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Topical Tacrolimus Nanocapsules Eye Drops for Therapeutic Effect Enhancement in both Anterior and Posterior Ocular Inflammation Models

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Abstract

Tacrolimus has shown efficacy in eye inflammatory diseases. However, due to the drug lability, its formulation into a stable ophthalmic product remains a challenge. Tacrolimus-loaded nanocapsules (NCs) were designed for ocular instillation. Further, the stability and effects of the formulation were analyzed under different experimental conditions. Physicochemical characterization of the NCs revealed suitable homogeneous size and high encapsulation efficiency. Moreover, the lyophilized formulation was stable at ICH long term and accelerated storage conditions, for at least 18 and 3 months, respectively. The tacrolimus NCs did not elicit any eye irritation in rabbits after single- and multiple-dose applications. Additionally, *ex vivo* penetration assays on isolated porcine cornea and pharmacokinetics analyses in various rabbit eye compartments demonstrated the superiority of the NCs in retention and permeation into the anterior chamber of the eye compared to the free drug dissolved in oil. Moreover, multiple dose ocular instillation of the NCs in rats allowed high tacrolimus levels in the eye with very low plasma concentrations. Finally, the developed delivery system achieved a significant decrease in four typical inflammatory markers in a murine model of keratitis, an anterior chamber inflammation. Furthermore, these NCs, applied as eye drops, displayed clinical and histological efficacy in the mainly posterior chamber inflammation model of murine, experimental autoimmune uveitis.

Graphical Abstract

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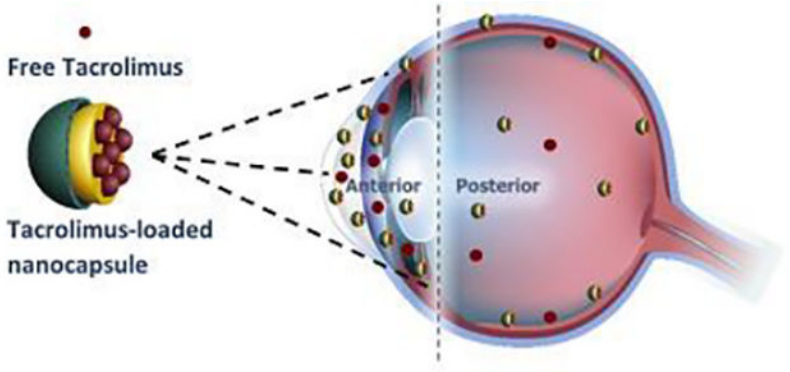
Conflict of interest

For transparency, Prof. Simon Benita is also the chairman of BioNanoSim.



Keywords

Tacrolimus; nanocapsules; ocular delivery; keratitis; auto-immune uveitis

1. Introduction

Ocular absorption of topically applied drugs is challenging. It was already reported that for hydrophilic compounds the corneal epithelium appears to be rate limiting to drug permeation, whereas for hydrophobic compounds, the corneal stroma is the rate limiting layer [1]. Indeed, the cornea represents the main route of access for drugs whose target is the inner eye, however, crossing the corneal barrier is a major obstacle for many active molecules. Considering the highly organized multilayer corneal epithelium with abundant tight junctions and the hydrophilic stroma, only compounds with a low molecular weight and moderate lipophilic character can moderately permeate through these barriers [2,3]. In recent decades, it has been demonstrated that some types of nanocarriers smaller than 1 μm, especially nanoemulsions, can overcome the eye-related obstacles[3,4]. The main drawback of these dosage forms is their inability to deliver aqueous-sensitive molecules prone to hydrolysis. To overcome these limitations, delivery systems based on polymeric nanoparticles were developed [5,6]. Such colloidal delivery systems have the capability to contain a wide array of compounds, including highly lipophilic ones, to diminish the degradation of labile drugs, extend their retention time in the external ocular tissues, improve their interaction with the corneal and conjunctival epithelia and consequently, their bioavailability as well [2,3].

Tacrolimus (FK506), a lipophilic, labile drug[7], does not easily penetrate the corneal epithelium and accumulates in the corneal stroma due to its poor water solubility of 1–2 μg/mL and relatively high MW[8,9]. It is a well-known macrolide, inhibiting calcineurin activity within T lymphocytes, yielding suppressed formation of cytokines by these immune cells and decreased stimulation of other inflammatory cells[10,11]. Microbial keratitis is a common ocular infection caused by bacteria, fungi, viruses, or parasites [12]. The usual treatment consists of aggressive antimicrobial drug regimens to clear the causative organisms from the cornea. However, in 50% of the cases, the conventional strategies are unable to prevent eye tissue damages caused by excessive inflammation. Thus, combination

therapy with anti-inflammatory drugs such as tacrolimus may improve the clinical outcomes [13].

Different forms and concentrations of tacrolimus have been successfully assessed clinically in the treatment of anterior segment inflammatory and allergic disorders [14]. Furthermore, an ongoing phase 2 clinical trial is preliminarily evaluating the effectiveness of a micellar tacrolimus formulation, Tacrosolv®, for the treatment of allergic rhinoconjunctivitis [15]. It should be emphasized that, currently, there is no globally marketed approved ocular product of tacrolimus for anterior chronic uveitis treatment. Thus, most of the clinical use of the drug for the treatment of anterior allergic disorders is in personal prescriptions with short shelf-life, an aqueous ophthalmic 0.1% tacrolimus suspension product, marketed only in Japan or off-label when in a pharmaceutical non-sterile ointment dosage form. We would like to point out that several attempts were made by many investigators to identify an appropriate ocular formulation of tacrolimus based on different approaches including nanotechnology [16–18] and ophthalmic tacrolimus preparations in olive oil and dextrin respectively [19,20]. Some of these preparations, nevertheless, may be associated with several side effects such as ocular irritation, redness, burning and itching sensation [19].

In addition, several studies demonstrated systemic tacrolimus efficacy in non-infectious uveitis even in refractory cases [21–23]. Uveitis is a potentially blinding disorder describing any inflammation within the eye including the uvea, sclera, retina, vitreous and optic nerve. Acute anterior uveitis is generally managed with topical corticosteroids while more posterior sight-threatening inflammations require aggressive systemic immunosuppression. Although these treatments can be effective, they may cause many severe side-effects if their therapeutic use is prolonged. Indeed, topical steroids induce cataract and rise the intraocular pressure whereas systemic immunosuppression treatments increase the risk of opportunistic infections and renal failure [24]. Therefore, yet, effective topical eye drops formulations to deliver drugs to chronically inflamed anterior chamber and reaching the posterior segment have not been achieved for the treatment of chronic uveitis [25]. While the exact cause of this orphan disease is still unknown, a wide array of studies, using different animal models, have established that the inflammation in uveitis is due to an autoimmune response to various ocular antigens. Accordingly, Experimental Autoimmune Uveitis (EAU) is typically induced by immunizing susceptible animals with one of the several antigens originating from the retina, causing severe inflammation. Most studies in the past three decades have focused on the role of T cells in the pathogenesis of uveitis [26–28]. This mechanism was further supported by the prevention of EAU development achieved with oral administration of calcineurin inhibitors, T cell-targeting agents such as Cyclosporine A [29]. Hence, this study aimed to develop a safe and stable ocular delivery system of tacrolimus that could significantly increase drug absorption through the eye and act locally to improve its therapeutic effect in experimental models of both keratitis, an anterior chamber disease, and auto-immune uveitis, a mainly posterior chamber inflammation model. To this end, different types of nanocarriers were designed and characterized. It should be noted that there is no novelty in the nano-encapsulation of tacrolimus. The originality, in the present investigation, resides in the combination of various formulations processes, including nano-encapsulation, lyophilization, an appropriate reconstitution approach prior to subject application and a choice of suitable protective excipients. Altogether, this comprehensive, distinctive,

approach led to the novelty of the final, stable, ocular tacrolimus preparation. Finally, tacrolimus formulations meeting the required criteria for ophthalmic preparations were verified *ex vivo* on porcine corneas to assess their potential to improve corneal penetration. The selected nanocarriers, based on the appropriate physicochemical properties, underwent additional *in vitro* stability evaluations. Then, their irritation potential in single and multiple-dose applications, along with pharmacokinetic and pharmacodynamic studies to assess ocular drug distribution and efficacy in different murine models, were performed.

2. Materials

Tacrolimus (as monohydrate) was purchased from TEVA (Opava, Komárov, Czech Republic); Castor oil and medium-chain triglyceride (MCT) were acquired from TAMAR Industries (Rishon LeTsiyon, Israel). Bovine Serum Albumin (BSA), Polysorbate 80 (Tween® 80), polyoxyl-35 castor oil (Cremophor® EL), *Pseudomonas aeruginosa* Lipopolysaccharides (LPS) Freund's complete adjuvant (H37Ra) and Pertussis toxin were acquired from Sigma-Aldrich (St. Louis, USA). S-antigen was obtained from Genosphere Biotechnologies (Paris, France). Xylazine (Rompun®) and Ketamine (Imalgene®) were purchased from Bayer Schering Pharma (Berlin, Germany) and Merial (Lyon, France), respectively. Tropicamide (Mydriaticum®) was from Thea Laboratories (Clermont-Ferrand, France). Lipoid® E80 was acquired from Lipoid GmbH (Ludwigshafen, Germany) and Glycerin from Romical (Be'er-Sheva, Israel). [^{3}H]-Tacrolimus, Ultima-Gold® liquid scintillation cocktail and Solvable® were purchased from Perkin- Elmer (Boston, MA, USA). Polyvinyl Alcohol (PVA, Mowiol 4–88) was acquired from Efal Chemical Industries (Netanya, Israel); PLGA (50:50 blend of lactic acid:glycolic acid) 50K (Mw 50 kDa) was purchased from Lakeshore Biomaterials (Birmingham, AL, USA) and PLGA 100K (Mw 100 kDa) from Lactel® (Durect Corp., AL, USA). Macrogol 15 hydroxystearate (Solutol® HS15) was kindly donated by BASF (Ludwigshafen, Germany). (2-Hydroxypropyl)-β-cyclodextrin (HPβCD) was from Carbosynth (Compton, UK). ELISA kits for cytokines determination were obtained from R&D Systems Inc. (Minneapolis, USA). Phosphate-buffered saline was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). All organic solvents were HPLC grade and purchased from J.T Baker (Deventer, Holland).

3. Methods

3.1. Preparation of the nanocarriers

To identify the optimal nanoformulation in terms of stability and high tacrolimus content, three different nanocarriers, nanoemulsions (NEs) [30], nanospheres (NSs) and nanocapsules (NCs) [31] were prepared. The differences in their formulation process resided in the lack of oil component in the NSs whereas the NEs did not contain polymer. These nanocarriers were formulated via the well-established solvent displacement method [32]. Briefly, poly (lactic-co-glycolic acid) (PLGA) (60 mg, 50/100 kDa) were dissolved in acetone (10 mL). Castor oil (100 mg) and the various surfactants Tween®80/ Cremophor®EL/Lipoid®E80, were introduced to the organic phase in varying concentrations and combinations together with Tacrolimus (5 or 10 mg). The organic phase was then poured into the aqueous phase containing Solutol® HS15 (25/50 mg) or PVA (140 mg). The

volume ratio between the organic and aqueous phases was 1:2 v/v. The colloidal dispersions were stirred at 900 rpm for 15 min and concentrated by reduced pressure evaporation to 10 mL. Blank (BLK) nanocarriers, without tacrolimus, were prepared by the same respective method. The most stable colloidal dispersions (without aggregates or debris) were then mixed with the cryoprotectant (2-Hydroxypropyl) - β-cyclodextrin (HPβCD) at a PLGA: HPβCD ratio of 1:10 and further lyophilized by an Epsilon 2–6D freeze-drier (Martin Christ GmbH, Germany). For particle size and distribution, drug content determination and encapsulation efficiency assay, lyophilized powder (20 mg) was first rehydrated in water (2.5 mL) and further analyzed as the NCs dispersions. For *ex vivo* corneal permeation studies, radiolabeled reconstituted NCs were prepared following the same protocol by mixing [^{3}H]-Tacrolimus (3 μL) in the organic phase. Radiolabeled control was obtained by mixing the same amount of [^{3}H]-Tacrolimus to castor oil.

3.2. Physicochemical characterization of the NCs

3.2.1. Size and PDI—The mean diameter and size distribution of the various NCs were measured by Malvern's Zetasizer instrument (Nano series, Nanos-ZS) at 25°C. Each formulation (10 μL) was diluted in water (990 μL) and measured in triplicate. The same protocol was used for the lyophilized powder after redispersion in water.

3.2.2. Morphology—Tacrolimus NCs were visualized by transmission electron microscopy (TEM) using a JEM-1400 plus 120 kV (JEOL Ltd., Tokyo, Japan) apparatus. Specimens were mixed with uranyl acetate for negative staining and dried at room temperature.

3.2.3. Osmotic pressure—Lyophilized NCs were reconstituted with 2.25% w/v glycerin in water, the isotonic vehicle used throughout this study, to obtain the final ophthalmic product. Osmotic pressure was determined by a 3MO Plus Micro Osmometer (Advanced Instruments Inc., Massachusetts, USA).

3.2.4. Drug content—The total tacrolimus content was quantified by mixing either fresh colloidal dispersion or the reconstituted one (100 μL) in acetonitrile (900 μL) (ACN) and injecting the samples into an HPLC system equipped with a UV detector (Dionex Ultimate 300, Thermo Fisher Scientific). Co-elution of the tacrolimus and the formulation components in the analytical method was rule-out (no difference before and after lyophilization was noted in the recovery of tacrolimus against the appropriate calibration curve). Acetonitrile allowed complete dissolution of the NCs before lyophilization. After lyophilization, the solvent successfully extracted tacrolimus when the sample was already reconstituted in water. Using a 5 μm Phenomenex C18 column (4.6×150 mm) (Torrance, California, USA) kept at 60°C, a 95:5 v/v mixture of ACN: water as mobile phase and a flow rate of 0.5 mL/min, tacrolimus was detected at the wavelength of 213 nm with a retention time of 5.1 min. The tacrolimus content was calculated using a calibration curve constructed from drug concentrations ranging from 0 to 200 μg/mL, which yielded a linear correlation (r^2 = 0.999). The reference standard was the drug dissolved at a concentration of 100μg/mL, giving a 100.1% assay, confirming the acceptable manufacturer specifications for tacrolimus assay, that were between 98% and 102%, including both tautomers.

3.2.5. Encapsulation efficiency (EE)—For encapsulation efficiency (EE) determination of fresh/reconstituted NCs, 1 mL formulation was placed in a 1.5 mL capped polypropylene tube (Beckman Coulter) and ultra-centrifuged at 45 000 rpm for 40 min at 4°C (Optima MAX-XP ultracentrifuge, TLA-45 Rotor, Beckman Coulter). Supernatant (100 μL) in ACN (900 μL) were injected into the HPLC system using the same analytical method. The percentage of the drug encapsulated in each formulation was calculated according to the following equation:

$$\mathrm{EE}\ (\%) = \frac{C_t - C_f}{C_t} \times 100\%$$

with C_t, the total drug concentration in the sample and C_f, the free drug concentration in each sample.

3.3. *Ex vivo* study of tacrolimus corneal permeability

To identify the most appropriate formulation for animal studies, ex *vivo* corneal permeability studies were performed using Franz diffusion cells (Permegear Inc., Hellertown, PA, USA) maintained at a constant temperature (35±1°C) with circulating water. The device had two diffusion pools and a diffusional area of 1 cm^2. Porcine eyes were obtained from Lahav Animal Research Institute (Kibbutz Lahav, Israel). The enucleated eyes were kept in a saline solution on ice during transportation and used within 3 hours. Freshly excised pig corneas were carefully placed between the donor and the receptor pool. Radiolabeled reconstituted NCs/castor oil solution (150 μL) was placed in the donor pool on the corneal epithelium. The receptor medium consisted of freshly prepared Dulbecco's phosphate-buffered saline (PBS) (8 mL) (pH = 7.0) mixed with 10% ethanol. 24 h from the beginning of the experiment, the distribution of radioactivity-labeled [^{3}H]-Tacrolimus was determined in several compartments. First, the remaining formulation on the corneal surface was collected by serial washes with the receptor medium. The cornea was then chemically dissolved with Solvable® in a water bath kept at 60°C until obtaining complete tissue disintegration. Finally, aliquots of the receptor fluid were collected. Radiolabeled tacrolimus was determined in Ultima-gold® scintillation liquid in a Tri-Carb 4910 TR beta counter (Perkin Elmer, USA).

3.4. Tacrolimus nanocapsules stability evaluation

Stability evaluations of both lyophilized nanocapsules (NCs) and reconstituted colloidal NCs dispersion in the isotonic vehicle (2.25% w/v glycerin in water), were performed. The NCs dried powder was divided into samples of 300 mg which were kept sealed and protected from light for 3 months in controlled conditions at 4±2°C, 25±2°C/60%RH±5%RH and 40±2°C/75% RH±5% RH. The powder was analyzed at the starting point (T_0) and after 1, 2 and 3 months. At the end of each period, powder from the relevant sample was re-dispersed in water. The NCs stability was assessed by measuring the mean diameter, particle-size distribution and drug content using the protocols described above.

The isotonic, reconstituted, colloidal NCs dispersion, was kept at 4°C and 25°C and analyzed for 28 days at different time points. After 1, 3, 7, 14 and 28 days, NCs stability was

evaluated by measuring the osmolarity of the colloidal dispersion and the various parameters described above. The actual HPLC method succeeded to differentiate the drug content results at different storage time and temperatures by analysis of the central tacrolimus peak.

3.5. *In vivo* PK studies

3.5.1. Single dose PK study—To assess the tacrolimus distribution and concentration in the various eye compartments, a single-dose PK study was carried out. Thirty healthy male New Zealand white rabbits weighing 2.0–2.5 kg, were provided by Eurofins Advinus Limited, Bengaluru, India. The study protocol was approved by the Institutional Animals Ethics Committee (IAEC) of said company (Proposal No. 020/Apr-2019). Rabbits were randomly assigned to groups, one receiving tacrolimus MCT oil solution (TAC SOL) and the other receiving reconstituted tacrolimus nanocapsules (TAC NCs). For this study, castor oil was not chosen due to the difficulty involved in its filtration and sterilization for rabbit eye instillation. Rabbits were administered a single topical ocular administration of TAC NCs or TAC SOL (0.1%) at a fixed dose of 50 μL in each eye (right and left). Blood samples were collected from the marginal ear vein at pre-determined time intervals at 1 h, 2 h, 4 h, 8 h and 24 h post-dose, from 3 animals at each sampling timepoint and stored at 2–8°C until analysis. Following blood collection, animals were euthanized by intravenous administration of thiopentone sodium solution (20 mg/kg). Both eyes were harvested and snap-frozen. The eye parts (Aqueous humor, Retina and Choroid, Iris and Ciliary body, Cornea and Conjunctiva) were collected and stored below −60°C until their bioanalysis. The whole-blood and tissue samples were evaluated for tacrolimus using the fit-for-purpose LC-MS/MS method described below, with a lower limit of quantification of 0.545 ng/mL. The HPLC system comprised of a Shimadzu Prominence HPLC equipped with an LC-10ADvp pump, SIL-HTC autosampler. All chromatograms were recorded using the Analyst 1.6.3 workstation software (AB SCIEX, USA). The stationary phase was reverse-phase C8 Hypurity Advance (4.6 × 50 mm, 5 μm, Thermo Scientific™, USA) column. The mobile phase consisted of 2 mM ammonium bicarbonate in water (A) and methanol (B) (30:70, V:V) at a flow rate of 1.0 mL/min. The column oven was set to 40°C. The injection volume was 10 μL. Tacrolimus and the Internal Standard (IS) Tacrolimus 13CD2 were monitored by an API 4000 triple quadrupole mass spectrometer (AB SCIEX, USA) with an electrospray ionization source (ESI). Multiple reaction monitoring (MRM) with positive ionization mode was used for the mass analysis. MS conditions were set as follows: capillary voltage 5500 V and nebulizer gas (nitrogen) pressure 45 psi. Gas temperature was set to 525°C. The *m/z* of ion pairs used for monitoring were 821.6 → 768.6 for tacrolimus and 824.5 → 771.5, 824.5→721.5 for tacrolimus 13CD2 (IS). The collision energies were 25.0 and 28.0 V for tacrolimus and 30.0 V for IS. The cell accelerator voltage for the drug was 20.0 V and 25.0 V for IS. The quantitative analysis of tacrolimus in rabbit ocular tissues was based on the peak area ratio (peak area of the drug versus peak area of IS). Drug pharmacokinetic parameters were calculated using the non-compartmental analysis tool of the validated Phoenix WinNonlin® (Version 8.0) software. The area under the concentration-time curve (AUC_{last}) was calculated by the linear trapezoidal rule from time zero to the time of last quantifiable concentration. Peak concentration (C_{max}) and time for the peak concentration (T_{max}) were the observed values. Tissue to whole-blood exposure ratios were calculated and reported.

3.5.2 Multiple -dose PK study—A multiple-dose PK study was also performed on rats. The rat was selected instead of the rabbit because of the attempt to correlate the tacrolimus levels in the various segments of the eye with the efficacy effects in the well-established Experimental Autoimmune Uveitis model carried out in female Lewis rats as described in the *in vivo* model method below. Twenty-four healthy female Sprague Dawley rats (Hylasco Bio-Technology Pvt Ltd, India) weighing 200–270 g, were used to evaluate and compare the pharmacokinetics of tacrolimus (TAC) in whole blood and ocular tissues following multiple ocular administration of 0.1% reconstituted TAC NCs. The study protocol was approved by the Institutional Animals Ethics Committee (IAEC) of said company (Proposal No. 019/Apr-2019). Rats were randomly divided into 4 groups corresponding to the evaluated time points 0, 2, 4 and 8 h (6 animals per time point, per group). 10 μL of 0.1 % (w/v) tacrolimus NC formulation, were topically instilled into each eye (right and left) for seven days, daily, three times a day (2.5 h±20 min) under mild isoflurane anesthesia. Blood samples were collected at 0, 2, 4 and 8 h, post last dose without pooling. At each time point, approximately 0.5 mL of blood were drawn from each animal's retro-orbital sinus and stored at 2–8°C until plasma separation by centrifugation, within 30 min of sample collection. After blood collection, animals were sacrificed through deep isoflurane anesthesia and both eyes were harvested and snap frozen. The eye parts (Aqueous humor, Iris and Ciliary body, Cornea and Conjunctiva) were pooled from three rats, so for every group, at each time point, there were two samples available for analysis. The plasma and tissue samples were stored below −60°C until their bioanalysis for tacrolimus, using the LC-MS/MS method described above with some modifications. All chromatograms were recorded using the Analyst 1.7 workstation software (AB SCIEX, USA). The injection volume was 5 μL. Tacrolimus and the Internal Standard (IS) Tacrolimus 13CD2 were monitored by an API 4500 triple quadrupole mass spectrometer (AB SCIEX, USA) with an electrospray ionization source (ESI). Multiple reaction monitoring (MRM) with positive ionization mode was used for the mass analysis. MS conditions were set as follows: capillary voltage 5500 V and nebulizer gas (nitrogen) pressure 35 psi. Gas temperature was set to 550°C. The *m/z* of ion pairs used for monitoring were 821.6→768.6, 821.6→786.6 for tacrolimus and 824.5→771.5, 824.5→721.5 for tacrolimus 13CD2 (IS). The collision energies were 25.0 V for tacrolimus and 20.0 V for IS. The cell accelerator voltage for both the drug and IS was 20.0 V. The quantitative analysis of tacrolimus in rat ocular tissues was based on the peak area ratio (peak area of the drug versus peak area of IS). The pharmacokinetic parameters of Tacrolimus were calculated using the same method described in the single dose administration study protocol.

3.6. *In vivo* efficacy studies

3.6.1. LPS induced keratitis—Forty wild type female C57BL/6 (10 weeks old) mice were obtained from the Jackson Laboratory. All mice were maintained in pathogen-free conditions and had free access to food and drinking water. The Cleveland Clinic's Institutional Animal Care and Use Committee (IACUC) approved all animal studies (Proposal No. 2020–2324). The anesthesia cocktail was a mixture of ketamine (50 mg/kg) and dexmedetomidine (0.38 mg/kg). Anesthesia reversal consisted of an atipamezole solution (3.75 mg/kg). Animals were randomly assigned to the five different study groups. The results of four combined experiments are presented. Each time, ten mice were randomly

divided into three groups (n=3/4) to evaluate the efficacy of the different treatments in decreasing the inflammation induced by LPS. Lyophilized Blank (BLK) NCs were reconstituted in iso-osmolar vehicle containing 2.25% glycerin in water. Lyophilized TAC NCs were reconstituted with the same vehicle to obtain a drug concentration of 0.1%. Free tacrolimus was dissolved in castor oil to obtain the same concentration. PBS/LPS-injected groups received the vehicle only.

Mice were anesthetized by intraperitoneal injection of the anesthesia cocktail (50 μL). Intrastromal injections of *Pseudomonas aeruginosa* LPS (0.5 μg) in PBS (1 μL) or PBS only were performed on mice, as previously described with slight changes [33]. Briefly, a small tunnel from the corneal epithelium to the central stroma was created using a 27-gauge needle. Another 33-gauge needle attached to a 5 μL Hamilton syringe (Hamilton Co., Reno, NV) was passed through the tunnel into the central cornea, where the contents of the syringe were injected. Mice were treated topically with the various formulations (2 μL) immediately after the PBS/LPS injections, and one hour after. 30 min after the second treatment, the mice were injected with an anesthesia reversal cocktail (50 μL). 24 h after LPS injection, mice were euthanized in CO_2 chambers and corneas were immediately dissected.

To measure cytokine production on the eye surface, individual corneas were homogenized in 180 μL PBS 1% BSA, using a Precellys 24 homogenizer (Bertin Technologies SAS, Montignyle-Bretonneux, France) for 30 seconds at 6500 rpm. A 25 μL aliquot was assayed by ELISA according to the manufacturer's directions (R&D Systems).

3.6.2. Experimental Autoimmune Uveitis (EAU)—Thirty female Lewis rats (8 weeks old), obtained from Charles River, were used for efficacy study assessment in EAU model. The study protocol was approved by Iris Pharma Internal Ethics Committee (DAP17). All animals were treated according to Directive 2010/63/UE of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were maintained in pathogen-free conditions and had free access to food and drinking water.

The animals used for this study were selected based on good health and absence of significant ocular defect using a scale previously described [34]. They were further assigned to the 3 study groups that were subjected to EAU induction and received either per os Cyclavance® (1 mL/kg), the positive control, topical reconstituted BLK NCs as negative control or 0.1% F1 TAC NCs from Day 7 to endpoint on Day 15. Oral treatment was administered once daily when topical ones (10 μL) were applied four times a day (q 2.5 h ±20 min) in both eyes.

Animals were anesthetized by an intramuscular injection of a mix Ketamine (25 mg/kg)/ Xylazine (5 mg/kg) before EAU induction. On Day 0, ocular inflammation was induced on anesthetized animals by a footpad injection of rat S-antigen (100 μg/100 μL) in Freund's complete adjuvant (2 mg/mL H37Ra) and an intraperitoneal injection of pertussis toxin (1 μg/100 μL). The onset of disease is generally observed between Days 9 and 12 after

immunization and the peak of the disease occurs in Days 16–20. The study was stopped on Day 16 when the control group showed inflammation with a mean severity score ≥3.5.

Clinical examinations and histological analysis were performed in a masked manner and scored using a semi-quantitative scale.

Both eyes of each rat were examined with a slit-lamp at baseline and then every day from Day 12 to Day 15 using adapted scale [28] provided in the SI, section 1.1.

After examination of anterior segment, one drop of Mydriaticum® (0.5%) was instilled for pupillary dilatation and posterior segment examination according to the scale in the SI, section 1.2. Visualization of the fundus was possible only if the anterior chamber did not become moderately to severely inflamed. Immediately after euthanasia on Day 16, both eyeballs of animals from each group were fixed in Davidson fixative (Iris Pharma, La Gaude, France), dehydrated and embedded in paraffin wax. They were further cut into three sections of 5–7 μm (one central, one temporal and one nasal), spanning over the whole retina, and stained with hematoxylin and eosin (H&E). Samples were examined under light microscope (Leica Microsystems, Wetzlar, Germany) at a 20x magnification and further analyzed by the Leica Application Suite software version 4.4. Retinal morphology and cell infiltration were evaluated and scored in the three sections for each eye, using the histolopathological examination scale described in the SI section 1.3.

3.7. Statistical analysis

The effects of treatments *ex vivo* in pig corneas and *in vivo* in LPS induced keratitis model were assessed using one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's test. In EAU model, evaluation of the clinical and histological scores was achieved using the Kruskal Wallis non-parametric test followed by post-hoc Dunn's test. All the statistical analysis was performed on the GraphPad Prism 8.3.0. software. The significant threshold was fixed at $P<0.05$.

4. Results and discussion

Over the last decade, tacrolimus efficacy has been clinically well assessed and confirmed in many topical ocular, allergic disorders studies, mainly in Japan, including a one year use report of a marketed 0.1% aqueous suspension product (Talymus®) in severe atopic keratoconjunctivitis [35].Tacrolimus is also widely used as an off-label marketed ointment product designed for atopic dermatitis, surprisingly found to be effective and safe in anterior segment inflammatory disorders [14]. Various other ocular delivery systems for tacrolimus, irrespective of the indication, were reported. In a novel nano-vesicle system (proglycosomes) containing 0.1% tacrolimus, the authors showed enhanced drug penetration following single dose administration in rabbits. However, no multiple dose ocular safety assessment or long-term stability study were exhibited [36]. Furthermore, there are reports on PEG-PLGA tacrolimus nanoparticles although no stability study was performed [37] and on tacrolimus entrapped in polymeric micelles where its chemical stability was assessed for a maximum of 4 weeks at 4°C and 25 °C. At the end of this period at 25 °C, the tacrolimus content decreased by 25% from the original drug concentration while it was still stable at

4°C [38]. Another delivery system developed was a solution of 0.03% tacrolimus in Semi-Fluorinated Alkanes, containing 1.4% ethanol, tested on uveitis[39]. However, no stability data or ocular tolerability were reported. Moreover, it should be mentioned that ethanol was shown to disrupt the integrity of corneal epithelium and induce inflammation in corneal cells[40]. Furthermore, very recently, a group of Spanish hospital pharmacists have developed non-toxic 0.02% tacrolimus ophthalmic aqueous formulations which could be safely prepared in hospital pharmacy departments for the treatment of uveitis and were shown to be stable for at least 3 months in refrigeration [41]. From these reports it can be deduced that, to the best of our knowledge there is still no tacrolimus formulation with enough prolonged stability to become in the future a commercially viable product.

In this study, following a thorough investigation of nanoemulsions, nanospheres and nanocapsules, only a stable tacrolimus-loaded nanocapsule delivery system meeting the adequate physicochemical properties for stability was identified. This nanocarrier, tested first *ex vivo* for corneal permeation as a screening test for the different formulations, improved the drug solubility in the oil core, corneal absorption and efficacy *in vivo*.

4.1. Physicochemical characterization of the NCs

Many formulations, differing in PLGA molecular weight, drug concentration and surfactant, were prepared to evaluate the influence of these different parameters on particle size and encapsulation efficiency (EE). All attempts to formulate tacrolimus in NSs (nanospheres) failed because tacrolimus could not be entrapped within the solid formed polymeric nanomatrix while NEs (nanoemulsions) could not retain tacrolimus within the oil nanodroplets, resulting in the formation of aggregates after a few hours. Thus, the use of oil to dissolve tacrolimus together with PLGA polymer envelop seemed to be essential to obtain a stable product. To this latter end, nanocapsules were prepared. The formulations presented in Table 1 were selected based on their ideal physicochemical parameters: their small particle size (106–166 nm), narrow polydispersity index (≤0.1) and their high encapsulation efficiency (61–80%). The tacrolimus loading was between 2.6–5.1%w/w, the highest being in F1. F1, F3 and F4 included Solutol® HS15 in the aqueous phase, whereas F2 had PVA. Other differences were in drug concentrations and PLGA molecular weight. The main parameters that affected the encapsulation efficiency were the type and concentration of the surfactants used. The lowest EE (61%) was obtained with the composition containing Lipoid® E80 in the organic phase, the highest concentration of tacrolimus in the oil and Solutol® HS15 in the aqueous phase as shown in F4 formulation (Table 1). This is probably due to drug micellization above the surfactant CMC, limiting its encapsulation within the oil core PLGA NCs. All the NCs had a size of under 200 nm. It should be noted that particles smaller than 1000 nm can permeate the cornea through endocytosis [25]. Additionally, microparticles of over 10 μm, intended for ocular use, cause itching and eye irritation [25]. Moreover, to achieve colloidal dispersion stability, the polydispersity index, representing the particles' size distribution, must be lower than 0.2. These critical parameters were all fulfilled by the selected formulations (Table 1), highlighting their potential to maintain stability, increase drug penetration through ocular tissues and avoid causing patients eye discomfort.

To prevent both tacrolimus and PLGA degradation in the aqueous environment over time, NCs were lyophilized [42,43]. An adequate lyophilization method would have to meet three necessary criteria: the intact cake is to occupy the same volume as the original frozen mass; the appearance of the reconstituted NC dispersion is to be homogeneous and without aggregates; and finally, upon appropriate aqueous reconstitution, the NCs' initial physicochemical properties are to be maintained [44].Numerous parameters affect the resistance of NCs to the stress imposed by lyophilization, including the type and concentration of the cryoprotectant [44]. Cryoprotectants at variable ratios were screened to satisfy the required criteria. The concentration of these compounds in the final reconstituted product, per tested ratio, was considered to comply with FDA requirements. Hydroxypropyl-β-Cyclodextrin (HPβCD) was the only compound to produce a good cake and a quick redispersion in aqueous solutions. The best lyophilization results were obtained for F1 (Table 1) with the PLGA: HPβCD ratio of 1:10. Indeed, similar sizes were obtained before and after the process, 143.9 and 172.8 nm respectively, along with a PDI under 0.2. In addition, the F1 encapsulation efficiency remained similar after freeze-drying with HPβCD as confirmed by the ultracentrifugation process described in the methods section 3.2.5, followed by the tacrolimus determination in the sediment before and after lyophilization. This result suggested that the drug remained in the NCs without distributing in the HPβCD.

Morphological examination by TEM revealed high resemblance before and after freeze-drying and redispersion in water (Figure 1A and 1B, respectively) without signs of aggregation. NCs displayed spherical aspect and nanometric size with an estimated coating thickness of few nanometers, characteristics which were maintained after freeze-drying, revealing the success of the process.

4.2. *Ex vivo* study of tacrolimus corneal permeability

In order to assess and differentiate the potential of these NCs to be retained in the cornea and permeate the tissue, radiolabeled formulations and oil solution control were applied *ex vivo* on porcine corneas having a morphology close to the human eye [45]. The results reported in Figure 2 exhibit the concentration of [^{3}H] -Tacrolimus in the cornea (Figure 2A) and in the receptor compartment (Figure 2B) following topical application of [^{3}H]-Tacrolimus-loaded reconstituted NCs and oil control after 24 h incubation. Although viability of the cornea lasts few hours, many investigators have used the excised corneas over different time intervals rather for comparing formulations than reflecting the actual penetration through a viable cornea [36,38,46].

Thus, the rationale behind this *ex vivo* cornea experiment was only to find the optimal formulation for the *in vivo* experiments. As expected, part of the tacrolimus dissolved in oil was retained in the cornea, most likely in the lipophilic layers of the epithelium, because of the relatively high octanol/water partition coefficient of the drug (logP 3.3). However, this amount was negligible in comparison to the NCs retained in the cornea by more than 40-fold (Figure 2A, $p<0.0001$), probably due to the small particles' ability to be trapped in the tight junctions connecting the corneal epithelial cells [2]. No significant differences were noted among the four formulations tested, differing mainly in the PLGA molecular weight and surfactant type. In the receptor part, representing a deeper compartment of the eye, NCs

concentration was 12-fold higher than that of the oil solution group (Figure 2B, $p<0.0001$) indicating their capacity to cross the cornea barrier. Similar results were obtained before the lyophilization of the NCs (data not shown), suggesting that HPβCD did not impact the drug absorption through the cornea. Therefore, this positive effect may be fully attributed to the nanocarrier. It should be pointed out, as previously shown, that the increase in the molecular weight of the polymer does not affect the penetration rate of the loaded drug even between 4KDa and 100KDa [47]. In addition, with the same PLGA polymer molecular weight, two different contents were compared, 5 mg (F3) and 10 mg (F4) meaning 0.05%w/v and 0.1%w/v tacrolimus, respectively. Surprisingly, the amounts of tacrolimus retained in the cornea or diffused in the receptor compartment were not significantly different. The probable reason for such a finding is the burst effect of tacrolimus once the nanocapsules are applied on the cornea. In fact, the recovery calculations after 24 hours showed that the amount of tacrolimus in the washed fractions of F1 (0.1%) and F4(0.1%) were similar but much higher than in the washed fractions of F3 with half concentration (0.05%). This was further confirmed in an animal study of keratitis, where three tacrolimus concentrations were tested 0.03, 0.05 and 0.1% and no significant differences in the efficacy were noted as shown in the SI section 2 (Figure S1).

Moreover, formulations 1, 3 and 4 were significantly better than formulation 2 in passing through the various layers of the cornea (depending on the NCs, $p<0.001$, $p<0.0001$) demonstrating that the surfactant used in the aqueous phase effectuated the extent of cornea permeation, where F2 was the only formulation containing PVA instead of Solutol® HS15. These two latter compounds have different chemical structures, which could affect corneal penetration. Indeed, PVA is a synthetic polymer whose hydrophobic fraction forms a network on the PLGA polymer surface [48], altering the surface hydrophobicity of the nanoparticles. This alteration can affect the cellular uptake of these particles [49], a mechanism involved in ocular penetration [50]. In contrast, Solutol® HS15 is a polyoxyethylene ester of 15-hydroxystearic acid whose monomers can partition within the membrane bilayer of the corneal epithelial cells, perturbing the packing order of the phospholipids and creating gaps for the nanocapsules to permeate the epithelium [51]. Therefore, the decreased penetration of F2 may be due to a reduction in corneal epithelium uptake occurring when PVA-containing colloidal drug delivery systems are applied topically to the eyes [52]. Among colloidal dispersions 1, 3 and 4, equivalent in terms of corneal retention and permeation, F1 was chosen for lyophilization and further analysis based on its small particle size (149 nm), and for its highest encapsulation efficiency (80%). Following reconstitution, tacrolimus concentration in the F1 colloidal dispersion oil core was 1000μg/ml, a 500-fold increase compared to the drug solubility in water[8], and higher than the solubilization of tacrolimus reaching 700μg/mL in the Marinosolv® micelle formulation[16]. The F1 TAC NCs allow the achievement of higher doses, in the same instilled volume, although these are not similar formulations.

4.3. Stability study

Following 3 months storage of the lyophilized formulation at 4, 25 and 40°C, the drug content remained essentially unchanged compared to the initial concentration, as shown in Table 2. Moreover, the NCs kept their initial physical characteristics with a mean diameter

of 172 nm and a PDI of 0.12 following reconstituted colloidal dispersion, revealing the long-term stability of the F1 NCs powder.

Previous studies, in which tacrolimus was formulated in nanospheres, reported maximal stability of the lyophilized powder at 25°C for one month [53]. The lyophilized powder formulation in this study elicited better results and protected tacrolimus more efficiently from external environmental conditions, probably because of the different surfactants and oil core in which our drug was dissolved. The actual stability data were confirmed by the immediate analysis of the reconstituted F1 colloidal dispersion using a USP stability indicating test described in the SI, section 3. The results presented, support the preliminary stability data reported in this manuscript not only for the tacrolimus NCs in the lyophilized powder but also for the Blank NCs which did not show any peak at the relevant retention times (Fig.S2). These findings indicate that the BLK NCs components did not elicit any alteration in the analytical method since a flat chromatogram was observed (Fig.S2, blue). In addition, the chromatogram exhibited in Fig. S3 showed that following 18 months storage at 25±2°C/60%RH±5%RH conditions, no impurity or degradation byproduct of tacrolimus, respectively, ascomycin and tacrolimus-8 epimer, were detected.

Ophthalmic formulations must have a similar osmolarity to that of tears, approximately 300 $mOsm{\cdot}L^{-1}$, to avoid eye tissue irritation. Thus, lyophilized F1 NC powder was re-dispersed in the isotonic vehicle, stored in the dispersion state and analyzed at different time points. For the 28 days of storage in controlled conditions, the slightly acidic pH of the dispersion did not change. Moreover, as reported in Table 3, osmolality, mean diameter, PDI and content were constant for 14 days at 25°C or 28 days at 4°C, demonstrating the required ocular stability of the final reconstituted product. These results could probably be due to the presence of the polymeric shell and oil core in the F1 TAC NCs, representing additional protective barriers to hydrolysis between the drug and the aqueous medium. In a recent study of an aqueous tacrolimus formulation stored at 25 °C, the drug concentration dropped at 7 and 28 days respectively to 88% and 41% of its initial value [18] whereas at the same time periods and storage temperature, the drug concentration in the F1 reconstituted colloidal NC dispersion was respectively 97.5% and 84.2% (Table 3).

4.4. *In vivo* PK studies

4.4.1. Single-dose PK study—To compare the ocular tissue distribution of tacrolimus oil solution (TAC SOL) and the encapsulated drug (F1 TAC NCs), a preliminary pharmacokinetic study was performed on male New Zealand White rabbits. Following a single topical ocular administration of the same drug amount, the tacrolimus concentration C_{max}, in the various tissues was different between the two groups as reported in Table 4. In the TAC SOL group, drug exposure (AUC), in ascending order, was in the Aqueous Humor (AH), Vitreous Humor (VH), Iris and Ciliary body, Retina and Choroid, Conjunctiva and Cornea. In the NCs group, however, the lowest AUC was in the Vitreous Humor, then, in ascending order, in the Aqueous Humor, Retina and Choroid, Conjunctiva, Iris and Ciliary body and the highest being in the Cornea.

Indeed, the NCs' small size allows better interaction with the corneal epithelial tissue [2], leading to their increased retention on the eye surface and to higher drug concentration in

this tissue (Figure 3A). Moreover, as reported [50], this property encourages transcellular transport through the corneal epithelium which leads to better absorption of the drug in deeper ocular compartments such as the aqueous humor (Figure 3B) and the iris and ciliary body (Figure 3C). Some researchers have suggested that a concentration of 2–10 ng/mL in the aqueous humor might be considered as the minimal effective concentration for controlling immune-mediated eye diseases [19]. Our NCs maintained drug concentration within that therapeutic range for almost 8 hours, contrary to the TAC SOL which did not reach such levels via topical instillation. In the TAC SOL group, despite achieving the highest cornea drug exposure among the different ocular tissues, the AUC value was three times lower in comparison to the NCs. Vitreous Humor (Figure 3E), Retina and Choroid (Figure 3F) and Whole blood (Figure 3G) were equally exposed to the drug after a single instillation. In view of the protocol used for the determination of the concentration of tacrolimus in the various eye compartments, it was not possible to make any statistical analysis comparison of the findings between the F1 TAC NCs and the TAC SOL. Nevertheless, it can be noted from the results depicted in Figure 3, A–C, that the NCs elicited higher drug levels than the oil solution with a very marked difference. The advantage of these NCs was more pronounced in the anterior chamber than in the posterior chamber where drug levels were approximately similar, as depicted in Figure 3 D–F. The formulations showed no differences regarding the tacrolimus Whole blood concentrations as shown in Figure 3G.

Our 0.1% F1 TAC NCs exhibited much higher C_{max} drug concentrations in the Cornea, Aqueous humor, Iris and Ciliary body, Vitreous humor and Retina and Choroid compartments than previously reported for a 10-fold more concentrated 1% tacrolimus suspension containing the free drug in a micronized form [54]. Another PK study of tacrolimus nanoparticles reported cornea drug levels of 56 ng/g after 24 h [53], with a tacrolimus concentration of 0.03%, while the F1 TAC NCs reached a concentration of 93 ng/g at the same timepoint.

Thus, from these preliminary results, F1 TAC NCs seem to have a better potential to improve therapeutic effect in anterior inflammatory diseases of the eye in comparison to the drug solution in oil. Furthermore, oil solutions such as the one used in this study, cannot serve as a therapeutic option because of vision blurring, eye irritation and patients' difficulty of use[55]. In contrast, the F1 TAC NCs exhibited good tolerability and safety in rabbits following single and multiple-dose instillations (SI, section 4 and 5 respectively). It should be emphasized that all the ingredients used in the F1 TAC NCs optimal formulation are FDA/EMA approved [56] and fall also in the allowed range of concentrations. In addition, reports have shown that drug-loaded nanoparticles can be less cytotoxic than the free dissolved drug on human retinoblastoma cell line. These results were further confirmed by ocular *in vitro* and *in vivo* tolerance assays [31].

4.4.2. Multiple - dose PK study—The multiple-dose PK study was performed in rats. The rat was selected instead of the rabbit because of the attempt to correlate the tacrolimus levels in the various segments of the eye with the efficacy effects in the well-established Experimental Autoimmune Uveitis model on female Lewis rats as described in the *in vivo* model method above. To assess and compare the pharmacokinetics of tacrolimus in plasma,

reflecting systemic drug absorption, and in anterior chamber ocular tissues, multiple ocular administrations of the F1 TAC NCs, were performed on Sprague Dawley Rats. As presented in Table 5, the overall drug exposure, AUC_{last}, consisted of a mere few units (3.43 h.ng/mL) in plasma whereas in the ocular tissues, hundreds were quantified.

Moreover, as showed in Figure 4, the drug concentrations in plasma remained very low and negligible after repeated dose administration, in agreement with human data following one-year use of tacrolimus suspension [57]. In contrast, in all the analyzed anterior chamber ocular tissues, detectable drug levels were measured. At T_0, on the 7^{th} day of administration, all the compartments had tacrolimus trough concentrations confirming the local drug accumulation after multiple dosing with steady-state levels. After last administration, as presented in Table 5, the highest concentrations, C_{max}, were reached after two hours in Cornea (378 ng/mL), Iris and Ciliary body (436 ng/mL), and Conjunctiva (740 ng/mL) and after four hours in the Aqueous Humor (243 ng/mL). Beyond this time point, tacrolimus concentrations decreased, although they remained, after 8 hours, equal to or higher than the tacrolimus trough concentrations (Figure 4). A previous study, evaluating tacrolimus ocular distribution after multiple-dose administration of a 0.3% suspension (q.i.d, at 3 h intervals), revealed the same steady-state effect reached after 7 days of drug topical instillation [48]. In contrast, even though the suspension used in said study was of higher tacrolimus concentrations and administered more times per day, our TAC NCs engendered a 3-fold increase in C_{max} drug levels in the Aqueous Humor (70 vs 240 ng/ml) and Conjunctiva (250 vs 740 ng/ml). In addition, slightly higher concentrations were found in the Iris and Ciliary body (250 vs 436 ng/mL). These results demonstrate the F1 TAC NCs' tendency to accumulate and act locally in the anterior eye chamber after multiple dose administration, exposing the various compartments to tacrolimus with little to no systemic drug absorption and subsequent adverse effects.

It should be emphasized that in the PK studies, we could only determine the total tacrolimus concentration. It was impossible to distinguish between the released drug and the remaining tacrolimus in the NCs in the various tissues. There was no other alternative to answer this issue but to carry out efficacy studies and evaluate the effect of progressive release and availability of tacrolimus from the NCs.

4.5. *In vivo* effect of the NCs on ocular inflammation

All the above results were the reason to carry out pharmacodynamical evaluations on murine keratitis and Experimental Autoimmune Uveitis (EAU) models to gain insight on the effective concentration of tacrolimus released from the NCs against the inflammation.

4.5.1. LPS induced keratitis—To evaluate the effect of the tacrolimus F1 NCs compared to the free drug dissolved in oil, a sterile corneal inflammation model induced by purified *Pseudomonas aeruginosa* LPS was performed on the wild type C57BL/6 mouse strain. *P. aeruginosa* is one of the most commonly found causative organisms in microbial keratitis. The results presented in Figure 5 are the concentrations of (A) KC, (B) MIP-2, (C) IL-6 and (D) GCSF, inflammatory cytokines induced in this model [58–60], measured in mice corneas lysates. Levels of all four markers were significantly reduced by the F1 TAC

NCs compared to the LPS untreated group ($p<0.01$ for MIP-2, $p<0.0001$ for KC, IL-6 and GCSF), whereas no statistically significant difference was noticed between LPS untreated and TAC OIL groups ($p>0.05$). Moreover, the data for TAC NCs was significantly different from the TAC OIL ($p<0.01$ for KC, MIP-2, GCSF and $p<0.05$ for IL-6).

In this animal model, *P. aeruginosa* LPS activates toll-like receptor 4 (TLR4)/MD-2 pathway on corneal epithelial cells and on resident and infiltrating immune cells in the corneal stroma [61], inducing expression of pro-inflammatory and chemotactic cytokines such as KC[62], MIP-2[63], IL-6 and GCSF [64]. MIP-2 and KC, thought to be functional analogs of IL-8 (human), are potent chemoattractants recruiting polymorphonuclear cells (PMN) from limbal capillaries to the corneal stroma in *P. aeruginosa*-infected corneas [62,64]. During infection, PMNs are required to clear bacteria from affected tissue, yet their persistence contributes to irreversible tissue destruction [63]. Indeed, these immune cells produce reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) disrupting the cornea's collagen matrix and leading to a loss of corneal clarity and damaged vision [65]. The persistence of PMN in C57BL/6 cornea is regulated by CD4 T cells. Both inflammatory cells play roles in tissue destruction leading to severe corneal ulceration and perforation [63].

While our model focused on assessing the early inflammatory response within 24 hours after bacterial ligand challenge, tacrolimus, which can suppress T cell activation, and subsequent inflammatory cytokines production [66], coupled with its ability to promote apoptosis and decrease the viability of PMNs and monocytes cells [67], has the pharmacological capacity to eliminate the various actors in the inflammation response induced in microbial keratitis. As seen in Figure 5, tacrolimus dissolved in oil slightly decreased inflammatory markers, probably by treating the inflammation response of the corneal epithelial cells and the resident macrophages and dendritic cells present there [68–73], yet this effect was not significant. Indeed, to obtain a major anti-inflammatory effect against the immune cells recruited to the stroma[58], the drug has to penetrate the highly selective epithelium barrier, a challenge possible to overcome when the tacrolimus is encapsulated in NCs, as revealed by their superior effect in comparison to the TAC oil solution (Figure 5). These NCs can either follow a paracellular pathway through the tight junctions between the corneal epithelial cells or undergo transcellular endocytosis due to their small size[2,25], providing higher drug exposure into the cornea as previously reported in Table 4.

In addition, in the present study, although a total resolution of inflammation and efficacy was not demonstrated, our NCs significantly diminished inflammatory markers of the innate immune host response found to be critical for inflammation resolution and subsequent decrease in the harmful effects to the cornea[64]. It can be deduced from the results of this experiment that TAC NCs released effective concentration of tacrolimus, resulting in a significant decrease in the inflammatory markers tested at the end of the experiment period. Altogether, our tacrolimus nanocapsules seem to have great potential to treat inflammation induced in LPS keratitis and possibly other anterior eye inflammatory disorders responsive to the drug.

4.5.2. Experimental Autoimmune Uveitis (EAU)—To assess the therapeutic effect of the reconstituted F1 TAC NCs on intraocular posterior inflammation, a rat model of EAU was induced. At baseline, no ocular findings were detected by slit lamp examination in all the groups. As exhibited in Figure 6A, administrations of the F1 TAC NCs significantly reduced ocular inflammation from Day 12 to Day 15 compared to the BLK NCs group ($p<0.05$). On day 15, the mean clinical scores of the F1 TAC NCs and BLK NCs groups were respectively 0.9±1.3 and 3.9±1.2 with an incidence of 25 and 70%. As expected, no ocular findings were observed for Cyclavance®, the positive control group. Cyclavance® inhibited EAU development, $P<0.05$ on Day 12, and $P<0.01$ from Day13 to Day 15 versus BLK NCs group.

Fundus examinations performed after slit lamp examinations have demonstrated as follows. In the BLK NCs group, normal fundus was observed in 17 eyes of 20 on Day 12, whereas on Day 15, it was only possible to observe a normal fundus in 7 eyes of 20, due to the substantial inflammation causing myosis. In contrast, in the F1 TAC NCs group, while 1 eye was not evaluated due to myosis, a normal fundus was observed in 14 out of 20 eyes during this study. 5 eyes exhibited abnormal retinal morphology elicited by the induction of the inflammation, where adequate tacrolimus therapeutic concentrations were not achieved locally. As expected, in Cyclavance® group, a normal fundus was noticed for all eyes.

In the late phase of EAU, the clinical score alone is not sufficient for efficacy evaluation since it pertains only to the anterior inflammation. In addition, visualization of the fundus is limited because of myosis. Histopathological scoring as described in the SI section 1.3, would therefore be a more suitable method to evaluate retinal pathology. As shown in Figure 6B, histological analysis of the eyes, taken 16 days after immunization, revealed a significant decrease in both inflammatory infiltration into retina as well as retinal damage in groups treated with the F1 TAC NCs (mean score: 1.4±1.4, $P<0.05$) and Cyclavance® (mean score: 0, $P<0.001$) compared to the BLK NCs group (mean score: 5.0±1.0). Indeed, apart from its known immunosuppressive effect, tacrolimus also modulates neuroprotective genes in the retina during ongoing EAU, leading to preservation of the retinal architecture [74]. Additionally, F1 TAC NCs and oral Cyclavance® groups were not found statistically different ($P>0.05$).

Nevertheless, it is worth mentioning that topical cyclosporine applications, at concentrations that would not cause to systemic side effects, did not inhibit EAU development [75]. Typical representative pictures of the histological evaluation for each treatment group are shown in Figure 6C. In the BLK NCs group, fibrous deposits with inflammatory cells were observed. Moreover, the retina layers were destroyed. In contrast, in the F1 TAC NCs group, histological preparations exhibited a normal retina with organized layers. The vitreous and sclera did not show any abnormalities as well. Similar findings were observed in the positive control Cyclavance® group.

The significant effect of TAC NCs on the retina should be attributed to a local rather than a systemic effect since the mean concentration-time profile of tacrolimus in the plasma following multiple topical ocular administration of F1 TAC NCs (0.1%) in rats was negligible (Figure 4). As previously exhibited in Figure 3F, single topical instillation of the

TAC NCs on rats, led to a tacrolimus concentration of over 50 ng/g in the retina and choroid for 24 hours. Consequently, we assume that sufficient therapeutic tacrolimus levels were reached in the eye posterior chamber by simple topical instillation of our NCs. By this mode of administration, there are two alternative pathways, driven by the physico-chemical properties of the drug, to attain adequate concentrations in the posterior segment of the eye. Hydrophilic compounds will prioritize the lateral route to penetrate through the highly vascularized conjunctiva, reach the sclera and uvea, and further back to the posterior chamber. Contrarily, small lipophilic compounds would predominantly permeate the anterior chamber via the transcorneal pathway, reach the aqueous humor and penetrate the tight junctions sealing the ciliary body and iridial pigment epithelium to arrive at the posterior chamber [76,77].

This hypothesis is further confirmed by the results of the multiple-dose PK in rats showing a concentration in the iris/ciliary body and conjunctiva of 436 and 740 ng/g (Table 5). Nevertheless, the previously described single-dose pharmacokinetic rabbit studies (Figure 3) of the lipophilic tacrolimus encapsulated in NCs, exhibited high drug levels in the Cornea, Aqueous humor, Iris and Ciliary body but also in the Conjunctiva. These results exhibit the characteristic of the NCs to hinder the drug properties enabling drug accumulation and possibly intraocular penetration through both pathways to reach the posterior part of the eye and deliver therapeutic tacrolimus levels. The overall findings clearly point out the contribution of the NCs to enhance tacrolimus penetration to the remote and hardly accessible posterior retinal tissue, enabling the delivery of therapeutic drug concentration in at least 70% of the treatment group eyes. Previous studies performed on another rat models of EAU revealed tacrolimus capacity to reduce both anterior and posterior inflammation when injected intravitreally as a solution or encapsulated in liposomes [78,79]. However, there is only one other report of topical tacrolimus efficacy in another rat model of EAU, difficult to compare to our data due to the different experimental conditions used [39].

As such, ocular clinical and histopathological findings both imply that simple topical application of our 0.1% tacrolimus nanocapsule eye drops significantly reduced EAU development under the experimental conditions of the study, a therapeutic effect that could avoid painful intravitreal injections or oral systemic treatments leading to dangerous adverse effects.

5. Conclusions

In the present study, we successfully designed and optimized nonirritant and stable tacrolimus PLGA nanocapsules exhibiting adequate characteristics for ocular application. This delivery system increased drug retention in the cornea and improved penetration to deeper ocular compartments *ex vivo* on porcine corneas and *in vivo* both on rabbit and rat eyes, enabling a superior anti-inflammatory effect on the anterior chamber LPS-induced keratitis model in comparison to the drug in oil solution. Further studies carried out to assess the effects of these NCs on the severe posterior chamber EAU model showed significant improved pharmacological and histological efficacy. These promising results could reduce the use of treatments like steroids, painful intravitreal injections and systemic oral treatments, all causing severe side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

NEs	Nanoemulsions
NSs	Nanospheres
NCs	Nanocapsules
PDI	Polydispersity index
AUC	Area Under the Curve
EAU	Experimental Autoimmune Uveitis

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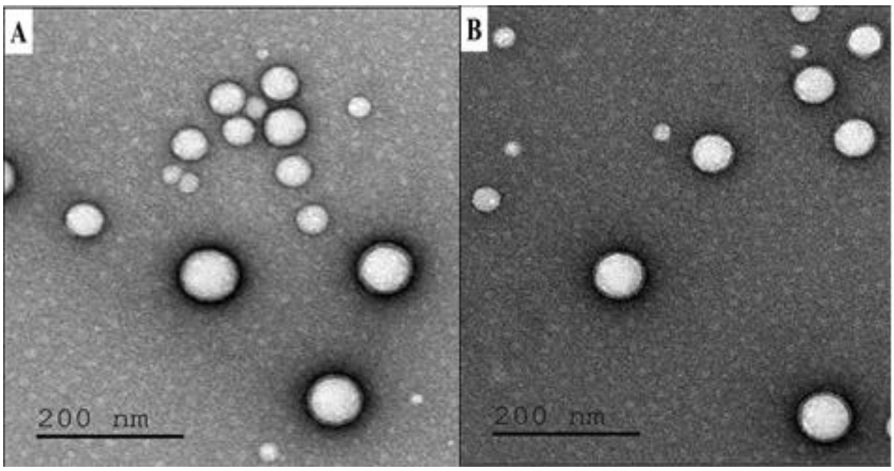

Figure 1.
TEM pictures of F1 tacrolimus-loaded nanocapsules before (A) and after (B) lyophilization following aqueous reconstitution.

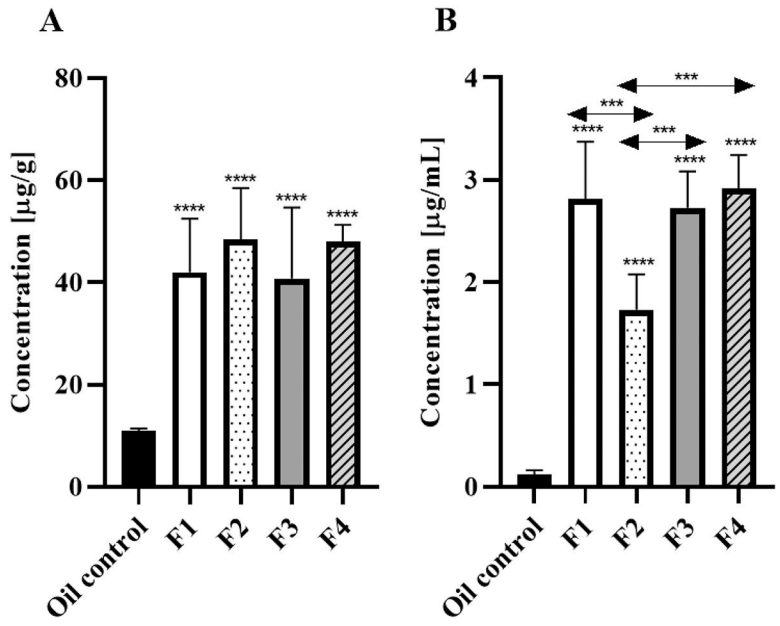

Figure 2.

Tacrolimus concentration in the cornea (A) and in the receptor fluid (B) 24 h following incubation of NCs and oil control. F1, F3, and F4 contain different combinations of surfactants whereas F2 contains one surfactant and PVA as described in Table 1. Values represent mean+SD based on six replicates. ****P<0.0001 between all the NCs and oil control. ***P<0.001 for other comparisons.

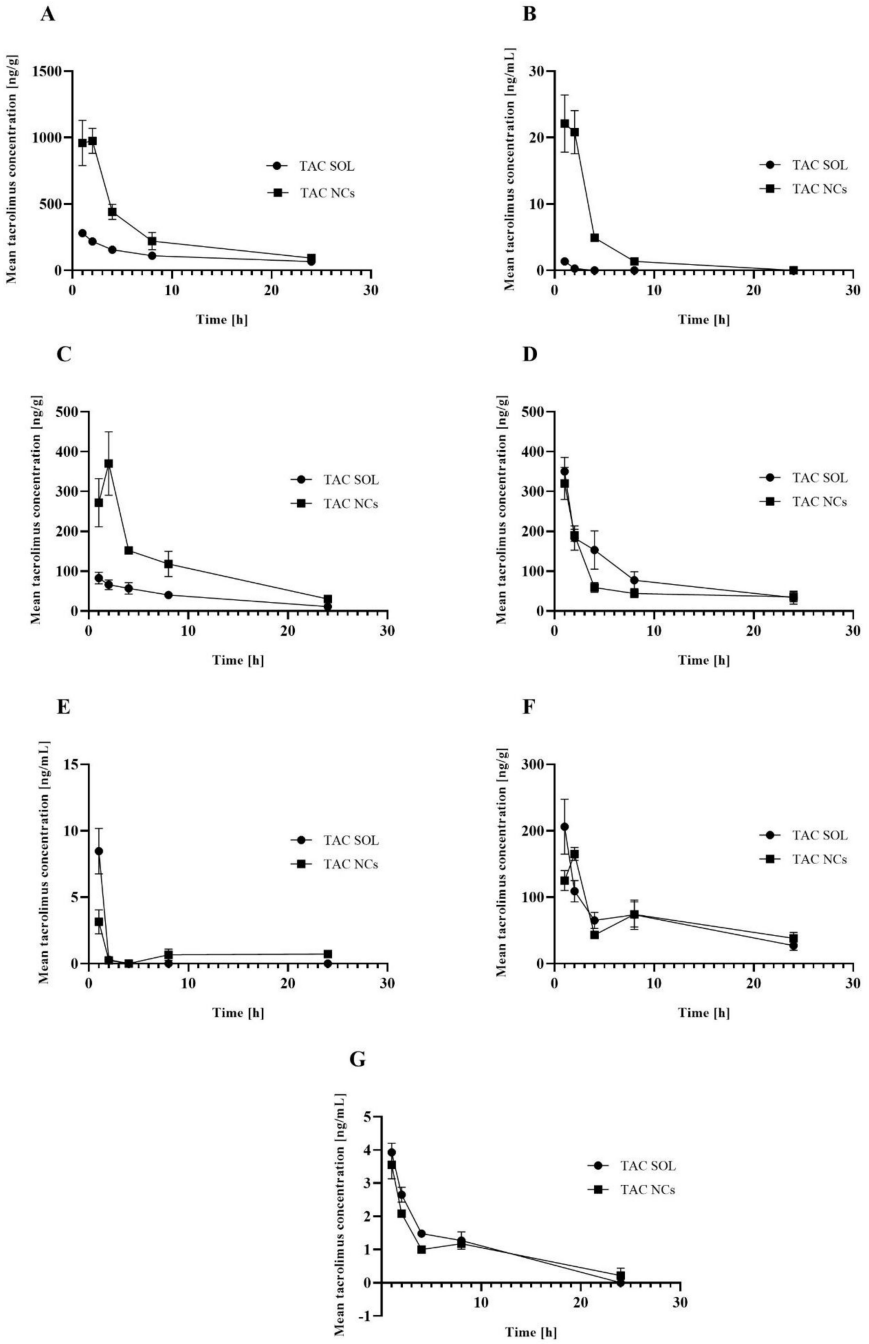

Figure 3.

Mean concentration-time profile of Tacrolimus in the various eye compartments: (A) Cornea; (B) Aqueous Humor; (C) Iris and Ciliary Body; (D) Conjunctiva; (E) Vitreous Humor; (F) Retina and Choroid and (G) in the Whole Blood, following single topical ocular administration of F1 TAC NCs and TAC SOL (0.1%) in male New Zealand White Rabbits. Error bars represent the SEM obtained from 3 animals at each time point.

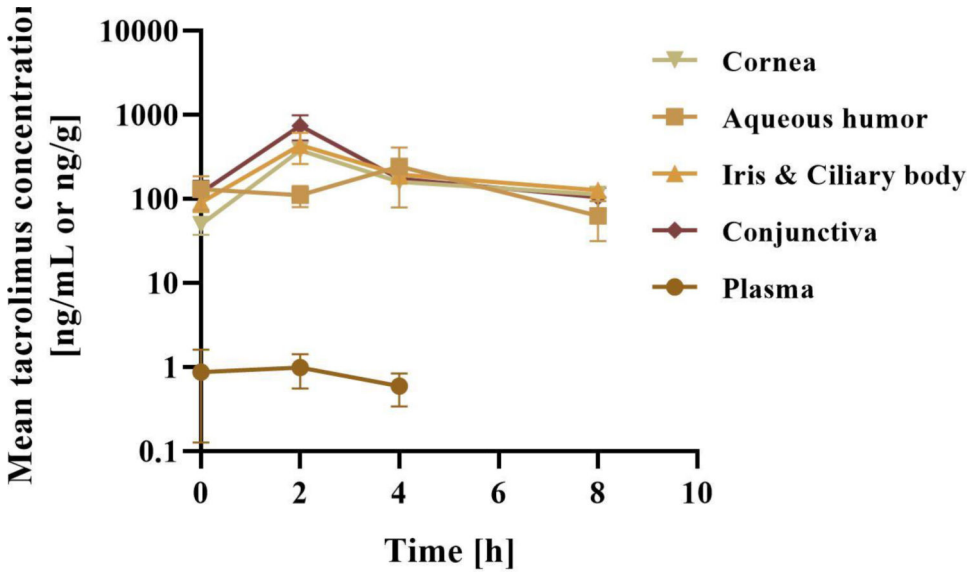

Figure 4.

Mean concentration-time profile of Tacrolimus in the various eye compartments: Cornea; Aqueous Humor; Iris and Ciliary Body; Conjunctiva and in the Plasma following multiple topical ocular administration of F1 TAC NCs (0.1%) in female Sprague Dawley Rats. Samples were collected at time points post last dosing on the 7th day. Error bars represent the SEM obtained from 6 animals in Plasma and from 2 pooled groups of 3 animals at each time point for the eye compartments.

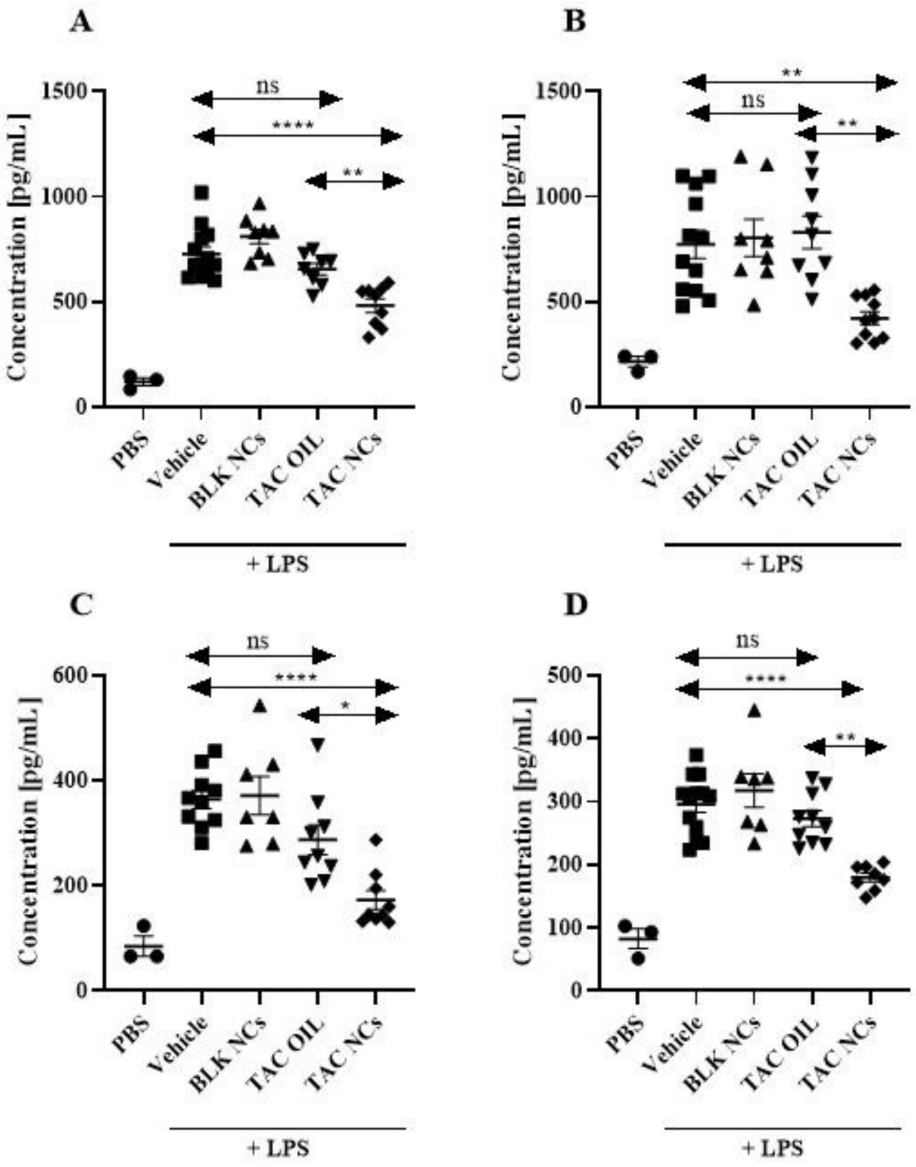

Figure 5.

Effect of 0.1% Tacrolimus formulations on (A) KC, (B) MIP-2, (C) IL-6, and (D) GCSF cytokines production in murine corneas after 24h. BLK NCs correspond to the blank nanocapsules without the drug, TAC NCs, the F1 TAC formulation These graphs represent the merged results of four repeated experiments. The overall P value by one-way ANOVA is <0.0001 for all cytokines. Other P values are derived from Tukey post hoc analyses. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data are shown as mean ± SEM

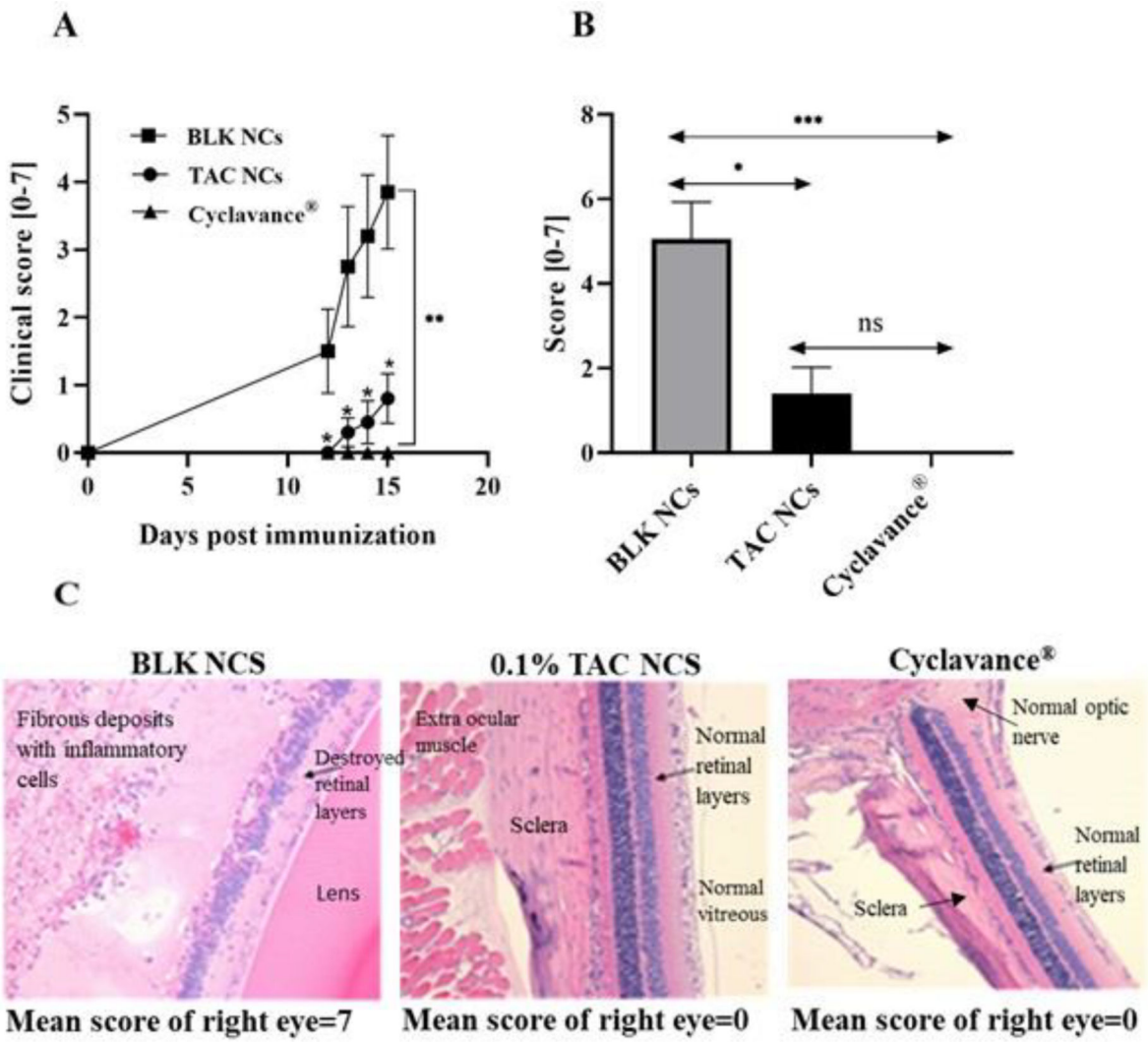

Figure 6.

Clinical and histological evaluations of S-Ag immunized rats treated with BLK NCs, TAC NCs or Cyclavance®. (A) Slit lamp biomicroscopy, *P<0.05 TAC NCs vs BLK NCs and **P<0.01 Cyclavance® vs BLK NCs. (B) Histopathological grading of retinal inflammation, *P<0.05 TAC NCs vs BLK NCs and ***P<0.001 Cyclavance® vs BLK NCs. Data represent mean ± SEM of 10 animals per group. (C) Representative light-microscopic images of the retina in the different treatment groups.

Table 1.

Composition and physicochemical parameters of the selected tacrolimus nanocapsules formulations (amounts in mg). All formulations contain 100 mg of castor oil.

Formulation	PLGA MW [kDa]	Tacrolimus	Tween® 80	Cremophor® EL	Lipoid® E80	Solutol® HS15	PVA	Mean diameter ±SD [nm]	PDI	EE [%]
F1	100	10	25	-	-	25	-	143.9±1.5	0.08	80
F2	50	5	-	25	-	-	140	165.7±0.9	0.08	79
F3	50	5	25	-	-	25	-	165.1±1.3	0.1	79
F4	50	10	-	-	30	50	-	106.5±2.1	0.09	61
BLK	100	-	25	-	-	25	-	147.7±1.2	0.1	-

Table 2.

Physicochemical characteristics of the lyophilized F1 NCs powder stored at 4, 25 and 40°C and reconstituted immediately prior to analysis. Drug content values represent the mean±standard deviation.

	T0	1 month			2 months			3 months		
		4°C	25°C	40°C	4°C	25°C	40°C	4°C	25°C	40°C
Content [%]	100±0.2	98.3±0.4	97.7±1.3	97.9±1.6	99.3±0.4	98.9±0.96	97.0±1.3	100.8±0.29	99.45±0.05	97.14±0.07
Diameter [nm]	172.8	149.4	153.1	191.6	188.1	167.8	173.9	153	147.1	172.3
PDI	0.123	0.108	0.106	0.125	0.132	0.121	0.135	0.097	0.082	0.122

Table 3.

Physicochemical characteristics of the colloidal F1 NCs dispersion reconstituted in the isotonic vehicle and kept at 4 and 25°C. Drug content values represent the mean±standard deviation.

	Day 0	Day 1		Day 3		Day 7		Day 14		Day 28	
		4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C
Content [%]	100±0.9	96.7±1.5	96.9±0.8	98.9±0.8	100.3±0.9	97.7±0.3	97.5±0.8	96.0±0.9	91.2±0.4	100.3±0.0	84.2±0.4
Mean diameter [nm]	159.2	157.9	158.6	158.7	161.3	161	161.2	157.3	164.1	162.2	166.3
PDI	0.11	0.11	0.099	0.12	0.12	0.11	0.11	0.12	0.07	0.11	0.1
Osmolality [mOsm/kg]	308	300	301	300	297	307	310	306	304	310	309

Table 4.

Average pharmacokinetic parameter values following single topical ocular administration of 0.1% F1 TAC NCs or 0.1%TAC SOL (50μL) in rabbits. *N=3* for each time point.

	0.1%w/v F1 TAC NCs				0.1%w/v TAC SOL			
	T_{max} [h]	**C_{max} [ng/mL] *a***	**AUC_{last} [h.ng/mL] *b***	**T/B AUC_{last} ratio**	**T_{max} [h]**	**C_{max} [ng/mL] *a***	**$AUCl_{last}$ [h.ng/mL] *b***	**T/B AUC_{last} ratio**
Whole blood	1	3.55±0.73	24.4	NA	1	3.93±0.477	14.9	NA
Aqueous humor	1	22.1±7.47	70.8	2.9	1	1.35±0.58	2.32	0.16
Retina and Choroid	2	165±17	1550	63.52	1	206±71.5	1520	102
Iris and Ciliary body	2	370±138	2700	110.6	1	82.9±25.6	843	56.5
Cornea	2	976±164	6690	274.1	1	281±47.8	2720	182.5
Conjunctiva	1	320±69.3	1740	71.31	1	420±74.1	2550	171.1
Vitreous humor	1	3.14±1.57	15.94	0.65	1	8.47±2.97	11.53	0.77

a) Considered ng/g unit for all tissues data represents mean± standard deviation

b) Considered h.ng/g unit for all tissues

T/B: Tissue to whole blood ratio of AUC_{last}; NA: Not Applicable

Table 5.

Average pharmacokinetic parameter values following repeated topical ocular administration of 0.1% F1 Tacrolimus NCs in rats at a dose of 10 μL t.i.d. (i.e., at 2.5h intervals) for 7 days. Each value of the various eye tissues represents the pooled mean of 2 groups of 3 animals. Plasma value represents the mean of 6 animals.

	0.1%w/v F1 TAC NCs			
	T_{max} [h]	C_{max} [ng/mL] [a]	AUC_{last} [h.ng/mL] [b]	T/P AUC_{last} ratio
Plasma	2	0.986±1.06	3.43	NA
Cornea	2	378±6.36	1506	439
Aqueous humor	4	243±232	1206	352
Iris and Ciliary body	2	436±250	1797	524
Conjunctiva	2	740±351	2324	678

a) Considered ng/g unit for all tissues, data represents mean± standard deviation

b) Considered h.ng/g unit for all tissues

T/P: Tissue to plasma ratio of AUC_{last}; NA: Not Applicable