alloimmune responses after LT. In the LEW to BN rat LT model, the lack of host cells capable of phagocytosing alloantigens and presenting them to host T cells during the early posttransplantation period resulted in an inferior degree of liver allograft acceptance. Under a therapeutic dose of TAC immunosuppression, histopathological analyses and in vitro T cell proliferative assays showed significant anti-donor alloreactivity in LP-CL-treated liver recipients compared to those in Control-LP-treated recipients. The finding was further substantiated in the model of spontaneous liver allograft acceptance: BN liver allografts, which normally were accepted in LEW rat recipients without immunosuppression,[36] were rejected in 57% of LP-CL-treated recipients.

After LT, Th1 cytokines have been shown to be up-regulated in the spleen mainly by host T cells.[37] In this study, the lack of host phagocytes in LP-CL-treated liver transplant recipients during the early posttransplantation period resulted in a significant reduction of mRNA levels for Th1 cytokines (INF-γ and IL-2) in the spleen. Likewise, allogenic cell infusion into LP-CL-treated recipients was associated with reduced Th1 cytokine up-regulation and less T cell proliferation compared to those in Control-LP-treated recipients. Thus, the elimination of host phagocytes early after allogenic organ/cell transplantation resulted in reduced host T cell activation and cytokine up-regulation, suggesting that early T cell activation after organ/cell transplantation might involve the indirect pathway of allorecogni-

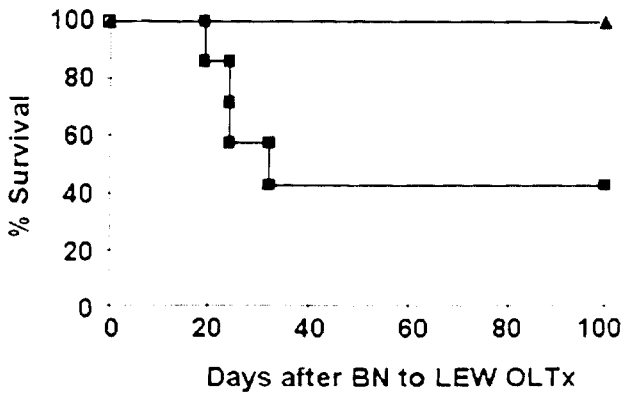

Figure 8. **BN liver allograft survival in LEW rats. All Control-LP-treated LEW recipients survived for >100 days after transplantation of BN liver allografts (n = 8). In contrast, the survival was significantly ($P < .05$) impaired, when recipient phagocytes were depleted with LP-CL treatment (−10 and −7 days before liver transplant), and 4 of 7 recipients pretreated with LP-CL died within 35 days after transplantation.**

tion via host phagocytes. The importance of indirect allorecognition in the acute phase of organ transplantation has been previously examined in several experimental studies using genetically engineered mice.[12-14,38] In a single MHC discordance model, cardiac or skin allografts from human leukocyte antigen-A2-transgenic C57BL/6 mice were acutely rejected in normal C57BL/6 mice, and it was suggested that indirect allorecognition of donor MHC class I molecules led to the development of alloantibodies and allograft rejection.[13,14] In other studies using MHC class II transactivator (CIITA)-deficient mice as the donor, cardiac or skin allografts from $CIITA^{-/-}$ mice were acutely rejected in unmodified and CD8-depleted recipients.[12,39] Because $CIITA^{-/-}$ allografts were not able to directly present class II alloantigens to host $CD4^+$ T cells, the rejection response was believed to be due to host $CD4^+$ T cells responding to indirectly presented alloantigens by host APC. In agreement with these studies in genetically engineered mice, the current study using normal inbred rats may suggest that host phagocytic cells and indirect allorecognition could play roles in acute phase alloimmune responses after LT.

An interesting finding in this study was the early loss of donor MHC class II^+ cells and the development of significant anti-donor alloreactivity in LP-CL-treated recipients. In control liver recipients with normal host phagocyte function, numerous donor MHC class II^+ cells persisted for >200 days in the spleen and portal triads of liver allografts, as we have previously reported.[26,35,40,41] Persisting donor MHC class II^+ cells in the liver allografts showed a similar frequency and distribution pattern as that seen in the normal liver and included $CD11c^+$ donor DC. However, in recipients treated with LP-CL, donor MHC class II^+ cells quickly disappeared from the liver allograft and host spleen, which is associated with the loss of typical hepatic tolerance. Because LP-CL was administered to recipients 7 days before LT, LP-CL unlikely had a direct effect in eliminating donor MHC class II^+ cells after transplantation. The previous study by Buiting et al.[29] showed that the concentration of clodronate in the blood, liver, spleen, and lung increased after LP-CL injection with the peak at 0.5 hour. However, clodronate levels quickly decreased thereafter and became baseline by 24 hours. Fleisch[42] also showed the rapid clearance of free clodronate from the body by renal excretion. The data suggests that there is almost no residual LP-CL in the recipient at the time of LT (7 days after LP-CL injection). Alternatively, the disappearance of donor MHC class II^+ cells could be due to anti-donor alloimmune reactions in LP-CL-treated recipients of liver allografts. However, LP-LC recipient treatment resulted in reduced early anti-donor immune reactions, determined by Th1 type cytokine up-regulation and T cell proliferation, thus the early loss of donor MHC class II^+ cells in LP-CL-treated recipients unlikely was a direct consequence of alloimmune responses.

Accordingly, the study might suggest that host phagocytes are required for the survival of donor leukocytes, in particular MHC class II^+ hepatic DC. Soluble and membrane-bound signals are require in maintaining the phenotype, maturation, and function of DC, and experimental studies have identified diverse varieties of cytokines, chemokines, and growth factors that can affect DC homeostasis.[43] DC are sensitive to environmental changes, and host phagocyte depletion with LP-CL treatment could significantly alter local microenvironment. Macrophages and DC produce numerous cytokines (e.g., IL-3, IL-6), chemokines, and growth factors [e.g., granulocyte-macrophage colony-stimulating factor (GMCSF)], which might be necessary to sustain donor cells after transplantation. GMCSF has been known to have antiapoptotic action on DC, and LP-CL-induced DC apoptosis was prevented with exogenous GMCSF.[21] Although further studies are required, it is tempting to speculate that early loss of donor MHC class II^+ cells in the environment lacking host phagocytes in LP-CL-treated recipients might be due to the lack of signals to sustain these cells.

Reduced early alloimmune responses in LP-CL-treated recipients and subsequent failure in developing tolerance might suggest an active role of early Th1 cytokines and T cell proliferation for tolerance induction. This finding is a reminiscence of previous studies using mice deficient for these cytokines. IL-2-deficient or INF-γ-deficient mice failed to engraft allografts or induce tolerance under immunosuppression, which otherwise resulted in long-term heart allograft acceptance.[44-46] The generation and function of alloantigen-reactive T regulatory cells for the development of operational tolerance to donor alloantigens were also shown to be impaired in INF-γ-deficient mice.[47]

Alternatively, the inferior level of liver allograft acceptance in recipients treated with LP-CL in this study may suggest the contributory role of recipient phagocytes in developing liver transplant tolerance. Macrophages/monocytes and DC are known to produce immunosuppressive molecules, such as IL-10 and tryptophan catabolizing indoleamine 2, 3-dioxygenase.[48-50] In this study, LP-CL treatment might abrogate immunosuppressive signals from macrophages and DC and fail to regulate T cells to develop graft acceptance.

The application of the LP-CL method to deplete phagocytic cells certainly has its limitations because the method targets only the liver and spleen and does not have efficacy in lymph nodes due to the inability of liposomes to cross capillary walls.[16,17] However, using an in vitro whole-organ transplant system, the study demonstrated the significant roles of host cells with phagocytic capability in the spleen. Although there are several possible mechanisms for how phagocytic cells contribute to hepatic allograft acceptance, the indirect pathway of allorecognition during early posttransplantation period might play roles in determining alloimmune reactions after LT.

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