Research Article

The Immunosuppressive Activity of Polymeric Micellar Formulation of Cyclosporine A: *In Vitro* and *In Vivo* Studies

Samar Hamdy,¹ Azita Haddadi,² Anooshirvan Shayeganpour,¹ Aws Alshamsan,^{1,3}
Hamidreza Montazeri Aliabadi,⁴ and Afsaneh Lavasanifar^{1,4,5}

Received 10 November 2010; accepted 2 February 2011; published online 19 February 2011

Abstract. We have previously developed micelles of methoxy poly(ethylene oxide)-*b*-poly(ϵ -caprolactone) as vehicles for the solubilization and delivery of cyclosporine A (CsA). These micelles were able to reduce the renal uptake and nephrotoxicity of CsA. The purpose of the current study was to test the efficacy of polymeric micellar formulation of CsA (PM-CsA) in suppressing immune responses by either T cells or dendritic cells (DCs). The performance of PM-CsA was compared to that of the commercially available formulation of CsA (Sandimmune®). Our results demonstrate that PM-CsA could exert a potent immunosuppressive effect similar to that of Sandimmune® both *in vitro* and *in vivo*. Both formulations inhibited phenotypic maturation of DCs and impaired their allostimulatory capacity. Furthermore, both PM-CsA and Sandimmune® have shown similar dose-dependent inhibition of *in vitro* T cell proliferative responses. A similar pattern was observed in the *in vivo* study, where T cells isolated from both PM-CsA-treated and Sandimmune®-treated mice have shown impairment in their proliferative response and IFN- γ production at similar levels. These results highlight the potential of polymeric micelles to serve as efficient vehicles for the delivery of CsA.

KEY WORDS: cyclosporine A; dendritic cells; polymeric micelles; T cells.

INTRODUCTION

Cyclosporine A (CsA) is a potent immunosuppressant drug which has been in use in the clinic since 1983. It is now widely approved for patients undergoing solid organ transplantation (mainly heart, liver, kidney, and lung) as well as bone marrow transplants. CsA markedly suppresses the patient's immune system, thus decreasing the risk of organ rejection and improving the long-term survival of transplant patients. The immunosuppressant properties of CsA are also useful in treating graft-versus-host disease, a variety of autoimmune diseases, as well as immune-related ophthalmic and dermatological disorders (reviewed in (1)).

Despite the initial efficacy of CsA in treating numerous local and systemic immune-related disorders, discontinuation of the therapy often leads to disease relapse. Therefore, CsA treatment has to be maintained for a long time, sometimes lifelong (1,2).

Long-term use of CsA is associated with serious side effects such as dose-dependent nephrotoxicity, hepatotoxicity, and hypertension. Other drawbacks of CsA include poor absorption owing to its low water solubility (27.67 $\mu\text{g/mL}$ at 25°C) (3) and high lipophilicity (log $P=2.92$ at pH 7.4). In addition, due to the very rigid cyclic structure of CsA (Fig. 1a) and its high molecular weight (1,203 kDa), CsA exhibits very low permeability across almost all the biological barriers, such as the gastrointestinal tract, skin, and cornea (1).

The first formulation of CsA, i.e., Sandimmune®, approved in 1983, is an emulsion pre-concentrate of CsA used for intravenous (i.v.) administration or oral use (in the form of soft gelatin capsules or an oral solution). Parenteral Sandimmune® consists of CsA with Cremophor EL and ethanol. Unfortunately, administration of Cremophor EL is associated with a wide range of adverse effects, including anaphylactic shock, hypersensitivity reactions, changes in blood pressure, tachycardia, hyperlipidemia, aggregation of erythrocytes, acute respiratory stress, and peripheral neuropathy (1,4). An extensive amount of research has been undertaken to optimize CsA formulations in order to come up with a delivery system that can improve CsA water solubility and bioavailability, and at the same time reduce the associated side effects of CsA (mainly nephrotoxicity) and eliminate the need for Cremophor EL. Various CsA formulations that have been developed and currently tested in animal models and/or clinical trials are listed in (1,4,5).

Our group has demonstrated the potential of poly(ethylene)-*block*-poly(ϵ -caprolactone) (PEO-*b*-PCL) micelles

¹ Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, 3133 Dentistry/Pharmacy Centre, Edmonton, Alberta T6G 2N8, Canada.

² College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

³ Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

⁴ Faculty of Engineering, Department of Chemical and Material Engineering, University of Alberta, Edmonton, Alberta, Canada.

⁵ To whom correspondence should be addressed. (e-mail: alavasanifar@pharmacy.ualberta.ca)

Fig. 1. Chemical structure of **a** cyclosporine A (CsA) and **b** poly(ethylene oxide)-block-poly(ϵ -caprolactone) (PEO-*b*-PCL). Degree of polymerization of PEO (x) and PCL (y) is 114 and 114

(Fig. 1b) for the solubilization and controlled delivery of CsA (6–8). We have previously shown that CsA-loaded PEO-*b*-PCL micelles are able to change the normal biodistribution of CsA by reducing accumulation of CsA in kidneys and increasing CsA levels in blood after single (6) or repeated dosing (8). It is worth mentioning that various drugs have shown decreased accumulation in kidneys when delivered in polymeric micelles (9–11). Interestingly, this decreased delivery of CsA to kidneys by the polymeric micelles has led to significant reduction of CsA-induced nephrotoxicity after multiple intravenous (i.v.) administration (8). These results highlight the significance of the polymeric micelles to improve the therapeutic outcome of CsA administration in clinic. However, the successful clinical development of CsA-loaded polymeric micelles (PM-CsA) requires direct demonstration and assessment of their immunosuppressive capacity.

Cyclosporin A effectively suppresses T-cell-mediated immune responses, the key effector cells involved in graft rejection and autoimmunity. CsA interacts with its cytoplasmic receptor in T cells, named cyclophilin, to form a complex. This complex inhibits the action of calcineurin, an essential phosphatase for the activation and translocation of nuclear factor of activated T cells (NFAT) transcription factor. NFAT regulates transcription of numerous genes involved in T cell activation and proliferation, such as IL-2, IL-4, and CD40 ligand (12). Failure to express those genes results in dramatic inhibition of T-cell-dependent immune responses. In addition to the well-known suppressing effect of CsA on T cells, recent studies have highlighted the ability of CsA to suppress other key cells of the immune system such as dendritic cells (DCs), which are the most potent antigen presenting cells (13–15). Although the underlying mechanism is not yet fully elucidated, CsA has been associated with impairment of several aspects of DC biological

activities such as migration, maturation, and allostimulatory capability (13–15).

The main objective of the current study was to assess the immunosuppressive activity of a polymeric micellar formulation of CsA making comparisons with its commercial i.v. formulation, Sandimmune®. In this context, the immunosuppressive effects of CsA as part of polymeric micellar or Cremophor EL formulation on the functional activity of T cells and DCs were evaluated *in vitro*. Moreover, the *in vivo* immunosuppressive activity of CsA in suppressing the proliferation of T cells from mice receiving i.v. CsA formulations in a mixed lymphocyte reaction (MLR) was evaluated.

MATERIALS AND METHODS

Mice

BALB/c and C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All experiments were performed using 6- to 12-week-old male mice. All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee (Biosciences, Health Sciences, or Livestock) of the University of Alberta.

Reagents

Stannous octoate (96%) was obtained from Aldrich (Milwaukee, WI, USA). Methoxy poly(ethylene oxide) (average molecular weight of $5,000 \text{ g mol}^{-1}$), ϵ -caprolactone and Cremophor EL were purchased from Sigma (St. Louis, MO, USA). CsA was supplied by Wuhan Zhongxin Company, China. Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Peprotech (Rocky Hill, NJ, USA). EasySep® murine T cell isolation kits were purchased from StemCell Technologies (Vancouver, BC, Canada). Murine IL-2 and IFN- γ ELISA kits were purchased from E-Bioscience (San Diego, CA, USA). TGF- β DuoSet ELISA Development kit was purchased from R&D Systems (Minneapolis, MN, USA). RPMI-1640, L-glutamine, and gentamycin were purchased from Gibco-BRL (Burlington, ON, Canada). Fetal calf serum (FCS) was obtained from Hyclone Laboratories (Logan, UT, USA). Anti-mouse CD16/CD32, CD40, and CD86, MHCII mAbs, and their respective isotype controls were purchased from BD Biosciences (Mississauga, ON, Canada). Acetone and water (all HPLC grades) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Preparation and Characterization of CsA-Loaded PEO-*b*-PCL Micelles

The PEO-*b*-PCL block copolymer with respective PEO and PCL molecular weights of $5,000$ and $13,000 \text{ g mol}^{-1}$ was synthesized as previously described (6). In brief, methoxy PEO (5 g), ϵ -caprolactone, and stannous octoate were added to a previously flamed 10 mL ampoule, nitrogen purged, then sealed under vacuum. The reaction product was dissolved in chloroform, precipitated, and washed with an excess of cold methanol, followed by centrifuge collection.

Assembly of block copolymers was achieved by co-solvent evaporation where 30 mg of PEO-*b*-PCL (with or without 9 mg CsA) was dissolved in acetone (0.5 mL) and added in a dropwise manner (1 drop/15 s) to stirring distilled water (3 mL). After 4 h of stirring at room temperature, vacuum was applied to ensure the complete removal of the organic solvent. The CsA-loaded micellar solution was then centrifuged at 11,600×*g* for 5 min, to remove CsA precipitates. Full characterization of CsA-loaded PEO-*b*-PCL micelles and the method for determination of CsA content were previously described (16).

Preparation of Murine Bone Marrow-Derived DCs

DC primary cultures were generated from murine bone marrow precursors from femurs of BALB/c mice or C57BL/6 in complete RPMI media in the presence of GM-CSF as described earlier in (17). Briefly, femurs were removed and cleaned from the surrounding muscle and fatty tissues. For disinfection, intact bones were put in 70% ethanol for 2 min (min) and then washed with phosphate-buffered saline (PBS). Afterwards, both ends of the femur were cut with sterile scissors and the marrow was flushed with PBS using an insulin syringe. After one wash in PBS, about 1–2×10⁷ leukocytes were obtained per femur. Leukocytes were plated in complete medium [RPMI-1640 supplemented with gentamycin (80 µg/mL), L-glutamine (2 mM), and 10% heat-inactivated FCS] at 2×10⁶ per 100 mm dish in 10 mL complete medium containing 20 ng/mL GM-CSF. At day 3, another 10 mL complete medium containing 20 ng/mL GM-CSF was added to the plates. At day 6, half of the culture supernatant was collected, centrifuged, and the cell pellet re-suspended in 10 mL fresh medium containing 20 ng/mL GM-CSF, and added back to the original plate. At day 7, cells were used. The purity of the DC population on day 7 was found to be between 70% and 75%, based on the expression of CD11c on the semi-adherent and non-adherent cell populations.

CsA-Mediated Inhibition of *In Vitro* DC Functions

On day 7, murine bone marrow-derived DCs (BMDCs; generated from femurs of BALB/c mice as described above) were treated with 1 µg/mL CsA either in soluble form (Sandimmune®) or in polymeric micellar formulation (PM-CsA). Untreated DCs and DCs treated with 1 µg/mL lipopolysaccharide (LPS) were used as negative control and positive control, respectively. Following 72 h incubation, DCs were harvested and tested for up-regulation of maturation surface markers (CD40, CD86, and MHC II) and for their

ability to stimulate allogenic T cells by flow cytometry and MLR, respectively. Culture supernatants were also collected at the end of the 72 h culture and assayed for the level of TGF-β secretion using ELISA available kits as per the manufacturer's recommendation. For flow cytometric studies, 2.5×10⁵ DCs were suspended in FACS buffer (PBS with 5% FCS, and 0.09% sodium azide) and incubated with anti-mouse CD16/CD32 mAb to block Fc receptors, then stained with appropriate fluorescent-labeled conjugated antibodies. All samples were finally acquired on a Becton-Dickinson FACSsort and analyzed by CellQuest software. For MLR, DCs were harvested, irradiated with 3,000 rd using a 137Cs irradiator, washed, and plated at graded doses in triplicates in 96-well microtiter plates (Costar, Cambridge, MA, USA). Allogenic T cells were isolated from C57BL/6 mice using an Easysep® T cell separation kit and were used as responders (0.1×10⁶ cells/well). DCs/T cell co-cultures were maintained for 72 h at 37°C. T cell proliferation was then assessed by [³H]-thymidine incorporation (1 µCi/well; Amersham, Oakville, ON, Canada) during an overnight incubation. Incorporation of [³H]-thymidine into DNA was measured by scintillation counting.

CsA-Mediated Inhibition of *In Vitro* T Cell Responses

In this experiment, an *in vitro* MLR was performed with T cells obtained from healthy C57BL/6 mice as responders and allogenic DCs (obtained from BALB/c mice) as stimulators. Briefly, day 7 DCs (generated from BALB/c mice) were harvested, irradiated with 3,000 rd using a 137Cs irradiator, washed, and plated in round-bottom 96-well microtiter plates (0.05×10⁶ DCs/well). T cells were isolated from the spleens of C56BL/6 mice using an Easysep® negative selection T cell isolation kit. Isolated T cells were then co-cultured with the allogenic DCs (0.1×10⁶ T cells/well) at a DC/T cell ratio of 1:2. T cell/DC co-cultures were then treated with varying concentrations (20–2,000 ng/mL) of CsA, either in the soluble form (Sandimmune®) or as a polymeric micellar formulation (PM-CsA). Empty polymeric micelles and Cremophor EL were similarly diluted and added to T cell/DC co-cultures as negative controls for PM-CsA and Sandimmune®, respectively. Co-cultures were incubated in RPMI 1640 complete medium for 72 h at 37°C. T cell proliferation was assessed by [³H]-thymidine incorporation as described above.

The CsA-mediated inhibition of T cell proliferation (TCP) was expressed as TCP % and calculated as described in the following equations:

$$TCP \% \text{ for PM-CsA} = (\text{cpm of PM-CsA} / \text{cpm of Empty micelles}) \times 100$$

$$TCP \% \text{ for Sandimmune}^{\circledR} = (\text{cpm of Sandimmune}^{\circledR} / \text{cpm of Cremophor EL}) \times 100$$

The dose–response curve of T cell proliferation % was plotted against CsA concentration and was calculated using four parameter logistic functions (SigmaPlot®version10.0).

Visual inspection of T cell proliferation has been performed after 72 h of DC/T cell co-culture using a Zeiss

Axio microscope (Carl Zeiss; Jena, Germany) with identical settings for each analysis. In addition, supernatants from different treatment groups were collected at the end of co-culture and assayed for IL-2 secretion using routine ELISA techniques as per the manufacturer's instruction.

CsA-Mediated Inhibition of *In Vivo* T Cell Responses

Three groups of BALB/c mice (three mice per group) were injected intravenously for three consecutive days with one of saline, Sandimmune®, or PM-CsA (CsA concentration = 20 mg/kg/day). Twenty-four hours after the last dose, T cells were isolated from the spleens of treated animals and co-cultured with allogenic DCs (generated from C57Bl/6 mice) at different ratios for 72 h. T cell proliferation was assessed by [³H]-thymidine incorporation as described above. In a parallel experiment, the extent of T cell inhibition was further assessed by comparing IFN- γ secretion in the co-culture supernatant between different treatment groups, using routine ELISA technique per the manufacturer's instruction.

Statistical Analysis

The significance of differences between groups was analyzed by unpaired Students *t* test or one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test for multiple comparisons. Before executing the ANOVA, data were tested for normality and equal variance. If neither of the latter criteria were met, data were compared using a Kruskal-Wallis one-way ANOVA on ranks. *P* value of ≤ 0.05 was set for the significance of difference between groups. The statistical analysis was performed with SigmaStat software (Systat Software Inc. San Jose, California, USA).

RESULTS

Characterization of Micellar Formulations of CsA

Preparation of PM-CsA and their *in vitro* release profile has been reported before (6,16). The final concentration of CsA in PM-CsA solution was 2.29 ± 0.23 mg/mL, corresponding to an encapsulation efficiency of $75.9 \pm 7.51\%$ and a loading level of $0.23 \pm 0.02\%$ (*w/w*). In addition, the *in vitro* release studies have shown that PM-CsA could achieve the controlled delivery of CsA, as evidenced by the retention of most of the drug (94%) inside the micellar concentration after 12 h incubation in the release media. At this time point (12 h), the Cremophor EL formulation (Sandimmune®) released 77% of its drug content (6). The average sizes for Empty micelles and PM-CsA were 63 ± 4.0 and 89.3 ± 15.3 nm, respectively, similar to what was reported previously.

Assessment of CsA-Mediated Inhibition of *In Vitro* DC Phenotype and Function

Analysis of the alteration in the expression level of different DC maturation markers following treatment with CsA formulations is shown in Fig. 2a. Our results demonstrate that both PM-CsA and Sandimmune® have markedly inhibited CD86 expression by BMDCs, as evidenced by the decrease in the percentage of CD86 positive cells by more than 4-fold, relative to the untreated groups (30.1%, 7.6%, and 5.7%, for the untreated, PM-CsA-treated, and Sandimmune®-treated groups, respectively). On the other hand, DCs treated with Sandimmune® has shown decreased expression of MHC II compared to both untreated and PM-CsA-treated groups (52.3%, 44.2%, and 13.7%, for the untreated, PM-CsA-

treated, and Sandimmune®-treated groups, respectively). However, neither CsA formulations inhibited the expression of CD40 on DCs (19.7%, 27.6%, and 20.9%, for the untreated, PM-CsA-treated, and Sandimmune®-treated groups, respectively; Fig. 2a). LPS treatment has resulted in marked increases in the expression of all maturation markers tested (75.6%, 50.2%, and 69.4% for MHCII, CD86, and CD40, respectively).

Treated DCs were also tested for their ability to secrete TGF- β , a potent immunoinhibitory cytokine. Figure 2b shows that relative to the control groups, both PM-CsA and Sandimmune® increased the level of TGF- β secretion by DCs. The amounts of TGF- β secreted by PM-CsA-treated and Sandimmune®-treated DCs ($1,192.6 \pm 47$ and $1,107.1 \pm 40$, respectively) were significantly higher ($P < 0.05$, ANOVA) than that secreted by untreated or LPS-treated DCs ($1,047.2 \pm 49.5$ and 996.9 ± 66.2 , respectively). However, no significant difference in the level of TGF- β secretion was observed between PM-CsA-treated and Sandimmune®-treated DCs ($P > 0.075$). On the other hand, LPS treatment led to a dramatic increase in IL-12 secretion, a potent immunostimulatory cytokine secreted by activated DCs (data not shown). Untreated, PM-CsA-treated, and Sandimmune®-treated DCs did not show any detectable level of IL-12 secretion (data not shown).

We have further assessed the inhibitory effect of CsA on the allostimulatory capability of DCs. As demonstrated in Fig. 3, both CsA formulations significantly inhibited the potential of DCs to stimulate allogenic T cells, compared with untreated DCs ($P < 0.05$, ANOVA). The suppressive effects of PM-CsA vs Sandimmune® on the allostimulatory capability of DCs were not statistically different at any of the ratios tested ($P < 0.05$, ANOVA).

Assessment of CsA-Mediated Dose-Dependent Inhibition of T Cell Responses

In this set of experiments, titrated doses of CsA were added to DC/T cell co-cultures for 72 h. Proliferation of allogenic T cells was then assessed in an *in vitro* MLR, as described previously in "MATERIALS AND METHODS". The results show that PM-CsA exhibits a strong inhibition of the T cell proliferative response (Fig. 4a) at concentrations as low as 50 ng/mL. Both PM-CsA (Fig. 4a) and Sandimmune® (Fig. 4b) have shown a similar pattern of dose-dependent inhibition of T cell proliferation over the tested concentration range (20–2,000 ng/mL). It is worth mentioning that the concentration of CsA needed for inhibiting *in vitro* T cell proliferative responses in a similar MLR assay was around 100 ng/mL for both free CsA and CsA in polylactide nanoparticles (18).

A slight reduction in T cell proliferation readout (cpm) was observed at higher concentrations of control groups; Empty micelles and Cremophor EL (> 100 ng/mL). However, compared to Empty micelles, PM-CsA induced significant inhibition of T cell proliferation at all concentrations ranging from 50 to 2,000 ng/mL ($P < 0.05$, unpaired Students *t* test). Similarly, the effect of Sandimmune® was significantly different from the effect of Cremophor EL at concentrations ranging between 100 and 2,000 ng/mL ($P < 0.05$, unpaired Students *t* test). The reason for the inhibitory effect of Cremophor EL and Empty micelles on the proliferation of T cells is not known, but it may be attributed to non-specific direct effects on the viability of T cells

Fig. 2. Effect of CsA formulations on dendritic cell maturation. DC primary cultures were generated from murine bone marrow precursors from femurs of BALB/c mice as described in “**MATERIALS AND METHODS**”. On day 7, DCs were treated with 1 $\mu\text{g/mL}$ CsA either in soluble form (*Sandimmune®*) or in polymeric micellar formulation (*PM-CsA*). Untreated DCs and DCs treated with 1 $\mu\text{g/mL}$ LPS were used as negative and positive controls, respectively. Following 72 h incubation, DCs were harvested and tested for up-regulation of maturation surface markers (MHC-II, CD40, and CD86) **a**, TGF- β secretion **b** by flow cytometry and routine ELISA technique, respectively

at high concentrations. For better assessment of CsA-induced inhibitory effects on T cell proliferative responses, T cell proliferation percentages were calculated for both PM-CsA-treated and Sandimmune®-treated groups, where the inhibitory effect of negative controls were taken into account. T cell proliferation % was then plotted against CsA concentration, giving a dose-response curve shown in Fig. 4c. The concentration of CsA that corresponds to 50% reduction in T cell proliferation % (IC_{50}) did not differ between PM-CsA and Sandimmune® (56.15 ± 3.35 and 65.46 ± 4.6 ng/mL, respectively; $P > 0.05$, unpaired Students t test).

At the end of DC/T cell co-culture studies, microscopic images were taken (Fig. 5) to further compare the inhibitory effect of PM-CsA and Sandimmune® on T cell proliferation. A representative well of each concentration used for PM-CsA and Sandimmune® is shown. For control groups, the images shown represent wells that have been treated with the highest concentration of Empty micelles or Cremophor EL (equivalent to 2,000 ng/mL of CsA in either PM-CsA or Sandimmune®). Photographs of the co-cultures with proliferating T-cells show the development of large colonies after 72 h incubation. A dose-dependent decrease in the number and size of T cell colonies

Fig. 3. Decreased allostimulatory capacity of DCs when treated with CsA formulations. DCs were generated from murine bone marrow precursors from femurs of BALB/c mice. On day 7, DCs were treated with 1 $\mu\text{g/mL}$ CsA either in soluble form (*Sandimmune®*) or in polymeric micellar formulation (*PM-CsA*). Untreated DCs were used as negative control. Following 72 h incubation, DCs were harvested and tested for their ability to stimulate allogenic T cells by MLR. In brief, harvested DCs were irradiated with 3,000 rd using a ^{137}Cs irradiator, washed and plated at graded doses in triplicates in 96-well microtiter plates. T cells were isolated from C57BL/6 mice using Easysep T cell separation kit and were used as responders (0.1×10^6 cells/well). DC/T cell co-culture was maintained for 72 h at 37°C . T cell proliferation was assessed by [^3H]-thymidine incorporation following a final 24-h pulse

was observed upon increasing CsA concentration for both *PM-CsA* and *Sandimmune®*-treated groups. Such an effect was not seen in wells treated with medium only (untreated) or treated with the highest concentration of Empty micelles or Cremophor EL. The dark central region in the photographs reflect the inability to focus on both the bottom of the round wells and the proliferating T cell colonies; however, the photographs give a sense of the large decrease in number of proliferating T cells with increase in dose of CsA.

Furthermore, supernatants were collected at the end of co-culture and assayed for IL-2 level (Fig. 6). Consistent with T cell proliferation results (Fig. 4), a dose-dependent inhibition of IL-2 secretion was observed with increasing CsA concentration. Both *PM-CsA* and *Sandimmune®* have shown similar potency in inhibiting IL-2 secretion at all concentrations tested. Although higher concentrations of Empty micelles and Cremophor EL caused slight inhibition in the T cell proliferation (Fig. 4), neither of them decreased the level of IL-2 secretion (data not shown). The level of IL-2 detected in the supernatant of untreated cells did not differ from the IL-2 level detected for cells treated with Empty micelles or Cremophor EL at concentrations equivalent to maximum CsA concentrations used in this study ($P > 0.05$, ANOVA; data not shown).

Assessment of *In Vivo* CsA-Mediated Inhibition of T Cell Responses

In this set of experiments, BALB/c mice were randomly assigned to one of the following treatment groups: saline, *Sandimmune®*, or *PM-CsA* (CsA dose, 20 mg/kg/day). Each group received the treatment intravenously for three consecutive days. The suppressive effect of CsA on T cells was

Fig. 4. CsA-induced inhibition of T cell proliferative responses in an *in vitro* mixed lymphocyte reaction. Day 7 DCs (generated from BALB/c mice) were harvested, irradiated with 3,000 rd using a ^{137}Cs irradiator, washed, plated in 96-well microtiter plates (0.05×10^6 DCs/well), and used as stimulators in an *in vitro* MLR. Responders consisted of allogenic T cells isolated from the spleens of C56BL/6 mice (0.1×10^6 T cells/well) and co-cultured with DCs. DC/T cell co-culture were then treated with varying concentrations (20–2,000 ng/mL) of CsA, either in the soluble form (*Sandimmune®*) or in a polymeric micellar formulation (*PM-CsA*). Empty micelles and Cremophor EL were similarly diluted and added to the DC/T cell co-culture as negative controls. The co-culture was incubated for 72 h at 37°C . T cell proliferation was then assessed by [^3H]-thymidine incorporation as described in “MATERIALS AND METHODS”. The values represent the mean counts per minutes (cpm) of triplicates wells $\pm$ standard deviation for the different treatment groups; **a** and **b** shows results from *PM-CsA* and *Sandimmune®*-treated groups (with their respective controls), respectively. Asterisks denote significant difference between test groups (*PM-CsA* or *Sandimmune®*) and control groups (Empty micelles or Cremophor EL). **c** T cell proliferation % was calculated at each dose by comparing counts per minutes (cpm) of test groups versus cpm of control groups, as described in “MATERIALS AND METHODS”. Dose-response curve of T cell proliferation % was plotted against CsA concentration using four parameter logistic functions. Data shown are representative of three independent experiments

Fig. 5. Microscopic examination of CsA-induced inhibition of *in vitro* T cell proliferative responses (magnification x50). T cell proliferation (from the experiment described in Fig. 4) was further visualized using a Zeiss Axio microscope with identical settings for all groups. For the control groups, pictures showing represent the wells that were treated with the highest concentration of either Empty micelles or Cremophor EL (equivalent dilution to the wells having 2,000 ng/mL CsA)

assessed 24 h following last treatment (outline of the experiment is given in Fig. 7a). Consistent with the *in vitro* T cell proliferation results (Figs. 3, 4, and 6), both PM-CsA and Sandimmune® showed a strong and comparable inhibition of T cell response in the treated animals, compared to saline-treated group (Fig. 7b). T cell proliferation was performed using three different stimulator/responder ratios, 1:5, 1:10, and 1:20, where the number of T cells per well was kept constant (100×10^3), whereas the number of DCs was titrated to 20×10^3 , 10×10^3 , and 5×10^3 . The inhibition of T cell

proliferative responses induced by PM-CsA was not statically different to that induced by Sandimmune® at all ratios tested. However, in the presence of a lower number of DCs (1:20 DC to T cell ratio), PM-CsA showed a greater inhibitory effect than saline-treated groups. Interestingly, at this ratio (1:20) there was no significant difference between Sandimmune®- and saline-treated groups (Fig. 7b).

To further assess the inhibitory effect of CsA on the extent of T cell activation, culture supernatants were collected for the middle ratio (DC/T cell ratio of 1:10) and assayed for IFN- γ , an immunostimulatory cytokine secreted from T cells upon activation and proliferation. Consistent with the inhibition observed in T cell proliferation, T cells isolated from animals treated with either Sandimmune® or PM-CsA have shown dramatic reduction in the level of IFN- γ , compared to saline-treated animals (Fig. 7c). No significant difference in the level of IFN- γ was observed between Sandimmune® and PM-CsA-treated groups.

Fig. 6. Dose-dependent reduction in IL-2 secretion by T cells treated with CsA formulations. At the end of the experiment described in Fig. 4 (after 72 h of co-culture), supernatants from the different treatment groups were collected and assayed for IL-2 secretion using routine ELISA techniques per the manufacturer's instructions. Data shown are representative of three independent experiments

DISCUSSION

Cyclosporine A is widely used for suppressing immunity in patients undergoing organ transplantation as well as by patients with immune-related disorders such as graft-host disease, atopic dermatitis, ulcerative colitis, and a wide variety of other autoimmune diseases. CsA administration, however, has been associated with acute and/or chronic renal dysfunction (19). Delivery systems that could decrease the accumulation of CsA in kidneys while maintaining its therapeutic level in blood and/or lymphatic organs may potentiate the therapeutic effect of CsA while sparing the kidneys from the nephrotoxicity of CsA. We have previously

Fig. 7. Assessment of *in vivo* CsA-mediated inhibition of T cell responses. Three groups of BALB/c mice (three mice per group) were injected intravenously for three consecutive days with saline, Sandimmune®, or PM-CsA (20 mg/kg/day). Twenty-four hours after the last dose, T cells were isolated from the spleens of treated animals and co-cultured with allogenic DCs (generated from C57Bl/6 mice) at different ratios for 72 h. The experiment design is outlined in **a**. T cell proliferation was then assessed by [³H]-thymidine incorporation **b**. At the end of the co-culture (at DC/T cell ratio 1:10), the extent of T cell inhibition was further compared between different groups by assessing IFN-γ secretion in the co-culture supernatant using routine ELISA techniques as per the manufacturer's recommendations **c**. Asterisks denote a significant difference between test groups (PM-CsA or Sandimmune®-treated animals) and control group (saline-treated animals)

shown a decreased accumulation of CsA in kidney tissue of rats following multiple i.v. injections of PM-CsA, compared to animals injected with Sandimmune® (8). This decrease in the biodistribution of CsA has resulted in a significant reduction of CsA-induced renal toxicity in PM-CsA-treated animals, as evidenced by unaltered creatinine clearance as well as normal histology of kidney tissues in PM-CsA-treated animals. In contrast, animals treated with Sandimmune® have shown signs of renal toxicity including 50% reduction in creatinine clearance and abnormal histological findings, such as vacuolated cytoplasm, which could be indicative of glomerular cellular defects (8). These results highlight the potential of polymeric micelles for the safe and effective delivery of CsA. However, direct assessment of the immunosuppression

potential of PM-CsA compared to Sandimmune® is a crucial requirement to fully evaluate the biological performance of PM-CsA. The goal of the current study was to test the efficacy of CsA formulations (PM-CsA and Sandimmune®) in suppressing T-cell-mediated responses both *in vitro* and *in vivo*. The effect of these formulations on other immune cells such as DCs has been also investigated.

Results in Fig. 2 have shown that both PM-CsA and Sandimmune® have comparable effects on inhibiting DC functions. The exact mechanism underlying CsA-mediated inhibition of DC functions is not fully understood. This is partly due to the conflicting findings among different studies investigating the effect of CsA on DCs (13,14,20–22). For example, earlier studies have shown that CsA significantly inhibited the

up-regulation of CD80 and CD86, but has no effect on the expression of CD40 and MHC-II (22). In contrast, other studies have demonstrated that CsA has no effects on the expression of any of the co-stimulatory molecules (CD40, CD80, CD86, or MHC-II) by DCs (13,14). The reason behind this discrepancy is not clear, but may be due to variation in experimental parameters such as the source of DCs, time of addition of CsA, and the duration of DC incubation with CsA.

Analysis of the cytokine secretion profile by CsA-treated DCs has revealed that both PM-CsA and Sandimmune® were capable of inducing TGF- β secretion to a comparable level (Fig. 2b). These results are consistent with earlier studies that have shown that treatment with CsA results in up-regulation of TGF- β expression in various cell types, including mesangial cells (23) and T cells (24). Both PM-CsA and Sandimmune®-treated DCs have also demonstrated comparable impairment in their allostimulatory capability (Fig. 2c), relative to untreated DCs. Such impairment in DC biological function is believed to be a consequence of lowered expression of co-stimulatory molecules and an increase in the level of TGF- β in DCs treated with CsA formulations. An alternative mechanism is suggested by Geng *et al.* (13), who reported that CsA is also capable of inducing the expression of regulatory molecules on DCs (such as B7-DC) resulting in significant inhibition in their allostimulatory potential (13).

It is worth mentioning that the superiority of PM-CsA over Sandimmune® in inhibiting DC functions could be observed under different culture conditions. In fact, an earlier study from our lab has assessed the immunosuppressive effect of rapamycin formulations on murin BMDCs (25). In these studies, DCs were generated from mouse bone marrow and exposed to particulate and soluble rapamycin without any additional treatment, or with pre- or post-treatment with LPS culture. Both soluble and particulate rapamycin were capable of inhibiting DC functions under the three different conditions. The superiority of rapamycin nanoparticles was more evident in LPS pre- or post-treated DCs than untreated DCs. Similar studies are required to fully unravel the effect cyclosporine formulations (Cremophor EL *versus* polymeric micellar formulation) on DC maturation and/or functions.

As discussed previously, data from Fig. 2 clearly demonstrate the immunoinhibitory effect of PM-CsA on DCs. One question that may arise is whether CsA was released from the micelles inside the cells (after the micelles were taken up by DCs) or was it released outside the DCs before it entered the cells and gave such an effect. Full characterization of the uptake profile of PEO-b-PCL-based micelles by BMDCs is currently under investigation in our lab and is beyond the scope of this paper. However, our preliminary results suggest that these two scenarios are not mutually exclusive and both can account for the delivery of CsA to DCs. In fact, earlier reports have demonstrated the ability of DCs to take up peptide cross-linked micelles made of thiolated poly(ethylene glycol) block copolymers (average size is 50 nm) (26). This is consistent with a more recent study by Boudier *et al.* (27), where they have found that polymethacrylic acid-*b*-polyethylene oxide/poly-L-lysine micelles (particle size of approximately 30 nm) could be efficiently internalized by DCs. Similarly, our preliminary results show that DCs are capable of internalizing PEO-*b*-PCL-based micelles. Following 24 h incubation with fluorescently labeled micelles, more than 50%

of CD11c⁺ DCs had taken up the formulation, as measured by flow cytometry (data not shown). Further studies are being undertaken in our lab to assess the preferential localization of the internalized micelles in different cellular compartments inside DCs (such as endosomes, lysosomes, and cytoplasm) and to assess the uptake profile under variable conditions (such as different time points).

The next set of studies was done to assess the direct effect of CsA formulation on T cell proliferation/activation in an *in vitro* mixed lymphocyte reaction (Figs. 4–6). Results in Figs. 2 and 3 have demonstrated that CsA formulations (PM-CsA and Sandimmune®) are capable of reducing the allostimulatory capacity of DCs. However, the inhibition of T cell proliferative responses seen in Figs. 4–6 is probably due to a direct effect of the CsA formulations on T cells rather than an indirect inhibitory effect on DCs, since DCs present in the co-culture have been irradiated prior to exposure to CsA treatment. Irradiation is believed to arrest alteration in the co-stimulatory molecules and cytokine secretion by DCs (28).

Results from *in vitro* studies have demonstrated that both PM-CsA and Sandimmune® are capable of inhibiting T cell proliferative responses (Figs. 4 and 5) as well as IL-2 secretion (Fig. 6) in a comparable dose-dependent fashion. Images in Fig. 5 represent microscopic examination of one representative well from each treatment group. Although these images did not provide quantitative data, visual inspection of wells treated with titrating doses of CsA has clearly shown that PM-CsA was as potent as Sandimmune® in inhibiting T cell proliferative responses at all concentrations tested.

Results from *in vivo* experiments (Fig. 7) were in agreement with the *in vitro* studies described in Figs. 2–4 and 6. Multiple injections of CsA formulations (20 mg/kg/day for 3 days) resulted in impaired alloreactive T cell responses, as measured by an *ex vivo* MLR. Both PM-CsA-treated and Sandimmune®-treated animals showed inhibition of *ex vivo* T cell proliferative responses (compared to animals treated with saline). The difference between PM-CsA and Sandimmune® was not statistically significant at any of DC/T cell ratios tested. Both formulations also have comparable effects on decreasing the level of IFN- γ secretion T cells isolated from treated mice (Fig. 7c). We have previously given a detailed characterization of the pharmacokinetic parameters of PM-CsA after single (6) or multiple (8) dose administration in rats. In those studies, we have demonstrated that delivering CsA in polymeric micelles (PM-CsA) resulted in higher levels of CsA in the blood (2.1-fold higher than Sandimmune®), while decreasing its level in kidney (2.6-fold lower than Sandimmune®) after repeated dosing. However, similar immunosuppressant effect was observed between PM-CsA and Sandimmune® in the current study (Fig. 7). In an earlier study, Varela *et al.* (29) demonstrated a similar *in vivo* immunosuppressant activity in animals orally treated with repeated doses of either Sandimmune® or CsA delivered in polycaprolactone nanoparticles, despite significantly higher CsA blood levels achieved by the later formulation (29). These results suggest the presence of a part of CsA associated with the delivery vehicle in systemic circulation. This proportion may not be available (or not yet released) to exert its immunosuppressive effect. Thus, a direct correlation between the total drug detected in the blood (released drug + remaining drug inside the formulation) and the immunosuppressive activity could not be achieved (29). This prolonged release of

CsA when delivered as PM-CsA could be attributed to the kinetics as well as thermodynamic stability of the PEO-*b*-PCL-based micellar formulation (8). In addition, the presence of the PEO shell in the polymeric micellar formulation hinder their rapid elimination by reticuloendothelial cells, and thus favors their prolonged circulation in the blood (as opposed to the Cremophor EL-based formulation Sandimmune®).

CONCLUSION

Our results demonstrate that PM-CsA could deliver functional CsA to immune cells both *in vitro* and *in vivo*. As a result, CsA as part of this polymeric micellar formulation could exert potent immunosuppressive effects similar to the commercial formulation of CsA, i.e., Sandimmune®. These results highlight the potential of polymeric micelles to serve as an alternative efficient vehicle for the delivery of CsA, with the additional advantages of prolonged drug release and reduced risk of renal toxicity.

ACKNOWLEDGMENTS

The authors would like to thank Elaine Moase for proof reading the manuscript. The authors would like to acknowledge financial support by research grant from Natural Sciences and Engineering Council of Canada (STPGP 336987).

REFERENCES

- Italia JL, Bhardwaj V, Kumar MN. Disease, destination, dose and delivery aspects of ciclosporin: the state of the art. *Drug Discov Today*. 2006;11(17-18):846-54.
- Faulds D, Goa KL, Benfield P. Cyclosporin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in immunoregulatory disorders. *Drugs*. 1993;45(6):953-1040.
- Ismailos G, Reppas C, Dressman JB, Macheras P. Unusual solubility behaviour of cyclosporin A in aqueous media. *J Pharm Pharmacol*. 1991;43(4):287-9.
- Beauchesne PR, Chung NS, Wasan KM. Cyclosporine A: a review of current oral and intravenous delivery systems. *Drug Dev Ind Pharm*. 2007;33(3):211-20.
- Czogalla A. Oral cyclosporine A—the current picture of its liposomal and other delivery systems. *Cell Mol Biol Lett*. 2009;14(1):139-52.
- Aliabadi HM, Mahmud A, Sharifabadi AD, Lavasanifar A. Micelles of methoxy poly(ethylene oxide)-*b*-poly(epsilon-caprolactone) as vehicles for the solubilization and controlled delivery of cyclosporine A. *J Control Release*. 2005;104(2):301-11.
- Aliabadi HM, Brocks DR, Lavasanifar A. Polymeric micelles for the solubilization and delivery of cyclosporine A: pharmacokinetics and biodistribution. *Biomaterials*. 2005;26(35):7251-9.
- Aliabadi HM, Elhasi S, Brocks DR, Lavasanifar A. Polymeric micellar delivery reduces kidney distribution and nephrotoxic effects of Cyclosporine A after multiple dosing. *J Pharm Sci*. 2008;97(5):1916-26.
- Nishiyama N, Kato Y, Sugiyama Y, Kataoka K. Cisplatin-loaded polymer-metal complex micelle with time-modulated decaying property as a novel drug delivery system. *Pharm Res*. 2001;18(7):1035-41.
- Kwon GS, Yokoyama M, Okano T, Sakurai Y, Kataoka K. Biodistribution of micelle-forming polymer-drug conjugates. *Pharm Res*. 1993;10(7):970-4.
- Zhang X, Burt HM, Mangold G, Dexter D, Von Hoff D, Mayer L, *et al.* Anti-tumor efficacy and biodistribution of intravenous polymeric micellar paclitaxel. *Anticancer Drugs*. 1997;8(7):696-701.
- Ho S, Clipstone N, Timmermann L, Northrop J, Graef I, Fiorentino D, *et al.* The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol*. 1996;80(3 Pt 2):S40-5.
- Geng L, Dong S, Fang Y, Jiang G, Xie H, Shen M, *et al.* Cyclosporin A up-regulates B7-DC expression on dendritic cells in an IL-4-dependent manner *in vitro*, which is associated with decreased allostimulatory capacity of dendritic cells. *Immunopharmacol Immunotoxicol*. 2008;30(2):399-409.
- Chen T, Guo J, Yang M, Han C, Zhang M, Chen W, *et al.* Cyclosporin A impairs dendritic cell migration by regulating chemokine receptor expression and inhibiting cyclooxygenase-2 expression. *Blood*. 2004;103(2):413-21.
- Duperrier K, Farre A, Biennu J, Bleyzac N, Bernaud J, Gebuhrer L, *et al.* Cyclosporin A inhibits dendritic cell maturation promoted by TNF-alpha or LPS but not by double-stranded RNA or CD40L. *J Leukoc Biol*. 2002;72(5):953-61.
- Aliabadi HM, Elhasi S, Mahmud A, Gulamhusein R, Mahdipoor P, Lavasanifar A. Encapsulation of hydrophobic drugs in polymeric micelles through co-solvent evaporation: the effect of solvent composition on micellar properties and drug loading. *Int J Pharm*. 2007;329(1-2):158-65.
- Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods*. 1999;223(1):77-92.
- Azzi J, Tang L, Moore R, Tong R, El Haddad N, Akiyoshi T, *et al.* Polylactide-cyclosporin A nanoparticles for targeted immunosuppression. *FASEB J*. 2010;24(10):3927-38.
- Vitko S, Vicky O. Cyclosporine renal dysfunction. *Transplant Proc*. 2004;36(2 Suppl):243S-7.
- Kalthoff F, Elbe-Burger A. RE: effects of cyclosporine on human dendritic cell subsets. *Transplant Proc*. 2005;37(10):4639-40.
- Ciesek S, Ringe BP, Strassburg CP, Klempnauer J, Manns MP, Wedemeyer H, *et al.* Effects of cyclosporine on human dendritic cell subsets. *Transplant Proc*. 2005;37(1):20-4.
- Tajima K, Amakawa R, Ito T, Miyaji M, Takebayashi M, Fukuhara S. Immunomodulatory effects of cyclosporin A on human peripheral blood dendritic cell subsets. *Immunology*. 2003;108(3):321-8.
- Akool el S, Doller A, Babelova A, Tsalastra W, Moreth K, Schaefer L, *et al.* Molecular mechanisms of TGF beta receptor-triggered signaling cascades rapidly induced by the calcineurin inhibitors cyclosporin A and FK506. *J Immunol*. 2008;181(4):2831-45.
- Li B, Sehajpal PK, Khanna A, Vlassara H, Cerami A, Stenzel KH, *et al.* Differential regulation of transforming growth factor beta and interleukin 2 genes in human T cells: demonstration by usage of novel competitor DNA constructs in the quantitative polymerase chain reaction. *J Exp Med*. 1991;174(5):1259-62.
- Haddadi A, Elamanchili P, Lavasanifar A, Das S, Shapiro J, Samuel J. Delivery of rapamycin by PLGA nanoparticles enhances its suppressive activity on dendritic cells. *J Biomed Mater Res A*. 2008;84(4):885-98.
- Hao J, Kwissa M, Pulendran B, Murthy N. Peptide crosslinked micelles: a new strategy for the design and synthesis of peptide vaccines. *Int J Nanomedicine*. 2006;1(1):97-103.
- Boudier A, Aubert-Pouessel A, Louis-Pence P, Gerardin C, Jorgensen C, Devoisselle JM, *et al.* The control of dendritic cell maturation by pH-sensitive polyion complex micelles. *Biomaterials*. 2009;30(2):233-41.
- Liao YP, Wang CC, Butterfield LH, Economou JS, Ribas A, Meng WS, *et al.* Ionizing radiation affects human MART-1 melanoma antigen processing and presentation by dendritic cells. *J Immunol*. 2004;173(4):2462-9.
- Varela MC, Guzman M, Molpeceres J, del Rosario Aberturas M, Rodriguez-Puyol D, Rodriguez-Puyol M. Cyclosporine-loaded polycaprolactone nanoparticles: immunosuppression and nephrotoxicity in rats. *Eur J Pharm Sci*. 2001;12(4):471-8.