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Local delivery of tacrolimus using electrospun poly-ε-caprolactone nanofibres suppresses the T-cell response to peripheral nerve allografts

V H Roberton[1,2], H N Gregory[1,2], U Angkawinitwong[1], O Mokrane[1], A S Boyd[2,3], R J Shipley[2,4], G R Williams[1] and J B Phillips[1,2,*]

[1] UCL School of Pharmacy, University College London, London, United Kingdom
[2] UCL Centre for Nerve Engineering, London, United Kingdom
[3] UCL Institute of Immunity and Transplantation, Royal Free Hospital, London, United Kingdom
[4] Department of Mechanical Engineering, UCL, London, United Kingdom
* Author to whom any correspondence should be addressed.

E-mail: jb.phillips@ucl.ac.uk

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Abstract

Objective. Repair of nerve gap injuries can be achieved through nerve autografting, but this approach is restricted by limited tissue supply and donor site morbidity. The use of living nerve allografts would provide an abundant tissue source, improving outcomes following peripheral nerve injury. Currently this approach is not used due to the requirement for systemic immunosuppression, to prevent donor-derived cells within the transplanted nerve causing an immune response, which is associated with severe adverse effects. The aim of this study was to develop a method for delivering immunosuppression locally, then to test its effectiveness in reducing the immune response to transplanted tissue in a rat model of nerve allograft repair. *Approach.* A coaxial electrospinning approach was used to produce poly-ε-caprolactone fibre sheets loaded with the immunosuppressant tacrolimus. The material was characterised in terms of structure and tacrolimus release, then tested *in vivo* through implantation in a rat sciatic nerve allograft model with immunologically mismatched host and donor tissue. *Main results.* Following successful drug encapsulation, the fibre sheets showed nanofibrous structure and controlled release of tacrolimus over several weeks. Materials containing tacrolimus (and blank material controls) were implanted around the nerve graft at the time of allograft or autograft repair. The fibre sheets were well tolerated by the animals and tacrolimus release resulted in a significant reduction in lymphocyte infiltration at 3 weeks post-transplantation. *Significance.* These findings demonstrate proof of concept for a novel nanofibrous biomaterial-based targeted drug delivery strategy for immunosuppression in peripheral nerve allografting.

1. Introduction

Injuries to the peripheral nerves can result in complete transection of the nerve, which requires surgical repair to allow regeneration of axons across the injury site and back to their targets. When the gap size is too long for direct anastomosis, bridging of the nerve gap is required. Currently the gold standard treatment for such injuries is the nerve autograft, requiring the sacrifice of a donor nerve from the individual which is considered less essential to function [1]. This demands an additional surgical site, with associated donor site morbidity. In addition, there is a limited supply of donor nerve tissue, and autografting tends to use small sensory nerves to repair large mixed or motor nerves, which means there is little opportunity to match tissue size and structure. An alternative source of viable tissue for nerve repair would be the use of nerve allografts from organ donors; however, even with some matching of donor

and recipient, grafts will not survive without suppression of the host immune system [2]. Rejection of allografts can be prevented with pharmacological immunosuppression, but this requires systemic delivery and frequent dosing to maintain a sufficient level of immunosuppression. Such treatment is associated with potentially severe adverse effects including nephrotoxicity, hepatotoxicity and haematologic malignancies, which vastly outweigh the benefits of the nerve repair [3]. Decellularization of allografts allows transplantation without immunosuppression, but the therapeutic benefits of viable cells within the graft are lost, again resulting in reduced efficacy.

Peripheral nerve grafts effectively act as a tissue bridge containing cells that promote and guide the growth of regenerating axons from the proximal nerve stump back to their targets following injury. Importantly, this provides a transplant scenario where chronic life-long immunosuppression is not necessary, since once regeneration across this bridge is complete, the transplant will become populated by host support cells and the donor cells within the transplant are no longer required [4, 5]. Local drug delivery can improve targeting of treatments, and alleviate issues with maintaining concentrations within the therapeutic window. Therefore sustained local delivery of immunosuppressants at the site of the transplant may be a suitable approach for peripheral nerve repair. This approach would potentially allow an efficacious dose to be maintained for the duration of regeneration through a nerve allograft, without the need for repeated systemic dosing and associated adverse effects.

A commonly used immunosuppressant in organ transplantation is tacrolimus (FK506), an immunophilin ligand which was first used clinically more than 30 years ago [6]. The immunosuppressive mechanism of action is the inhibition of T-cell activation, via inhibition of calcineurin signalling and interleukin-2 synthesis, leading to failure of T-cell proliferation and maturation [7, 8]. Tacrolimus is also well established as an enhancer of nerve regeneration and therefore has been used to improve nerve repair, where doses lower than those required for immunosuppression can have a beneficial effect (reviewed comprehensively in [9–11]). The pro-regenerative effects of tacrolimus are thought to be mediated via reduced scarring and activation of the ERK pathway in neural cells [12]. Enhanced nerve regeneration has been demonstrated in animal models using local delivery of tacrolimus from polymer nanospheres [13], conduits [14] and poly(lactic-co-glycolic acid) (PLGA) microspheres trapped in fibrin gel [15]. The PLGA microsphere approach was also recently shown to improve regeneration in a 20 mm common peroneal nerve allograft model, with local tacrolimus treatment resulting in allografts being comparable to isografts in terms of neurite growth after 4 weeks [16].

Local delivery of tacrolimus from micro and nanoparticles has been explored experimentally as a means to prevent transplant rejection in other models including islet cell and liver allografting [17, 18]. Another method which is often used to fabricate biomaterials for controlled local delivery of small molecules is electrospinning—this process can generate nanofibers with high drug loading and encapsulation efficiencies which allow modulation of drug release rate. This is particularly well suited to applications which require sustained high drug dose delivery at the target site to maintain efficacy [19]. Coaxial electrospinning to produce fibres can achieve such sustained drug release and can form flexible nanofiber mats for implantation around the target tissue. This is particularly appealing in the case of nerve grafting, where the simple cylindrical geometry of the transplanted nerve provides a straightforward structure around which a mat can be wrapped. In coaxial electrospinning, two solutions are dispensed simultaneously through concentric needles in a 'spinneret' towards a collector. The application of an electric field between spinneret and collector results in rapid drying and the generation of a non-woven nanofibrous mesh.

In one commonly-explored embodiment of the coaxial electrospinning approach, a polymer shell solution and a core drug solution are employed, resulting in high encapsulation of drug within the polymer fibre which allows control of drug release rate. Previously, such electrospun fibres have demonstrated sustained zero order release characteristics, using a poly-ε-caprolactone (PCL) shell [20]. Indeed, the use of electrospinning to fabricate nerve repair constructs containing encapsulated drug is increasing, and a number of formulations containing growth factors in particular have been successful in rat sciatic nerve injury models [21–23]. PCL is a biodegradable polymer which recently has become the most widely used biomaterial for making synthetic nerve conduits in published research studies [24]. Nerve wraps of biodegradable polymers including PCL have been used for the delivery of drugs to accelerate nerve regeneration [25]. We therefore sought to test whether tacrolimus could be successfully loaded into fibre mats to wrap around peripheral nerve allografts and whether this would result in reduced T-cell infiltration into mismatched rat allografts. Since the main aim was to investigate the host immunological response to the mismatched allograft tissue, a primary end point of 3 weeks was used to explore the infiltration of T-cells and macrophages into the graft tissue. Additionally, some animals were maintained for 8 weeks to examine whether the local release of tacrolimus from the fibre mats had any negative effects on animal health or nerve regeneration.

2. Method

2.1. Material preparation and characterisation

2.1.1. Preparation of tacrolimus-loaded fibre mats

Tacrolimus (Generon, UK) loaded PCL (Sigma-Aldrich, UK) fibre mats were produced by coaxial electrospinning. A shell solution of 10% w/v PCL in 90% v/v TFE:deionized water and core solution of 133.3 mg ml^{-1} tacrolimus in ethanol were loaded into 5 ml syringes and mounted on separate syringe drivers (79–9100 C, Cole-Parmer, UK). Syringes were attached to a coaxial spinneret (Linari Engineering, Italy) with internal needle diameters of 0.51 and 1.37 mm. Flow rates were set at 1.5 and 0.3 ml h^{-1} for the shell and core solutions respectively. A 15 kV voltage was supplied to the spinneret using a high voltage power supply (HCP, 35-35000, FuG Elektronik, Germany) and a grounded, aluminium foil-coated collector plate was positioned 15 cm from the spinneret tip. Fibres were collected at room temperature for 2 h to generate a fibre mat of approximately 80 cm^2, resulting in a predicted dosage of 1 mg of tacrolimus per cm^2 of fibre mat. As a control material, blank polymer-only fibre mats were produced by monoaxial electrospinning of the PCL solution alone. All electrospun materials were stored at −20 °C until use.

2.1.2. Material characterisation

A Phenom Pro G6 Desktop scanning electron microscope (Thermo Scientific, USA) was used to investigate the morphology of tacrolimus loaded and blank fibres. Fibre diameter was measured using ImageJ, with 25 fibres measured in each of 6 separate fields.

Differential scanning calorimetry (DSC) was performed on a Q2000 instrument (TA instruments, USA). Blank and tacrolimus loaded fibres (2–3 mg) were sealed into Tzero aluminium pans using Tzero hermetic lids which had been pin-holed. The pans underwent heating from −70 to 150 °C with an N_2 purge at 50 ml min^{-1}.

Thermogravimetric analysis (TGA) was carried out using a Discovery TGA (TA instruments, USA). Blank and tacrolimus loaded fibres (3–5 mg) were heated isothermally for two hours at 30 °C, and the furnace purged with N_2 at 25 ml min^{-1}.

2.1.3. Quantification of tacrolimus release from fibres

Tacrolimus loaded PCL fibre mats were cut into 1 cm^2 pieces and incubated with 1 ml of a release buffer (0.05 M ammonium bicarbonate with 0.02% NaN_3). Experiments were performed in glass vials at 37 °C with shaking at 75 RPM for up to 38 days. Every two days, all release media was removed and replaced. Release samples were stored at −20 °C until analysis. Tacrolimus concentration was determined using an Agilent 1260 Infinity liquid chromatography system with an Agilent Pursuit 5 μm C8 50 × 2.1 mm column at 40 °C, coupled to an Agilent 6460 triple quadrupole mass spectrometer. Gradient elution at 0.8 ml min^{-1} was used with water (A) and methanol (B) mobile phases both containing 10 mM ammonium formate and 0.05% formic acid (Thermofisher Scientific) (supplementary table 1). The fragmentation of the tacrolimus ammonium adduct (m/z 821.52) to the product ion (m/z 768.47) was detected via multiple reaction monitoring (supplementary table 1). Analysis parameters were partly based on an established protocol [26].

2.2. Testing local release of tacrolimus in a rat sciatic nerve allograft model

2.2.1. Ethical statement

All animal procedures were conducted according to the UK Animals (Scientific Procedures) Act (1986)/the European Communities Council Directives (86/609/EEC) under Home Office Licence No. P50F86A60 with the approval of the UCL Animal Welfare and Ethical Review Body.

2.2.2. Surgical methods

Adult male inbred Lewis (LEW) rats (200–220 g, Charles River, UK) were used for transplant hosts (n = 31), and allogeneic donor nerves were taken from adult male Dark Agouti (DA) rats (210–230 g, Envigo, NL, n = 8). These different inbred rat strains were chosen due to a mismatch in their major histocompatibility complex (LEW $RT1^l$, DA $RT1^{av1}$) [27, 28]. Host animals were anaesthetised in an induction chamber with 5% isoflurane in O_2, and anaesthesia maintained throughout surgery at 1.5%–2.5% isoflurane in O_2 (0.8 l min^{-1}). The sciatic nerve of LEW rats was exposed at mid-thigh level and, for autografts, a 1 cm nerve section was resected, reversed and replaced via suturing with 10/0 epineurial sutures at the proximal and distal stumps. For allografts, DA rats were culled with sodium pentobarbital (i.p.) and a 1 cm piece of donor nerve dissected from each sciatic nerve for transplantation into LEW hosts. Following suturing of nerve grafts, a 1 cm^2 section of tacrolimus loaded or blank PCL fibre sheet was wrapped around the transplant, the muscle layer was sutured with 4/0 absorbable sutures, and the skin closed with wound clips. All animals received analgesia (Rimadyl 4 mg kg^{-1}, subcutaneous injection) and wounds were treated with veterinary wound powder after closure (Battles, UK). Animals were left to recover for 3 or 8 weeks after transplantation following random assignment to groups (1) autograft with blank wrap, (2) autograft with tacrolimus wrap, (3) allograft with blank wrap and (4) allograft with tacrolimus wrap (n = 3–4 per group at 3 weeks and n = 4 per group at 8 weeks). Animal health was monitored throughout the recovery period and weight was recorded weekly for 3 weeks post-surgery. Additionally, the effects of the different treatments on neurite growth were explored after 8 weeks using electrophysiology and histology. Note that the 10 mm gap

Figure 1. Nerve sample processing.

and 8 week time point does not represent a critical length nerve injury model in the rat [29, 30] therefore the extent of regeneration was not expected to differ between autograft and allograft animals.

2.2.3. Electrophysiology

At 8 weeks post-transplantation, animals were anaesthetised with isoflurane, and the contralateral and repaired ipsilateral nerves exposed. Ground and reference electrodes (Natus) were placed in the tail and hip respectively, and a recording needle (Ambu Neuroline) was inserted into the gastrocnemius muscle. The nerve was stimulated above the graft site with a stimulating electrode (Neurosign Bipolar Probe 2 × 100 mm × 0.75 mm). The distance between stimulating and recording electrodes was standardised, and nerves were stimulated using a bipolar stimulation constant voltage configuration and muscle response recorded (Natus Synergy Electromyography).

2.2.4. Tissue collection and processing

Rats were culled with sodium pentobarbital (i.p.) and the sciatic nerves (ipsilateral and contralateral) harvested and transferred to 4% paraformaldehyde (PFA) for 24 h. Ipsilateral and contralateral gastrocnemius muscles were collected prior to fixation in 4% PFA. After washing with phosphate-buffered saline (PBS), nerves were divided into samples for further processing by cutting with a surgical blade 2 mm into the proximal and distal stumps and 2 mm into either end of the graft (figure 1). After incubation in 15% and 30% sucrose for 24 h, nerve samples were snap frozen with liquid nitrogen cooled isopentane in NEG-50 frozen section compound (Richard-Allan Scientific) and 30% sucrose (1:1) in cryomoulds. Longitudinal cryosections were cut from the centre of the nerve grafts at 5 μm thickness, and transverse sections of the nerve stumps were cut at 15 μm thickness on a HM525 NX cryostat (Thermo Scientific) and collected directly on SuperFrost microscope slides (Thermo Scientific).

2.2.5. Immunohistochemistry

All washes and incubations were carried out in immunostaining buffer (0.2% Triton X-100 in PBS). After washing, sections were blocked in 5% normal serum for 20 min at room temperature followed by incubation in primary antibodies overnight at 4 °C. Primary antibodies were anti-neurofilament (Eurogentec SMI-35, 1:1000), anti-CD4 (HycultBiotech HM3001, 1:500), anti-CD8 (Bio-Rad MCA48GA, 1:500) and anti-ED-1 (Merck MAB1435, 1:100). Sections were washed and incubated with secondary antibodies (DyLight 488 or 594, 1:200) for 45 min at room temperature. After washing, nuclei were stained with 4′, 6-diamidino-2-phenylindole for 5 min at room temperature before final washing and mounting with Vectashield Hardset Antifade Mounting Medium (Vector Laboratories).

2.2.6. Imaging and quantification

Sections were imaged using fluorescence microscopy. T-Cell infiltration was assessed in longitudinal sections using three fields obtained from pre-defined sampling locations (Zeiss AxioLabA1) and counting the number of $CD4^+$ and $CD8^+$ cells observed in each case. Macrophage and monocyte presence was assessed using tile-scans of longitudinal sections (Zeiss AxioScan Z1 slide scanner), with the area of ED-1 immunofluorescence expressed as a percentage of the total area of the section. Neurites were quantified in transverse sections taken 2 mm into the distal nerve stump, expressed as the total number of neurites in the entire nerve cross section. Where appropriate, statistical testing was performed using analysis of variance (ANOVA) with Tukey's post-test, having first established data were normally distributed (GraphPad Prism).

3. Results

The tacrolimus loaded PCL fibre mat (total 80 cm^2) was prepared in 1 cm^2 pieces for release assays and implantation (figure 2(A)). Fibres were non-uniform (figure 2(B)), with a mean diameter of 390 ± 199 nm (figure 2(C)). DSC performed on the blank and tacrolimus-loaded fibres demonstrated a sharp endothermic peak around 56 °C in both thermograms (figure 2(D)), consistent with the melting of PCL [31]. Tacrolimus has a melting point of 128 °C [32, 33]; this is not observed in the DSC traces, suggesting that the drug is present in the fibres in the amorphous form. This is typical for electrospun

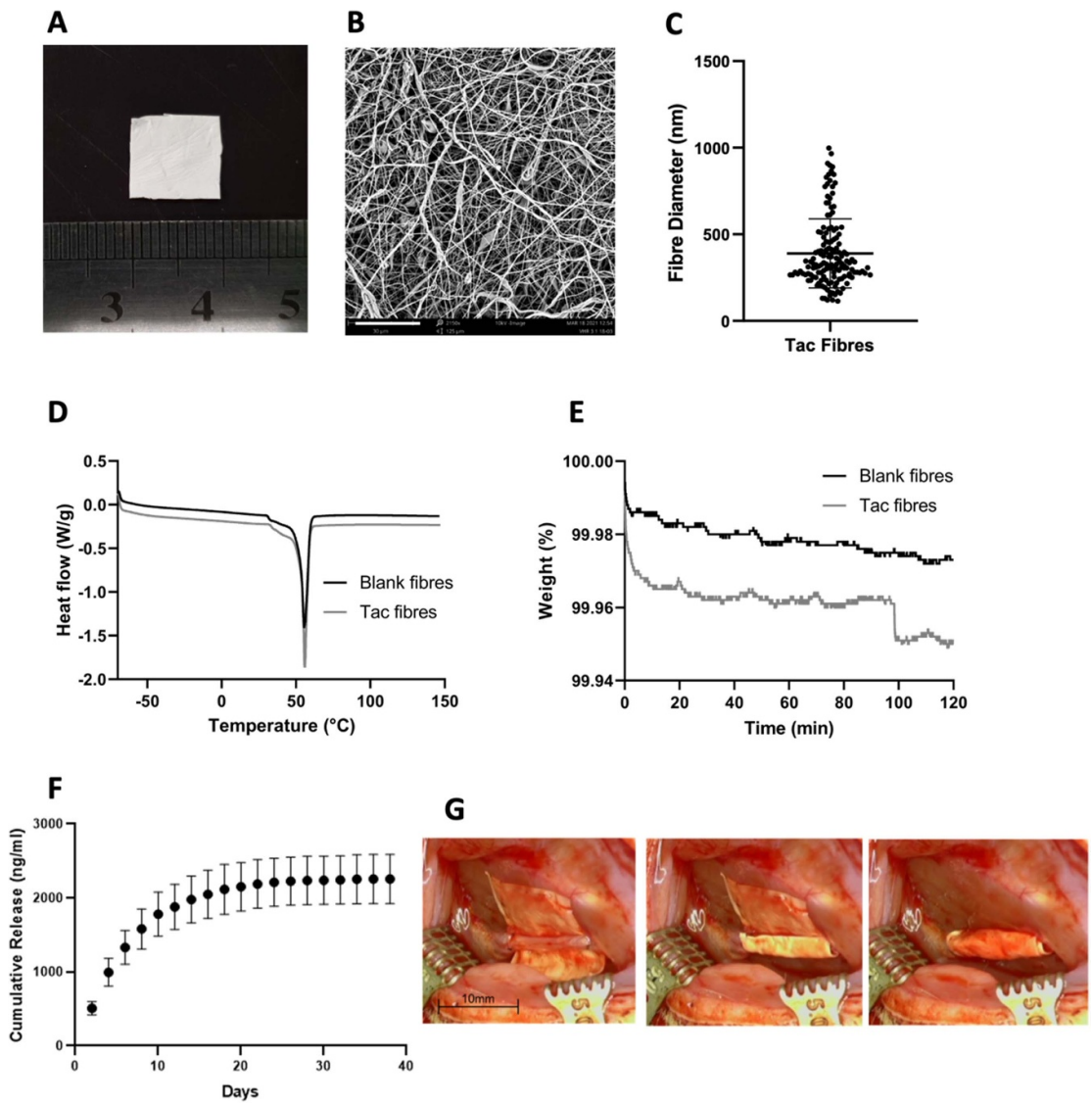

Figure 2. Electrospun coaxial PCL nanofibre mats for controlled release of tacrolimus. After collection, mats were cut into 1 × 1cm squares (A) of mass 6.6–8.6 mg (mean 7.9 ± 1.1 mg). Scanning electron micrograph (scale bar 30 μm) shows a nanofibrous structure (B) with mean ± SD fibre diameter 390 ± 199 nm (C). Differential scanning calorimetry of tacrolimus-loaded and blank fibre mats showed only a melting peak for PCL around 56 °C (D), and thermogravimetric analysis at 30 °C indicating that no residual solvent was present after fabrication (E). Release of tacrolimus into solution was monitored over 38 days using LC-MS (F), data expressed per mg fibres, mean ± SEM, $n = 3$. Mats could be wrapped around a 10 mm rat sciatic nerve graft during the microsurgical procedure (G).

formulations and arises owing to the rapid drying process preventing recrystallisation [34, 35]. However, actual encapsulation of tacrolimus into the fibres was relatively low by mass, and as such the drug may be present in amounts too low to detect. TGA of the fibres was similar for the tacrolimus-loaded and blank formulations (figure 2(E)). With heating at 30 °C, any mass loss from the fibres can be attributed to the vaporisation of residual solvent from the electrospinning process. Both sets of fibres had a mass loss of less than 0.05% and since the sensitivity of the Discovery instrument is ±0.1% [36], this corresponds to virtually zero change in weight. This conclusion is reinforced by the lack of broad endotherm around the boiling points of the solvents in the DSC thermograms. Extended release of tacrolimus was observed for >20 days (figure 2(F)). Fibre sheets (1 cm^2) could be easily wrapped around transplanted nerve allografts *in vivo* using standard microsurgical instruments (figure 2(G)). At the end of the study the material appeared unchanged and remained in position in all animals.

Longitudinal sections from within the graft tissue were stained for $CD4^+$ and $CD8^+$ putative T-cell populations to assess the lymphocyte response to nerve grafts 3 weeks post-transplantation. Very low densities of both lymphocyte populations were observed in the autografts, whereas a greater number of $CD4^+$ and $CD8^+$ cells had infiltrated the allografts (figure 3). $CD4^+$ T-cell infiltration was significantly higher in the allografts which were wrapped with control material without tacrolimus than those

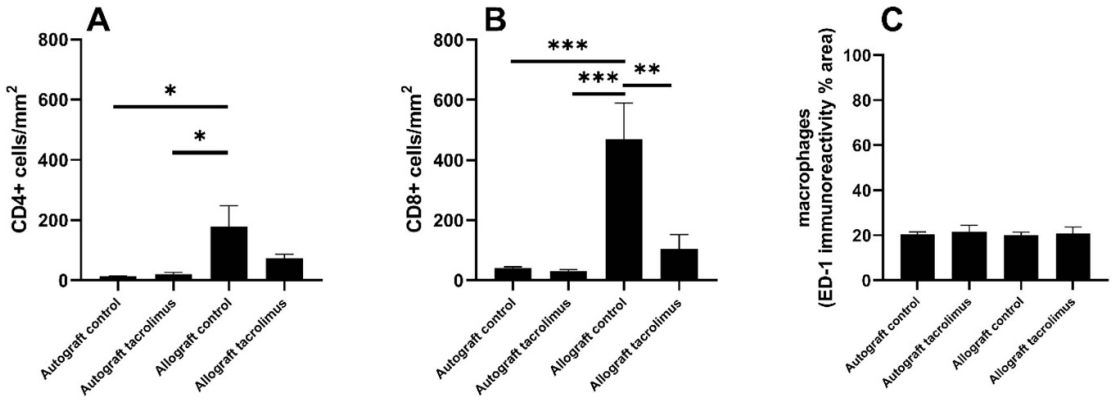

Figure 3. T-cell and macrophage presence in sciatic nerve autografts and allografts. Rat sciatic nerve gaps (10 mm) were repaired using autografts or mismatched allografts wrapped with PCL mats containing tacrolimus or control mats. After 3 weeks the T-cells present in longitudinal sections through the graft tissue were assessed histologically and the number of cells showing immunoreactivity for CD4 (A) and CD8 (B) were quantified throughout the longitudinal sections. The presence of ED-1^+ monocytes and macrophages was detected in longitudinal sections of the graft tissue, expressed as a % of the total area (C). Data are means ± SEM, $n = 3$-4). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA with Tukey's post-test).

receiving untreated autografts ($p < 0.05$), or autografts with tacrolimus loaded material ($p < 0.05$). A reduction in $CD4^+$ T-cell infiltration was observed in treated allografts. This was not significantly lower than untreated allografts ($p = 0.11$). However, no difference was detected between treated allografts and either autograft group (figure 3(A)). Infiltration of $CD8^+$ T cells was increased more than 10-fold in untreated allografts compared with both autograft groups ($P < 0.001$) and was significantly reduced by about 80% ($P < 0.05$) in the group that received allografts with local tacrolimus delivery (figure 3(B)). Monocytes/macrophages were present throughout the engrafted tissue and the area of immunofluorescence for ED-1 constituted approximately 20% of the total area of the longitudinal sections. There were equivalent amounts of ED-1 immunoreactivity present in all groups (figure 3(C)).

In addition to looking at the host immune and inflammatory response at 3 weeks, additional data were collected from animals 8 weeks after surgery to explore any differences in health and nerve regeneration through the grafts. Animals in both treated and untreated groups showed a similar increase in weight over time (figure 4(A)), confirming that there was no weight loss associated with the administration of the tacrolimus. All animals in the treated and untreated allograft and autograft groups showed successful regeneration through the grafts at 8 weeks. This was determined by the presence of neurites detected in the distal nerve stump, and contraction of the gastrocnemius and tibialis anterior muscles in response to electrophysiological stimulation of the sciatic nerve proximal to the graft. The number of neurofilament positive neurites in the distal stumps was 2000–3000 in all cases, with no significant differences detected between any of the groups (figure 4(B)). This was consistent with the electrophysiological results which also showed similar restoration of compound muscle action potential amplitude in both the gastrocnemius (figure 4(C)) and tibialis anterior (figure 4(D)) muscles, with similar latency (data not shown), in all four groups.

4. Discussion

In this study, locally implanted flexible sheets of electrospun tacrolimus-eluting PCL nanofibers were demonstrated to deliver a therapeutic dose sufficient to reduce the host immune response to a sciatic nerve allograft in rats. This approach was distinct from previous research where tacrolimus-eluting microparticles and other materials have been used to deliver tacrolimus to improve regeneration [14, 15]. PCL is a widely-used degradable biomedical material available for production at a clinical grade and electrospinning is a scalable method for drug encapsulation and nanofibre fabrication, making this approach suitable for future clinical translation [37].

The electrospun mat yielded sustained release of a therapeutically relevant concentration of tacrolimus from 1 cm^2 sheets over >2 weeks *in vitro*. Previous *in vivo* studies have indicated that the nerve regeneration enhancement of tacrolimus can be elicited using lower doses than are required for effective immunosuppression [38], although the optimum concentration of tacrolimus reported for promoting regeneration varies between models [39]. There are challenges in determining the optimal local dose of tacrolimus required for immunosuppression because most studies have used systemic immunosuppression and focussed on maintaining a minimum plasma trough level of 5–15 ng ml^{-1} [39], rather than reporting local tissue levels.

Thermal analysis on the tacrolimus loaded and blank materials using DSC showed only a peak for the melting transition of crystalline PCL and was consistent with the literature. The onset of melting was

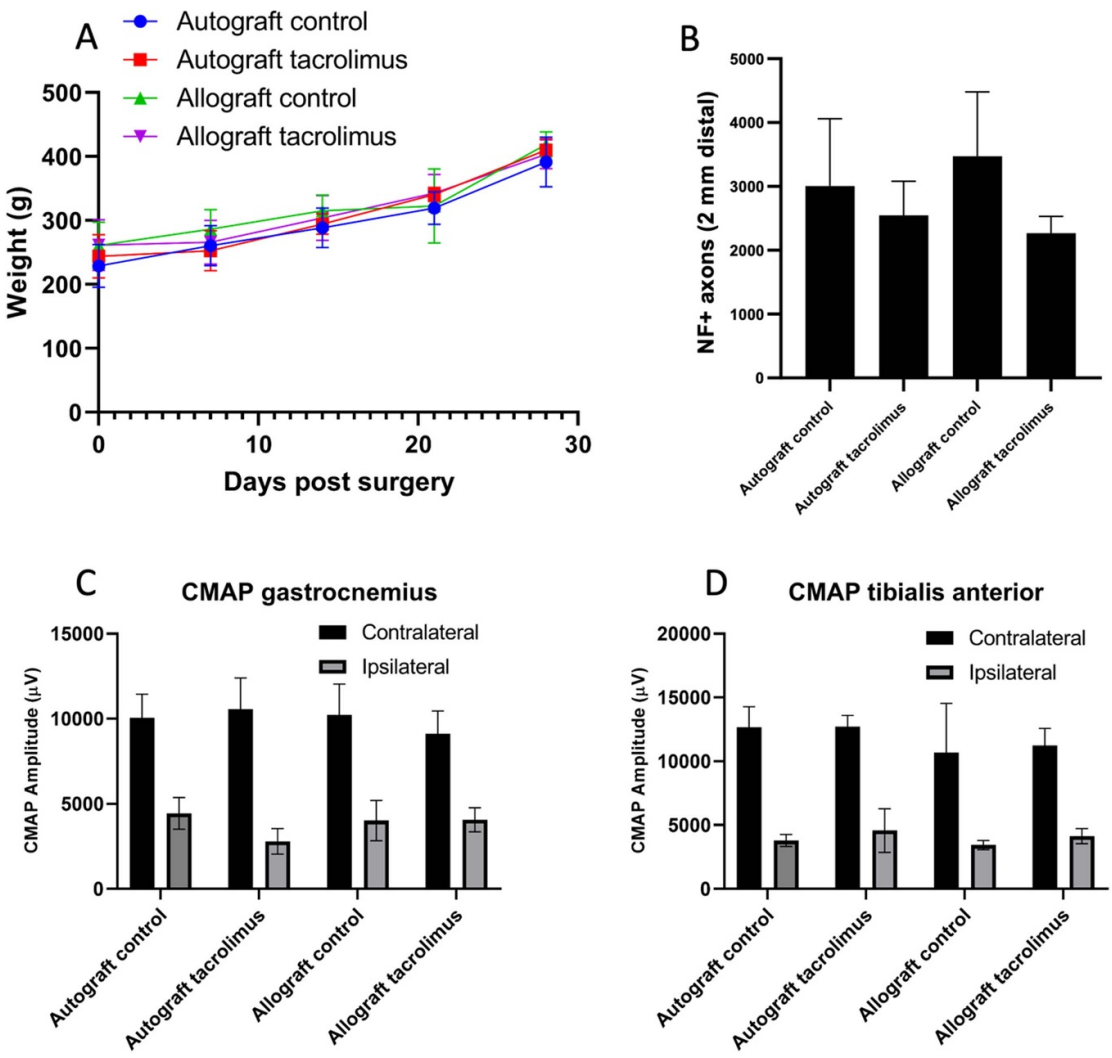

Figure 4. Animal health and nerve regeneration. Rat sciatic nerves were repaired using autografts or mismatched allografts wrapped with nanofibrous PCL mats containing tacrolimus or control nanofibrous PCL mats ($n = 4$). There were no differences between groups in terms of weight gain following surgery (A), and number of neurofilament-positive (NF+) axons detected in transverse sections 2 mm distal to the graft at 8 weeks (B). Electrophysiological testing indicated some restoration of function in all animals by 8 weeks, with no differences in between groups in recovery of gastrocnemius (C) and tibialis anterior (D) compound muscle action potential (CMAP).

around 38 °C, which is above normal core body temperature. No peak for the melting of tacrolimus was evident—this may indicate that the drug is amorphous within the fibres [40], or simply that insufficient drug was present to generate a signal. TGA was performed isothermally below the melting temperatures of the fibre components, and the stable sample mass suggested that the solvents used in manufacture had fully evaporated. The material was simple to wrap around the nerve following microsurgical grafting and stayed in place without any additional adhesive application and did not cause any observable additional fibrosis.

The nerve allograft model presented here is similar to that used previously in which allografting was performed between different inbred rat strains, Lewis and Dark Agouti, in an attempt to mimic a human clinical nerve allograft scenario [27]. The elevation in levels of $CD4^+$ (helper) T-cells and $CD8^+$ (cytotoxic) T-cells in the untreated allograft group at 3 weeks confirms that in this model allotransplantation of nerve tissue elicited a distinct host immune response, providing a suitable system in which the effect of local immunosuppression could be investigated. Differences between the four groups were assessed using ANOVA with Tukey's post test to compare between all groups, with a statistically significant increase in $CD4^+$ and $CD8^+$ T-cell infiltration in the untreated allograft group compared with the autograft groups. Treatment of allografts with tacrolimus delivered locally via the nanofibrous biomaterial was highly effective in abrogating the host immune response, reducing T-cell numbers significantly to near baseline levels. Macrophage/monocyte presence within the nerve grafts was equivalent between the different treatment groups at 3 weeks, although this study was limited to simply assessing the monocyte and macrophage fraction, as measured by ED-1 expression,

rather than exploring the more specific phenotype of the cells that were present e.g. whether they were pro- or anti-inflammatory. Macrophages play a key role in immune and inflammatory responses and in this model they are likely to respond to the nerve damage, the inflammation arising from the grafting procedure, in addition to the presence of the biomaterial itself, making it difficult to interpret the macrophage data without understanding which influence is likely to be dominant at the 3 week time point [41]. Furthermore, it has been shown previously that calcineurin inhibitors such as tacrolimus may modulate macrophages but cannot suppress their activation to the same extent as achieved in T-cells [42], which may account for the lack of effect on macrophages seen here with tacrolimus.

None of the animals which received tacrolimus exhibited side effects such as weight loss, which is consistent with previous evidence that local delivery of tacrolimus to nerves did not result in significant levels being detected in plasma and other organs [15]. The amount of tacrolimus loaded in each 1 cm^2 piece of nanofibrous material was theoretically 1 mg, which would potentially provide a maximum total dose of approximately 5 mg kg^{-1} in treated animals. LC-MS analysis suggests that the cumulative mass of tacrolimus released over 38 days was in the order of 2 μg per mg fibres (which is $\sim$16 μg per 1 cm^2 piece), which equates to $\sim$64 μg kg^{-1} per animal in total. By comparison, conventional systemic immunosuppression can involve doses in the range of 2–10 mg kg^{-1} per day in experimental animals [43, 44]. It will be important in future studies to establish the optimal dose of tacrolimus that is required for effective local immunosuppression, since a minimal dose was tested in this study. Further, development of the electrospinning process to improve encapsulation efficiency may refine the accuracy of dosage predictions.

In their recent study, Zuo *et al* showed that a single dose of 420 μg tacrolimus encapsulated in PLGA microspheres within a fibrin hydrogel was sufficient to improve neuron growth through nerve allografts [16]. This was associated with reduced serum interleukin 12, indicating an immunosuppressive effect, although immunosuppression in terms of T-cell response was not measured directly. In addition to establishing an optimal local dose for effective immunosuppression it will be important in future studies to understand the biodistribution of tacrolimus in other tissues and organs and the long-term response of local tissues to the drug and the biomaterial as the PCL gradually degrades.

Nerve function was restored in all animals by the 8 week time point, with no detectable differences between groups in terms of histological and electrophysiological outcome measures. The 10 mm graft length used here is not a critical gap size in the rat which means that regeneration is possible through sub-optimal environments. It is apparent that even in the untreated allograft groups where an immune response was present the allograft environment remained sufficiently permissive to support regeneration. Interestingly, the lack of difference detected between groups at 8 weeks indicates that any regeneration enhancing effect of tacrolimus was minimal in this particular model scenario, perhaps due to the local dose of tacrolimus not being optimal for that purpose [39]. The ability of nerve allografts to support regeneration even in the absence of immunosuppression has been noted previously, as reviewed recently by Roballo *et al* [45]. In that review a number of interesting observations are made about differences between living nerve allografts and other allogeneic transplanted tissues, and evidence is discussed that may indicate the enhanced ability of nerve tissue allografts to survive for long enough to support regeneration.

The main conclusion that can be drawn from this study is that local delivery of tacrolimus from nanofibrous electrospun PCL can ameliorate the T-cell response in a rat model of nerve allograft transplantation. Future studies should explore the effect this might have on nerve regeneration in a critical length nerve gap model, where a viable living graft is essential for functional recovery [46, 47]. Additional studies should be conducted to optimise the dose and release rate of the drug, to measure local tissue levels within and adjacent to the repair site following local delivery, and to assess systemic biodistribution and toxicity. It will also be important to investigate the long-term results of this approach. In the nerve repair scenario, transient survival of allograft tissue may be sufficient to promote regeneration, with subsequent gradual clearance of transplanted cells as host cells populate the graft. Translation of this approach to clinical application will require careful consideration of the timing and the distances involved, the matching of drug-eluting material quantity to length and diameter of grafts, and the safe degradation profile of the biomaterial. If successful, the concept of using transient local immunosuppression to enable living allogeneic tissue to be used in nerve repair, has the potential to overcome the serious limitations associated with the current reliance on the nerve autograft.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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ORCID iDs

V H Roberton https://orcid.org/0000-0002-0404-6984
A S Boyd https://orcid.org/0000-0002-4191-8155
R J Shipley https://orcid.org/0000-0002-2818-6228
G R Williams https://orcid.org/0000-0002-3066-2860
J B Phillips https://orcid.org/0000-0001-8117-3074

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