Targeting and Blocking B7 Costimulatory Molecules on Antigen-Presenting Cells Using CTLA4Ig-Conjugated Liposomes: *In Vitro* Characterization and *in Vivo* Factors Affecting Biodistribution

Chung-Gyu Park,[1,3,4] Natalie W. Thiex,[1] Kyung-Mi Lee,[2] Gregory L. Szot,[3] Jeffery A. Bluestone,[3,5] and Kyung-Dall Lee[1,5,6]

Received April 16, 2003; Accepted April 30, 2003

Purpose. CTLA4Ig, a fusion protein of CTLA-4 and Fc of immunoglobulin (Ig) heavy chain, inhibits the essential costimulatory signal for full T cell activation via blocking the interaction between CD28 and B7 molecules and renders T cell nonresponsiveness. CTLA4Ig has been used to control deleterious T cell activation in many experimental systems. We hypothesized that by conjugating CTLA4Ig to liposomes the efficacy of CTLA4Ig could be enhanced through multivalent ligand effect, superior targetability, and modification of the fate of ligated costimulatory molecules.

Methods and Results. Consistent with this hypothesis, liposome-conjugated CTLA4Ig bound to B7 and blocked their binding sites more efficiently than free CTLA4Ig, lowering the half maximal dose for B7 blocking by an order of the magnitude. These results were similar both in B7-1 expressing p815 cells and in activated macrophages. Moreover, CTLA4Ig-liposomes underwent rapid internalization upon cell surface binding through B7 molecules. In allogenic mixed lymphocyte reaction assays, the CTLA4Ig-liposomes were tested to show effective inhibition of T cell proliferation. In vivo, however, when CTLA4Ig-liposomes were injected into mice, a significant fraction was localized to the reticuloendothelial system (RES), presumably because of its binding to Fc receptors expressed on tissue macrophages. The Fc receptor-mediated uptake could be alleviated by coinjection of anti-FcR monoclonal antibody. In the mouse engrafted with pancreatic islets of Langerhans underneath the capsule of one kidney, despite the increased localization in RES, enhanced accumulation of CTLA4Ig-conjugated liposome was observed in the engrafted kidney compared to the contralateral kidney.

Conclusion. We show that the conjugation of CTLA4Ig to liposome could increase the efficiency of the targeting by increasing the binding avidity at cellular level and by increasing the concentration at the target site in *in vivo* system. The biodistribution and circulation time data suggested that the CTLA4Ig-liposomes could be improved upon minimizing the FcR-mediated uptake by Fc receptor-bearing cells. Thus, the strategy of conjugating CTLA4Ig to liposomes could be exploited for immune intervention in transplantation and autoimmune diseases for the efficient blocking of costimulation.

KEY WORDS: B7 costimulatory molecules; targeting; CTLA4Ig; liposomes; islet transplantation.

INTRODUCTION

Two distinct signals are essential for full activation of T cells: 1) the antigen-specific signal that is generated upon the engagement of T cell receptor (TCR) by the peptide bound to the major histocompatibility complex (MHC) on antigen-presenting cells (APCs); and 2) the costimulatory signal through the ligation of costimulatory receptors by their ligands expressed on APCs (1).

One of the most potent costimulatory signals known to date is generated through CD28, a molecule expressed constitutively on the surface of T cells. CD28 interacts with two ligands, B7-1 (CD80) and B7-2 (CD86), both of which are expressed on the surface of APCs (1). CD28 and CTLA-4 are two T cell surface receptors for B7-1 and B7-2. Upon binding to its ligand, CD28 provides an essential costimulatory signal required for full activation of T cells (1). However, CTLA-4, a structural homologue of CD28 expressed on T cells, binds to the same B7 ligands but inhibits T cell activation. This suggests that CTLA-4 regulates T cell activation through a distinct pathway. The affinity of CTLA-4 for B7 molecules is 20-fold higher than that of CD28 for B7 (2,3). Using these characteristics, the B7-binding region in the extracellular domain of CTLA-4 was fused to the Fc portion of IgG to generate a soluble protein CTLA4Ig as an agent to inhibit CD28/B7-mediated costimulation; although the CTLA-4 ectodomain can bind monovalently and block B7 molecules, fusion to the Fc portion of IgG generates a bivalent molecule that possesses higher protein stability with relative ease of manufacturing processes for pharmaceutical applications. Blocking B7 binding sites with CTLA4Ig has been reported to compete with CD28 binding to B7 molecules and thus to prevent antigen-specific activation of T cells, thus rendering T cells unresponsive or anergic (4). Specifically, CTLA4Ig has been used to prevent organ transplant rejection (5,6), lupus-like autoimmune disease (7), experimental autoimmune encephalomyelitis (8,9), autoimmune oophoritis in experimental models (10), and asthmatic lung inflammation (11). The efficacy of CTLA4Ig has also been tested in phase I clinical trials for patients with psoriasis (12).

Despite the success in using the soluble CTLA4Ig fusion protein in clinical settings, its full potential has yet to be achieved. Current treatment regimens with CTLA4Ig solution involve multiple injections of high doses at a high frequency, which may be burdensome to patients and may lead to undesirable side effects. The problems associated with the current formulation of soluble CTLA4Ig can be largely solved by a delivery strategy to enhance the pharmacokinetic and pharmacodynamic parameters (13–15). We hypothesized in this report that the efficacy of CTLA4Ig could be altered and greatly improved by conjugation to sterically stabilized, long-circulating liposomes.

Liposomes have been used widely as carriers for a range of both hydrophilic and hydrophobic diagnostic or therapeu-

[1] Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109-1065.

[2] The Committee on Immunology, Department of Pathology, University of Chicago, Chicago, Illinois 60637.

[3] UCSF Diabetes Center, University of California, San Francisco, California 94143-0540.

[4] Department of Microbiology and Immunology, The Transplantation Research Institute, Seoul National University College of Medicine, Seoul 110-799, Korea.

[5] These authors made equal contributions, and are co-senior authors of this article.

[6] To whom correspondence should be addressed. (e-mail: kdlee@umich.edu)

tic agents (16). Formulation in liposomes provides a larger drug payload per particle, protects encapsulated agents from metabolic processes, and increases affinity through multivalent binding to target cells. The lipid composition of liposomes can be modified for specific needs thus acquiring additional desirable properties, such as prolonged circulatory half-life in plasma, ability to complex with nucleic acids to mediate gene delivery or genetic regulation, and capacity to deliver encapsulated contents to the cytosol of cells through the endosomal/lysosomal pathway (17,18). Conventional liposomes have been shown to be rapidly cleared from plasma circulation by the reticuloendothelial system (RES). Sterically stabilized liposomes containing 5–10 mol % PEG-PE, polyethylene glycol (PEG) of 50–250 chain length conjugated to phospatidylethanolamine (PE), however, exhibit retarded uptake by the RES and thus have a prolonged circulatory half-life (16,19). Additionally, a functional maleimide can be attached to the terminal end of the PEG for conjugating proteins or other molecules to the liposome for targeted drug delivery (20,21). Conjugation of ligands, functional proteins, or monoclonal antibodies has been extensively explored, primarily for targeting specific cells or tissues. The concept of the so-called "immunoliposomes" is one example of such approaches (22,23). One of the most advanced immunoliposome formulations is the anti-HER2 mAb conjugated to PEG-liposomes carrying antitumor compound; PEGylated liposomes were reported to accumulate in solid tumors, but mostly extracellular in the interstitial space with no intracellular distribution unless anti-HER2 antibodies were conjugated (24). The antitumor effect, mediated by ligation of HER2 receptors by the mAb, was reported to be augmented by liposomal conjugation. Our approach using CTLA4Ig is analogous to that used for the anti-HER2 mAb-PEGylated liposome strategy. One major difference of our system, and perhaps an advantage in comparison with other systems, is the fact that the intended target cells for CTLA4Ig-liposomes are primarily antigen presenting cells like macrophages in which liposomes have a natural propensity to accumulate even without targeting.

In this study, we generated CTLA4Ig-liposomes and tested its efficacy in blocking and downregulating B7 molecules as compared to free CTLA4Ig. We investigated its efficiency using B7-transfected P815 cells and activated bone marrow-derived macrophages (BMMs). Additionally, the fate of B7-bound CTLA4Ig-liposomes was determined to support the hypothesis that B7 molecules multivalently ligated by liposomal CTLA4Ig are induced to be internalized rapidly along with the liposomes; B7 molecules have a long cell surface half-life once expressed, and B7 ligated by soluble CTLA4Ig are believed to stay most likely on the cell surface while B7 bivalently ligated by anti-B7 antibody have been reported to be internalized at 37°C (25).

Subsequently, limited *in vivo* experiments were conducted to assess the targeting capability, biodistribution and circulation time of CTLA4Ig-liposomes in a mouse model as well as pancreatic islet transplant model. These studies demonstrated the potential therapeutic advantages of liposomal CTLA4Ig but an unexpected negative effect associated with liposomal conjugation. Twenty-four hours after injection, high levels of CTLA4Ig-liposome were accumulated in the liver and spleen, which could be alleviated by simultaneous injection of anti-FcR mAb. Regardless of circulation time, however, twice as much CTLA4Ig-liposome was accumulated in the kidney engrafted with pancreatic islets of Langerhans compared to the contralateral control kidney. A careful mechanistic investigation suggested that the circulation time, and subsequently targetability and efficacy, of CTLA4Ig-liposomes could be improved by modifying the extent of interaction of Fc moiety of the CTLA4Ig with Fc receptors.

The results in this report suggest that this approach might be useful for controlling the pharmacokinetic and biodistribution profiles of CTLA4Ig, applicable to treatments of organ transplantation scenarios and delivery of drugs site-selectively within the immune system. Thus, liposomal formulation of CTLA4Ig may become a potential strategy to improve the function of CTLA4Ig by combining with the intrinsic characteristics of liposomes.

MATERIALS AND METHODS

Cell Culture

A hybridoma cell line, HB-197 secreting 2.4G2 mAb (American Type Culture Collection, Manassas, VA, USA), B7-1 transfected P815 cells and nontransfected P815 parental cells (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's modified Eagles Medium (DMEM, Gibco/BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) supplemented with non-essential amino acids, β-mercaptoethanol (β-ME), HEPES and penicillin/streptomycin (Gibco/BRL).

Mouse bone marrow-derived macrophages were obtained from the femurs of female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA) and cultured *in vitro* as described (26). In brief, the mice were euthanized according to institutional guidelines and dissected to expose the whole femur. The femur was removed and the marrow was irrigated twice with 5 mL of DMEM containing 10% FBS. The harvested cells were washed with 10% FBS-DMEM by centrifugation for 5 min at 200 × g. The cells were resuspended in BMM media (DMEM containing 10% FBS, 30% L cell conditioned media (the source of Macrophage Colony Stimulating Factor), and β-ME), counted, and 4×10^6 cells were plated onto 100-mm flasks.

Animals

C3H, BALB/c and C57BL/6 mice were purchased from the Jackson Laboratory and were kept at the Pathogen-Free animal facilities of the University of Michigan and the University of Chicago. *In vivo* experimental protocols were used according to approved institutional guidelines.

Liposome Preparation

Liposomes composed of egg phosphatidylcholine (PC, Avanti Lipid Inc., Alabaster, AL, USA) and cholesterol (Calbiochem, La Jolla, CA, USA) in a 2:1 molar ratio were prepared as described before (19,20,24). Where indicated, 2 mole % of maleimideparabenzoic-PE (MPB-PE), a PE derivative with a functional maleimide, was incorporated into the lipid mixture to allow conjugation of CTLA4Ig to the surface of the liposome. After mixing, solvent was evaporated (Rotovapor R-200, Buchi), and the multilamellar lipid film was hydrated with either HBSE (10 mM HEPES, 140 mM

NaCl, 1 mM EDTA, pH 7.0) or 5-hydroxypyranine trisulfonate (HPTS; 35 mM in HBSE, pH 7.0, Molecular Probes, Eugene, OR, USA). The lipid solution was then subjected to four freeze-thaw cycles and four cycles of membrane extrusion (Lipex, Vancouver, BC, Canada) through 0.1-μm pore polycarbonate filters (Nucleopore Corning, Acton, MA, USA). After extrusion, the liposomes were kept at 4°C under argon until the time of the experiment. For animal studies, liposomes were prepared by lipid film hydration as above except the lipid composition was 10 mole % PEG-PE, PC, cholesterol, 1,2 Diacyl-SN-Glycero-3-Phosphoethanolamine-N-{Methoxy-(polyethyleneglycol)-2000} (PEG-PE, Avanti Polar Lipids), and maleimide-PEG-PE (MAL-PEG-PE) in a 9:5:0.75:0.25 molar ratio. The control liposomes without conjugated protein were composed of PC, cholesterol, and PEG-PE in a 9:5:1 molar ratio. Both CTLA4Ig-conjugated and non-conjugated liposomes contained 50 μCi of ^{3}H-labeled cholesteryloleate ether (Amersham Biosciences, Piscataway, NJ, USA) per μmole of phospholipid. The lipid films were hydrated with HBSE, freeze/thawed as above and extruded sequentially through 0.2-μm, 0.1-μm and 0.05-μm polycarbonate filters (four times each) to make liposomes of approximately 100-nm average diameter.

CTLA4Ig Conjugation to Liposomes

Modification of CTLA4Ig with Succinimidyl-S-Acetylthioacetate (SATA)

The human CTLA4Ig (obtained as a gift from Repligen Corporation, Cambridge MA, USA; Ref. 15) was modified with SATA following the protocol described previously (27). Briefly, CTLA4Ig in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 9 mM $CaCl_2{\cdot}2H_2O$, 5 mM $MgCl_2{\cdot}6H_2O$, pH 7.4) at a concentration of 2 mg/mL was incubated with SATA solubilized in dimethyl formamide for 25 min at 25°C with gentle mixing. The molar and volume ratios of CTLA4Ig to SATA were 1:20 and 1:100, respectively. Unreacted SATA was removed by dialysis at 4°C overnight. Deacetylation was performed by incubating with deacetylation solution (0.5 M hydroxylamine, 50 mM sodium phosphate, 25 mM EDTA, pH 7.0) for 2h at 25°C right before mixing with liposomes.

Coupling to Maleimide-PE-Containing Liposome and Separation of Free CTLA4Ig from Liposomes

Maleimide-containing liposomes were added to the deacetylated CTLA4Ig-linked SATA (CTLA4Ig-SATA) solution with the lipid to CTAL4Ig molar ratio of 290:1, and incubated overnight with gentle mixing at 4°C under Argon gas. Following conjugation, free unconjugated CTLA4Ig was removed by gel filtration (1 × 20 cm Sepharose CL-4B column, Amersham Biosciences, Piscataway, NJ, USA), eluant was collected. The amount of conjugated CTLA4Ig and phospholipid in the liposomal fraction from the column was assayed by quantitative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and phosphate assay (17,27), respectively. The number of conjugated CTLA4Ig molecules per liposome was calculated based on liposome size of 100 nm.

Purification of anti-FcR mAb 2.4G2

A hybridoma cell line HB-197 was cultured in DMEM with 4 mM glutamine, 10% FBS, and penicillin/streptomycin in a roller bottle, and the antibody from the culture supernatant was affinity purified using a Protein G Sepharose column (Amersham Biosciences, Piscataway, NJ, USA)

Assay for Available B7 Molecules: Targeting of CTLA4Ig-Liposomes to B7 Molecules on the Cell Surface

Cultured P815 cells and B7-1 transfected P815 cells were harvested and washed twice with PBS with 0.5% bovine serum albumin. Nonspecific Fc receptor binding was blocked by the addition of 2.4G2 mAb to 0.5×10^6 cells and incubated for 10 min at room temperature. Ten microliters of CTLA4Ig-liposomes or controls at different dilutions were added to the cells in suspension and incubated for 30 min at 4°C. Ten microliters of murine CTLA4Ig-FITC (500 μg/mL) were added to the cells and further incubated for 45 min at 4°C. Cells were then washed twice with 0.5% bovine serum albumin PBS and were analyzed by flow cytometry.

Internalization Study Using CTLA4Ig-Liposomes Containing Encapsulated HPTS

Murine bone marrow-derived macrophages were plated on cover slips in 24-well culture plates on culture day 7 at a density of 20,000 cells per coverslip. After 24 h, the cells were activated with 25 μg/mL of lipopolysaccharide from *Escherichia coli* (Sigma, St. Louis, MO, USA) and 250 U/mL of interferon-γ (Genzyme, Cambridge, MA, USA) for 24 h. Cells were first incubated with CTLA4Ig-liposomes encapsulating HPTS for 45 min at 4°C (28,29). Where indicated, an excess amount of free CTLA4Ig (125 μg/ml) was added to compete binding to B7s. After washing three times, cells were further incubated for 1 h either at 4°C or at 37°C to allow internalization. The cells were observed under a fluorescence microscope (Zeiss, Axiovert 135TV) equipped with alternating excitation filters (405 nm and 450 nm with 10 nm bandpass) and the images were monitored and analyzed by Metamorph Image Analysis System (Universal Imaging, West Chester, PA, USA).

Allogenic Mixed Lymphocyte Reaction (MLR).

Lymph node cells from BALB/c mice were harvested and used as responder T cells for allogenic MLR. The stimulator cells were prepared from the spleen of C57BL/6 mice irradiated (2000 rad) and depleted of T cells. Primary MLR cultures were performed in 96-well plates consisting of 2×10^5 responder BALB/c T cells and 2×10^5 B6 splenic cells in a total volume of 0.2 mL. Where indicated, free CTLA 4 Ig, liposomal CTLA 4 Ig, or empty liposomes were added to the well. Mixed cells were incubated for 96 h at 37°C and pulsed with ^{3}H-thymidine for the last 16 h. The amount of ^{3}H-thymidine uptake by proliferating T cells in each well was monitored by harvesting cells at the end of 16 h pulse and scintillation counting.

Mouse Islet Isolation and Transplantation

Mouse Islet Isolation

Murine islets were isolated from BALB/c as described previously (30). Briefly, animals were killed by cervical dislocation, and the pancreas was exposed and injected with

Hanks' balanced salt solution (Mediatech, Herndon, VA, USA) containing 0.5 mg/mL collagenase P (Roche Diagnostics Corporation, Indianapolis, IN, USA) via the common bile duct until distension was achieved. Digestion was performed at 37°C for 17 min with gentle shaking and terminated by the addition of cold RPMI containing 10% FBS and 2 mM L-glutamine (GIBCO, Carlsbad, CA, USA). Islets were purified on Euro-Ficoll (Sigma, St. Louis, MO) gradients by centrifugation at 900 × g for 15 min, routinely yielding preparations of >90% purity. Islets were hand-picked, counted, and scored for size. Subsequently, islets were rested and stabilized by culturing in RPMI 1640 medium supplemented with 11 mM glucose, 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium) for 2 h before transplantation.

Islet Transplantation under the Kidney Capsule

The isolated islets from BALB/c mice were transplanted under the capsule of the left kidney of recipient C57BL/6 mice. 500 handpicked isolated islets were pelleted by brief centrifugation at 200 × g. With the mouse under isoflurane anesthesia, the left kidney was exposed through a lumbar incision. A capsulotomy was performed in the lower pole of the kidney using a 30-gauge needle, and the tip of the tubing was advanced under the capsule of the upper pole, the site of final injection. The capsulotomy was cauterized after delivery of the islets, and the animal was closed using 5-O silk suture. Animals were allowed to recover under a heat lamp and blood glucose levels were checked.

Biodistribution of CTLA4Ig-Liposomes in Mice

One day after transplant surgery, mice were injected with radio-labeled CTLA4Ig-conjugated PEGylated liposomes via the tail vein. Where indicated, mice were injected intraperitonealy with 100 μg 2.4G2 mAb 90 min before liposome injection, and 50 μg 2.4G2 was also co-injected with the liposome formulation. Blood samples of approximately 100 μL in volume were taken at 1 h, 4 h, and 24 h postinjection by retroorbital puncture. At 24 h, animals were weighed, euthanized according to the institutional protocol, and the major organs were removed and weighed. Portions of each organ (approximately 0.1 g) were solubilized with Solvable (Packard Instruments, Meridan, CT, USA) for 2–24 h at 60°C. Subsequently, the samples were decolorized using 30% H_2O_2 (Fisher, Fair Lawn, NJ), and the radioactivity was counted using Hionic Fluor Scintillation Cocktail (Packard Instruments, Meridan, CT, USA) on a Beckman LS6000SC Beta counter. The total blood volume was estimated to be 7.7% of total body weight (31). The total radioactivity of the blood and other organs was extrapolated from the portions that were counted, and radioactivity due to the blood content of each tissue was accounted for according to a previously reported method (32). The radioactivity measured in each organ was divided by the total dose injected into each animal to calculate the percent injected dose in each organ.

RESULTS

CTLA4Ig-Liposomes Bind to B7-Expressing Cells with Higher Affinity

To assess targeting of CTLA4Ig-conjugated liposomes and their ability to block the binding sites of B7 molecules expressed on the cell surface, we prepared, tested and compared CTLA4Ig-liposomes with soluble CTLA4Ig by performing competitive binding assays using two cell culture systems: 1) parental and B7-1 transfected P815 cells; and 2) activated and nonactivated BMMs. The average number of CTLA4Ig molecules conjugated per liposome used in this study was approximately between 100 and 300, which was according to the calculation based on the experimentally determined conjugation efficiency of CTLA4Ig and the average liposome size of 100 nm in diameter (data not shown).

Cells were pre-incubated with either soluble free CTLA4Ig or CTLA4Ig-liposomes at varying concentrations, and subsequently incubated with FITC-labeled CTLA4Ig at 4°C to monitor the remaining unblocked B7 or the fraction of the prebound B7 that would be competed off by a high concentration of CTLA4Ig-FITC. The binding of CTLA4Ig-FITC was monitored by flow cytometry. As shown in Fig. 1A, CTLA4Ig-FITC bound to P815 cells expressing B7-1 and showed a single peak (curve p, Fig. 1A, upper panel). CTLA4Ig-FITC did not bind to P815 parental cells not expressing B7-1 (curve n, Fig. 1A), confirming that CTLA4Ig-FITC binds to B7-1 in a specific manner. Pre-incubation of cells with 50 μg/mL of liposomal CTLA4Ig completely blocked the binding of additional CTLA4Ig-FITC to B7 molecules (curve p shifts to curve a, Fig. 1A, upper panel); whereas preincubation with free CTLA4Ig (62.5 μg/mL) blocked B7 binding sites only partially (curve b, Fig. 1A, upper panel), which showed that liposomal CTLA4Ig has a higher affinity or avidity to B7-1 than free CTLA4Ig. The increased binding of liposomal CTLA4Ig to B7-1 was specific only to liposome-conjugated CTLA4Ig as only 50% of B7-1 in transfected P815 cells could be blocked by pre-incubating the cells with 62.5 μg/ml free or 62.5 μg/ml free CTLA4Ig mixed with blank liposomes (curve c, Fig. 1A, lower panel). Control, blank liposomes without conjugated CTLA4Ig showed no blocking (curve d, Fig. 1A, lower panel).

A similar result was obtained using primary cultures of bone marrow-derived macrophages (Fig. 1B), which often are classified as professional APCs. Upon activation with LPS and IFNγ, the level of B7 molecules on the BMM surface increased as detected by CTLA4Ig-FITC binding (from curve a to b, Fig. 1B). Preincubation of cells with 50 μg/ml of liposome-conjugated CTLA4Ig for 1h at 4°C blocked nearly 100% of the B7 molecules on activated BMMs (curve c, Fig. 1B).

To further investigate dose dependency, specificity, and binding avidity of liposomal CTLA4Ig, B7-1-transfected P815 cells were preincubated with varying concentrations of either free CTLA4Ig, liposomal CTLA4Ig, CTLA4Ig mixed with non-CTLA4Ig-liposomes (i.e., blank liposomes), liposomes conjugated with a non-specific antibody 10A6, or blank liposomes alone, and subsequently followed by staining with CTLA4Ig-FITC (Fig. 2). Free CTLA4Ig blocked 50% of cell surface B7 molecules at approximately 100 μg/ml, as monitored by CTLA4Ig-FITC staining (filled circle, Fig. 2). The liposome-conjugated CTLA4Ig had significantly enhanced blocking efficiency, showing 50% blockage at approximately 10 μg/ml CTLA4Ig (filled triangle, Fig. 2). This enhanced avidity could not be achieved by simply mixing free CTLA4Ig with empty liposomes as the CTLA4Ig mixed with blank liposomes behaved the same as free CTLA4Ig (open circle, Fig. 2), demonstrating that conjugation of CTLA4Ig onto li-

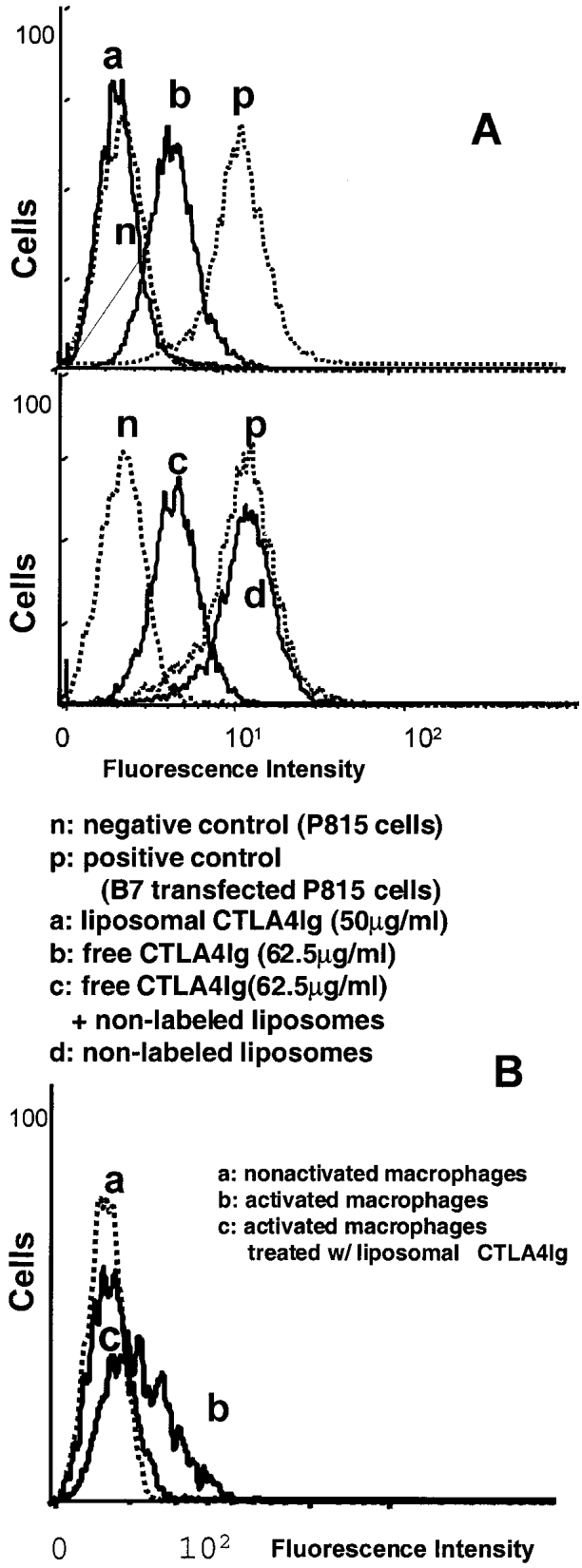

Fig. 1. A, Flow cytometric data showing CTLA4Ig labeled with FITC (CTLA4Ig-FITC) binding to P815 cells expressing of B7 and blocking of this interaction by liposomal CTLA4Ig formulations. The negative control (curve n) shows the basal level of CTLA4Ig-FITC binding to P815 cells, and the positive control (curve p) shows the level of CTLA4Ig-FITC binding to P815 cells transfected with a B7-1 gene.

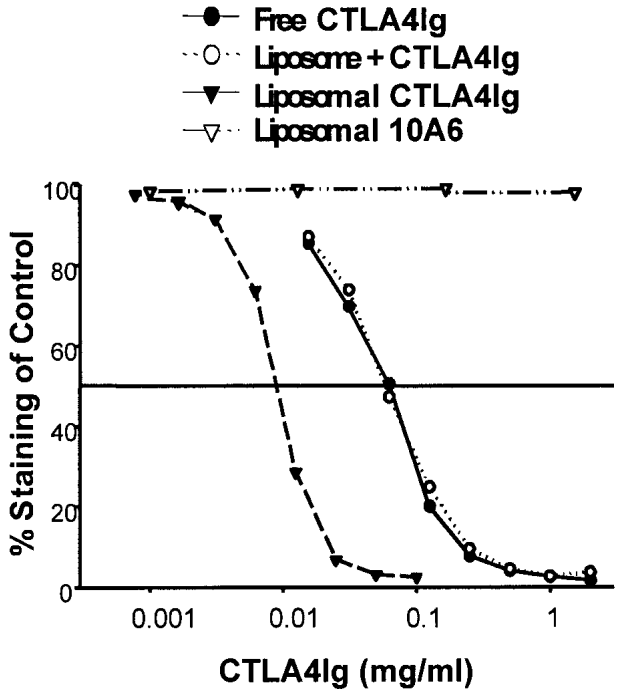

Fig. 2. A comparison of blocking of B7 expressed on P815 cells by free CTLA4Ig and liposomal CTLA4Ig. P815 cells were preincubated with free CTLA4Ig (filled circle), liposomal CTLA4Ig (filled triangle), mixture of empty liposomes and free CTLA4Ig (open circle), or control mAb 10A6 conjugated to liposomes (open triangle). Subsequently unblocked B7 was detected with CTLA4Ig-FITC staining and flow cytometry.

posomes was required. The B7 specificity of the CTLA4Ig-liposome-mediated blocking was shown by the lack of B7 blocking using the liposomes conjugated to irrelevant antibodies, 10A6 mAb (open triangle, Fig. 2). These data quantitatively support the hypothesis that liposomal CTLA4Ig is superior to free CTLA4Ig in blocking the B7 molecules, presumably through a multivalent ligand effect, and that CTLA4Ig-conjugated liposomes can be used to efficiently target liposomes to B7 expressing cells and block B7 molecules.

Liposomal CTLA4Ig Undergo Rapid Internalization upon B7 Ligand Binding

To further monitor binding morphologically and determine the cellular fate of CTLA4Ig-conjugated liposomes, a

Fig. 1 (continued). Curve a shows blocking of CTLA4Ig-FITC binding to B7-transfected P815 cells by liposomal CTLA4Ig (50 μg/mL); curve b shows blocking of CTLA4Ig-FITC binding to B7-transfected P815 cells by free CTLA4Ig (62.5 μg/mL); curve c shows blocking of CTLA4Ig-FITC binding to B7-transfected P815 cells by free CTLA4Ig (62.5 μg/mL) and empty liposomes administered simultaneously; and curve d shows blocking of free CTLA4Ig binding to B7-transfected P815 cells by empty liposomes. B, Flow cytometric data showing CTLA4Ig-FITC binding to B7 expressed by activated macrophages and blocking of this interaction by liposomal CTLA4Ig. Curve a shows the basal level of CTLA4Ig-FITC binding to resting macrophages without activation; curve b shows the level of CTLA4Ig-FITC binding to activated macrophages, and curve c shows blocking of CTLA4Ig-FITC binding to activated macrophages by liposomal CTLA4Ig.

pH-dependent fluorescent dye, HPTS, was encapsulated inside the liposomes, and their intracellular trafficking was investigated using epifluorescence microscopy. At physiologic pH, HPTS has fluorescence excitation peaks at both 405 nm and 450 nm. The fluorescent intensity with 450 nm excitation, however, decreases as pH drops while that with 405 nm excitation increases (29,33). The CTLA4Ig-liposomes were incubated with BMMs for 1 h at 4°C for binding, and cells were washed and monitored on a live cell stage. CTLA4Ig-liposomes showed minimal binding to nonactivated BMMs (Fig. 3a), which was consistent with the low expression level of B7 on BMM observed by flow cytometry (Fig. 1B). When cells were activated, as the expression of B7 was induced by activation, binding of CTLA4Ig-liposomes to BMMs was clearly augmented (Fig. 3b). The similar intensity of the cell-associated HPTS fluorescence at 405 nm and at 450 nm excitation wavelength indicates the CTLA4Ig-liposomes remained on the cell surface at 4°C and were not internalized. CTLA4Ig-liposome binding to BMMs was abrogated by preincubating cells with excess free CTLA4Ig, which supported the idea that the CTLA4Ig-liposome binding seen in this microscopy study was predominantly mediated by the CTLA4Ig/B7 interaction (Fig. 3c). When cells were warmed to 37°C and incubated for 1 h, the fluorescence signal previously seen on the cell surface migrated to the perinuclear region of the cells. Additionally, the HPTS signal at 450 nm excitation weakened while the signal at 405 nm became distinct (Fig. 3d), indicating that CTLA4Ig-liposomes were internalized into low pH compartments upon specific binding to B7 molecules on BMMs.

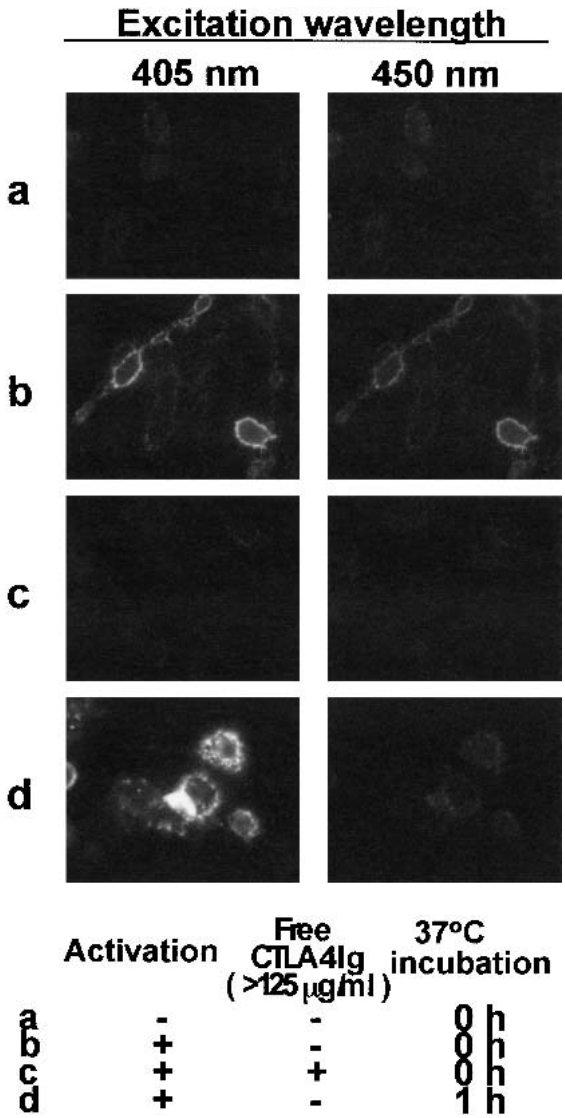

Fig. 3. Epifluorescence microscopic images of macrophages treated with CTLA4Ig-conjugated liposomes containing 5-hydroxypyranine trisulfonate. Cells were incubated with HPTS-containing CTLA4Ig-liposomes at 4°C for 1 h, washed, and followed by various additional treatments noted in the caption including activation, free excess CTLA4Ig, and warming up the cells to 37°C. The cell-associated HPTS-containing CTLA4Ig-liposomes were visualized in an epifluorescence microscope. Fluorescence images of macrophages with excitation at 405 nm and at 450 nm are shown next to each other.

Blockade of Allogenic MLR with CTLA4Ig-Liposomes

The effectiveness of CTLA4Ig-liposomes in blocking the surface expressed B7 molecules on APCs, thereby attenuating T cell activation, was investigated. In order to do this, the proliferation and consequent incorporation of ^{3}H-thymidine in the T cells of BALB/c mouse lymph nodes were monitored when the T cells were mixed with the APCs of C57BL/6 spleen. The ^{3}H-thymidine uptake was effectively inhibited by CTLA4Ig-liposomes incubated during the 96 h mixed lymphocyte culture (Fig. 4). The inhibition was concentration-dependent, with 50% attenuation at ≤100 ng/ml CTLA4Ig concentration, while blank liposomes without CTLA4Ig conjugation did not have any effect on the MLR. The effective-

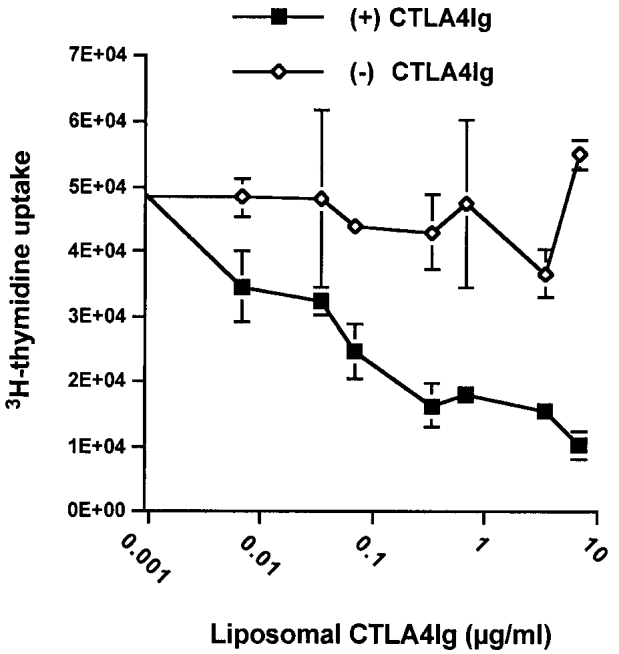

Fig. 4. Inhibition of mixed lymphocyte reaction by CTLA4Ig-liposomes. Liposomes with and without conjugated CTLA4Ig were added to mixed lymphocyte culture (2×10^5 responder BALB/c T cells and 2×10^5 B6 splenic cells in a total volume of 0.2 mL in a 96-well microplate well) at various concentrations during 96 h culture period. The cells were pulsed with ^{3}H-thymidine for the last 16 h, and the amount of ^{3}H-thymidine uptake by proliferating T cells in each well was monitored by harvesting cells and scintillation counting.

ness of CTLA4Ig, however, was not significantly improved over free CTLA4Ig as monitored by this assay.

Blood Circulation and Biodistribution of CTLA4Ig-Liposomes

To determine plasma circulation time and biodistribution, CTLA4Ig-conjugated and control PEG-PE-containing liposomes (i.e., stealth) liposomes were labeled with ^{3}H-cholesteryl oleate ether and injected into C57BL/6J mice with allogeneic pancreatic islet transplants in one kidney. Islets were transplanted under the capsule of the left side kidney while the right side kidney was left untouched as control side. At 1 h, 4 h and 24 h postinjection, 100–200 μl of blood was sampled, and at the 24 h postinjection, the radioactivity remaining in the blood and that accumulated in major organs were measured.

Investigation of the plasma levels and biodistribution of liposomes with and without CTLA4Ig conjugation showed that the CTLA4Ig conjugation resulted in increased accumulation in RES and correspondingly decreased circulation time in relative comparison with the control stealth liposomes (Table I); the half-life of the CTLA4Ig-conjugated liposomes in the blood circulation was approximately 0.5 h in comparison with that of the non-CTLA4Ig-liposomes, which was approximately several hours (Fig. 5). In mice injected with liposomes without conjugated CTLA4Ig (control liposomes), 18.6% of injected dose of liposomes was circulating in the blood (Table I). In contrast, only 2.2% of CTLA4Ig-liposomes were circulating in the blood. Reduced blood circulation of CTLA4Ig-liposomes correlated with increased accumulation in RES including the liver (65.5% vs. 21.7%). Based on these data, we hypothesized that the recognition of the Fc portion of CTLA4Ig by Fc receptor expressed on macrophages in RES, rather than liposomal CTLA4Ig binding to B7 on APCs, was the major mechanism for the increased uptake by RES of the liposomes with CTLA4Ig conjugation and the consequent short circulation time. This hypothesis was tested by comparing the blood circulation of CTLA4Ig-liposomes in wild-type mice and in B7-1/B7-2 knockout (KO) mice. The results shown in Table I strongly indicate that CTLA4Ig binding to B7 is not the major interaction for the enhanced uptake by the RES. Regardless of B7 presence in mice, the blood circulation profiles of these liposomes were not affected (Fig. 5). The biodistribution of CTLA4Ig-stealth liposomes at 24 h postinjection was also very similar in wild-type and B7 KO mice (Table I). High levels of CTLA4Ig-conjugated liposomes were localized to the liver and spleen at 24 h postinjection, whereas, much more of the control liposomes remained in circulation and less accumulated in RES.

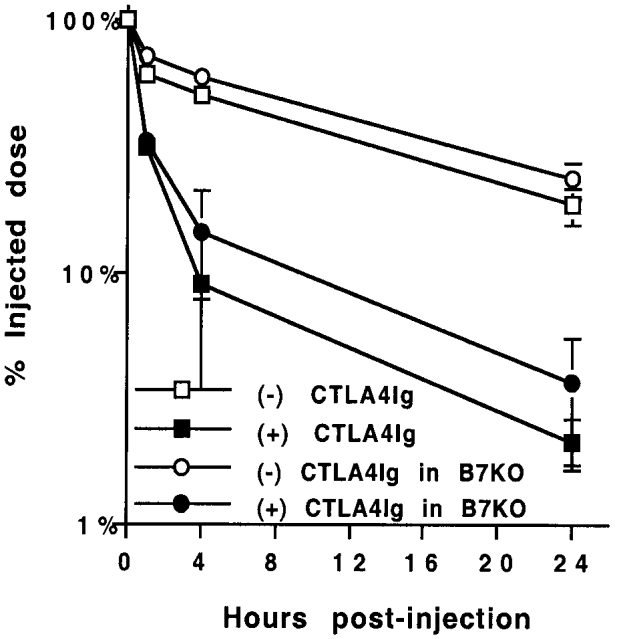

Fig. 5. Percent injected dose of liposomes in blood circulation at 1, 4, and 24 h postintravenous injection. Liposomes with (filled square and circle) and without (open square and circle) CTLA4Ig conjugation were labeled with ^{3}H cholesteryloleate ether and injected into either normal mice (squares) or B7 knockout mice (circles). The amounts of radioactivity in blood samples collected at various timepoints post-injection are plotted as percent of injected dose. Data are plotted as mean ± SD, n = 4.

To further confirm that the decreased circulation time of CTLA4Ig liposomes was due to Fc receptor-mediated uptake, mice were pre-injected with the FcR-blocking 2.4G2 mAb before the administration of CTLA4Ig-stealth liposomes. In both wild-type and B7 KO mice, animals preinjected with 2.4G2 showed increased CTLA4Ig-liposome levels in circulation at 1 and 4 h after injection (Table II), supporting the hypothesis that decreased circulation of the CTLA4Ig-stealth liposomes was predominantly due to Fc receptor-mediated uptake.

Circulation of liposomes in mice with allogeneic islet cell transplants was not different from that in mice without transplants. The percentage of CTLA4Ig-liposomes localized to the transplanted kidney was also reduced as compared to control liposomes, presumably due to the lower level of blood circulation. As the circulation time of control liposomes was

Table I. Tissue Distribution of Liposomes at 24 h Post-Injection

	B7 knockout		Wild type	
	– CTLA4Ig	+ CTLA4Ig	– CTLA4Ig	+ CTLA4Ig
Blood	23.4% (±3.7%)	3.7% (±1.9%)	18.6% (±3.1%)	2.2% (±0.5%)
Liver	21.5% (±1.1%)	64.9% (±2.8%)	21.7% (±2.7%)	65.5% (±10.6%)
Spleen	0.6% (±0%)	0.2% (±0.1%)	0.5% (±0.1%)	0.1% (±0%)
Lung	2.0% (±0.3%)	4.2% (±1%)	2.2% (±0.1%)	6.2% (±0.5%)
Kidney (transplant)	2.4% (±0.4%)	0.7% (±0.1%)	2.0% (±0.3%)	0.7% (±0.1%)
Kidney (control)	0.9% (±0.2%)	0.3% (±0%)	0.9% (±0.1%)	0.3% (±0.1%)

Note: Values are expressed as mean (± SD)

Table II. Percent Injected Liposome in Circulation at 1 and 4 h Post-Injection

	Wild type[a]		B7 Knockout[b]	
	−2.4G2 mAb (n = 3)	+2.4G2 mAb (n = 3)	−2.4G2 mAb (n = 1)	+2.4G2 mAb (n = 4)
1 h	9.0% (±1.1%)	16.3% (±2.4%)	15.50%	20.9% (±1.3%)
4 h	0.6% (±0.2%)	1.3% (±0.3%)	4.72%	9.7% (±2.1%)

Note: Values are expressed as mean (± SD).
[a] Balb/c mice
[b] C57BL/6J mice

significantly longer, approximately three times more control stealth liposomes accumulated in the kidney than CTLA4Ig-stealth liposomes (2.0% vs. 0.7%, Table I). Regardless of whether CTLA4Ig was conjugated or not, however, the liposomes accumulated approximately 2-fold more in the transplant side kidney than in the non-transplant control side, and this trend was observed both in wild type and B7 KO mice (Table I).

DISCUSSION

In this study, we explored the feasibility of generating immunoliposomes using CTLA4Ig and further examined their effectiveness *in vitro* and *in vivo*. Our results show that conjugating CTLA4Ig onto the surface of liposomes improves the avidity of CTLA4Ig binding to B7 molecules and induces their internalization upon binding. When the CTLA4Ig molecules are conjugated to phospholipids in the bilayers of liposomes, at approximately 100–300 CTLA4Ig per liposome, the multivalent effect leading to increased avidity is anticipated. As the lipid-conjugated CTLA4Ig molecules possess lateral mobility within the lipid membrane of liposomes, they are able to ligate multiple B7 molecules on cell surface beyond the initial binding of the first CTLA4Ig molecule to a B7 molecule. Consistent with this, the data from the cell culture study demonstrated that CTLA4Ig-liposomes could block 100% of B7 molecules on either B7 transfectant cells or activated bone marrow-derived macrophages at a concentration 10-fold lower than the level needed using free soluble CTLA4Ig, in which 5-fold excess amount of CTLA4Ig-FITC could not compete off the prebound liposomal CTLA4Ig. This was only observed upon covalent conjugation of CTLA4Ig onto liposomes as the simple mixture of CTLA4Ig and liposomes behaved identically to free CTLA4Ig. The blocking was specific as the irrelevant antibody 10A6 conjugated to liposomes did not block the subsequent binding of FITC-CTLA4Ig.

One of the distinct features of the liposome-conjugated ligand would be its characteristic to induce internalization, upon cell surface binding, of the liposomal ligand-receptor complex. This propensity is increasingly powerful not only for achieving long-term depletion of receptors upon internalization into endolysosomal compartments, but also for obtaining intracellular delivery of drugs that can be encapsulated inside liposomes. The receptor level on the cell surface can be down-regulated, beyond a simple blockage of the receptor binding sites, if the liposomal ligands are co-internalized with the receptors and delivered to the lysosomal degradation pathway. Thus for blocking B7 sites on macrophage surface, the liposomal CTLA4Ig could have an added effect beyond simple binding-mediated blockade of B7 by soluble CTLA4Ig. The data using HPTS, a pH-sensitive fluorophore, inside the CTLA4Ig-liposomes strongly support this possibility.

The biologic activity of CTLA4Ig-liposomes in attenuating the allogenic MLR-mediated T cell activation and proliferation in the ex vivo cell culture assay was effective at liposomal CTLA4Ig concentrations ≤ 1 μg/mL. This functional test of CTLA4Ig conjugated to liposomes is important to demonstrate the effectiveness beyond binding assay in cell culture and to move the formulation to animal models. The enhanced inhibition of B7-mediated costimulation, however, was not observed using this 96 h continuous incubation protocol of the standard allogenic MLR assay. This is mostly likely due to a diminished effect of avidity differences between free and liposome-conjugated CTLA4Ig during the long incubation period, which perhaps does not accurately mimic the *in vivo* situation.

By conjugating CTLA4Ig molecules to the terminal end of PEG in the PEG-PE-containing liposomes (i.e., stealth liposomes), we also tested if the prolonged circulation characteristic of PEG-PE-containing liposomes is retained. One apparent disadvantage of the liposomal CTLA4Ig in its current formulation used in this study was its binding to RES, especially liver, thus lowering the level of blood circulation. The data presented show that the CTLA4Ig conjugation significantly reduced the circulation time of PEG-PE-containing stealth liposomes and increased their uptake by liver and spleen. This was observed both in wild type and in B7 KO mice indicating the uptake by RES is most likely not mediated by the specific interaction between CTLA4Ig and B7. The half-life of the CTLA4Ig-conjugated liposomes in the blood circulation of both B7 KO and wild type mice was approximately 0.5 h, whereas the half-life of the non-CTLA4Ig-liposomes was several hours both in wild type and in B7 KO mice (Fig. 5). By 24 h after injection, almost no CTLA4Ig-conjugated liposomes remained in circulation in B7 KO and wild type mice; however, approximately 25% of the non-conjugated liposomes were in circulation in B7 KO and wild type mice. The nature of enhanced RES uptake is probably the interaction of the Fc moiety of CTLA4Ig on the liposome surface with FcR expressed on tissue macrophages in RES as supported by the 2.4G2 anti-FcR mAb experiment shown in Table II. Several modes of molecular interaction between immunoliposomes with cells exist: 1) nonspecific liposome-cell interactions that might be reduced to minimal upon incorporation of PEG-PE; 2) specific interaction of targeting motifs, B7-binding site of CTLA4Ig in our case, with corresponding cell surface receptors; and 3) general interaction of the Fc portion of liposome-conjugated immunoglobulins with cell surface Fc receptors predominantly expressed on antigen presenting cells. The last interaction (3) is modulated by the type of Fc portion of the immunoglobulin used, the density of IgG conjugated per liposome, and the orientation of IgG on the liposome surface. As it has been reported that the number of IgG per liposome in the range used in our report may induce enhanced recognition by Fc receptors (21), a careful investigation into the rate of liposome clearance as a function of CTLA4Ig density in our system will be necessary. Moreover, the CTLA4Ig molecules on liposomes, using the current conjugation protocol, are randomly oriented thus resulting in non-preferential binding between CTLA4 and Fc

regions to their corresponding receptors on the macrophage cell surface. This FcR-mediated uptake of immunoglobulin-conjugated liposomes has been suggested previously in the studies of immunoliposomes (34). One approach to reducing the Fc-mediated uptake, in addition to controlling the density of CTLA4Ig per liposome, would be to use other means of conjugation to preferentially orient the CTLA4Ig molecules on the liposomes. In addition, molecular engineering of CTLA4Ig to reduce FcR binding will be necessary for increasing blood circulation as well as specific targeting to the desired sites.

Specific uptake of liposomes into the kidney having transplanted islets, despite lower blood circulation, over that into the contralateral control kidney, is notable and promising. CTLA4Ig-liposomes, even though they did not circulate as long as non-CTLA4Ig liposomes, accumulated approximately 2-fold more in the islet-containing side than in the contralateral, control kidney. This higher uptake into the experimental kidney was also observed without CTLA4Ig conjugation, which indicates passive targeting of liposomes in general to inflammatory sites, rather than specific ligand-receptor interaction-mediated active targeting, as suggested by other models (19). The results *in vivo* using KO mice further support that this increased kidney accumulation is not mediated by CTLA4Ig/B7 interactions, but is due to passive targeting as seen in other tumor targeting studies. The enhanced uptake into the experimental kidney with transplant could be due to inflammation-mediated processes at the transplantation site and enhanced extravasation of liposomes into transplanted islets. Upon extravasation and passive accumulation within the transplant tissue, the B7/CTLA4Ig-liposome interaction would be critical for the localized targeting to B7-expressing APCs within the region. These properties make liposomes ideal for targeting to B7-expressing APCs within engrafted sites. The typical volume of transplanted islets in the kidney was approximately 15 μL. If 1% of injected CTLA4Ig-liposomes accumulate in the transplanted islets of the experimental side kidney, the local concentration of CTLA4Ig that can be achieved with a 50 μg dose would be approximately 25 μg/mL, which is at a sufficient level to induce complete blockade of B7 binding sites. This suggests a potential advantage of CTLA4Ig-liposomes, even in their current formulation reported in this study, over free CTLA4Ig. These results overall suggest the feasibility and the enormous potential for significant increase in targeting to transplanted islets. Taken together, our data provide a basis for a new strategy to improve the efficacy of CTLA4Ig by formulating it into the liposomes. Prolongation and optimization of circulation of CTLA4Ig-PEG-liposomes using modified CTLA4Ig will be a critical step to maximizing the benefit of CTLA4Ig-liposomes. The data presented suggest that the optimization of CTLA4Ig-stealth liposomes can be first achieved by modifying the CTLA4Ig-liposomes for reduced FcR-mediated uptake by the RES for the prolonged blood circulation time and consequently enhancing the probability of specific targeting via CTLA4/B7 interactions.

CONCLUSION

We have shown in this report the enhanced B7-blocking capacity of CTLA4Ig when conjugated with liposomes using cell culture studies. The CTLA4Ig-liposomes are shown to be internalized into endocytic pathways upon binding to macrophages. The blood circulation time of CTLA4Ig-liposomes in mice, however, was significantly shorter that that of the long-circulation liposomes without CTLA4Ig conjugation due primarily to the high uptake by the RES. The avid uptake by the RES was not dependent on B7 expression in mice and was most likely due to FcR-mediated recognition of liposomal CTLA4Ig. The *in vivo* biodistribution results suggest a great potential for augmenting the local concentration of CTLA4Ig-liposomes in the transplant of islets, as well as an obvious improvement strategy to reduce the uptake by the RES, through the modification of the Fc region of the CTLA4Ig, which can ultimately accomplish better pharmacokinetics and further uptake into tissues containing transplants.

ACKNOWLEDGMENT

We would like to thank Dr. Chester Provoda for careful reading of the manuscript, Dr. Manas Mandal for technical help in animal work, and members of Lee and Bluestone lab for helpful discussions. This work was partially supported by the NIH grants R29AI42084 and R01AI47173 (K.-D. Lee) and JDRFI Center Grant 4-1999-841 (J. A. Bluestone), Vahlteich Research Fund from the College of Pharmacy, University of Michigan, and Good Health R & D Project grant (HMP-01-PJ1-PG1-01CH09-0006) of the Ministry of Health and Welfare, Republic of Korea (C.G. Park).

REFERENCES

1. D. J. Lenschow, T. L. Walunas, and J. A. Bluestone. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* **14**:233–258 (1996).
2. P. S. Linsley, W. Brady, M. Urnes, L. S. Grosmaire, N. K. Damle, and J. A. Ledbetter. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* **174**:561–569 (1991).
3. A. V. Collins, D. W. Brodie, R. J. C. Gilbert, A. Iaboni, R. Manso-Sancho, B. Walse, and D. I. Stuart. P. A. v. d. Merwe, and S. J. Davis. The interaction properties of costimulatory molecules revisited. *Immunity* **17**:201–210 (2002).
4. P. S. Linsley, P. M. Wallace, J. Johnson, M. G. Gibson, J. L. Greene, J. A. Ledbetter, C. Singh, and M. A. Tepper. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science* **257**:792–795 (1992).
5. D. J. Lenschow, Y. Zeng, J. R. Thistlethwaite, A. Montag, W. Brady, M. G. Gibson, P. S. Linsley, and J. A. Bluestone. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* **257**:789–792 (1992).
6. L. A. Turka, P. S. Linsley, H. Lin, W. Brady, J. M. Leiden, R. Q. Wei, M. L. Gibson, X. G. Zheng, S. Myrdal, D. Gordon, T. Bailey, S. F. Bolling, and C. B. Thompson. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc. Natl. Acad. Sci. USA* **89**:11102–11105 (1992).
7. B. K. Finck, P. S. Linsley, and D. Wofsy. Treatment of murine lupus with CTLA4Ig. *Science* **265**:1225–1227 (1994).
8. S. D. Miller, C. L. Vanderlugt, D. J. Lenschow, J. G. Pope, N. J. Karandikar, M. C. Dal Canto, and J. A. Bluestone. Blockade of CD28/B7-1 interaction prevents epitope spreading and clinical relapses of murine EAE. *Immunity* **3**:739–745 (1995).
9. P. J. Perrin, D. Scott, L. Quigley, P. S. Albert, O. Feder, G. S. Gray, R. Abe, C. H. June, and M. K. Racke. Role of B7:CD28/CTLA-4 in the induction of chronic relapsing experimental allergic encephalomyelitis. *J. Immunol.* **154**:1481–1490 (1995).
10. N. D. Griggs, S. S. Agersborg, R. J. Noelle, J. A. Ledbetter, P. S. Linsley, and K. S. Tung. The relative contribution of the CD28 and gp39 costimulatory pathways in the clonal expansion and pathogenic acquisition of self-reactive T cells. *J. Exp. Med.* **183**:801–810 (1996).

11. N. Harris, C. Campbell, G. Le Gros, and F. Ronchese. Blockade of CD28/B7 co-stimulation by mCTLA4-Hgamma1 inhibits antigen- induced lung eosinophilia but not Th2 cell development or recruitment in the lung. *Eur. J. Immunol.* **27**:155–161 (1997).
12. J. R. Abrams, S. L. Kelley, E. Hayes, T. Kikuchi, M. J. Brown, S. Kang, M. G. Lebwohl, C. A. Guzzo, B. V. Jegasothy, P. S. Linsley, and J. G. Krueger. Blockade of T lymphocyte costimulation with cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (CTLA4Ig) reverses the cellular pathology of psoriatic plaques, including the activation of keratinocytes, dendritic cells, and endothelial cells. *J. Exp. Med.* **192**:681–694 (2000).
13. M. G. Levisetti, P. A. Padrid, G. L. Szot, N. Mittal, S. M. Meehan, C. L. Wardrip, G. S. Gray, D. S. Bruce, J. R. Thistlethwaite Jr., and J. A. Bluestone. Immunosuppressive effects of human CTLA4Ig in a non-human primate model of allogeneic pancreatic islet transplantation. *J. Immunol.* **159**:5187–5191 (1997).
14. A. D. Kirk, D. M. Harlan, N. N. Armstrong, T. A. Davis, Y. Dong, G. S. Gray, X. Hong, D. Thomas, J. H. Fechner Jr., and S. J. Knechtle. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc. Natl. Acad. Sci. USA* **94**:8789–8794 (1997).
15. N. R. Srinivas, R. S. Weiner, G. Warner, W. C. Shyu, T. Davidson, C. G. Fadrowski, L. K. Tay, J. S. Lee, D. S. Greene, and R. H. Barbhaiya. Pharmacokinetics and pharmacodynamics of CTLA4lg (BMS-188667), a novel immunosuppressive agent, in monkeys following multiple doses. *J. Pharm. Sci.* **85**:1–4 (1996).
16. D. D. Lasic and D. Papahadjopoulos. Liposomes revisited. *Science* **267**:1275–1276 (1995).
17. K.-D. Lee, Y. K. Oh, D. A. Portnoy, and J. A. Swanson. Delivery of macromolecules into cytosol using liposomes containing hemolysin from Listeria monocytogenes. *J. Biol. Chem.* **271**:7249–7252 (1996).
18. C. Provoda and K.-D. Lee. Bacterial pore-forming hemolysins and their use in the cytosolic delivery of macromolecules. *Adv. Drug Deliv. Rev.* **41**:209–221 (2000).
19. D. Papahadjopoulos, T. M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K. D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, and F. J. Martin. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA* **88**:11460–11464 (1991)
20. D. Kirpotin, J. W. Park, K. Hong, S. Zalipsky, W. L. Li, P. Carter, C. C. Benz, and D. Papahadjopoulos. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry* **36**:66–75 (1997).
21. C. B. Hansen, G. Y. Kao, E. H. Moase, S. Zalipsky, and T. M. Allen. Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. *Biochim. Biophys. Acta* **1239**:133–144 (1995).
22. T. M. Allen. Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trends Pharmacol. Sci.* **15**:215–220 (1994).
23. T. Ishida, D. L. Iden, and T. M. Allen. A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. *FEBS Lett.* **460**:129–133 (1999).
24. J. W. Park, D. B. Kirpotin, K. Hong, R. Shalaby, Y. Shao, U. B. Nielsen, J. D. Marks, D. Papahadjopoulos, and C. C. Benz. Tumor targeting using anti-her2 immunoliposomes. *J. Control. Release* **74**:95–113 (2001).
25. G. Girolomoni, G. Zambruno, R. Manfredini, V. Zacchi, S. Ferrari, A. Cossarizza, and A. Giannetti. Expression of B7 costimulatory molecule in cultured human epidermal Langerhans cells is regulated at the mRNA level. *J. Invest. Dermatol.* **103**:54–59 (1994).
26. E. L. Racoosin and J. A. Swanson. Macrophage colony-stimulating factor (rM-CSF) stimulates pinocytosis in bone marrow-derived macrophages. *J. Exp. Med.* **170**:1635–1648 (1989).
27. D. D. Spragg, D. R. Alford, R. Greferath, C. E. Larsen, K. D. Lee, G. C. Gurtner, M. I. Cybulsky, P. F. Tosi, C. Nicolau, and M. A. Gimbrone Jr. Immunotargeting of liposomes to activated vascular endothelial cells: a strategy for site-selective delivery in the cardiovascular system. *Proc. Natl. Acad. Sci. USA* **94**:8795–8800 (1997).
28. D. L. Daleke, K. Hong, and D. Papahadjopoulos. Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay. *Biochim. Biophys. Acta* **1024**:352–366 (1990).
29. R. M. Straubinger, D. Papahadjopoulos, and K. L. Hong. Endocytosis and intracellular fate of liposomes using pyranine as a probe. *Biochemistry* **29**:4929–4939 (1990).
30. P. E. Lacy and M. Kostianovsky. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* **16**:35–39 (1967).
31. T. Allen and A. Chonn. Large unilamellar liposomes with low uptake into the reticuloendothelial system. *FEBS Lett.* **223**:42–46 (1987).
32. T. Allen. Stealth liposomes: avoiding reticuloendothelial uptake. In *Liposomes in the Therapy of Infectious Diseases and Cancer*, Vol. 89, UCLA Symposium on Molecular and Cellular Biology, Alan R. Liss, Inc., New York, 1989 pp. 405–415.
33. K.-D. Lee, S. Nir, and D. Papahadjopoulos. Quantitative analysis of liposome-cell interactions in vitro: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes. *Biochemistry* **32**:889–899 (1993).
34. K. Maruyama, N. Takahashi, T. Tagawa, K. Nagaike, and M. Iwatsuru. Immunoliposomes bearing polyethyleneglycol-coupled Fab' fragment show prolonged circulation time and high extravasation into targeted solid tumors in vivo. *FEBS Lett.* **413**:177–180 (1997).