ORIGINAL ARTICLE

Evaluation of PLGA microspheres with triple regimen on long-term survival of vascularized composite allograft – an experimental study

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SUMMARY

Systemic immunosuppression is indispensable for vascularized composite allotransplantation (VCA). Daily administration of standard triple therapy regimen of tacrolimus (FK506), mycophenolate mofetil (MMF), and steroid has severe side effects and reduces the compliance of VCA recipients. To overcome these hurdles, FK506/MMF/prednisolone (PDNN) was loaded into PLGA microspheres (PGLA MS). A single injection of FK506/MMF/PDNN-PLGA MS significantly prolonged the survival time of allograft in a rat hind limb transplantation model with a median survival time (MST) of more than 150 days compared to 34.5 days in the group treated orally with FK506/MMF/PDNN and 11 days in the nontreatment allograft and MS control groups. Analysis of showed that FK506/MMF/PDNN-PLGA MS could maintain relatively higher plasma and tissue drug concentrations for a long time. Moreover, histopathology and flow cytometry of circulating mononuclear cells revealed significantly prolonged immunosuppression by the FK506/MMF/PDNN-PLGA MS compared with the orally given FK506/MMF/PDNN. In conclusion, a single injection of FK506/MMF/PDNN-PLGA MS may provide a new approach for long-term prevention of immune rejection in VCA.

Transplant International 2020; 33: 450–461

Key words
immunosuppression, microsphere, sustained release, triple regimen, vascularized composite allotransplantation

Received: 30 August 2019; Revision requested: 23 September 2019; Accepted: 8 January 2020;
Published online: 29 January 2020

Introduction

Vascularized composite allotransplantation (VCA) is a promising option for severe injuries and defects involving multilayered functional tissues, which cannot be repaired to the level of fine aesthetic and functional recovery through conventional reconstruction with autologous tissues or prostheses [1,2]. However, since VCA consists of complex tissue types such as skin, muscle, bone, nerves, and blood vessels, severe immunological rejection occurs after transplantation. After the introduction of cyclosporine—a highly effective immunosuppressive agent—both solid organ transplantation (SOT) and VCA have developed rapidly [3,4]. Severe side effects due to long-

doi:10.1111/tri.13574

term intake of immunosuppressive drugs leading to complications, such as diabetes, opportunistic infections, and malignancies, are common in transplantation patients [5]. A combination of FK506/MMF/corticosteroid regimen has been proposed for better management of transplant patients, and studies have shown that this combination not only increased the survival rate of transplants but also resulted in relatively lower toxic side effects [6]. So far, the FK506/MMF/corticosteroid regimen is widely used as induction therapy in SOTs and VCAs. Nevertheless, daily medication and long-term cumulative effects of the drug are still a heavy burden on patients. Therefore, a novel drug delivery strategy is urgently needed to avoid the cumbersome routine of daily administration and to reduce the requirement of systemic immunosuppression.

Ravindra *et al.* [7] reported the benefits of topical application of clobetasol and FK506 ointment in hand transplantation. In addition, local application of FK506 increased the local drug concentration while maintaining a relatively low blood concentration [8], suggesting a potential for local drug administration in VCA. Biodegradable polymers are widely used as sustainable drug delivery vehicles due to their advantages such as easily fabricated, encapsulation of a variety of drugs, and predictable biodegradability [9–11]. Moreover, many studies have confirmed that biodegradable polymer-encapsulated immunosuppressants prolong the survival of allograft without significant adverse effects [12–14], indicating that biodegradable microspheres could be a novel drug delivery system for VCA site-specific immunosuppression. Poly lactic-co-glycolic acid (PLGA) is widely used as a biodegradable material for fabrication of polymeric nanoparticles [15].

Based on the above concepts, we encapsulated FK506/MMF/corticosteroid in PLGA microspheres for sustained delivery, thus, eliminating the need for daily and consequently intake of systemic administration, and improving patient compliance. Moreover, the formulation parameters were systematically and thoroughly studied to optimize the characteristics of the microspheres. Assessment of allograft survival and tissue and blood drug concentration were performed in the rat hind limb transplantation model after the injection of the triple immunosuppressant-MS formulation.

Materials and methods

Materials

Poly lactic-co-glycolic acid (LA:GA = 75:25) was purchased from Jinan Daigang Co., Ltd. (Jinan, China). Analytical grade polyvinyl alcohol (PVA) and methylene chloride were obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). FK506, MMF, and prednisolone (PDNN) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd.

Fabrication of FK506/MMF/PDNN-PLGA MS

FK506/MMF/PDNN (6 mg/300 mg/60 mg) was mixed with 250 mg PLGA, and then, the mixture was dissolved in 5 ml methylene chloride as oil phase (O); the oil phase was dripped into 25 ml of 1% PVA solution (W) at a constant rate and stirred at 600 rpm to yield a W/O system. The oil phase was loaded with 10 kV high voltage for electrospraying. After the oil phase was completely added, the mixture was stirred at 300 rpm for 3 h at room temperature to evaporate methylene chloride and obtain the FK506/MMF/PDNN-PLGA MS. The drug-loaded MS were washed with deionized water three times and centrifuged at 838.5 *g* for 10 min before use. The prepared microspheres were frozen, dried, and aliquoted into small injection bottles, sealed and sterilized with ethylene oxide. Blank control microspheres without FK506/MMF/PDNN were prepared as above.

Characterization of FK506/MMF/PDNN-PLGA MS

Morphology of PLGA MS with and without FK506/MMF/PDNN was examined under a scanning electron microscope (SEM, Phenom XL, Shanghai, China), and diameter distribution of the samples was calculated by the Image J software. Raw PLGA polymer, raw FK506, MMF, PDNN, and prepared FK506/MMF/PDNN-PLGA MS were characterized by Fourier transform infrared spectroscopy (FTIR, Avatar 380 FTIR spectrometer).

Release behavior of drugs in the FK506/MMF/PDNN-PLGA MS was performed in vitro. Briefly, MS sample (1 mg) was soaked in 10 ml of phosphate buffered saline (PBS) in glass vial and incubated at 37 °C. At each time points (days 2–28, every 2 days), 2 ml of the supernatant was removed and replaced by an equal volume of fresh PBS. The released drugs were measured in the supernatants by high-performance liquid chromatography/mass spectrometry (HPLC-MS) [16] to generate drug release curves. The drug release curves were fitted by BoxLucas1 function in the ORIGIN 8.0 software.

Animals

Male Brown-Norway (donors) and Lewis (recipients) rats weighing between 350 and 450 g were obtained from

Xipu'er-bikai Experimental Animal Co. Ltd (Shanghai, China) and housed under conditions published by the National Institutes of Health.

Rat hind limb transplantation model

Under aseptic conditions, femoral vessel of the recipient rat was exposed, and the hind limb was amputated from the middle of thigh. Similarly, the hind limb of donor rat was removed and transplanted to the recipient. An 18 gauge needle was used as intramedullary rod to perform femoral osteosynthesis. 12-0 nylon sutures were used to anastomose blood vessels. Finally, the muscle and skin were sutured intermittently with 3-0 nylon thread.

Treatment groups and drug administration

The control group (I) consisted of 6 Brown-Norway rats as hind leg donors and 6 Lewis rats as recipients of hind leg transplantation. The control group did not receive any medication after surgery. General health and indications of acute immune rejection were monitored and recorded daily. Graft rejection was evaluated macroscopically and graded as a consistent sequence of no rejection (grade 0), erythema and edema (grade 1), progressive epidermolysis and exudation (grade 2), and desquamation, necrosis, eschar formation and mummification (grade 3). Animals were euthanized at grade 3 immune rejection. Samples were fixed, embedded, sectioned, and stained for histology.

MS group (II): Surgical treatment was same as in the control group. Following surgery allografts were injected with 1 ml of 50 mg/ml blank microsphere suspension. Postoperative observations, sampling, and detection were the same as those in the control group. The MS group was used to detect the effect of sustained release system on allograft rejection.

FK506/MMF/PDNN oral administration group (III): Surgical treatment was the same as in the control group. FK506 1 mg/kg, MMF 50 mg/kg, and PDNN 10 mg/kg were orally administered daily for two weeks postoperation. Drug concentration, plasma cytokine levels, and plasma hepatic and renal toxicity markers were assessed in blood samples collected on days 1, 3, 7, 11, 14, 17, 21, 24, and 28 after surgery. Two weeks after surgery, about 5 × 5 mm sample of skin and muscle tissue was taken from the site above the anastomotic incision (recipient side) of the transplanted limb to detect tissue drug concentration. Other observations, sampling and testing were identical to the control group. This group was used to evaluate the effect of daily systemic drug administration on graft rejection.

FK506/MMF/PDNN-PLGA MS injection group (IV): Surgical treatment was same as in the control group; allografts were postoperatively injected with 1 ml of 50 mg/ml FK506/MMF/PDNN-PLGA MS suspension. Blood was collected on days 3 and 7 after surgery, and once a week for the next two weeks. Three weeks later, blood was collected every two weeks. Postoperative observation, sampling, and detection were the same as those in FK506/MMF/PDNN oral administration group. This group was employed to evaluate the effect of drug-loaded sustained release system on allograft rejection.

FK506/MMF/PDNN-PLGA MS contralateral injection group (V): Surgical treatment was same as in the control group; the contralateral limb was postoperatively injected with 1 ml of 50 mg/ml FK506/MMF/PDNN-PLGA MS suspension. Blood collection, postoperative observation, sampling, and detection were same as in the FK506/MMF/PDNN-PLGA MS injection group. And this group was applied to assess the effect of drug-loaded sustained release system on allograft rejection in contralateral limbs.

Histology

Skin and muscle biopsies were preserved in 4% buffered formaldehyde and stained with HE for morphology.

Flow cytometry for peripheral blood mononuclear cells

Peripheral blood was collected from the tail vein of recipient rats and incubated with red blood cell lysate for 15 min. PBMCs were isolated and stained with the following antibodies: PerCP-Cy5.5-CD45RA (BD Pharmingen, Franklin Lake, WI, USA), APC-CD3 (BD Pharmingen), PE/Cy7-CD4 (BD Pharmingen), and PE-CD8a (BD Pharmingen), V500-CD25 (BD Pharmingen). For Treg analysis, PBMCs were fixed and permeabilized in Foxp3/Transcription Factor Staining Buffer (eBioscience, San Diego, CA, USA) and incubated with isotype control and anti-FoxP3 monoclonal antibody (eBioscience). After staining, samples were washed twice, resuspended in PBS, and analyzed using flow cytometry (BD FACSCanto II) and BD Diva software. Data were analyzed using the FlowJo software. Isotype controls were used to set the cutoff for the Treg analysis.

Assessment of plasma markers of kidney and liver damage

Plasma level of liver and kidney damage markers—Creatinine, blood urea nitrogen (BUN), aspartate

aminotransferase (AST), and alanine aminotransferase (ALT)—in groups III and IV were compared.

Detection of FK506, MMF, and PDNN levels in plasma and skin

EDTA plasma specimens were gathered at postoperative days (PODs) 3, 7, 11, 21, 35, 49, 63, 77, 94, and 100 and stored at –80 °C till further use. Under general anesthesia, hair-free skin was sterilized with 70% alcohol before surgical excision of a 5 × 5 mm skin biopsy from the allograft and homogenized in Qiagen TissueLyser II. In brief, samples were homogenized at 30 Hz for 2 min in 100 ml radioimmunoprecipitation buffer with protease inhibitor mixture (Sigma) and centrifuged for 10 min at a speed of 1677 *g* at 4 °C. Concentration of FK506, MMF, and PDNN was analyzed in the supernatant by HPLC-MS.

Statistical analysis

Statistical analysis was carried out using the GRAPHPAD PRISM 5. All results are expressed as mean ± standard deviation. Kaplan–Meier analysis was used estimate the survival rate of allografts and logarithmic rank test was used to compare each group. Multiple comparisons were done by Bonferroni post hoc test and one-way ANOVA, and the data of pairwise comparisons were analyzed by *t*-test. $P < 0.05$ was statistically significant.

Results

Fabrication and characterization of FK506/MMF/PDNN-PLGA MS

The FK506/MMF/PDNN-PLGA MS were fabricated by electrospraying and freeze-drying method (Fig. 1). Morphology of the empty PLGA MS and FK506/MMF/PDNN-PLGA MS is shown in Fig. 2. SEM revealed that both the samples had smooth and uniform microsphere morphology. The diameter of PLGA MS was 14.32 ± 2.60 μm, and drug loading did not alter the diameter of FK506/MMF/PDNN-PLGA MS (13.92 ± 2.53 μm). In addition, the magnified SEM images (Fig. 2b,e) of a single microsphere showed lots of pores on the surface of the microsphere. It might be due to the volatilization of organic solvents during freeze-drying. The formation of porous structure can significantly improve the porosity and specific surface area of PLGA microspheres. This is more conducive to drug release and material degradation.

The FTIR evaluation of raw PLGA polymer, raw FK506, MMF, PDNN, and FK506/MMF/PDNN-PLGA MS is shown in Fig. 3a. The FTIR spectrum of pure PLGA MS showed typical absorption peaks at 1091 cm^{-1} ascribed to C–O stretching, 1171 cm^{-1} due to C–O–C symmetric stretching, and 1751 cm^{-1} assigned to C=O stretching. The absorption peaks at 1740, 1690 and 1637 cm^{-1} were observed for FK506, corresponding to the chain saturated C=O, diketone and pyridine ring group. MMF exhibited absorption peaks at 1742 (carboxylic ester group), 1704 (C=O stretching on carboxyl), and 1625 cm^{-1} (C=C stretching on olefins). And for PDNN, bands at 1707 and 1667 cm^{-1} were attributed to the existence of C=O on carboxyl and benzoquinone group. The FK506/MMF/PDNN-PLGA MS showed all the aforementioned absorption peaks, which confirmed the loading of FK506/MMF/PDNN on PLGA MS. In vitro drug release analysis showed that each of the three drugs had a stable and sustained release behavior from the FK506/MMF/PDNN-PLGA MS, for 28 days (Fig. 3b). It showed that there was a burst release of drugs from PLGA microspheres in the first day. It might because of the rapid diffusion of drugs on the surface of PLGA microspheres. And the burst release of drugs may guarantee the sever immune rejection in the early stage. The fitting results of drug release curve were shown in

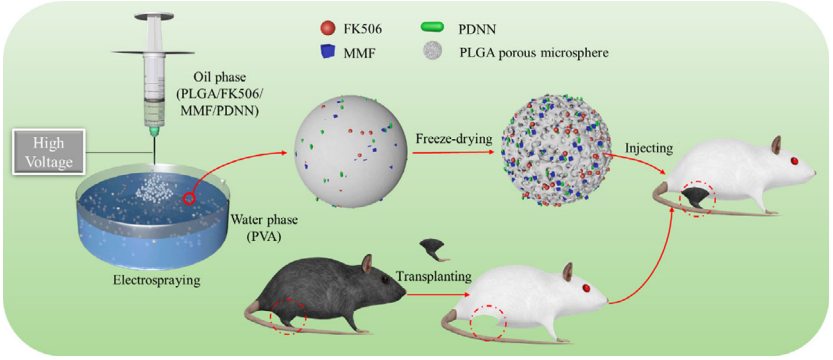


Figure 1 Schematic of FK506/MMF/PDNN-PLGA MS preparation.

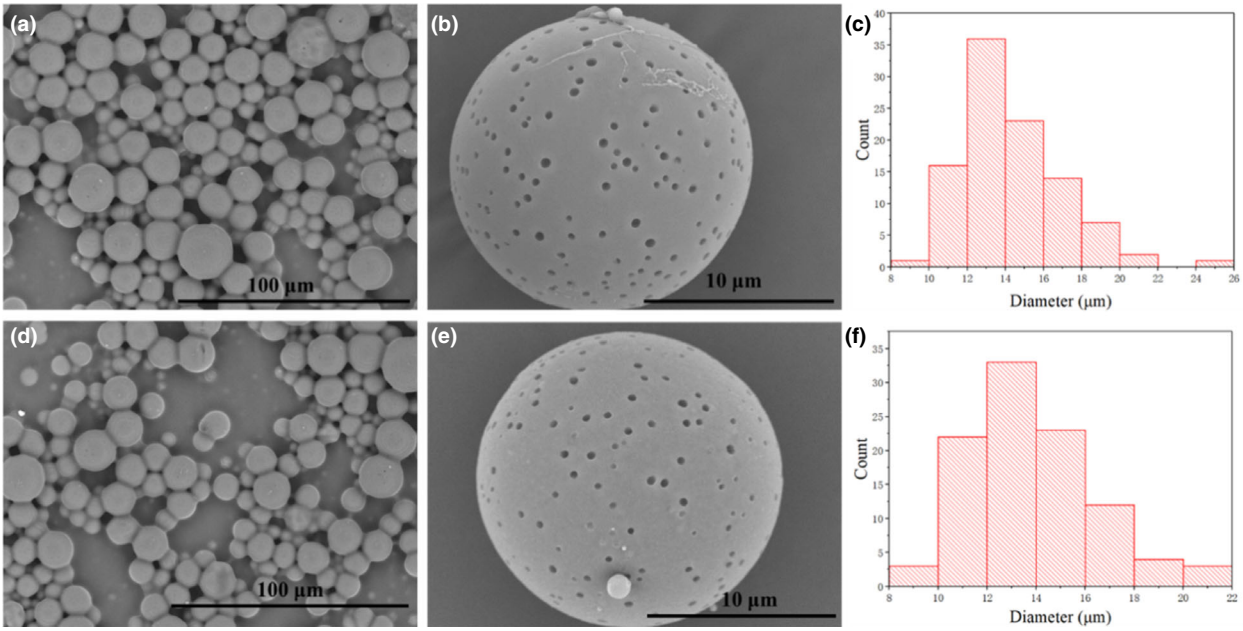


Figure 2 Representative SEM images of (a) PLGA MS and (b) single PLGA MS. (c) Histogram of PLGA MS diameter distribution. Representative SEM image of (d) FK506/MMF/PDNN-PLGA MS and (e) single FK506/MMF/PDNN-PLGA MS. (f) Histogram of FK506/MMF/PDNN-PLGA MS diameter distribution.

Fig. 3c; it indicated that the release kinetics of the three drugs are in accordance with the first-order kinetics equation.

VCA survival and macroscopic assessment

Following hind limb transplantation from Brown-Norway to Lewis rats, FK506/MMF/PDNN-PLGA MS was injected intramuscularly and subcutaneously into the graft. After the surgery, animals were monitored; graft rejection was evaluated macroscopically and graded. Transplanted limbs of groups I and II had a MST of 11 days (Fig. 4a,b), and grafts were acutely rejected with desquamation and necrosis in these groups (Fig. 4c), although the MST of allograft in group III (FK506/MMF/PDNN oral administration group) was 34.5 days. However, all grafts were rejected on oral immunosuppressant withdrawal. Interestingly, the macroscopic signs of acute rejection in group III were different from those seen in groups I and II. Signs of rejection initially involved hair loss, mild edema, and dry skin, but later on progressed to epidermolysis, exudation, and necrosis of allograft (Fig. 4c). In group V, the MST of the allograft was 117 days, which was significantly higher than that of group I and II (Fig. 4a). Most importantly, the MST in group IV (Fig. 4a) was more than 150 days, significantly higher than that in all other groups.

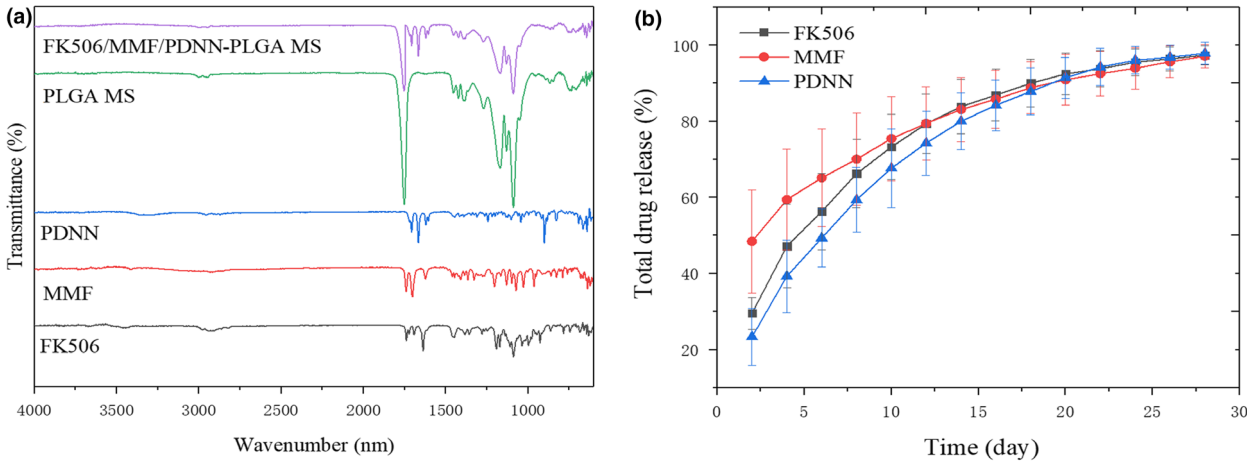
Histology

In groups I and II (no treatment and MS controls), intense diffuse mononuclear cells infiltration and severe disruption of skin and muscle tissue architecture were observed at the time of rejection (Fig. 4d,e). Severe infiltration of mononuclear cells into the dermis, especially the perivascular area was observed in the skin. Compared with the groups I and II, although histopathological changes were less severe in Group III (FK506/MMF/PDNN oral administration group), mononuclear cells infiltration and edema were more evident. No evidence of monocyte infiltration and immune rejection was observed up to 150 days in group IV (Fig. 4).

Assessment of hepatotoxicity and nephrotoxicity

To test whether intragraft injection of FK506/MMF/PDNN-PLGA MS could damage liver and kidney, histological examination, plasma level of creatinine and BUN—indicators of kidney damage, and AST and ALT—the markers of liver damage—were measured in groups III and IV. Histological analysis of liver and kidney revealed no significant damage in the groups (Fig. S1). All the observed values were within the expected normal range in rats [17], and there were no statistically

Transplant International 2020; 33: 450–461



(c)

Model	Equation	Drugs	M_∞	k	R-Square
First-order equation	$M_t = M_\infty*(1 - \exp(-k*t))$	FK506	98.08055 ± 0.8125	0.14929 ± 0.0068	0.99301
		MMF	95.79926 ± 1.54629	0.17386 ± 0.01928	0.85658
		PDNN	102.7223 ± 0.61839	0.11013 ± 0.00259	0.99786

t is time, M_t is the accumulative release rate at time t.

Figure 3 (a) FTIR of different samples, (b) release behavior of drugs from FK506/MMF/PDNN-PLGA MS, and (c) Fitting results of drug release kinetics.

significant differences between the two groups at any time point (Fig. S1), demonstrating stable kidney and liver functions.

Plasma and tissue levels of FK506/MMF/PDNN

Plasma levels of FK506/MMF/PDNN (Fig. 5a and Table S1) were compared between groups III, IV, and V. With daily oral administration of FK506/MMF/PDNN, the plasma drug level gradually increased in the initial few days (PODs 1–3), stabilized form PODs 4–14, and finally gradually reduced to undetectable level from PODs 15–28, while approaching the MST of the group. In contrast, the plasma levels of FK506/MMF/PDNN experienced a sharp decline and a steady decline in groups IV and V. It is noteworthy that there was no significant difference in FK506, MMF, and PDNN plasma levels between the groups IV and V; however, they were significantly higher than group III in the first week and after the fourth week.

The concentrations of FK506, MMF, and PDNN in skin (Fig. 5b and Table S1) and muscle (Fig. 5c and Table S1) of transplanted rats were studied in groups III, IV, and V. Similar to the plasma drug concentration levels, tissue drug concentration levels in group III were stable from PODs 4–14 and then gradually decreased from PODs 15–28. This was different from the trends seen in groups IV and V. Injection of FK506/MMF/PDNN-PLGA MS into the contralateral leg led to a relatively gentle decline. Correspondingly, the changes in tissue concentration were relatively stable in group IV. At the time of rejection, both skin and muscle concentrations of FK506, MMF and PDNN in group III and group V were low; hence, higher concentrations of the drugs in the skin and muscle of the transplanted rats maintained long-term survival of the grafts.

FK506/MMF/PDNN-PLGA MS induces graft survival through long-term immunosuppression

To determine the effectiveness of FK506/MMF/PDNN, we measured the dynamic changes in PBMCs, T lymphocytes and its three subsets (T helper cells (Th), cytotoxic T lymphocytes (CTL), and regulatory cells (Treg)) in groups III and IV at different time points. As shown in Fig. 6, the proportions of circulating PBMCs, T cells, CTL, and Th significantly decreased from POD 3 to POD 14 in both the groups and stabilized within the following few days. However, in group III, they showed a significant leap on POD 28 during the emergence of immune rejection (The MST of the FK506/MMF/PDNN oral administration group is 34.5 days). There were no significant changes in the proportions of PBMCs, T cells, CTL, and Th between the two at any time point except at POD 28 ($n = 6$; $P < 0.05$, respectively, POD 28, ANOVA with Bonferroni post-test for the proportions

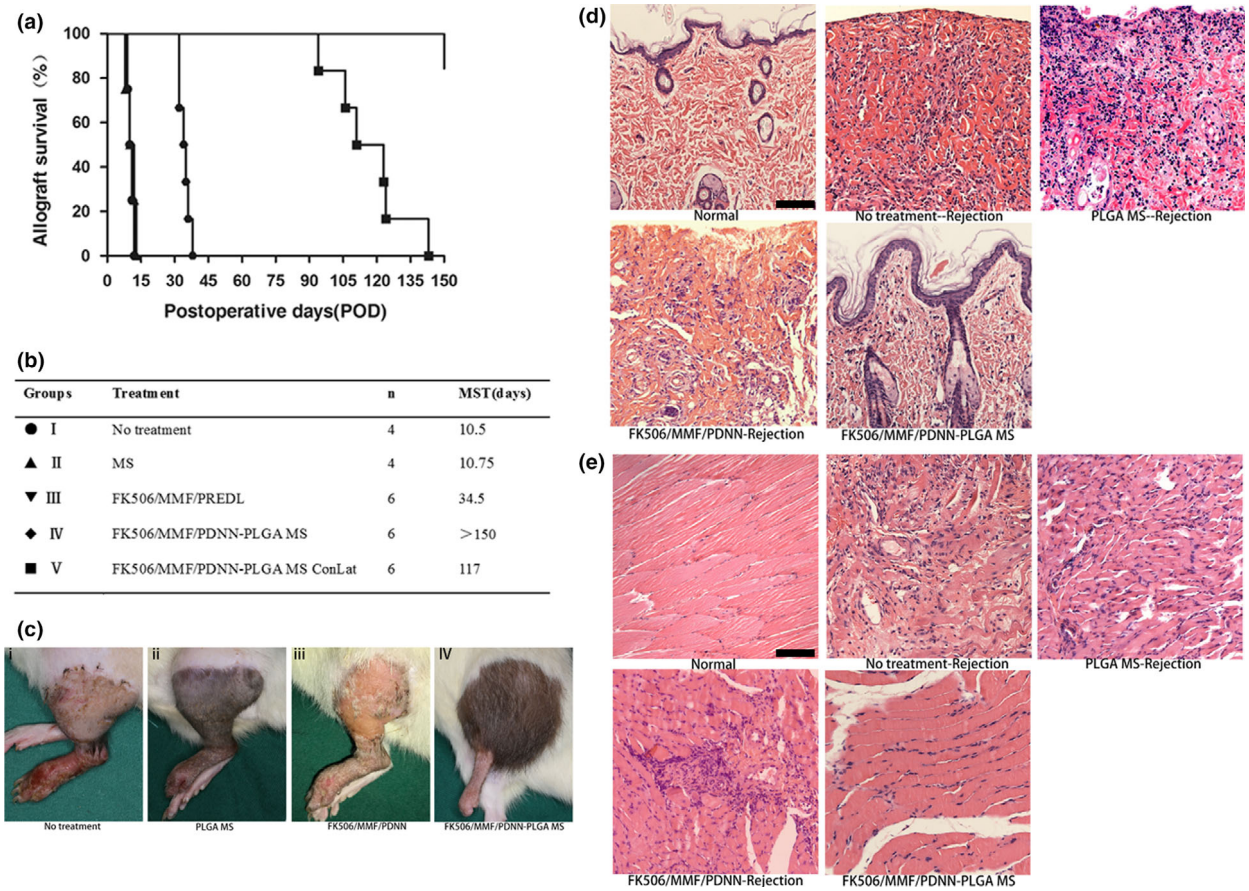


Figure 4 Vascular composite allograft survival curve, groups, and gross and histological evaluation. (a,b) Graft survival curves with *P* values calculated by log-rank (Mantel–Cox), group III versus group IV, $P = 0.0005$; group III versus group V, $P = 0.0013$; group IV versus group V, $P = 0.0005$. (c) Representative macroscopic images of hind limb allografts. (i) Acute rejection in groups I and II. (ii) Allografts were rejected with an MST of 33.5 days in group III. (iii) No signs of graft rejection in group IV at day 150. (d,e) HE staining of skin (d) and muscle (e) from normal, no treatment, PLGA MS, FK506/MMF/PDNN oral, and FK506/MMF/PDNN-PLGA MS groups. Scale bars, 200 μm.

of PBMCs, T cells, CTL, and Th). The number of Tregs was not significantly different between the groups. Notably, an increase in Tregs was observed in group IV with long-term surviving allograft ($n = 6$; $P < 0.05$, POD 28 versus POD 98, ANOVA with Bonferroni post-test for the proportions of Treg).

Plasma level of pro-inflammatory cytokines

Several pro-inflammatory cytokines act as markers for immune rejection. In fact, interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) have been considered as indicators of anti-transplant immune response in hand transplant recipients [18]. In this study, circulating IL-2, TNF-α, IFN-γ, and IL-1β generally showed a downward trend in groups III and IV (Fig. S2), even in subsequent follow-ups, they decreased or remained constant in group IV. It is worth noting that at POD 35 cytokine levels in group III did not increase significantly, which was consistent with the MST of 34.5 days.

Discussion

VCA is not a lifesaving treatment strategy; there is high controversy surrounding the widespread use of VCA and lifelong administration of immunosuppressive drugs, especially due to the complex composition of VCA, which, in addition to its stronger immunogenicity than solid organs, requires a larger dose of immunosuppressant's [19]. Although attempts to induce long-term immune tolerance in VCA may be a promising option for the future, but it is still in its infancy. Therefore, drug toxicity following daily systemic administration cannot be ignored. On the other hand, some steroid-free immunosuppressive strategies, such as the combination of FK506 and MMF, have failed due to the significant increase in acute rejection episodes during the first

Transplant International 2020; 33: 450–461

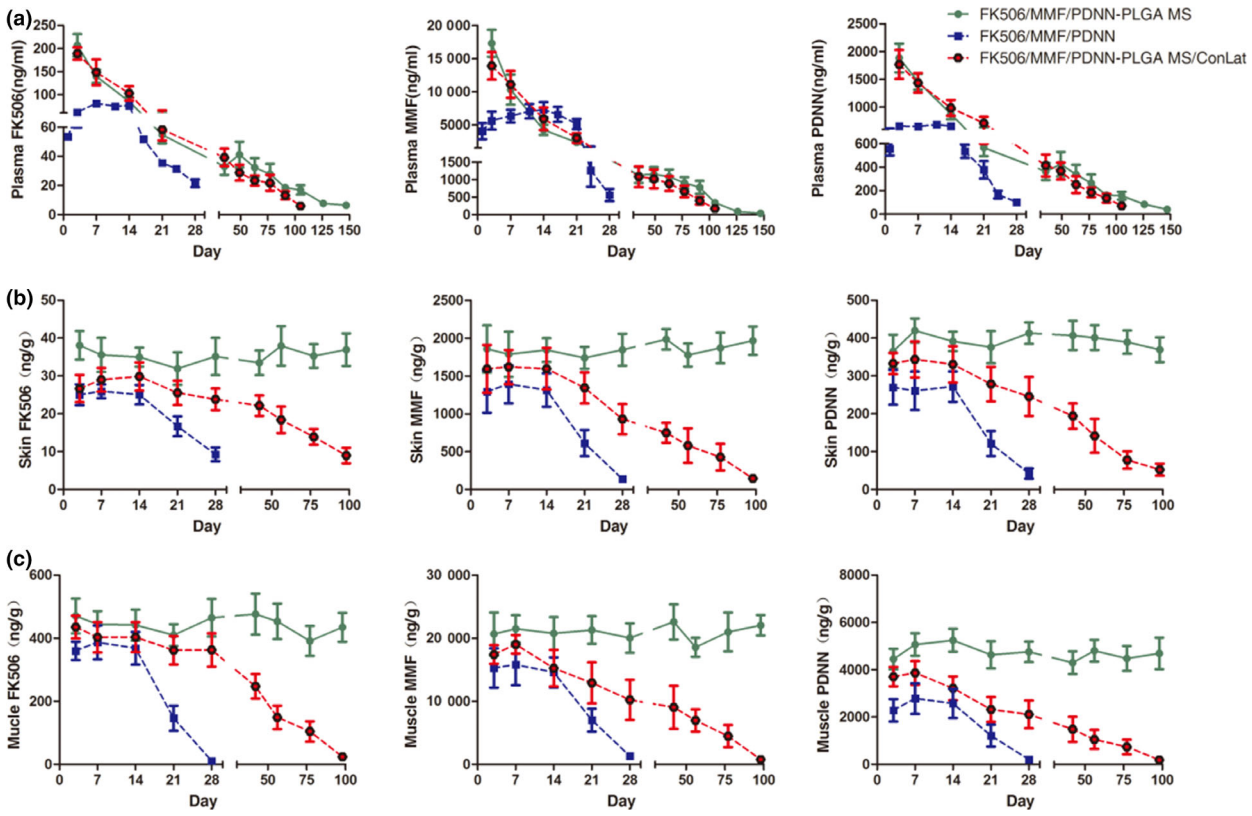


Figure 5 Concentrations of FK506, MMF, and PDNN in blood, skin, and muscle. (a) Plasma levels of FK506/MMF/PDNN in groups III, IV, and V. (b) Skin levels of FK506/MMF/PDNN in groups III, IV, and V. (c) Muscle levels of FK506/MMF/PDNN in groups III, IV, and V.

2 years, leading to reintroduction of steroids [20]. Currently, the triple drug regimen of FK506, MMF, and steroid remains the most effective maintenance therapy for VCA. Nevertheless, daily oral administration of the drug combination can seriously jeopardize patient compliance. Therefore, newer strategies should be explored to reduce adverse side effects and improve patient compliance. Currently, studies have confirmed that long-term immunosuppression can be achieved with biodegradable tacrolimus disk [12] and enzyme-responsive hydrogel [13] in rat hind limb transplantation model. Biodegradable polymers are particularly interesting as a drug delivery system due to the predictable biodegradability, ease of fabrication, ability to encapsulate a variety of drugs, and relatively stable and sustained release characteristics [9–11]. This prompted us to evaluate an injectable PLGA MS drug delivery system encapsulating a standard triple therapy regimen of FK506, MMF, and steroid in VCA.

In this study, a combination of FK506/MMF/PDNN was encapsulated in PLGA microspheres to explore a new sustained delivery method to significantly reduce drug dosage, improve the daily intake of drugs to better the synergistic release of drugs, and flatter blood concentration. Our data indicated that single intragraft injection of FK506/MMF/PDNN-PLGA MS significantly prolonged the survival of allograft for more than 150 days compared to 34.5 days following daily oral administration of FK506/MMF/PDNN at doses of 1, 50, and 10 mg/kg, respectively. It was estimated that recipients with body weight of 400 g had received about 5.6 mg of FK506, 280 mg of MMF, and 56 mg of PDNN during the 14-day oral administration. Correspondingly, 6 mg of FK506, 300 mg of MMF, and 60 mg of PDNN encapsulated in PLGA MS and administered as a single injection achieved a MST of more than 150 days, suggesting that FK506/MMF/PDNN-PLGA MS significantly reduced drug dosage. Compared with a previous report where 7 mg of FK506 encapsulated in enzyme-responsive hydrogel was used to achieve more than 100 days of MST [13], we achieved longer-term graft survival with lesser amount of FK506 combined with MMF and PDNN. In another study, 40 mg tablets of FK506 resulted in more than 180 days graft survival time [12]. It is clear that our sustained release system with reduced drug dosage was effective in

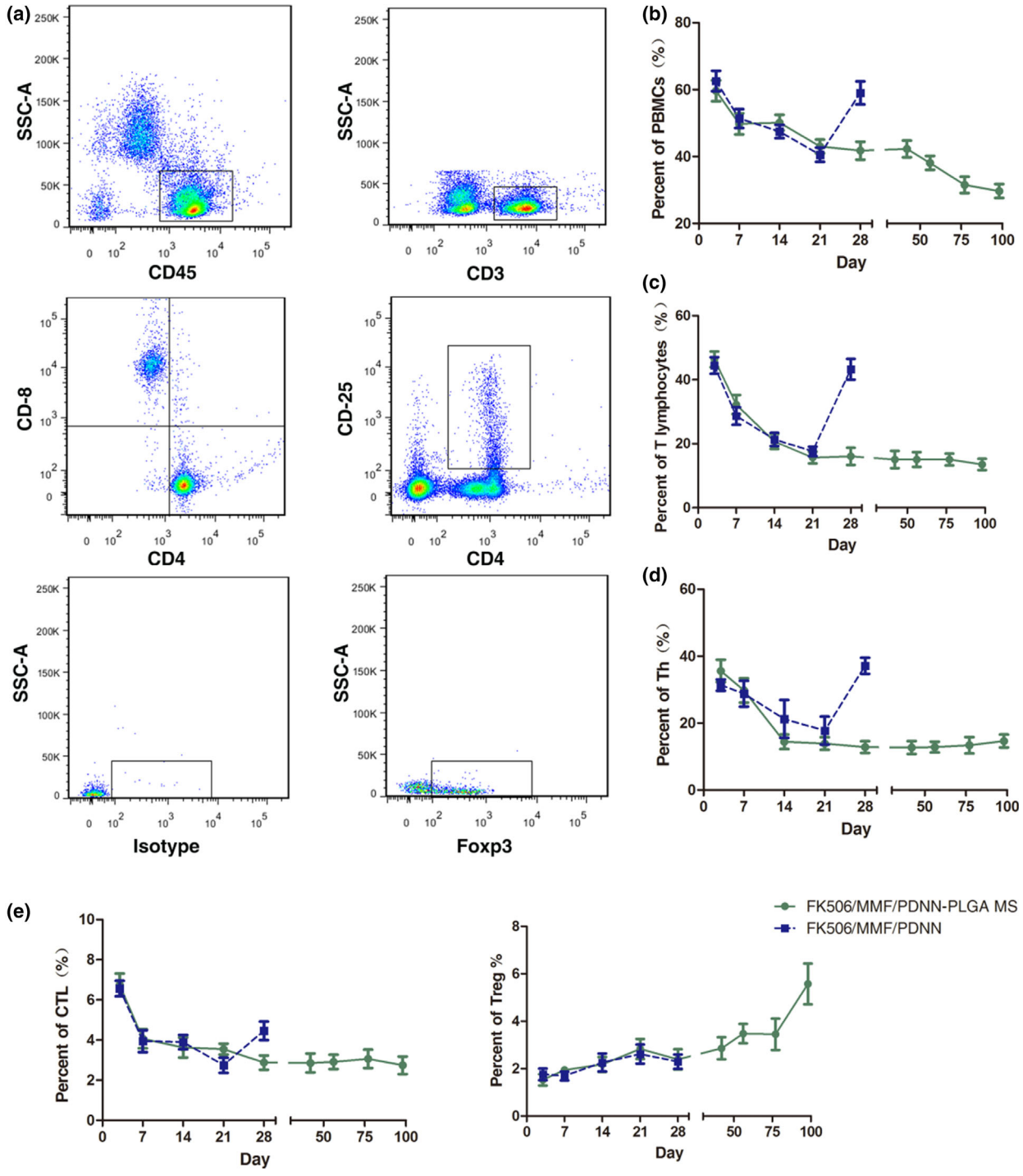


Figure 6 Gating strategy and percentages of PBMCs, T cells, Th, CTL, and Treg. (a) Gating strategy for PBMCs ($CD45^+$), T cells ($CD3^+$), Th ($CD4^+$ $CD3^+$), CTL ($CD8^+$ $CD3^+$), and Treg($CD4^+$ $CD25^+$ $Foxp3^+$) in the peripheral blood of recipients. Percentage of cells compared between group III and IV at different time points. (b) Representative percentage of PBMCs. (c) Representative percentage of T cells. (d) Representative percentage of Th. (e) Representative percentage of CTL. (f) Representative percentage of Treg.

achieving similar MST and also reduced the occurrence of adverse side effects.

Histological evaluation of the muscles and skin obtained from the FK506/MMF/PDNN-PLGA MS group showed only mild cellular infiltration, compared with severe disruption of tissue architecture and intense monocyte infiltration in the control and MS groups. These observations are consistent with previous reports

[], yet quite different from a study by Hautz *et al.* [21] in which tacrolimus (FK506) was ceased after PODs 50, and intensive infiltration and necrosis were noticed in the rejected graft skin and muscle at PODs 60.

Initially, in vivo drug release curves revealed release of high concentrations of drugs in group IV, and group V until POD 21, indicating a burst in release in the initial few weeks after transplantation. A high plasma drug concentration is beneficial in the first stages after surgery, in order to mitigate the higher changes of acute rejection at this stage [22]. Following that, a long-term lowering and flatting of plasma drug concentration curves and relatively high skin and muscle drug concentration occurred at the later time points, which is ideal in the latter stage as low toxicity might be related to low plasma drug level. However, high plasma levels with daily fluctuation were observed in intermittent oral administration group [12]. This may be due to the fact that injecting FK506/MMF/PDNN-PLGA MS can first released drugs into the graft tissue and then into the blood, while in oral administration, drugs are first absorbed into the blood and then transported to the graft tissue. Collectively, a long-term low plasma drug concentration makes the FK506/MMF/PDNN-PLGA MS a favorable sustained release system for long-term survival of VCA grafts.

Skin and muscle drug concentration measurements showed lower tissue drug concentration in the contralateral injection group consistent with the graft survival time which was shorter when FK506/MMF/PDNN-PLGA MS was injected in the contralateral leg compared to the transplanted leg injection group. The reason for faster graft rejection in the of FK506/MMF/PDNN-PLGA MS contralateral injection group remains unclear. We speculate that delay in drug transportation to the affected site along with the degradation of the microsphere contributed to the gradual decline in plasma drug concentration to a level that was insufficient for continued immunosuppression. However, injecting of FK506/MMF/PDNN-PLGA MS directly into the transplanted limb could suppress graft rejection and prolong survival time. In addition, Unadkat *et al.* [12] revealed that FK506 concentration in the draining lymph nodes of transplanted limbs was significantly higher than the contralateral groin lymph nodes, and T cells from draining lymph nodes were hyporesponsive to donor and nonspecific antigen stimulation, whereas T cells from the spleen or the contralateral native leg lymph nodes were hyperproliferative. The authors further speculated that the mechanism of long-term survival in the transplanted limb injection group was due to the local high concentration of FK506 mediated modulation of dendritic cell/T-cell interaction leading to inhibition of T-cell maturation and proliferation.

A new sustained release drug delivery system is also important to improve patient compliance. It has been reported that many hand transplanted patients lost their hands mostly because of poor medication compliance [23,24], highlighting the importance of medication compliance for long-term graft survival [25]. Our study showed that a single injection of FK506/MMF/PDNN-PLGA MS can maintain an effective plasma drug concentration for a long period of time and prolong VCA graft survival for more than 150 days. Thus, subcutaneous and intramuscular injection of FK506/MMF/PDNN-PLGA MS after transplantation can significantly reduce the frequency of administration and increase medication compliance during maintenance therapy.

Downward trends observed in pro-inflammatory cytokines in the groups III and IV were consistent with the study on hand transplant patients, where the levels of IL-2, TNF-α, IFN-γ decreased to pretransplant levels at the initial stage and continued to drop post-transplant [16]. We did not detect elevated levels of IL-2, TNF-α, IFN-γ, and IL-1β at POD 7, which were contrary to a previous study with on enzyme-responsive hydrogel encapsulated 7 mg of FK506 in as new drug sustained delivery system [13]. Besides, no significant correlation was found between plasma pro-inflammatory cytokines and graft rejection. Perhaps intragraft cytokines are more related to graft rejection and dysfunction [26] as was also reported in facial allotransplantation [27].

In terms of immunological outcomes, there was no difference in the proportion of PBMCs, T cell, CTL, Th, and Treg between FK506/MMF/PDNN-PLGA MS and systemic treatment, suggesting that both local and systemic applications were equally effective at immunosuppression to achieve long-term graft survival. In contrast to Gajanayake *et al.* [13], cytokines in the sustained release group and systemically treated groups showed similar declining trends which were not significant. However, increase in Tregs in the long-term survival group was not observed in enzyme-responsive hydrogel study [13], which was found in our study. The upregulation of Tregs is considered an important clinical marker of long-term graft acceptance [28].

In our study, there was a burst of drug release in the initial few weeks after FK506/MMF/PDNN-PLGA MS injection in the transplanted limb resulting in inconsistent blood drug concentration (higher concentration in the early stage and lower in the later stage). This

warrants for future research on development of better drug delivery strategy to prevent burst release and maintain stable plasma concentration. Although FK506/MMF/PDNN-PLGA MS significantly reduced the drug dosage compared to the daily oral administration of FK506/MMF/PDNN, the dose of the immunosuppressant is still high, A smaller groin flap transplantation model can be used to test the efficacy of a low-dose immunosuppressive sustained release drugs delivery and further clarify the minimum dose of systemic immunosuppressant required to prevent graft rejection.

Conclusion

In summary, three immunosuppressive drugs—FK506, MMF, and PDNN—were encapsulated in PLGA MS as immunosuppressive therapy for VCA. A single injection of FK506/MMF/PDNN-PLGA MS led to more than 150 days of allograft survival time. This new approach could eliminate daily systemic drug administration to improve patient compliance, reduce drug dosage, and stabilize drug concentration in blood and tissue to minimize toxicity and complications of long-term use of immunosuppressive drugs.

Authorship

SW and XY: operated the study and wrote paper. JC and YW: collected and analyzed the data. JY and BS: designed the study and revised the manuscript. All authors have read and approved the final version of the manuscript for publication.

Funding

The authors have declared no funding.

Conflicts of interest

The authors have declared no conflicts of interest.

Acknowledgements

This work was supported by grants 81871576, 81802131 from the National Natural Science Foundation of China.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. HE staining of liver (a) and kidney (b) from normal, groups III and IV at POD3 and POD14. Scale bars, 100 μm. (c) Plasma Creatinine, BUN, AST and ALT in groups III and IV at different time points.

Table S1. Plasma (ng/ml) and tissue (ng/g) levels of FK506, MMF and PDNN at different time points.

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