molecular pharmaceutics

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Targeted Drug Delivery to Lymphocytes: A Route to Site-Specific Immunomodulation?

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Received August 8, 2010; Revised Manuscript Received October 9, 2010; Accepted October 19, 2010

Abstract: Lymphocytes are central to the progression of autoimmune disease, transplant rejection, leukemia, lymphoma and lymphocyte-resident viral diseases such as HIV/AIDs. Strategies to target drug treatments to lymphocytes, therefore, represent an opportunity to enhance therapeutic outcomes in disease states where many current treatment regimes are incompletely effective and promote significant toxicities. Here we demonstrate that highly lipophilic drug candidates that preferentially access the intestinal lymphatics after oral administration show significantly enhanced access to lymphocytes leading to improved immunomodulatory activity. When coadministered with such drugs, lipids enhance lymphocyte targeting via a three tiered action: promotion of drug absorption from the gastrointestinal tract, enhancement of lymphatic drug transport and stimulation of lymphocyte recruitment into the lymphatics. This strategy has been exemplified using a highly lipophilic immunosuppressant (JWH015) where coadministration with selected lipids led to significant increases in lymphatic transport, lymphocyte targeting and IL-4 and IL-10 expression in CD4+ and CD8+ lymphocytes after ex vivo mitogen stimulation. In contrast, administration of a 2.5-fold higher dose of JWH015 in a formulation that did not stimulate lymph transport had no effect on antiinflammatory cytokine levels, in spite of equivalent drug exposure in the blood. The current data suggest that complementary drug design and delivery strategies that combine highly lipophilic, lymphotropic drug candidates with lymph-directing formulations provide enhanced selectivity, potency and therapeutic potential for drug candidates with lymphocyte associated targets.

Keywords: Lymphocyte; lymphatic transport; drug targeting; lipid based drug delivery system; lipophilic drugs

Introduction

Lymphocyte abnormalities, pathologies and malignancies are central to the progression of disease pathologies including autoimmune and immunodeficiency disorders, organ transplant rejection, allergies, leukemia and lymphoma. A significant proportion of the viral load of HIV is also present within CD4+ T lymphocytes particularly in the gut associated lymphoid tissue (GALT), in both the latent and active stages of HIV/AIDs.[1,2] Enhanced delivery of immunomodulatory, cytotoxic and anti-HIV drugs to lymphocytes may therefore provide an opportunity to selectively improve treatment efficacy and simultaneously reduce the dose-limiting side effects that are a common cause of treatment failure with many of these therapies.[3,4] Despite this, little attention has been directed toward lymphocyte targeted drug delivery strategies and the majority of attempts have employed parenteral, nanoparticle-based systems,[5−7] and/or structural motifs, peptides and antibodies directed toward

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10.1021/mp100259a
Published on Web 10/19/2010

lymphocyte surface molecules and receptors (e.g., lymphocyte adhesion molecules such as ICAM and CD receptors).[8−10] To this point, however, these efforts have met with limited success. In contrast, we describe here a complementary drug design and delivery approach to lymphocyte targeting, which is applicable to orally administered small molecule drug candidates, and has the potential for generic utility across a range of lymphocyte-related disorders.

Specifically, we have explored whether highly lipophilic drug candidates, in combination with lipid-based drug delivery systems, can be utilized to promote drug targeting to lymphocytes via enhanced drug transport into the intestinal lymphatics. We have further examined whether this targeting effect can be utilized to enhance the pharmacodynamic effect of an experimental immunomodulator. This hypothesis is based on the realization that (i) the majority (∼95%) of lymphocytes are present within the lymph and lymphoid tissues, (ii) up to 50−70% of these are present within the gut-associated lymphoid tissue (GALT) and intestinal lymphatics[11−13] and (iii) highly lipophilic drug molecules can be preferentially transported into the intestinal lymph after oral administration when coadministered with long chain lipids.[14]

The approach we describe is the antithesis of drug design strategies that follow traditional dogma with respect to drug candidate optimization[15] and therefore has the potential to signal a conceptual change in the use of drug delivery principles to guide medicinal chemistry for lymphocyte-related therapeutic indications. Indeed decision gates for drug candidate selection typically preclude the progression of highly lipophilic drug candidates on the basis that their low aqueous solubility is a barrier to oral absorption from traditional dose forms.[15] While highly lipophilic drug candidates require formulation in nontraditional delivery systems to facilitate absorption, this can be readily achieved with the use of lipid based formulations, which not only enhance absorption but also have the potential to promote lymphatic transport and lymphocyte recruitment and therefore direct lymphocyte drug targeting.

The current studies have therefore evaluated the impact of promotion of intestinal lymphatic drug transport on the kinetics of drug delivery to lymphocytes in the intestinal lymph, the subsequent effect on drug exposure to lymphocytes in the systemic circulation and the potential for this approach to enhance immunosuppression using an investigational lipophilic immunosuppressant (JWH015). The data suggest that promoting drug transport via the lymph is a highly efficient conduit to enhanced lymphocyte access and to improved immunosuppressant activity.

Methods

Materials. 1,1-Bis(4-chlorophenyl)-2,2,2-trichloroethane (DDT), 4,4′-DDT-Ring-UL-^{14}C, Diazepam, JWH015, triacetin, 30% hydrogen peroxide solution, normal mouse IgG, disodium EDTA, bovine serum albumin, RPM1640, phorbol 12-myristate 13-acetate, calcium ionophore A23187, sodium azide, sodium phosphate, sodium hydrogen phosphate and ammonium hydroxide were all purchased from Sigma-Aldrich Australia. Halofantrine (GlaxoSmithKline, India), ^{3}H cyclosporin A (Amersham Biosciences, U.K.), cyclosporin A (Novartis, Switzerland), sirolimus (Toronto Research Chemicals, Canada), JWH018 (Alpha Research Co., Hong Kong), Intralipid (Baxter, Australia), *N*,*N*-dimethylacetamide (Prolabo, France), To-PRO-3 iodide (T3605, Molecular Probes, Invitrogen, Carlsbad, CA), Lymphosep (MP Biomedicals, Australia) and Erythrolyse (AbD Serotec, U.K.), ^{3}H-diazepam, ^{14}C-oleic acid and Starscint scintillation fluid (Perkin-Elmer Life Sciences, Waltham, MA, USA) and FITC mouse anti-rat CD3, PE mouse anti-rat CD8, PE-Cy5 mouse anti-rat CD4, PE mouse anti-rat IL-4, PE mouse anti-rat IL-10, BD Trucount tubes, Stain Buffer FBS, Cytofix/Cytoperm solution Perm/Wash buffer and Golgi stop (BD Biosciences, Franklin, NJ) were obtained from the listed suppliers. Acetonitrile and methanol were analytical grade.

Drugs and Formulations. Lymphatically transported drugs are typically highly lipophilic as evidenced by both

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high log $D_{7.4}$ (>5) and high solubility in long-chain triglyceride lipids (>50 mg/g).[16] All the drugs utilized in the current studies were therefore lipophilic, however, a range of compounds with differing lipophilicities and therefore differing propensities for intestinal lymphatic transport were chosen to enable examination of the role of lymphatic transport in drug delivery to lymphocytes. Halofantrine (log $D_{7.4}$ 6.74,[17] long chain TG solubility 48 mg/g)[18−22] and DDT (log $D_{7.4}$ 5.92,[16,17] long chain TG solubility 97.5 mg/g[16]) were chosen as model lymphatically transported drugs as they are substantially absorbed via the intestinal lymphatic system. Diazepam (log $D_{7.4}$ 2.92, long chain TG solubility 18.3 mg/g[23]) was chosen as a lipophilic, but nonlymphatically transported drug.[24,25] Cyclosporin (log *P* 2.92, long chain TG solubility 44.9 mg/g[24]), sirolimus (log *P* 5.8,[26] long chain TG solubility <1 mg/g[27]) and JWH015 (clog *P* 5.8, long chain TG solubility unknown) were evaluated as they are lipophilic immunosuppressants for which lymphatic transport may offer the potential to enhance efficacy. However, the log *P* of cyclosporin and the long chain TG solubility of sirolimus suggest that they are unlikely to be transported into the lymph in significant amounts. Indeed, previous studies have examined the lymphatic transport of cyclosporin and shown that it is low (<3%)[28,29] but have suggested a relationship between the extent of lymphatic transport and activity for cyclosporin despite only finding modest transport via the lymph.[30,31]

Drug formulations contained 1 mg of drug (DDT, halofantrine, JWH015, diazepam, cyclosporin A or sirolimus) and 4 or 40 mg of oleic acid dispersed in 5.6 mL of 0.2% w/v Tween 80 in normal saline. ^{14}C-Oleic acid was used to label exogenous (formulation derived) lipids. The cyclosporin and diazepam formulations also contained 2 μCi of methyl-β-^{3}H-cyclosporin A and 5 μCi of ^{3}H-diazepam. Formulations were prepared and assessed for stability as described previously.[32]

Animal Studies. All experiments were approved by the local animal ethics committee and were conducted in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines. Male Sprague−Dawley rats (280−320 g) maintained on a standard diet and then fasted overnight with access to water were used in all experiments. Anesthetized rats[33] had cannulae inserted into the duodenum (for formulation administration and rehydration),[32] mesenteric lymph duct (for lymph collection),[32,34] jugular vein (for intravenous infusion)[32] or carotid artery (for blood collection).[18] After surgery, rats were rehydrated for 0.5 h via intraduodenal infusion of normal saline at 2.8 mL/h. Unless otherwise described, lipid and drug formulations were then infused into the duodenum at a rate of 2.8 mL/h for 2 h, after which the infusion was changed back to 2.8 mL/h normal saline for the remainder of the experiment.

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Lipid, Lymphocyte and Drug Flux into Mesenteric Lymph. Lipid based drug formulations were infused intraduodenally (as above), and lymph was collected continuously into tared tubes to allow gravimetric quantification of the volume of lymph collected. Triglyceride and phospholipid concentrations in the lymph were measured using a commercial enzymatic kit,[32] and mass transport was determined from the lipid concentrations in lymph and the volume of lymph collected. Total fatty acid transport was calculated by assuming that each mole of triglyceride and phospholipid contained 3 and 2 mol of fatty acid, respectively.[32] Lymph was centrifuged for 3 min at 1600*g* to pelletize the cellular fraction, and drug and exogenous oleic acid concentrations in total lymph and the cell pellet (lymphocytes) were determined by scintillation counting, HPLC or LC−MS. 99% of the cell pellet consisted of lymphocytes (as described previously[35]), and the supernatant did not contain any cellular material when viewed by light microscopy or examined by flow cytometry. Lymphocyte flux into the lymph was determined by staining lymphocytes, CD3/CD4+ lymphocytes and CD3/CD8+ lymphocytes[36] and identifying lymphocytes by flow cytometry as below.

Drug Targeting to Lymphocytes in the Systemic Circulation. Lipid based drug formulations were infused intraduodenally and blood samples (0.7 mL) collected at −5 min, 1 h, 2 h, 3 and 4 h and 5 h. In one group, plasma was separated from the blood samples via centrifugation at 3000*g* for 5 min to enable measurement of plasma drug concentrations. In a second parallel group, lymphocytes were separated using lymphocyte separation media (Lymphosep)[37] to enable measurement of drug concentrations in lymphocytes. To allow calculation of absolute bioavailability of JWH015, 1 mL of an intralipid formulation containing 300 μg of JWH015 (prepared as described previously[38]) was infused over 5 min and blood samples (0.3 mL) were collected and plasma separated via centrifugation. Drug concentrations in plasma and resuspended lymphocyte samples were determined by scintillation counting, HPLC or LC−MS.

Effect of Targeting JWH015 to Lymphatic Lymphocytes on Immunomodulator Activity. To determine whether lymphatic drug transport had an impact on immunomodulator activity, JWH015 was employed as an experimental immunosuppressant and administered with a low (4 mg of oleic acid) and high (40 mg of oleic acid) lipid dose in order to differentially stimulate lymphatic drug transport. The dose of JWH015 administered with each formulation was also adjusted such that overall systemic exposure was the same but lymphatic transport was greater after administration with 40 mg of lipid.

Immunomodulatory activity was evaluated by examining intracellular cytokine (IL-4 and IL-10) production by lymphocytes collected from rats administered JWH015 with 4 mg or 40 mg of lipid (or after administration of saline or 40 mg of lipid alone (i.e. without drug) as controls) and stimulated with mitogens. In these studies, the carotid artery and duodenum of rats were cannulated, and rats were infused with 5.6 mL of normal saline (as a control) or 1 or 0.4 mg/kg JWH015 dispersed in 4 mg or 40 mg of oleic acid (respectively) and blood samples collected over 8 h. 100 μL of blood was reserved for measuring intracellular IL-4 and IL-10 expression in lymphocytes following mitogen stimulation, and plasma was separated from 350 μL of blood for determination of JWH015 plasma concentrations by LC−MS.

Intracellular production of IL-4 and IL-10 in the blood samples was measured following in vitro stimulation with mitogens (phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187) and lymphocyte CD3, CD4, DNA and IL-4 or IL-10 stained as described below. IL-4 and IL-10 positive lymphocytes, CD3/CD4+ lymphocytes and CD3/CD8+ lymphocytes were counted using flow cytometry.

Analytical Methods

HPLC Analysis. *Preparation of Lymph Samples.* To measure DDT, halofantrine, sirolimus and JWH015 concentrations in lymph, lymph samples were diluted either 1/40 or 1/80 with acetonitrile (as described previously[32,39]), vortexed for 1 min and centrifuged at 1800*g* for 3 min. The supernatant was analyzed by HPLC. The efficiency of extraction was >95% for all drugs when blank lymph samples were spiked with low, medium or high drug concentrations.

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Preparation of Blood and Lymphocyte Samples. DDT, halofantrine, JWH015, sirolimus and diazepam were extracted from lymphocyte samples (450 μL) via addition of 1 mL of acetonitrile followed by vortexing for 1 min and centrifugation at 4200*g* for 5 min. Plasma samples (100 μL) containing DDT, halofantrine and JWH015 were processed in the same way. For both plasma and lymphocyte samples, 8 mL of *tert*-butyl methyl ether (TBME) was then added and samples vortexed for 1 min and centrifuged for 5 min at 4200*g*. The supernatant was transferred to a new polypropylene tube using a glass pipet and the contents were evaporated to dryness under a stream of nitrogen using a N-EVAP 112 evaporator (Organomation, Berlin, MA, USA). The residue was reconstituted with 50−200 μL acetonitrile.

Chromatography. HPLC−UV Assays. All assays utilized a HPLC system consisting of a Waters 486 tunable absorbance detector and Waters alliance 2695 pump and autosampler under the control of Waters Empower software (Millford, MA). Detailed assay conditions are tabulated in the Supporting Information. All assays were validated by assay of replicate (n = 4) quality control samples at low, medium and high concentrations in each different sample type. The assays were accurate (±10% of target concentration) and precise (coefficient of variation <10%) in these concentration ranges.

LC−MS Assay for JWH015. JWH015 concentrations in lymph and lymph lymphocytes were measured via HPLC whereas the lower JWH015 concentrations found in plasma and lymphocytes in the systemic circulation required quantification by LC−MS. JWH015 samples were prepared for LC−MS analysis as described above, and 100 ng/mL of internal standard (JWH018) was added prior to extraction. The LC−MS system consisted of two LC-20AD pumps, an online DGU-20A5 solvent degasser, a CTO-20A column oven and a single quadrupole mass spectrometer with an electrospray ionization (ESI) interface in positive ion mode and LCMS Solutions software (Shimadzu, Kyoto, Japan). Conditions for analysis were as follows: heat block and curved desolvation line (CDL) 200 °C, interface and CDL voltages 4.5 kV and −50.0 V and nebulizing and drying gas flow rates 1.5 and 10 L/min. 5 μL samples were injected onto a Gemini C18 column (3 μm particle size, 50 mm × 2.00 mm, Phenomenex, CA) at 22 °C and the mobile phase flow rate was 0.4 mL/min. Mobile phase A (MPA) was 60:40 (v/v) ACN:water with 0.1% ammonium hydroxide and mobile phase B (MPB) was 90:10 (v/v) ACN:water with 0.1% ammonium hydroxide. The mobile phase gradient sequence (v/v) was initiated with 25% MPB, then linearly increased to 95% MPB over 8 min, prior to holding at 95% MPB for 2.5 min, returning to 25% MPB over 0.5 min and holding 25% MPB for 3 min. JWH015 and JWH018 (internal standard) were detected by selective ion monitoring (SIM) of the 328.15 and 342.2 mass/charge ion peak (m/z) ($[M + H]^+$). JWH015 and JWH018 eluted at 6.8 and 8 min.

The LC−MS method for JWH015 was validated by assay of replicate (n = 4) quality control samples at low, medium and high concentrations. The assays were accurate (within 10% of target concentration) and precise (coefficient of variation <10%) for concentration ranges 0.5−500 ng/mL in plasma and 0.02−20 ng/mL in lymphocyte samples.

Scintillation Counting. The concentrations of radiolabels in lymphocytes and lymph were determined by scintillation counting after addition of 2 mL of Starscint scintillation fluid to 100 μL of resuspended lymphocytes or lymph. Methods were validated by spiking blank lymphocyte pellets or lymph in triplicate with known amounts of radiolabels. The measured concentrations were within 5% of the nominal concentration.

FACS Analysis of CD4+ and CD8+ T Lymphocytes. Lymphocytes in lymph and blood samples were counted and identified using a method described previously for granulocytes and lymphocytes in blood.[36] Briefly, 20 μL of lymph or blood samples were blocked at room temperature for 30 min with 1% w/v aggregated mouse IgG and incubated at room temperature for 15 min with 2 μL of 20 μM To-PRO-3 iodide (a DNA stain), 0.1 μL of 0.5 mg/mL FITC mouse anti-rat CD3, 0.1 μL of 0.2 mg/mL PE mouse anti-rat CD8, 0.5 μL of 0.2 mg/mL PE-Cy5 mouse anti-rat CD4 and 7.3 μL of staining buffer (0.02 M disodium EDTA and 2% bovine serum albumin in 0.01 M phosphate buffer saline pH 6.0). Cells were permealized via incubation with 400 μL of Erythrolyse for 10 min and washed twice with 200 μL of cold wash buffer (0.1% sodium azide, 1% bovine serum albumin in 0.01 M phosphate buffered saline pH 6.0), centrifuging at 4200*g* for 5 min between washes. Cells were finally resuspended in 300 μL of cold wash buffer, and 200 μL was pipetted into a BD Trucount Tube containing a known number of fluorescent beads.

Total lymphocytes, CD3/CD4+ lymphocytes, CD3/CD8+ lymphocytes and fluorescent beads were counted by using a Becton-Dickinson FACs-Calibur equipped with two laser excitation (488 and 633 nm). Unstained negative controls and single fluorophore stained controls were used to set up laser intensity and compensation parameters for each rat. Lymphocytes were identified from characteristic light scatter patterns, CD3/CD4+ lymphocytes from positive staining in the FITC and PE-Cy5 channels and CD3/CD8+ lymphocytes from positive staining in the FITC and PE but not PE-Cy5 channel.[36,40]

The number of lymphocytes, CD4+ T lymphocytes and CD8+ T lymphocytes in lymph samples was calculated from the total volume of lymph collected over each 2 h time period (determined gravitrimetrically) and the number of lymphocytes counted taking into account the volume of lymph that was stained (20 μL), the fraction of lymphocytes from the stained volume that was added to the BD Trucount tubes (200 μL/300 μL) and the fraction of lymphocytes in the BD Trucount tubes that were eventually counted (calculated from the ratio of the total number of fluorescent beads that were counted compared to the total number of fluorescent beads initially in the BD Trucount tubes).

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Quantification of Intracellular Cytokine Production by Mitogen Stimulated Lymphocytes. Intracellular production of IL-4 and IL-10 in blood samples taken from animals administered JWH015 in different lipid formulations was measured following in vitro stimulation with mitogens (phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187) according to the protocol 'immunofluorescent staining of intracellular cytokines for flow cytometric analysis" (BD Biosciences Web site; http://www.bdbiosciences.com/pharmingen/protocols/Intracellular_Cytokines.shtml) with some modifications. Briefly, 50 μL whole blood samples were diluted with 50 μL of RPM1640 medium, mixed by gentle inversion and incubated in a 5% CO_2, 95% O_2 atmosphere at 37 °C for 6 h following addition of 50 ng/mL PMA, 1 μg/mL A23187 and 6.6 μL of 1:100 diluted Golgistop. The CD3, CD4 and DNA of lymphocytes were then stained as described above except that the volumes used were 2.5-fold greater and the samples were not incubated with PE mouse anti-rat CD8 IgG since the cells were resuspended in 100 μL of BD perm/wash buffer containing 1 μL of 0.2 mg/mL PE mouse anti-rat IL-4 or 0.2 mg/mL PE mouse anti-rat IL-10 and incubated for 30 min at 4 °C to stain IL-4 or IL-10. Finally, the cells were washed twice with 1 mL of BD perm/wash buffer, centrifuging at 4200*g* for 5 min between washes, and resuspended in BD stain buffer FBS. IL-4 and IL-10 positive lymphocytes, CD3/CD4+ lymphocytes and CD3/CD8+ lymphocytes were counted using flow cytometry as described above. The proportions of IL-4 and IL-10 positive lymphocytes, CD3+/CD4+ lymphocytes and CD3+/CD4− lymphocytes (which are assumed to consist almost entirely of CD3+/CD8+ lymphocytes) were determined from the fraction of each type of lymphocyte with positive staining in the PE channel.

Calculations

The area under the plasma concentration−time profiles (AUC^{0-tz}_{plasma}, ng·h/mL) and area under the profiles for the mass of drug associated with lymphocytes obtained from 1 mL whole blood versus time profiles (AUC^{0-tz}_{WBC}, ng·h/mL) were calculated using the linear trapezoidal method. The bioavailability of JWH015 was subsequently calculated as the ratio of the AUC values following intraduodenal and IV administration, i.e.,

$$F_{total} = (AUC_{ID}/D_{ID})/(AUC_{IV}/D_{IV}) \times 100\% \quad (1)$$

where D_{ID} and D_{IV} are the total doses administered via the intraduodenal and intravenous routes, respectively, and AUC_{ID} and AUC_{IV} represent the $AUC_{0-\infty}$ in plasma following intraduodenal and IV dosing, respectively. The relative lymphocyte exposure of drug in the systemic circulation was determined from eq 2 as follows:

$$\text{Relative systemic lymphocyte exposure (as a \%)} = \frac{AUC^{0-tz}_{WBC}}{AUC^{0-tz}_{plasma}} \times 100\% \quad (2)$$

Statistical Methods. Statistically significant differences were determined by ANOVA followed by Tukey's test for multiple comparisons at a significance level of $\alpha = 0.05$ using SPSS for Windows V11.5.0 (SPSS Inc., Chicago, Il).

Results

Lipid Formulations Enhance Lymphocyte Flux into Mesenteric Lymph. Administration of relatively small quantities (4−40 mg) of a long chain lipid (oleic acid) to rats (equivalent on a mg/kg basis to ingestion of approximately 1−10 g of lipid in humans) stimulated a large increase in lymphocyte flux into mesenteric lymph (Figure 1A). The response was reproducible and dose dependent, and administration of 40 mg of oleic acid increased lymphocyte flux approximately 4−5-fold when compared to administration of normal saline (Figure 1A). The flux of lymphocytes into the lymph, however, did not correlate linearly with the rate of lipid transport into the lymph (Figure 1B) where lipid flux into the mesenteric lymph was similar after administration of normal saline or the low (4 mg) oleic acid dose and only increased significantly after administration of the higher (40 mg) lipid dose. The type of cells transported

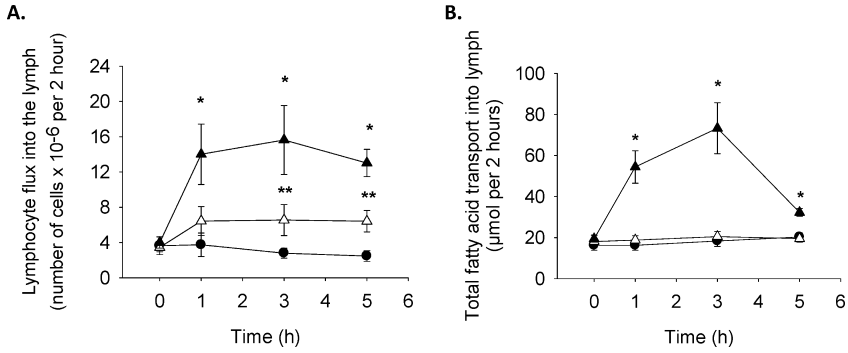

Figure 1. Effect of lipid dose on lymphocyte and lipid (fatty acid) flux into mesenteric lymph. (A) Lymphocyte flux into mesenteric lymph (number of cells $\times$ 10^{-6} transported over 2 h) and (B) total fatty acid transport into the lymph (μmol over 2 h) following intraduodenal administration of ● normal saline, Δ 4 mg of lipid or ▲ 40 mg of lipid to mesenteric lymph duct cannulated anesthetized rats. Data represent mean ± SEM, $n = 4-5$ rats. *Significantly greater than 4 mg of lipid group, **significantly greater than saline group.

into lymph was unchanged by the administration of the different lipid doses: 65−69% were CD4+ lymphocytes, 10−12% CD8+ lymphocytes and 20−24% other lymphocyte types following administration of all lipid quantities.

High Drug Lipophilicity and Coadministration with Lipids Is Required To Promote Drug Targeting to Lymphocytes. Significant lymphatic transport was only evident after administration of the most highly lipophilic compounds (DDT, halofantrine and JWH015) and was negligible after administration of diazepam and the currently marketed immunosuppressants cyclosporin and sirolimus (Figure 2 and Table 1). Administration with the higher (40 mg) when compared to the lower (4 mg) lipid dose enhanced lymphatic transport for all drugs.

A linear relationship ($r^2 = 0.99$) was evident between lymphatic drug transport and cumulative drug recovery in lymph lymphocytes (Figure 2C). Drug targeting to lymphocytes was therefore greater following administration of the more lipophilic drugs and after administration with the higher (40 mg) lipid dose (Figure 2B). The increase in drug delivery to lymphocytes with increasing drug lipophilicity was substantial, and, for example, the extent of lymphocyte targeting of DDT was 170-fold greater than that of diazepam. For most drugs, 2.4−5.8% of the mass of drug in the lymph was associated with lymphocytes (Table 1) regardless of the lipid dose with which they were administered. For cyclosporin, the mass of drug associated with lymphocytes, although low, was less closely related to the mass of drug in the lymph and 13.4% of the quantity in the lymph was associated with lymphocytes after administration of the lowest lipid dose (Table 1).

Enterocyte-based drug metabolism may reduce lymphatic drug transport by decreasing drug lipophilicity prior to lipoprotein association.[39] Data were therefore generated for cyclosporin after preadministration of ketoconazole (50 mg/kg ketoconazole at −24, −12, and −0.5 h prior to administration of cyclosporin) in order to inhibit cytochrome P450 mediated intestinal metabolism. This approach significantly enhanced the lymphatic transport of cyclosporin administered with 40 mg of lipid from 1.31 ± 0.08 to 2.82 ± 0.7% of the dose, however, the absolute extent of lymphatic transport remained low and did not appear to lead to increases in lymphocyte uptake (0.08 ± 0.02 and 0.07 ± 0.02% of the dose recovered in lymph lymphocytes, respectively after administration without and with ketaconazole).

Lymphocytes that are exposed to high concentrations of drug in the lymph subsequently drain back into the systemic circulation and ultimately recirculate between the blood, lymphoid tissues and lymph. In order to probe the longevity of the lymphocyte targeting effect, drug exposure to lymphocytes in the systemic circulation was also addressed for diazepam, halofantrine and DDT.

The maximum plasma concentrations of diazepam, DDT and halofantrine occurred at 2, 3, and 4 h, respectively (Figure 3A), and the AUC^{0-5h}_{plasma} was in the order DDT > diazepam > halofantrine (Table 2). However, drug association with lymphocytes in the systemic circulation was not simply dependent on the plasma concentration since the lymphocyte concentration time profiles (Figure 3B) and AUC^{0-5h}_{WBC} (Table 2) were in the order DDT > halofantrine > diazepam. Indeed, the profiles for drug association with lymphocytes in the

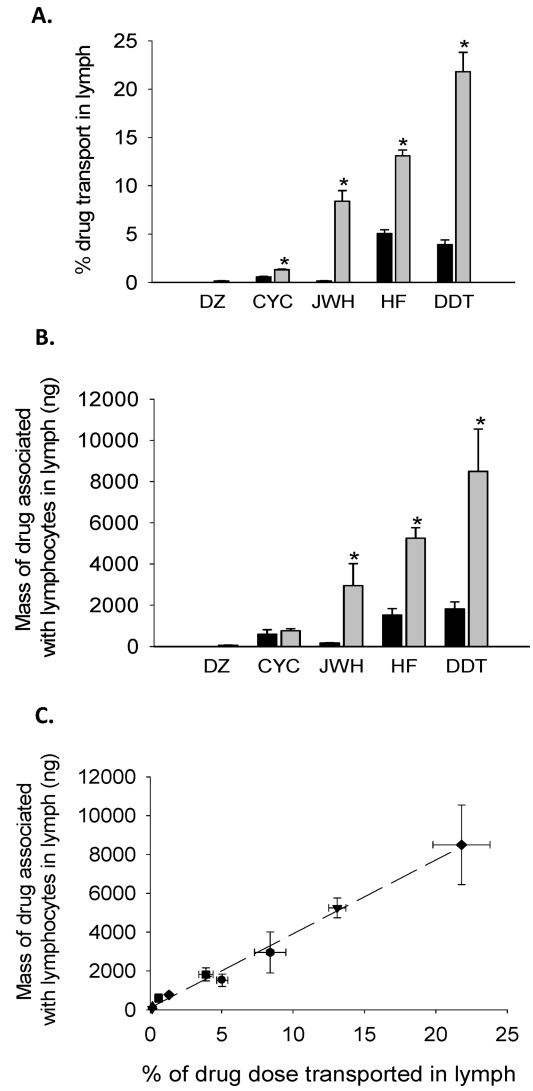

Figure 2. Effect of drug lipophilicity and lipid coadministration on drug targeting to lymphocytes in mesenteric lymph. (A) % of the administered drug dose transported into the intestinal lymph, and (B) the cumulative total mass of drug associated with lymphocytes in lymph (ng) and (C) the correlation between the mass of drug associated with lymphocytes in lymph and % of the drug dose transported into the intestinal lymph, over 6 h following initiation of intraduodenal administration of 1 mg of drug (diazepam (DZ), cyclosporin (CYC), JWH015 (JWH), halofantrine (HF) or DDT (DDT), in ascending order of drug lipophilicity) and 4 mg of lipid (filled bars in panel A and B) or 40 mg lipid (open bars in Panel A and B). Data represent mean ± SEM for $n = 4$ or 5 rats. *Significantly higher than 4 mg of lipid group.

Table 1. Effect of Lipid Dose and Drug Lipophilicity on Drug Transport into the Intestinal Lymph and Drug Targeting to Lymph Lymphocytes[a]

		% of drug dose		
formulation	drug	transported in lymph	in lymphocytes in lymph	fraction (as a %) of drug in lymphocytes
40 mg of oleic acid	DDT	21.8 ± 2.0	0.85 ± 0.20	3.1 ± 0.6
	halofantrine	13.1 ± 0.6	0.53 ± 0.05	3.9 ± 0.2
	JWH-015	8.4 ± 1.1	0.30 ± 0.11	3.9 ± 1.4
	diazepam	0.13 ± 0.04	0.005 ± 0.001	5.6 ± 2.5
	cyclosporin	1.31 ± 0.08	0.08 ± 0.02	5.8 ± 0.5
	sirolimus	<1%, some samples below LOQ	below LOQ	below LOQ
	oleic acid	48.9 ± 5.0	1.75 ± 0.02	3.6 ± 0.4
4 mg of oleic acid	DDT	3.9 ± 0.5	0.18 ± 0.03	4.6 ± 0.2
	halofantrine	5.1 ± 0.4	0.15 ± 0.03	3.0 ± 0.2
	JWH-015	0.16 ± 0.01	0.0017 ± 0.0001	1.05 ± 0.08
	cyclosporin	0.58 ± 0.04	0.08 ± 0.01	13.4 ± 2.2
	oleic acid	44.18 ± 0.05	3.5 ± 1.1	7.9 ± 2.6

[a] % of the administered drug and ^{14}C oleic acid doses transported into the intestinal lymph and associated with lymphocytes in lymph and the fraction (as a %) of the mass in the lymph that is associated with lymphocytes. Data collected over 6 h following initiation of intraduodenal administration of lipid-based drug formulations to mesenteric lymph-cannulated anesthetized rats. Formulations consisted of 1 mg of drug (DDT, halofantrine, JWH015, diazepam, cyclosporin or sirolimus) and 4 or 40 mg of oleic acid dispersed in 5.6 mL of 0.2% Tween 80 and were administered at a rate of 2.8 mL/h over 2 h. The DDT containing formulations also contained 5 μCi of ^{14}C oleic acid to enable determination of values for ^{14}C oleic acid. Data represent mean ± SEM for $n = 4$ or 5.

systemic circulation (Figure 3B) more closely mirrored the profiles for the rate of drug transport into lymphatic lymphocytes (Figure 3C) than the plasma concentration−time profiles (Figure 3A). To provide a single measure of systemic lymphocyte targeting the data describing drug uptake into lymphocytes in the blood were normalized for differences in total systemic exposure. The lymphocyte exposure ratios were highest for DDT followed by halofantrine and diazepam (Table 2) again reflecting differences in lymphatic drug transport and exposure to lymphocytes in lymph (Table 1, Figure 2).

Lymphocyte Targeting of the Experimental Immunosuppressant JWH015. Lack of appreciation of the targeting strategy described here has dictated that none of the currently marketed immunosuppressants (e.g., cyclosporin, sirolimus) are sufficiently lipophilic to facilitate lymphatic transport. The current targeting strategy was therefore explored in detail using a highly lipophilic experimental immunosuppressant, JWH015. JWH015 is a cannabinoid 2 (CB2) receptor agonist that has anti-inflammatory, neuroprotective and immunomodulatory activity[41−43] and activity in mouse models of multiple sclerosis.[44] The immunomodulatory effects of JWH015 include reduced production of "pro-inflammatory" cytokines (IL-2, TNFα, IFNα), increased production of "anti-inflammatory" cytokines (IL-4, IL-10), modulation of monocyte migration and promotion of lymphocyte apoptosis.[42,43,45]

Consistent with the other drugs, lymphatic transport and recovery of JWH015 in lymph lymphocytes was significantly greater after administration with the higher (40 mg) when compared to the lower (4 mg) lipid dose (Figure 2). Bioavailability and plasma exposure was also 2.7-fold higher following administration of JWH015 with 40 mg rather than 4 mg of lipid (reflecting an increase in drug absorption on coadministration with lipids) (Figure 4A, Table 3). Importantly, however, exposure to lymphocytes was much higher (12.8-fold, Figure 4B) after stimulation of lymphatic transport and therefore approximately 5-fold higher than would be expected based simply on differences in plasma exposure (Table 3).

Since the plasma exposure of JWH015 was increased after administration with the higher lipid dose (Table 3), a series of dose ranging studies were conducted (over slightly longer time periods (8 h)) to determine the dose required to match systemic JWH015 exposure at different lipid doses. Similar plasma exposure was achieved by administration of 1 mg/kg JWH015 with 4 mg of lipid and 0.4 mg/kg JWH015 with

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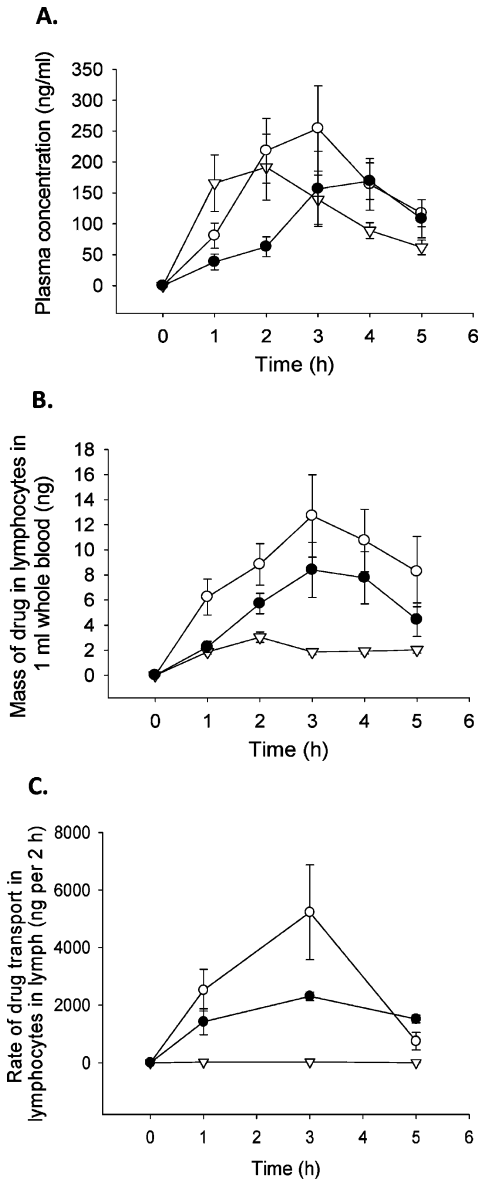

Figure 3. Targeting of DDT, halofantrine and diazepam to lymphocytes in the lymph and systemic circulation. (A) Plasma drug concentration (ng/mL) versus time profiles. (B) Mass of drug associated with lymphocytes in 1 mL of whole blood (ng) versus time profiles. (C) Rate of drug transport into lymphocytes in lymph (ng per 2 h, plotted at midpoint of collection time) following intraduodenal infusion of 1 mg drug (DDT ○, halofantrine (Hf) ● or diazepam (▽) and 40 mg lipid over 2 h. Data represent mean ± SEM for $n = 4$ or 5 rats.

40 mg of lipid (Figure 4C,D). While plasma exposure was not significantly different using this dosing regime, systemic lymphocyte exposure was substantially (~5-fold) higher ($\alpha < 0.05$) when JWH015 was administered with 40 mg of lipid (Figure 4D), presumably reflecting greater transport via the lymph (Figure 2).

Lymphocyte Targeting Enhances the Immunomodulator Activity of JWH015. To investigate whether targeting JWH015 to lymphocytes using a lymph-directing formulation improved immunomodulatory activity, we examined changes to IL-4 and IL-10 expression by mitogen stimulated CD4+ and CD8+ lymphocytes. Lymphocytes were collected from animals administered 1 mg/kg JWH015 with 4 mg of lipid or 0.4 mg/kg JWH015 with 40 mg of lipid. In both groups overall systemic exposure was similar, however animals administered the higher lipid dose were previously shown to have a greater proportion of the dose delivered to the lymph (>50-fold, Table 3), to lymphocytes in lymph (>50-fold, Table 3), and to lymphocytes in plasma (~5-fold, Figure 3). Consistent with high lymphocyte exposure, lymphocytes collected following administration of 0.4 mg/kg of JWH015 with 40 mg of lipid showed a significant increase in mitogen stimulated release of IL-4 (both CD4+ and CD8+ lymphocytes) and IL-10 (CD4+ lymphocytes but not CD8+ lymphocytes) (Figure 5). In contrast, a 2.5-fold higher dose of JWH015 after administration with 4 mg of lipid was essentially inactive and failed to increase the number of IL-4 positive CD4+ and CD8+ T lymphocytes and IL-10 positive CD4+ T lymphocytes when compared to administration of normal saline (Figure 5). Importantly administration of 40 mg of lipid alone (i.e. without drug) had no effect on IL-4 and IL-10 levels. Stimulation of lymphatic transport and lymphocyte targeting of JWH015 was therefore essential for immunomodulatory activity in the current studies and provides evidence of the potential for this approach to facilitate more effective pharmacodynamic outcomes.

Table 2. Effect of Drug Lipophilicity on Drug Targeting to Lymphocytes in the Systemic Circulation[a]

	DDT	halofantrine	diazepam
plasma AUC^{0-5h}_{plasma} (ng·h/mL)	3879 ± 378[b]	2405 ± 219	3084 ± 232
lymphocyte AUC^{0-5h}_{WBC} (ng·h/mL)	213 ± 24[c]	132 ± 7[d]	48.4 ± 2
systemic lymphocyte exposure (%)	5.5 ± 0.2[d]	5.5 ± 0.4[d]	1.6 ± 0.3

[a] The plasma exposure (AUC^{0-5h}_{plasma}, ng·h/mL), area under the profile for the mass of drug associated with lymphocytes in the systemic circulation (AUC^{0-5h}_{WBC}, ng·h/mL) and lymphocyte exposure ratio (as a %) of AUC^{0-5h}_{WBC} and AUC^{0-5h}_{plasma} following administration of 1 mg of drug (halofantrine, DDT or diazepam) with 40 mg of lipid via intraduodenal infusion. Data represent mean ± SEM, $n = 4$. [b] Statistically greater than the same parameter in the group administered halofantrine ($\alpha < 0.05$). [c] Statistically greater than the same parameter in the groups administered diazepam or halofantrine ($\alpha < 0.05$). [d] Statistically greater than the same parameter in the group administered diazepam ($\alpha < 0.05$).

Discussion

Strategies to promote drug targeting to lymphocytes within the lymph, the systemic circulation and ultimately the lymphoid tissues have the potential to benefit the treatment of a number of pathological conditions in which the lymphatics and lymphocytes play a major role in disease progression. These include autoimmune disease, transplant rejection, immunodeficiency, allergy, leukemia, lymphoma and lymphocyte and/or intestinal lymph-resident infections such as HIV,[2,46] hepatitis B,[47] hepatitis C,[48] severe acute

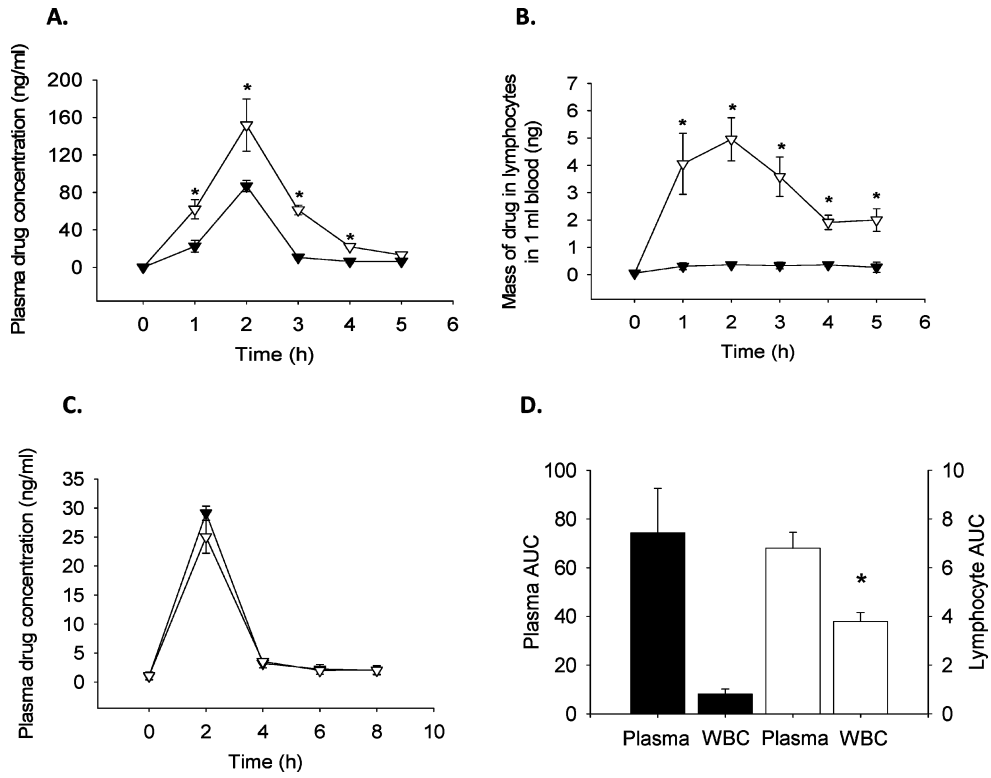

Figure 4. Effect of lipid coadministration on JWH015 targeting to lymphocytes in the systemic circulation. (A) Plasma drug concentration (ng/mL) versus time profiles and (B) mass of drug associated with lymphocytes in 1 mL of whole blood (ng) versus time profiles following intraduodenal infusion of 1 mg of JWH015 and 4 mg (▼) or 40 mg (▽) of lipid. (C) Plasma drug concentration (ng/mL) versus time profiles and (D) plasma exposure (AUC^{0-8h}_{plasma}, ng·h/mL) and area under the profile for the mass of drug associated with lymphocytes (WBC) in the systemic circulation (AUC^{0-8h}_{WBC}, ng·h/mL) after intraduodenal administration of 1 mg/kg JWH015 with 4 mg of lipid (▼ or filled bars) or 0.4 mg/kg JWH015 with 40 mg of lipid (▽ or open bars). Data represent mean ± SEM for $n = 4$ or 5 rats. *Significantly greater than 4 mg of lipid group.

Table 3. Effect of Lipid Dose on JWH015 Targeting to Lymphocytes[a]

	4 mg of lipid JWH015	40 mg of lipid JWH015	fold increase
mass of drug transported in lymph (μg)	1.6 ± 0.1	84 ± 11[b]	52.5
mass of drug in lymph lymphocytes (μg)	0.017 ± 0.001	3.0 ± 0.1[b]	176
plasma AUC^{0-5h}_{plasma}	113 ± 16	307 ± 26[c]	2.7
absolute bioavailability	5.3 ± 0.4	14.5 ± 2.1[c]	2.7
lymphocyte AUC^{0-5h}_{WBC}	1.3 ± 0.2	16.7 ± 2.7[c]	12.8
systemic lymphocyte exposure (%)	1.1 ± 0.3	5.5 ± 0.2[c]	5

[a] Summary of the mass of drug transported in lymph (μg), mass of drug transported into lymphocytes in the lymph (μg), plasma exposure (AUC^{0-5h}_{plasma}, ng·h/mL), absolute bioavailability, area under the profile for the mass of drug associated with lymphocytes in the systemic circulation (AUC^{0-5h}_{WBC}, ng·h/mL) and lymphocyte exposure in the systemic circulation (ratio of AUC^{0-5h}_{WBC} and AUC^{0-5h}_{plasma} as a %) for rats administered 1 mg of JWH015 with 4 mg or 40 mg of lipid via intraduodenal infusion. Data represent mean ± SEM, $n = 4$. [b] Statistically greater than the same parameter in the group administered 4 mg of lipid ($\alpha < 0.01$). [c] Statistically greater than the same parameter in the group administered 4 mg of lipid ($\alpha < 0.05$).

respiratory syndrome (SARS),[49] morbillivirus[50] and tuberculosis.[51] Here we provide evidence of the potential for lymphatic transport to enhance drug targeting to lymphocytes and further show that this targeting effect can enhance the pharmacodynamic effect of an experimental immunosuppressant.

Lymphocyte targeting was achieved by the use of drugs with physicochemical properties consistent with lymphatic transport and by coadministration with lipids. Coadministration with lipid resulted in three coincident and synergistic effects: promotion of drug absorption, enhancement of

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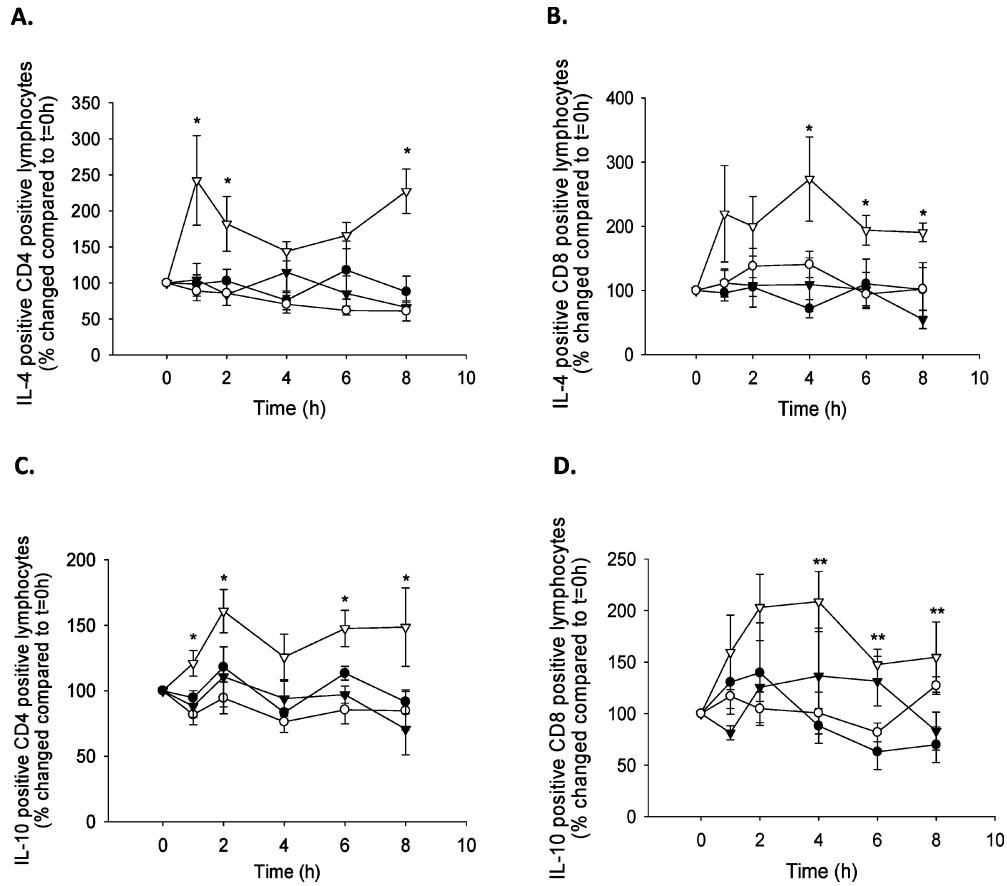

Figure 5. Impact of lymphatic transport on the immunomodulating effect of JWH015. (A) IL-4 positive CD4 positive T lymphocytes, (B) IL-4 positive CD8 positive T lymphocytes, (C) IL-10 positive CD4 positive T lymphocytes and (D) IL-10 positive CD8 positive T lymphocytes (expressed as a % change compared to time = 0 h) vs time after intraduodenal administration of 1 mg/kg JWH015 with 4 mg lipid (▼), 0.4 mg/kg JWH015 with 40 mg of lipid (▽), 40 mg of lipid alone (○) or normal saline (●). Collected blood samples were stimulated with mitogens and IL-4 and IL-10 expression detected via flow cytometry. Data represent mean ± SEM for $n = 4-6$ rats. *Significantly higher than normal saline, 40 mg of lipid alone and 4 mg of lipid with JWH015; **significantly higher than normal saline group.

lymphatic drug transport and recruitment of lymphocyte trafficking into mesenteric lymph.

Drug access to the intestinal lymphatics is facilitated by drug integration into lipid absorption and transport pathways and, in particular, by drug association with developing lymph lipoproteins in the enterocyte during drug and lipid absorption. The physical size of these colloidal lipoprotein structures subsequently precludes facile diffusion across the vascular endothelium and, instead, drug loaded lipoproteins are transported from the intestinal submucosa to the systemic circulation via the lymphatic capillaries.[52] Lymphatic transport is therefore only possible for drugs that have high affinity for lymph lipoproteins[17,53] and is enhanced by coadministration of lipids, in the form of either a fatty meal or a lipid formulation.[14,52] Historically, the potential for lymph lipoprotein association and lymphatic transport was thought to be well predicted by broad indicators of lipophilicity. As such drugs with log *D*'s in excess of 5 and triglyceride solubilites in excess of 50 mg/mL were typically thought to be potential candidates for lymphatic transport.[16] Recent studies, however, have suggested that these indicators may be too narrow and that even compounds with moderate triglyceride solubilites may have sufficient affinity for lymph lipoproteins to drive significant lymphatic transport if, for example, an affinity for the lipoprotein interface is evident.[38,53] In the current studies, however, significant lymphatic transport was only apparent after administration of the most lipophilic drug molecules: halofantrine, DDT and JWH015 (and not, for example, with current immunosuppressants such

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as cyclosporin and sirolimus) but was enhanced by administration of larger quantities of lipid.

The central finding in the current studies was that enhancement of lymphatic drug transport and stimulation of lymphocyte recruitment into the lymph via the use of lipid-based vehicles led to a profound increase in drug targeting to lymphocytes. A strong correlation was evident between the extent of drug transport into the lymph and drug uptake into lymphocytes and as such, coadministration of more highly lipophilic drugs with higher lipid doses (40 mg) led to significant (>100-fold) enhancements in lymphocyte drug targeting. Drug exposure to lymphocytes in the systemic circulation (rather than the lymph) was also greater when lymphatic drug transport and drug exposure to lymphocytes in the mesenteric lymph was enhanced and more closely mirrored changes in lymphatic lymphocyte exposure than plasma concentrations of drug. Enhanced drug targeting to systemic lymphocytes therefore appears to reflect drug uptake into lymphocytes in the intestinal submucosa and mesenteric lymph and subsequent transport to the systemic circulation, rather than de novo drug association with systemic lymphocytes in the plasma.

Coadministration of as little as 4 mg of lipid was capable of increasing recruitment of lymphocytes into the mesenteric lymph, however the effect was most significant after administration of larger quantities of lipid (40 mg), which also supported lymphatic drug transport. Previous studies have shown a similar enhancement in lymphocyte flux into the lymph of rats after lipid ingestion (although after ingestion of lipid doses in excess of those used here) and in animals that had been prefed a high fat diet.[35,54−56] Lymphocytes stimulated to enter the mesenteric lymph are derived from three major sources (Peyer's patches, the intestinal lamina propria and intestinal epithelial lymphocytes)[57] and lymphatic recruitment results from enhanced lymphocyte activation and proliferation (which in turn is stimulated by the presence of lipid-rich lipoproteins)[58,59] or improved lymphocyte extravasation.[35,54]

Previous in vitro evidence suggests that lipoprotein (HDL, LDL, VLDL) interaction with lymphocytes involves docking to the lymphocyte in a process that is mediated by receptor binding (e.g., via a HDL,[60] LDL[61] or VLDL receptor) or may occur independent of receptor binding.[61] Following docking, lipoproteins are hydrolyzed by lipoprotein lipase[58] and may be internalized via an endocytotic process. For

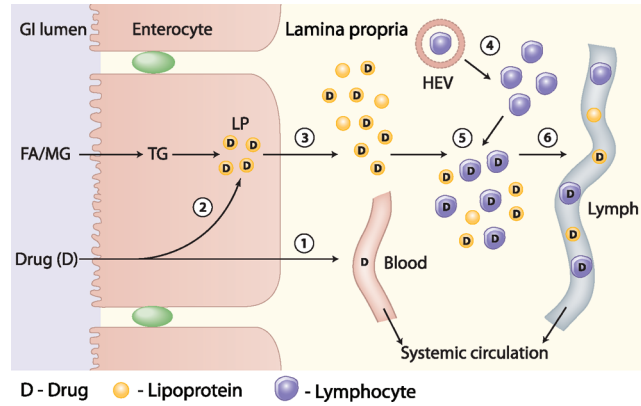

Figure 6. Proposed mechanism by which promotion of lymphatic drug transport, via administration of lipophilic drugs with lipids, facilitates targeted delivery to lymphocytes. Following oral administration, drugs (D) usually pass from the gastrointestinal (GI) lumen across intestinal absorptive cells (enterocytes) and drain via the blood capillaries to the systemic circulation (step 1). Some highly lipophilic drugs, however, may associate with developing lipoproteins (LP, yellow spheres) in the enterocyte (step 2), leading to exocytosis of a drug lipoprotein complex from the enterocyte into the underlying lamina propria (step 3). (Triglyceride (TG) forms the core lipid of developing lipoproteins in the enterocyte and is resynthesized from absorbed lipid digestion products, e.g. fatty acid (FA) and monoglyceride (MG).) Lipoprotein synthesis also stimulates lymphocyte (blue spheres) recruitment, for example, via increased trafficking through high endothelial venules (HEV) and intestinal microvilli (step 4). The high concentration of lipoproteins and lymphocytes in the lamina propria provides for significantly enhanced drug targeting to lymphocytes (step 5). Lymphocytes and drug loaded lipoproteins subsequently enter the lymphatic capillaries (step 6) and ultimately drain to local lymph nodes providing further opportunity for drug−lymphocyte interaction within the lymph or lymph nodes.

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example, the uptake of plasma LDL containing lipophilic pesticides (DDT, benzopyrene) into lymphocytes and macrophages[62,63] has previously been observed. Drug association with plasma lipoproteins therefore facilitates drug uptake into lymphocytes in vitro, and the patterns of drug association with lymph lymphocytes reported here might therefore be expected to have occurred, at least in part, via a similar mechanism. Regardless of the mechanisms of drug association with lymphocytes, however, the current data suggest that, at least in the case of JWH015, pharmacological activity is retained and effective potency significantly enhanced by the lymphotropic targeting strategy. Thus, administration of JWH015 with a lymph directing formulation led to a significant increase in the expression of antiinflammatory cytokines (IL-4 and IL-10) in mitogen stimulated lymphocytes. In contrast, administration of a 2.5-fold higher dose in a non-lymph directing formulation was inactive, even though drug exposure to the systemic circulation was equivalent.

In conclusion, the current study has demonstrated that promoting lymphatic drug transport via the coadministration of highly lipophilic drugs with lipids increases drug targeting to lymphocytes and improves the activity of an experimental immunomodulator, JWH015. This lymphotropic effect results from stimulation of lymphatic drug transport and simultaneous recruitment of lymphocytes into the lymph (Figure 6). The data suggest that drug (or prodrug) synthetic strategies that "design in" physicochemical properties consistent with intestinal lymphatic transport (i.e., high lipophilicity) may increase the efficacy of orally administered drugs that act on lymphocytes, such as immunomodulators, anticancer agents or antiinfectives. Importantly, drug treatments in these therapeutic areas are commonly limited by off-target toxicities and the current approach has the potential to significantly widen the therapeutic window or safety margin of prospective drug candidates by providing selective, targeted delivery and equivalent or increased activity at lower dose.

Supporting Information Available: Table S1 of conditions used for HPLC−UV analysis of drug concentrations in lymph, plasma and lymphocyte samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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