

Targeting CD34⁺ cells of the inflamed synovial endothelium by guided nanoparticles for the treatment of rheumatoid arthritis

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ABSTRACT

Despite the advances in the treatment of rheumatoid arthritis (RA) achieved in the last few years, several patients are diagnosed late, do not respond to or have to stop therapy because of inefficacy and/or toxicity, leaving still a huge unmet need. Tissue-specific strategies have the potential to address some of these issues. The aim of the study is the development of a safe nanotechnology approach for tissue-specific delivery of drugs and diagnostic probes. CD34⁺ endothelial precursors were addressed in inflamed synovium using targeted biodegradable nanoparticles (tBNPs). These nanostructures were made of poly-lactic acid, poly-caprolactone, and PEG and then coated with a synovial homing peptide. Immunofluorescence analysis clearly demonstrated their capacity to selectively address CD34⁺ endothelial cells in synovial tissue obtained from human, mouse, and rat. Biodistribution studies in two different animal models of rheumatoid arthritis (antigen-induced arthritis/AIA and collagen-induced arthritis/CIA) confirmed the selective accumulation in inflamed joints but also evidenced the capacity of tBNP to detect early phases of the disease and the preferential liver elimination. The therapeutic effect of methotrexate (MTX)-loaded tBNPs were studied in comparison with conventional MTX doses. MTX-loaded tBNPs prevented and treated CIA and AIA at a lower dose and reduced administration frequency than MTX. Moreover, MTX-loaded tBNP showed a novel mechanism of action, in which the particles target and kill CD34⁺ endothelial progenitors, preventing neo-angiogenesis and, consequently, synovial inflammation. tBNPs represent a stable and safe platform to develop highly-sensitive imaging and therapeutic approaches in RA targeting specifically synovial neo-angiogenesis to reduce local inflammation.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease affecting joints and extra-articular structures characterized by persistent synovial tissue inflammation [1–3] that leads to joint swelling and pain, as the main clinical symptoms, associated with cartilage damage and bone destruction followed by joint ankylosis [4,5]. The use of disease-modifying anti-rheumatic drugs (DMARDs), in particular methotrexate (MTX) [6,7], and the introduction of biological therapies have significantly improved RA outcomes [8]. Despite the success of these therapeutic approaches, some patients have to stop treatment due to inefficacy or side effects [9,10]. This leads to the discontinuation of therapy [11,12] and indicates the clinical need of new therapeutic targets, but also to improve drug bioavailability in order to decreasing total dose and/or injection frequency with the potential to reduce toxicity and improve compliance to long-term treatment.

Nanotechnology approaches have already been attempted as possible solutions to satisfy these medical needs [13–15] by employing anti-rheumatic drug-encapsulated nanoparticles that exploit the enhanced permeability retention (EPR) effect for their biodistribution [16]. Although this EPR effect increases the concentration of nanoparticles at sites of inflammation where blood vessels are leaky, while the lymphatic system has reduced draining function, it does not ensure selective biodistribution to deliver the contents to pathological tissues only. Guided nanoparticles coated with the peptide RGD, which is capable of targeting the α V β 3 integrin [17–19], have already been investigated in order to circumvent this problem. However, the expression of this integrin is not joint specific [20,21], which consequently allows for extra-articular nanoparticle accumulation [18].

Here, we report the use of targeted biodegradable nanoparticles (tBNPs) functionalized with a cyclic peptide that are able to specifically target the microvasculature of inflamed synovial tissue. The peptide was identified by L. Lee et al. [22] and subsequently developed for selective delivery of recombinant antibodies to inflamed synovial tissue [23]. We demonstrate, in two different animal models, that the tBNPs delivering system is superior to non-functionalized nanoparticles, and able to detect early inflammatory processes otherwise unnoticeable by physical evaluations. Furthermore, MTX-loaded tBNPs were an effective treatment in both animal models, increasing drug bioavailability, allowing reduction in both administration frequency and dosage compared to MTX and thus reducing potential drug toxicity. Lastly, we demonstrated a novel mechanism of action of tBNPs-MTX for RA treatment, which involves the ability of these particles to target CD34⁺ endothelial cell progenitors, inhibiting their proliferation and therefore blocking the process of neoangiogenesis that is essential to initiate and sustain inflammation in the joints.

2. Materials and methods

2.1. Nanoparticle production

Core-shell biodegradable nanoparticles were produced under class 100 clean-room conditions using a proprietary electrohydrodynamic technology (Bio-Target Inc., Chigaco, IL, USA; LNK Chemsolutions LLC, Lincoln, NE, USA). Their structure was composed of carboxylic acid-terminated biodegradable polymers (PLA-b-PEG-COOH and PCL-COOH). To produce tBNPs, a fraction of PLA-b-PEG-COOH was activated by N-hydroxysuccinimide to conjugate the targeting molecule by exploiting its primary amino groups. The method to produce these nanoparticles was extensively described in the patent US20080187487A1. Briefly, an organic solution containing the polymers of the shell flowed in the external wall of a hollowed tube, while the core fluid containing MTX (TEVA, UK) flowed in the interior tube. Nanoparticles were formed at the apex of the tube, which was under an electric potential. Nanoparticles produced were then resuspended in an aqueous buffer solution to obtain a stable suspension.

2.2. Statistical analyses

Measures of the mean, standard deviation (SD), standard error of the mean (SEM) and statistical analyses are indicated in each figure legend. P-value of < 0.05 is considered statistically significant.

2.3. Animal models and ethical approval

All the procedures were carried out in accordance with the national and international laws on the use of experimental animals (L.D. 26/2014, Directives 2010/63/EU) and with the ARRIVE guidelines. Sample sizes for all animal experiments were predetermined using G-Power [24] software providing a power of 0.8, 10% signal differences between groups and assuming 5% of SD using two-tailed t-tests. Few animals were excluded from the studies due to a poor arthritis development (in our experience we obtained 80–90% of arthritis incidence) or abnormalities. All the animals were randomly enrolled in the experimental groups and data collected blindly by two operators. Number of animals enrolled in the experiments are indicated in each figure legend.

2.4. AIA model

AIA was induced in male Wistar Hannover rats (Envigo, San Pietro al Natisone, Italy) weighing 120–150 g. All the experimental studies have been conducted under total anesthesia by intraperitoneal injection of Zoletil (Virbac, Sintra, Portugal)(25 mg/kg) and Xylazine (Pharmavet, Sassari, Italy)(7.5 mg/kg). The immunization was performed with two intradermic injections (the second one after one week) of 150 μ g of mBSA (Sigma) in a 0.9% NaCl physiologic solution and emulsified with 200 μ L of complete Freund adjuvant (CFA) (Sigma). Fourteen days after the second immunization, inflammation was induced by injecting 100 μ g of mBSA (diluted in 100 μ L of physiologic solution) in the intraarticular right knee of the rats [25]. The contralateral knee joint was injected with physiological solution as a control.

To verify that rats enrolled in the study developed an immune response against mBSA, an ELISA was used. mBSA (Sigma, 5 μ g/mL, 100 μ L/well) was used for well 4 μ m. Plate was blocked with 2% skim milk in PBS before incubation with serum samples (diluted 1:2000). Rat IgG binding was analyzed using biotin-labeled rabbit anti-rat (Dako, Santa Clara, USA-1:4000), horseradish peroxidase-conjugated streptavidin (Sigma-1:2000) and 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma).

2.5. Study of particle distribution in the AIA rat model

The Explore Optix MX pre-clinical optical imaging system (GE Healthcare) equipped with a pulsed laser diode and a time-correlated single-photon detector was used to evaluate the biodistribution of 4.8 nmol of BNP-Cy5.5 and tBNPs-Cy5.5 diluted in physiologic solution injected in the tail vein 2 h after the intraarticular injection of mBSA. A blank image was acquired before intravenous injection of labeled nanoparticles. The animals were monitored at multiple time points using a 670 nm pulsed laser diode with a repetition frequency of 80 MHz and a time resolution of 12 light pulses. Fluorescence emission was collected at 700 nm and detected through a fast photomultiplier tube and a highly sensitive time-correlated single-photon counting system. Two-dimensional scanning regions of interest were selected and laser power, integration time and scan step were optimized according to the signal emitted. The data were recorded as temporal point-spread functions, and the images have been reconstructed as fluorescence intensity and fluorescence lifetime. After 23 days, the animals were euthanized under total anesthesia. The organs were finally analyzed *ex vivo* and then embedded in biocompatible gelatinous matrix Tissue Tek OCT (BioOptica, Milano, Italy) and stored at -80 °C. Blood samples were collected for subsequent analysis.

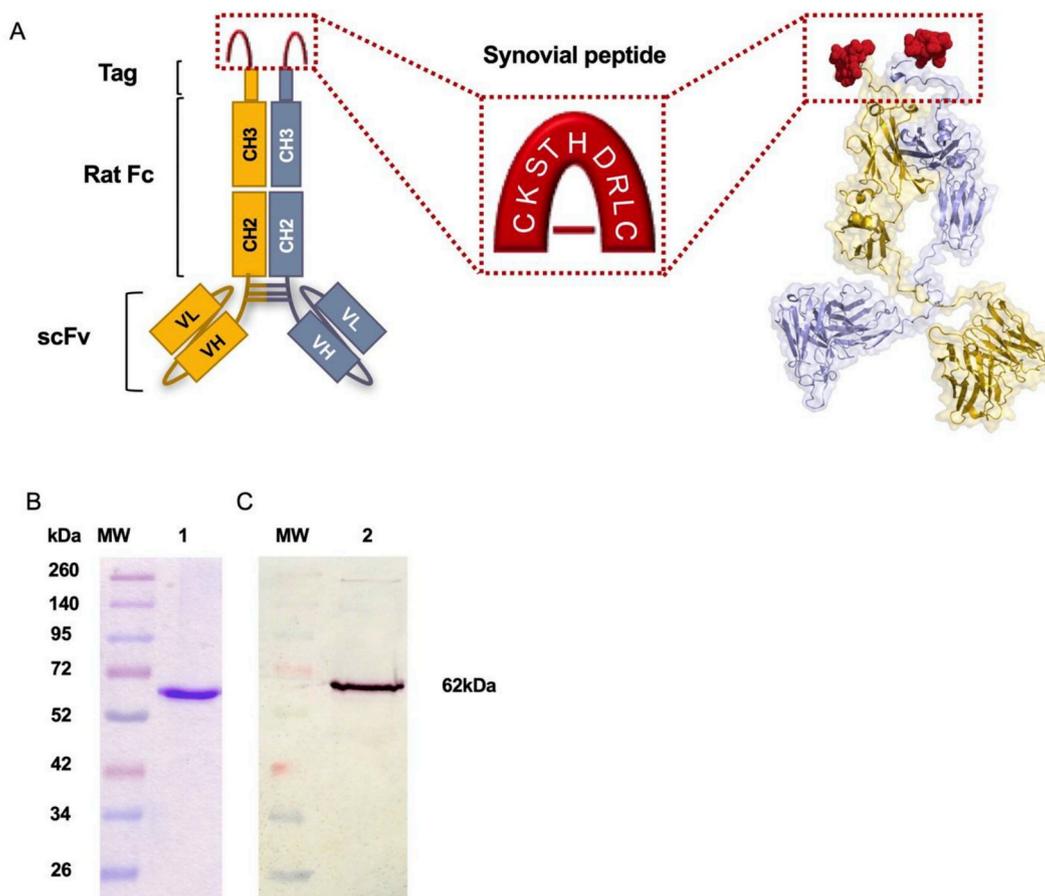


Fig. 1. Schematic representation of the targeting agent and the nanoparticles. (A) Left: schematic representation of the targeting molecule made of a non-correlated ScFv (VL-VH) linked through a hinge to the rat Fc IgG2b (CH2-CH3). In turn, the Fc is fused with an SV5 tag (stripes motif), which ends with the synovial peptide (red). Middle: schematic representation of the synovial peptide, which assumes the circular shape due to the presence of the flanking cysteines. (Red box). Right: 3D model of the targeting molecule modeled with Modeller. The two chains of the dimer are colored in light blue and gold, respectively. In the red boxes the synovial peptide. SDS-PAGE conducted in reducing conditions and analyzed by Coomassie staining (B) and Western Blot (C) of the targeting molecule (lane 1 and 2) show a single band with the expected molecular weight (62 kDa). (D) 3D structure of one monomer of the targeting molecule. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.6. Therapeutic efficacy in the AIA rat model

MTX (1 mg/kg/day) and tBNPs-MTX diluted in physiologic solution were administered i.p. in rats developing AIA. The swelling of the knees was measured with a caliper. At the end of the study, animals were euthanized under total anesthesia, and the articular cavities of the right and left knees were washed with PBS (2 mL) in order to collect synovial fluids and infiltrated cells. Part of the synovial membranes was collected, embedded in Tissue Tek OCT and stored at -80°C , while the whole articulation was embedded in paraffin for histological analysis. Blood samples were collected for subsequent analysis.

2.7. CIA animal model

Male DBA/10IaHsd mice (7 weeks old, Envigo) were housed in a temperature and humidity-controlled facility with a 12-h light/dark cycle and with water and food freely available throughout the experimental period.

Arthritis was induced by immunization with type II collagen (Chondrex Inc., Redmond, WA, USA) dissolved in 10 mM acetic acid and emulsified with a 1/1 v/v of complete Freund adjuvant, as described by Brand et al. [26]. Each mouse was anesthetized with 3–4% Sevoflurane gas (O_2 95.0%). Mice were immunized at day 0 with 50 μL of type II collagen and were boosted with the same antigen at day 15. The progression of CIA was evaluated by macroscopic scoring 3 times

per week. A numeric score was applied for each paw using the following scale (0: no inflammation; 4: severe inflammation): 0 = no evidence of erythema and swelling, 1 = erythema and mild swelling confined to the tarsals or ankle joint, 2 = erythema and mild swelling extending from the ankle to the tarsals, 3 = erythema and moderate swelling extending from the ankle to metatarsal joints, and 4 = erythema and severe swelling encompassing the ankle, foot and digits, or ankyloses of the limb. An ELISA was performed in order to verify that all mice enrolled in the study developed an immune response against bovine collagen type II.

2.8. Study of particle distribution in the CIA mouse model

On day 28, after the first immunization with type II collagen, two groups received in the tail vein BNPs-Cy5.5 (0.5 nmol Cy5.5/mouse) as a control compound ($n = 3$) or the same amount of tBNPs-Cy5.5 ($n = 4$). Optical imaging acquisitions were performed before these injections and at the following time points post injection: 1, 6, 24, 72, 96, and 168 h. IVIS[®] Spectrum (PerkinElmer Inc., USA) was used for image acquisition (excitation wavelength at 675 nm and emission at 720 nm), and illumination settings were as follows: f-stop 2, field of views 6.6, binning medium, acquisition time 1 s (time points of 1, 6, 24, and 72 h) or 15 s (time points of 96 and 168 h). Images were acquired and analyzed with Living Image Software (Caliper Life Sciences).

All the animals were anesthetized under 3–4% Sevoflurane gas (O_2

95.0%). A region of interest was drawn by the operator over each paw of the mouse, and data were collected as radiant efficiency values. The fluorescent signal value was obtained by subtracting the radiant efficiency value measured before the injection from the signal measured post injection.

2.9. Therapeutic efficacy in the CIA mouse model

Five different groups of CIA mice (scores of 2–3) received: saline, MTX 1 mg/kg/3 times per week or 3 mg/kg/once per week, tBNPs-MTX 3 mg/kg/once per week or 1 mg/kg/once per week. All the treatments were administered i.p. for 25 days. Animals were then euthanized under total anesthesia with Zoletil (Virbac-25 mg/kg) and Xylazine (Pharmatech-7.5 mg/kg). The paws, brain, liver, kidneys, spleen, lungs and heart were embedded in Tissue Tek OCT (BioOptica) and stored at -80°C , except for the paws, which were previously fixed in 10% neutral buffered-formalin (BioOptica) for 24 h and then decalcified for 24 h with Decalcifying Solution-Lite (Sigma). Blood samples were collected for subsequent analyses.

3. Results

3.1. Design and structural characterization of biodegradable nanoparticles

To prepare the tBNPs we initially generated a targeting molecule that consists of a synovial homing peptide fused to a scFv-Fc (single chain fragment variable – fragment crystallizable) (Fig. 1A). More specifically, the synovial homing peptide was made of 9 amino acids (CKSTHDRLC) containing two flanking cysteines (Fig. 1A), which constrain the peptide in a circular shape. Coomassie staining and Western blot analysis of the molecule (Fig. 1B–C) revealed the expected molecular weight (62 kDa) and absence of contaminations or protein degradation.

Immunofluorescence analysis of rat synovial tissues incubated with the targeting molecule confirms our previous finding that it binds to inflamed synovium but not to the normal tissue (Fig. S1). Since the synovial homing peptide was first reported to bind preferentially inflamed RA synovial vasculature, synovial tissues from RA patients were double stained with both the targeting molecule and von Willebrand Factor (vWF) as a marker of endothelial cells (Fig. 2A). The results show diverse co-staining with some vessels positive for both markers, while others stained for either vWF or the targeting molecule, which was also detected on isolated cells (Fig. 2B). Interestingly, staining of the synovial tissue for CD34, which is highly expressed on vascular endothelial progenitors [27,28], revealed a strong co-localization between the targeting molecule and CD34⁺ cells (Fig. 2C–E). In particular, CD34⁺ cells were bound by the targeting molecule in close proximity to blood vessels.

The targeting molecule was used to produce two different types of core-shell BNPs made of COOH-poly(ethylene glycol)-b-poly(lactide) (PEG-b-PLA) and poly(caprolactone)-COOH (PCL) [29,30], one of which was loaded with MTX (tBNPs-MTX), while the other contained an empty core (tBNPs). Two additional types of untargeted nanoparticles containing either an empty core (BNPs) or MTX (BNPs-MTX) were generated and used as controls (Fig. 3A).

The 3D structure of the targeting molecule was modeled in silico to predict its possible orientation on the surface of the nanoparticles. In the obtained model, some primary amino groups are exposed on the molecule surface and are available for covalent binding to the nanoparticle. In Fig. 3A, three possible sites for the binding of scFv-Fc to the nanoparticle are schematically shown, corresponding to the most solvent accessible primary amines (the side chains of Lys355 and Lys369 in the Fc-CH2 domain, and the protein N-terminus). All these sites are far from the targeting peptide and are compatible with its exposure on the particle surface and, consequently, with its availability for interaction with synovial tissue.

These nanoparticles were investigated by NanoTrack analysis (Fig. 3B), which showed homogeneous average hydrodynamic diameters (≈ 170 nm) and monodispersity ($\text{Pdl} \approx 0.2$) and a very similar nanoparticle concentration (approximately $10^{10}/\text{mL}$). These results were in line with the ones obtained by dynamic light scattering (DLS, Fig. 3C). A slightly negative ζ -potential (-8 mV) was measured for all the samples. Moreover, the particle showed a round shape and a uniform polymer distribution in both transmission electron microscopy (TEM, Fig. 3D) and scanning transmission electron microscopy (STEM, Fig. 3E–F).

To further characterize the structure of MTX-loaded BNPs, we analyzed the spontaneous release of MTX from the nanoparticles, showing that, after an initial burst release in which 40% of the MTX is dispersed from the tBNPs, more than 50% of the MTX was stably encapsulated for 24 h (Fig. 3G). The same results were obtained when analyzing particles stored at -20°C for 1 year, demonstrating the stability of the nanosystems and suggesting that nanoparticles, once injected and delivered to the target, would release 50% of the drug over a period longer than 24 h (data not shown).

3.2. Functional characterization of tBNPs

The functional characterization of the BNPs started in vitro using the EA.hy926 cell line that expresses the synovial homing peptide target. These cells incubated with fluorescently labeled BNPs and tBNPs were followed for 3 h by live imaging microscopy (example in Video S1) in order to investigate their interaction with tBNPs. To assess this, the binding time of BNPs or tBNPs with endothelial cells was calculated through tracking analysis. After 1 h (Fig. 4A), an increased time of interaction was reported for the tBNPs compared to BNPs. Similar results were obtained after 3 h (Fig. 4B). These data were confirmed by ELISA, in which we incubated endothelial cells with both nanosystems for 1 h (Fig. 4C). Finally, tBNPs were visualized inside EA.hy926 cells by confocal microscopy analysis, proving that tBNPs are already internalized after 1 h of incubation (Fig. 4D and Video S2). Binding and internalization of tBNPs-MTX were found to cause the death of endothelial cells to an increased degree over free MTX, and most importantly, residual MTX that remained in the BNPs after dialysis was still able to induce the same extent of endothelial cell death (Fig. 4E).

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jaut.2019.05.016>.

The superior cell binding of fluorescent tBNPs was confirmed in rat synovial tissues, where stronger staining was found with tBNPs in inflamed synovium than in either non-inflamed synovial tissues or untargeted particles (Fig. S2).

3.3. Biodistribution of tBNPs in animal models of arthritis

After completing the in vitro characterization, tBNP behavior was studied in two animal models of RA. First, the biodistribution of tBNPs and BNPs was compared in a rat antigen-induced arthritis (AIA) model, in which monoarthritis is induced in the right knee using the left knee as control. Three days after the induction of inflammation, Cy5.5-labeled tBNPs or BNPs were injected into the animals' tail vein, and their biodistribution was followed for 23 days (Fig. 5A). The animals were scanned by near-infrared optical imaging, and a higher accumulation of fluorescent nanoparticles was observed in the right knee of animals receiving tBNPs-Cy5.5 compared to the contralateral non-inflamed knee or to the inflamed knee of animals treated with BNPs-Cy5.5. Measuring the fluorescence intensity at various time points, a progressive accumulation of tBNPs-Cy5.5 was observed in the inflamed joints, reaching a maximum value after 7–8 days (Fig. 5B). The localization of both tBNPs-Cy5.5 and BNPs-Cy5.5 in the non-inflamed left knee was negligible (Fig. 5B). The fluorescence intensity detected in the knees was related to the joint swelling (Fig. 5C) and was used as a marker to evaluate inflammation in AIA. It is important to note that

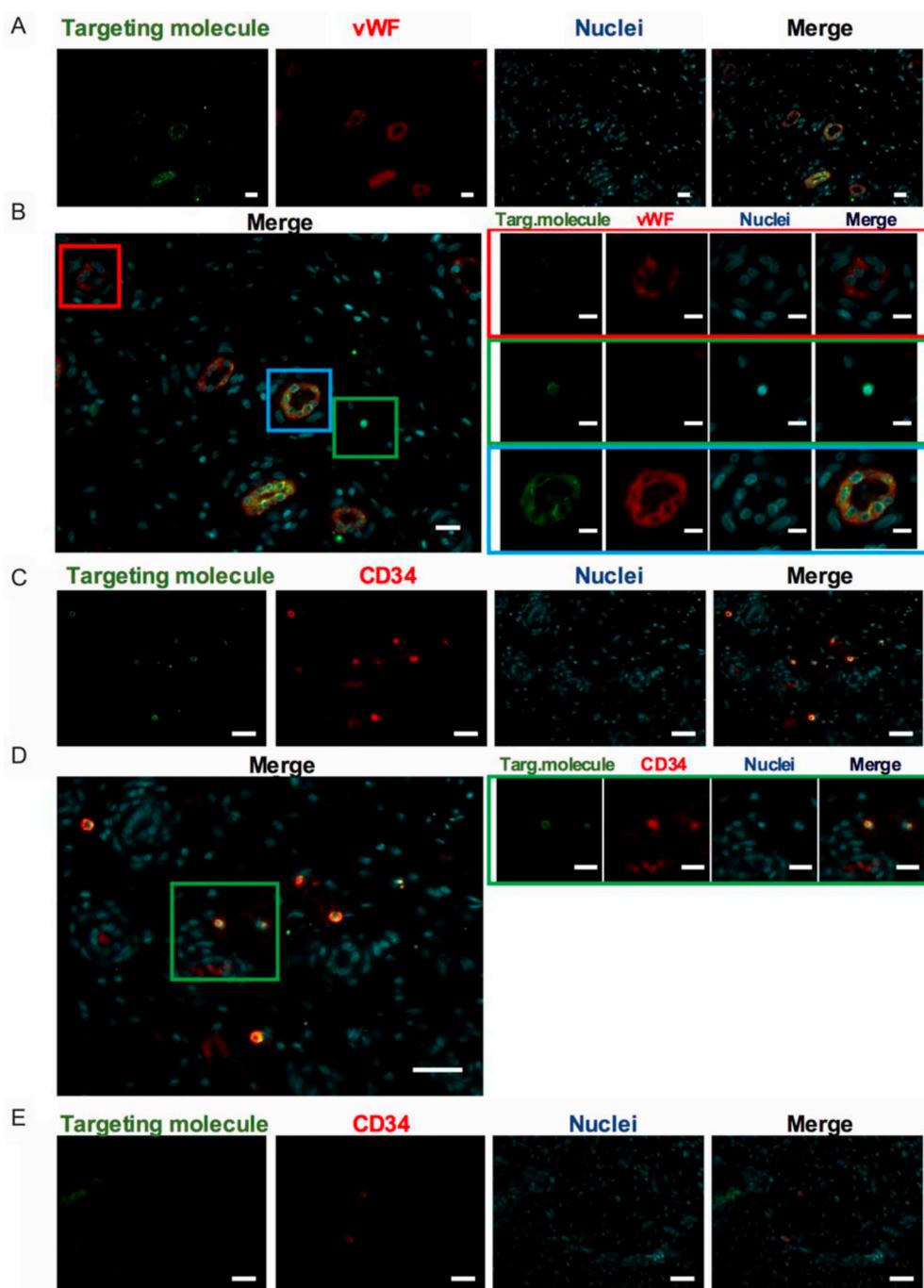


Fig. 2. The targeting agent interacts with CD34⁺ cells in RA inflamed synovial tissue. (A) RA human synovial tissue incubated with the targeting molecule (detected with goat anti-rat Alexa 488) and with anti-vWF (detected with goat anti-rabbit Alexa 594). Nuclei have been stained using Dapi. Scale bar 40 μ m. (B) The merge highlights a vessel positive for the vWF and negative for the targeting molecule (red box, scale bar 20 μ m), a cell positive for the targeting molecule and negative for the vWF (green box, scale bar 20 μ m) and a vessel positive both for the targeting molecule and for the vWF (blue box, scale bar 20 μ m). (C) RA human synovial tissues incubated with both the targeting molecule and anti-CD34. Nuclei have been stained with Dapi. From the merge it is possible to observe co-localization between the targeting molecule and the CD34⁺ cells. Scale bar 40 μ m. (D) Merge of the staining performed with the targeting molecule and the anti-CD34 highlights the co-localization (green box, scale bar 20 μ m). (E) Untargeted molecule (rat Fe) did not show any staining nor co-localization for the CD34 in RA human synovial tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

both groups of animals receiving the two types of nanoparticles showed the same degree of knee swelling (Fig. 5C) as a consequence of a similar IgG response to bovine serum albumin (BSA) (Fig. S3).

To analyze particle clearance, residual fluorescence was measured at the end of the experiment in the paws and in the main organs, including the kidneys, liver, lungs, brain, heart and spleen. The results presented in Fig. 5D show an increased accumulation of nanoparticles in the liver, while low intensity of fluorescence was detected in the other main organs.

The biodistribution of tBNPs was then tested in collagen-induced arthritis (CIA), which shares several features with human RA, involving most joints, and is considered the gold standard to test new compounds at pre-clinical level for the imaging and treatment of RA. Two groups of mice showing similar signs of arthritis received either tBNPs-Cy5.5 or BNPs-Cy5.5 into their tail veins. The animals were scanned for 7 days,

and the images of the limbs of two representative mice are presented in Fig. 6A. To correlate the fluorescence intensity with the degree of joint inflammation, disease severity was measured by assigning an arthritis score ranging from 0 (no arthritis) to 4 (severe arthritis). Interestingly, paws with the same scores in the two groups of mice showed different fluorescence intensities, and the highest level was detected in the paws of mice 24 h after injection of either type of nanoparticle (Fig. 6B). Surprisingly, an increased presence of tBNPs was also observed in paws with a score of 0, which should not be inflamed. Histological analysis of these samples, however, indicated features of an early inflammatory process, such as leukocyte infiltration (Fig. 6C).

3.4. Treatment of CIA using MTX-loaded tBNPs

Having established the selective delivery of tBNPs to inflamed

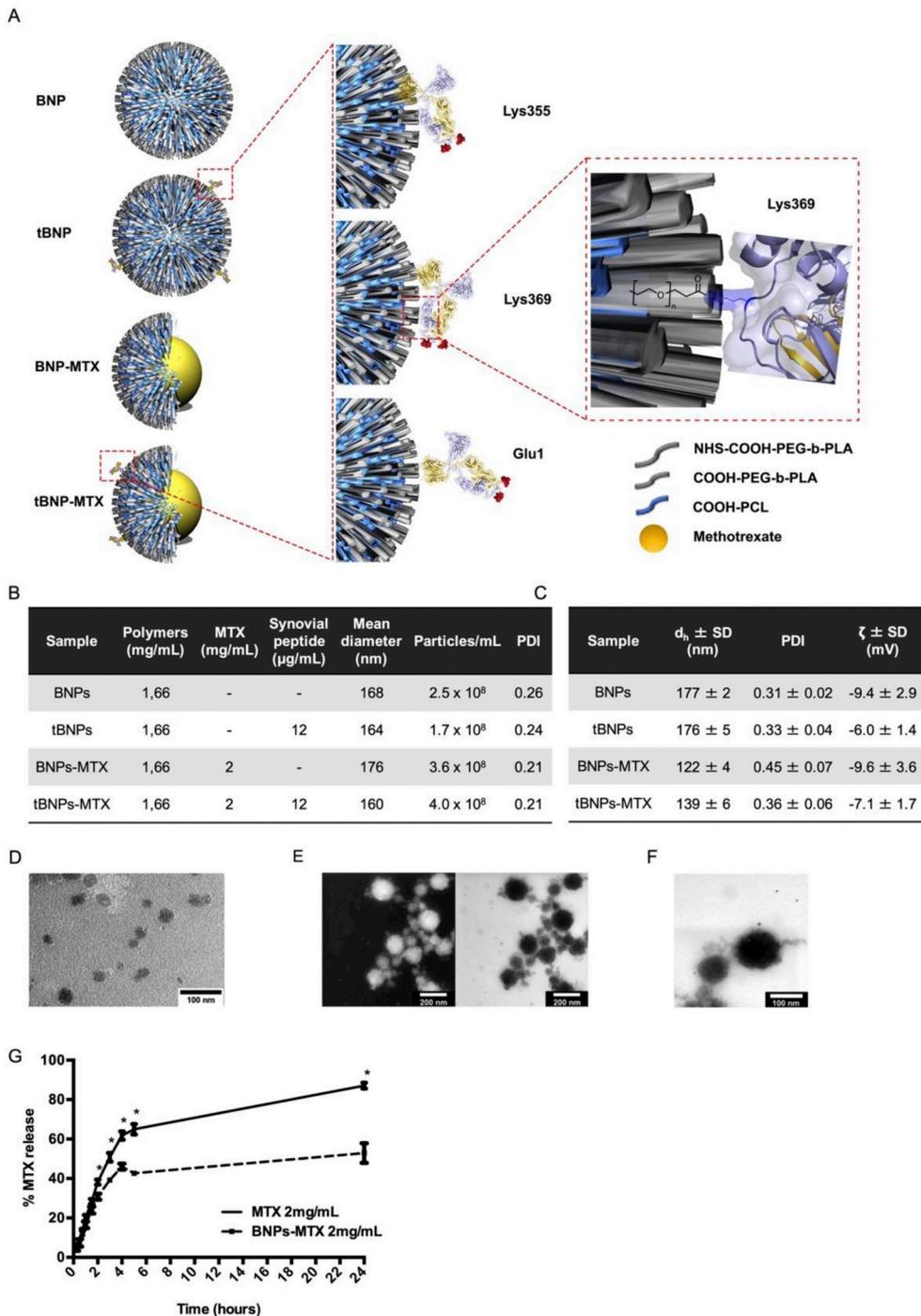


Fig. 3. Characterization of the nanoparticles. (A) Left: starting from the top: BNPs with the polymeric shell (blue/grey) made of PEG-b-PLA, PCL and an empty core; tBNPs: identical to the BNPs except for the target molecule linked to the surface; BNPs-MTX: identical to the BNPs but with MTX loaded into the core; tBNPs-MTX: represent the complete format of the nanoparticles, loaded with MTX and provided with the targeting molecule on the surface. Right: schematic representation of the nanoparticles surface with three hypothetical orientations of the targeting molecule exploiting highly solvent accessible primary amines: the protein N-terminus (on Glu1) and the side chains of Lys355 and Lys369 (on the Fc-CH₂ domain). The tables summarize the main features of the nanoparticles and the data obtained by NanoTrack (B) and DLS (C) analysis. (D) TEM image of tBNPs (scale bar 100 nm). (E) STEM image of tBNPs in dark field (left) and bright field (right) (scale bar 200 nm). (F) Enlargement of the bright field STEM image of the tBNPs (scale bar 100 nm). (G) Release studies of MTX from tBNP-MTX in PBS at 37 °C for 24 h. Samples have been analyzed in triplicate and data reported as the mean \pm SD. **** = $P \leq 0.0001$ by Two-way ANOVA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

synovium, we sought to determine whether MTX-loaded targeted nanoparticles were able to control the development of CIA in mice treated weekly for 3 weeks and followed for 25 days after disease onset. To this end, one group of mice was treated with collagen to induce arthritis and was then administered a single weekly injection of 3 mg/kg tBNPs-MTX that resulted in substantial amelioration of the joint inflammation and reduced arthritis progression compared to saline-treated animals (Fig. 7A). Similar beneficial effects were obtained in mice that received 1 mg/kg of free MTX three times a week, while a single injection of 3 mg/kg of free MTX was ineffective (Fig. 7A). The timing and dosing of free MTX administration were critical for efficacy because the drug is cleared rapidly with a half-life of 34 min [31]. In contrast, the persistence of the nanoparticles in the inflamed synovium for approximately a week explains the beneficial effects associated with a single injection

of tBNPs-MTX. Interestingly, tBNPs-MTX also proved to be therapeutically effective at a single dose of 1 mg/kg per week (Fig. 7A).

As shown in Fig. 7B, histological analysis of the joints from treated animals indicated reduced leukocyte infiltration and bone degradation. Histological scores of the inflammatory parameters, summarized in Fig. S4, revealed a reduction in cartilage and subchondral bone leukocyte infiltration as well as decreased stromal cell proliferation in mice treated with a single weekly injection of tBNPs-MTX.

We have also evaluated the potential toxic effects of tBNPs-MTX compared to the free drug by analyzing body weight and changes in blood cell counts in the treated animals. Injection of tBNPs-MTX did not affect body weight, whereas free MTX administered once a week at the dose of 3 mg/kg caused a 60% reduction in mice growth (Fig. 7C), despite its failure to produce a therapeutic effect. Likewise, red blood

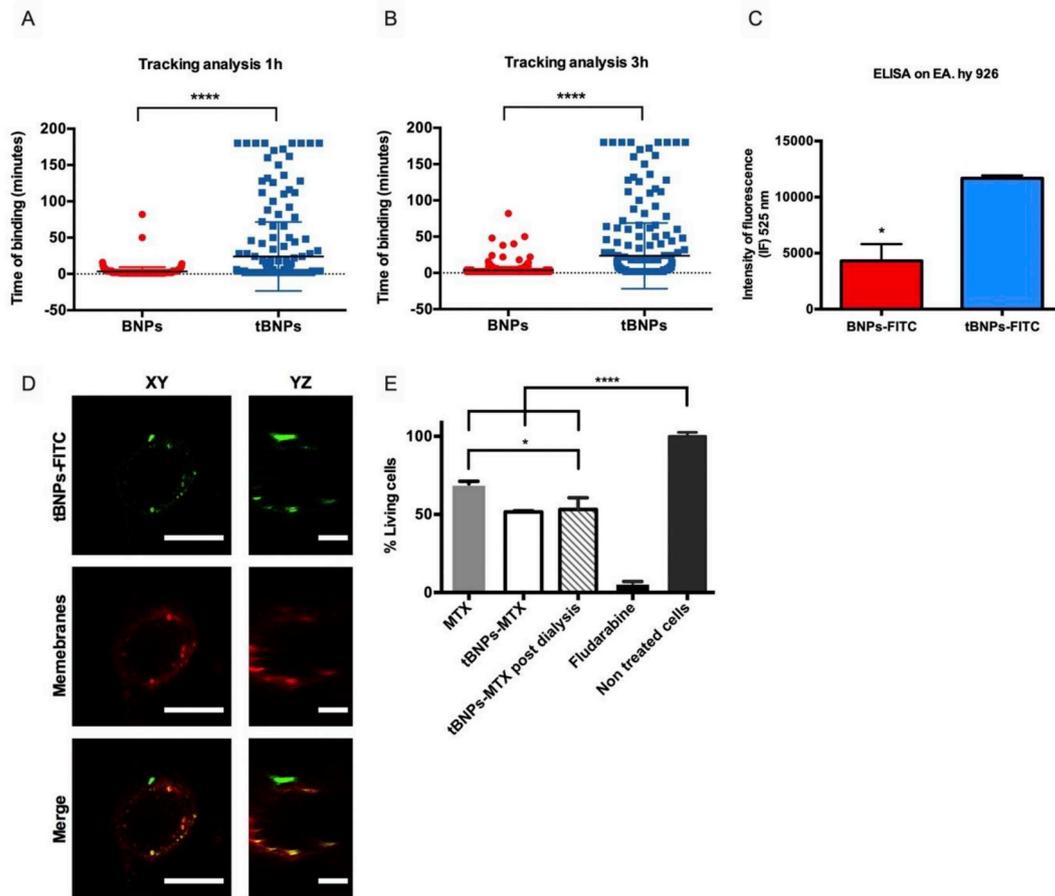


Fig. 4. The targeting of nanoparticles enhances binding interaction with endothelial cells. Tracking analysis at 1 h (A) and 3 h (B) performed on living EA.hy926 cell line incubated with BNP-Cy5.5 and tBNP-Cy5.5. Data are expressed as minutes of binding of a single particle and were analyzed by unpaired t-test. **** = $P \leq 0.0001$. (C) ELISA test performed on living EA.hy926 cells: tBNPs-FITC and BNP-FITC have been incubated for 1 h with EA.hy926. Data are expressed as intensity of fluorescence at 525 nm (mean of 3 biological replicates \pm SD) and were analyzed by unpaired t-test. * = $P \leq 0.05$. (D) Cropped image obtained by confocal microscopy shows internalized tBNPs-FITC into EA.hy926 cell after 1 h of incubation. Scale bars 10 μ m for XY and 5 μ m for YZ. (E) Cytotoxicity analysis of EA.hy 926 cell line incubated with free MTX, tBNP-MTX, tBNP-MTX after 24 h dialysis or fludarabine (as positive control). Data are presented as % of living cells compared to not treated cells and reported as the mean of biological triplicate \pm SD. Statistical significance was calculated with One-way ANOVA multiple comparison test. * = $P \leq 0.05$, **** = $P \leq 0.0001$.

cell, leukocyte and platelet counts (Fig. 7D–F) in mice treated with tBNPs-MTX did not change, while mice receiving 1 mg/kg of free MTX three times a week presented a significant decrease in the red blood cell and platelet counts. Subsequently, morphological analysis of various organs of the specimens highlighted heterogeneous tissue damage related to the treatment toxicity in mice treated with saline, tBNPs-MTX, or free MTX. In all treatment conditions, the specimens presented a disarray of the pulmonary parenchyma consisting of a variable grade of increased interstitial cellularity and fibrosis as well as blood extravasation. A variable grade of decreased cortical glomerular density or rarefaction associated with necrosis, rarely also involving the tubules, was detected in the renal parenchyma. Both the histopathological alterations observed in the lung and kidney presented at a higher severity in free MTX samples than in tBNPs-MTX samples (Fig. S5A). No significant histopathological alterations of the liver, spleen, brain or heart were detected in any of the treatment conditions (Fig. S5B).

3.5. Treatment of AIA using MTX-loaded tBNPs to prevent neoangiogenesis

To investigate the mechanism of action of MTX-tBNPs we turn again to the AIA model. MTX-tBNPs, unlike free MTX, reduced knee swelling and the number of polymorphonuclear (PMN) cells in the synovial fluid compared to controls (Fig. 8A and B). The treated rats did not show any sign of toxicity (Fig. S6) and had levels of anti-methylated BSA (mBSA)

IgG and C3 deposition in synovial tissues that were comparable to the controls (Figs. S7A and B), thus ruling out differences in the induction of the inflammatory model between the two groups of rats.

Taking into account that tBNPs target endothelial cell precursors, it was reasonable to investigate their effect on the control of neoangiogenesis, which is usually associated with joint inflammation in these models and in RA patients [32]. To this end, synovial tissues obtained from untreated animals or animals treated with free MTX or tBNPs-MTX were stained for the vWF to visualize the vessels. Representative figures are shown in Fig. S8. The vessel density data, expressed as intensity of vWF fluorescence/tissue area and summarized in Fig. 8C, show a reduced number of vessels in rats treated with tBNPs-MTX compared to both control- and MTX-treated rats, supporting the hypothesis that tBNPs-MTX interfere with the formation of new vessels in inflamed synovia. Given the above demonstrated binding of tBNPs to CD34⁺ cells, it is tempting to conclude that the selective delivery of MTX to CD34⁺ endothelial cells precursors causes cell death, preventing the development of new vessels in the inflamed synovial tissue and the local recruitment of leukocytes (Fig. 8D).

4. Discussion

Efforts are being made to develop diagnostic and therapeutic approaches that preferentially localize to tissues principally affected by

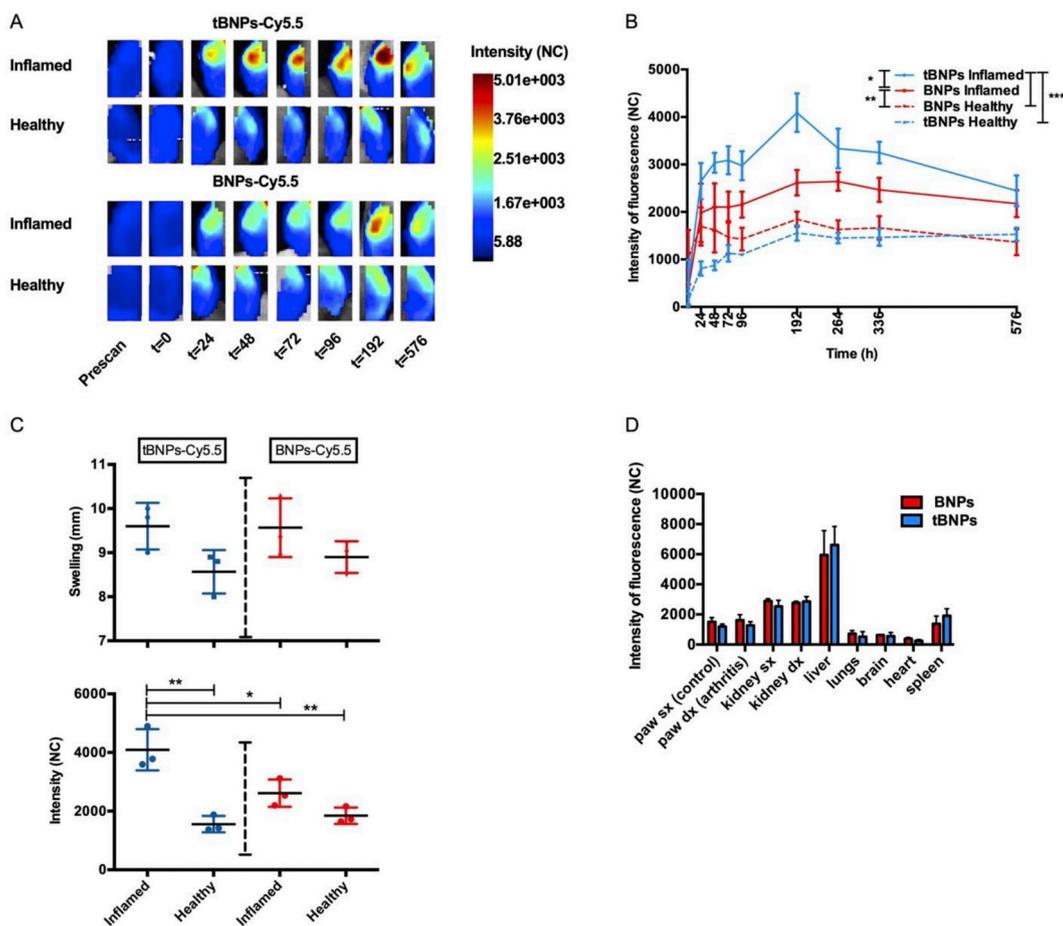


Fig. 5. Biodistribution analysis of BNP and tBNPs in AIA. (A) Images acquired by time domain optical imaging of knees from two representative AIA rats treated i.v. with labeled tBNPs (first rows) and BNPs (second rows). The fluorescence intensity has been reported as normalized counts (NC). (B) Data obtained from 2 groups of rats ($n = 3$) were expressed as mean of fluorescence intensity \pm SEM of the right and left paws of the animal. Statistical significance has been tested by two way ANOVA. (C) The graph shows the correlation between swelling (mean \pm SEM) and intensity of fluorescence (mean \pm SEM) recorded for each paw at day 7. Blue bars represent data from rats treated with tBNPs-Cy5 ($n = 3$), while red bars data from BNPs-Cy5.5 treated rats ($n = 3$). (D) The graph shows ex-vivo fluorescence intensity of main organs (express as mean NC \pm SEM). Statistical significance has been tested by unpaired t-test* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the pathologic process. This was mainly proposed for cancer treatment, but this strategy was also described for the treatment of rheumatoid arthritis, with the aim to locally inhibit effector molecules usually present in the circulation and in the body fluids. The selective delivery of mAbs [23] initially showed the potential validity of this approach as well as the selective delivery of chemical drugs using guided nanoparticles coated with the peptide RGD [17], though the expression of the alpha V beta 3 integrin is not tissue specific and cause an extra-articular nanoparticle accumulation [17].

The treatment of RA remains anchored to the use of MTX and biological drugs, although some patients have to stop treatments due to their side effects [33], leaving the management of RA still problematic. For this reason, the development of novel therapeutic approaches targeting different mechanisms of action from standard drugs are desirable. Although the autoimmune response in RA patients is triggered at various organs and that systemic treatments clearly demonstrated their efficacy, the pathogenic paths driving inflammation take place in the synovial tissues, and therefore their specific control remains a priority.

Here, we present a novel drug delivery system for the management of rheumatoid arthritis. It is based on the use of drug-loaded polymeric biodegradable nanoparticles functionalized with a targeting agent specific for endothelial cells precursors in the inflamed synovial tissue. We demonstrated that such treatment blocks the progression of the inflammatory process through inhibition of tissue-specific

neovascularization.

Several types of structures have been previously proposed for drug delivery. Each of them has been extensively characterized in vitro for their physicochemical properties. tBNPs solutions, developed in this study are monodisperse and stable for long-term storage. The drug release showed that after an initial burst release, a described phenomenon associated with the shape and pore size of the polymeric nanoparticles [34], in which 40% of the MTX is dispersed from the tBNPs, $\approx 50\%$ of MTX is still encapsulated after 24 h. This data results particularly important because demonstrated the capacity of residual loaded drug to kill endothelial cells even after 24 h of distribution in the body.

Particular attention was also devoted to the characterization of the targeting agent. *In silico* studies showed the three most probable orientations of the targeting molecule on the surface of the nanoparticles, all ensuring the exposure of the synovial peptide far from the nanoparticles surface and from PEG branches. These predictions were confirmed by immunofluorescence staining between tBNPs and inflamed rat synovial tissues demonstrating that the targeting agent is needed to specifically target the inflamed synovial membrane and confirming nanoparticles functionalization. In addition, as demonstrated by tracking analysis studies, we found that the targeting agent is important to increase the time of interaction between tBNPs and endothelial cells, emphasizing the important role of the targeting peptide at the beginning of the interaction. This finding suggests that even *in vivo* the

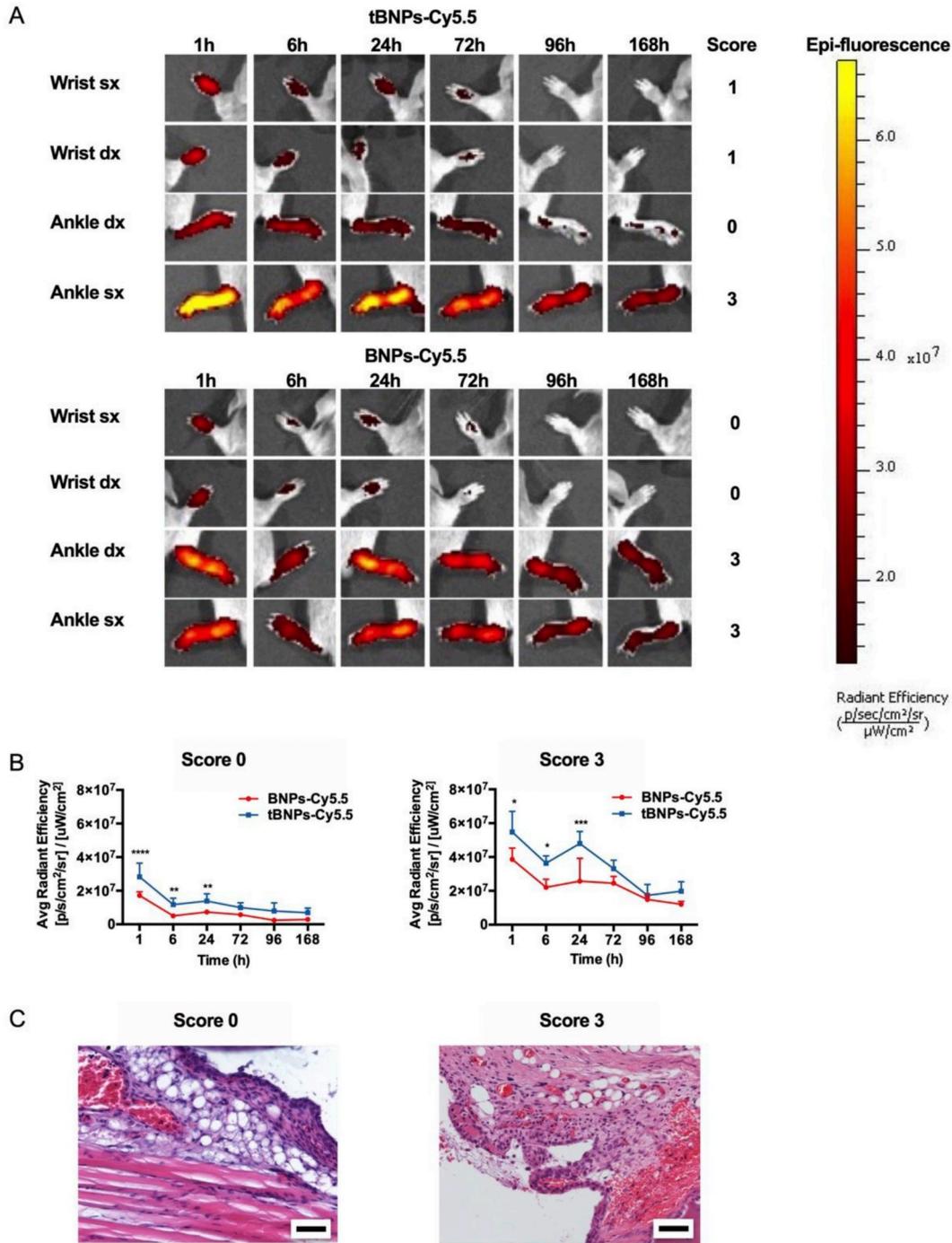


Fig. 6. Biodistribution analysis of BNP and tBNPs in CIA. (A) Images acquired by time domain optical imaging of two representative CIA mice treated intravenously with tBNPs-Cy5.5 (first panel) or BNPs (second panel), respectively. The score of each paw has been reported on the right of each row. (B) The graphs show the mean intensity of fluorescence (as average radiant efficiency \pm SD) detected for a total of 7 days in paws with score 0 or score 3. In this study has been enrolled 7 mice: 4 for tBNPs-Cy5.5 and 3 for BNPs-Cy5.5. The graph regarding the score 0 takes into account $n = 5$ paws of tBNPs-Cy5.5 treated mice and $n = 7$ of BNPs-Cy5.5 treated animals. Instead, the graph of the score 3 takes into account $n = 4$ paw of tBNPs-Cy5.5 treated mice and $n = 4$ of BNPs-Cy5.5 treated animals. Two-way ANOVA multiple comparisons test has been used for statistical analysis. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$. (C) Haematoxylin and Eosin staining of CIA mouse joints with score 0 and 4. In particular, joints usually scored by 0 (no arthritis) by physical examination reveals an initial inflammatory state if analyzed by histology. The initial inflammatory state explains the different fluorescence intensity detected after 1 h in mice treated with tBNPs-Cy5.5. Scale bar 100 μ m.

retention time of tBNPs is increased compared to BNPs. In order to determine the specific binding of the synovial peptide, the targeting molecule was stained in combination with endothelial markers, such as vWF and CD34. A colocalization with the CD34⁺ was detected on inflamed human synovial membranes; in particular, many individual CD34⁺ cells were recognized by the targeting molecule in close

proximity to blood vessels. This staining pattern has been previously reported [35,36], demonstrating the great variability in the expression and distribution of endothelial cell markers among different types of vascular tissues and different anatomic compartments. These findings confirmed that the synovial peptide exploits its target as a signature of the inflamed synovial membrane, highlighting its potentiality as a

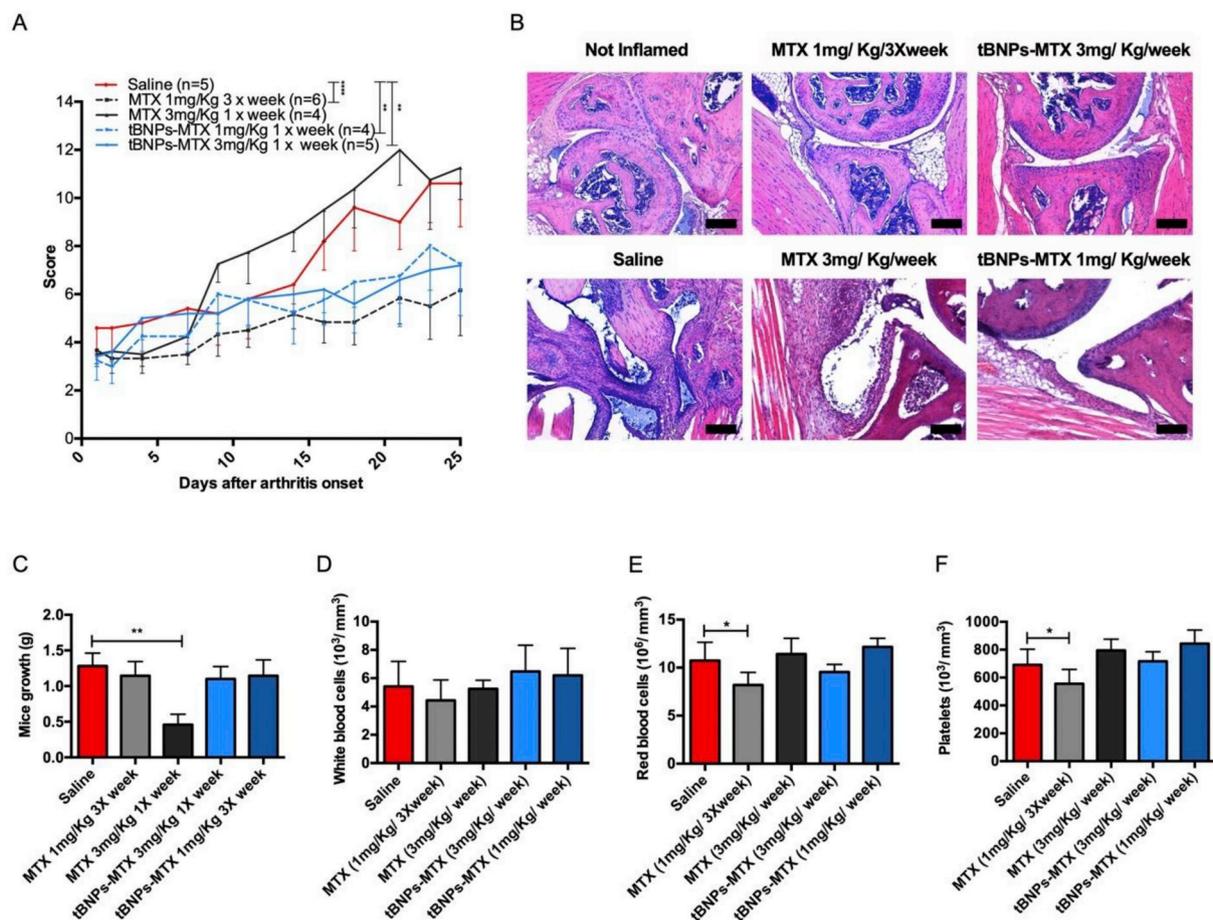


Fig. 7. Effect of tBNP-MTX in CIA. (A) The graph shows arthritic scores of CIA DBA/1 mice treated i.p. with different doses of tBNPs-MTX and free MTX. Groups of mice have been enrolled as follow: saline as control (n = 5, red line); MTX 1mg/Kg three times per week (n = 6, continuous black line); MTX 3 mg/kg once per week (n = 4, dotted black line); tBNPs-MTX 3mg/Kg once per week (n = 5, continuous blue line); tBNPs-MTX 1mg/Kg once per week (n = 4, dotted blue line). The scores have been reported as the mean of the sum of the scores \pm SEM assessed for each paw of the mouse. Statistical analysis have been assessed by Two-way ANOVA test. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$. (B) Histological images representative of each group of mice treated in this study. An additional image of a healthy paw was added as a negative control. Scale bar 200 μ m. (C) The graph represents mice Δ growth in g (weight day 25 – weight day 0) expressed as mean \pm SEM. (D) (E) (F) The graphs show WBC, RBC, PLT counts in blood, respectively. Data have been reported as the mean \pm SD. Statistical significance has been assessed by t-test. * = $P \leq 0.05$; ** = $P \leq 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

disease-specific targeting agent.

Through the biodistribution studies conducted in AIA, we demonstrated that targeted tBNPs were found to be more efficient than untargeted particles in discriminating between inflamed and non-inflamed joints, further supporting the conclusion that the higher tBNP accumulation in the inflamed knee was the result of active targeting. At the end of this study, we measured the residual amount of nanoparticles in other organs, confirming data obtained with similar particles by our group [29,37] and indicating that the structures are mainly cleared through the liver, bile and intestine. The undetectable presence of nanoparticles in the lungs confirmed that our nanosystems are not non-specifically bound by macrophages.

Moreover, this study shows an increased accumulation of tBNPs into inflamed joints of mice that, even though were scored as 0, present tracts of an early inflammatory process by histological analysis. For this reason, these data underline the potential use of tBNPs as a diagnostic tool able to reveal inflammatory processes that cannot be detected by physical examination. These results obtained *in vivo* prove again the superior accumulation of targeted nanoparticles compared with untargeted nanoparticles and demonstrate that the targeting molecule actively guides nanoparticles to inflamed joints. Moreover, this is a further proof that the use of targeted nanoparticles is a potential way to overcome the lack of efficacy of untargeted nanocarriers based on

passive delivery.

CIA represent the animal model sharing more features with chronic human pathology. The treatment with MTX-loaded tBNPs reduced the severity of the disease compared to control animals. In particular, a lower dose of tBNPs-MTX maintains its efficacy compared to the same dose of the free drug. These results confirmed the initial hypothesis that, through targeted delivery system, it is possible to increase drug bioavailability at the disease site, while reducing systemic side toxicity secondary to decrease in dose reduction and administration frequency. Indeed, no toxic effects were associated with tBNPs-MTX treatment while mice receiving the standard dose of MTX three times a week presented a significant decrease in the red blood cell and platelet counts. These data confirm previous observations of cytopenia associated with frequent injections of MTX [33,38].

Based on our finding that tBNPs are able to deliver MTX inside CD34⁺ cells in synovial tissue and considering that different regimens of free MTX and MTX-loaded tBNPs were needed to obtain similar results in the treatment of CIA, we hypothesized that free MTX and MTX-loaded tBNPs may have different mechanisms of action. To address this point, we used the AIA model, which is dependent on the production of immune complexes in the joint microenvironment; this production in turn activates the complement system, eventually leading to recruitment of immune cells [23,39]. This model develops in 3–4 days and is

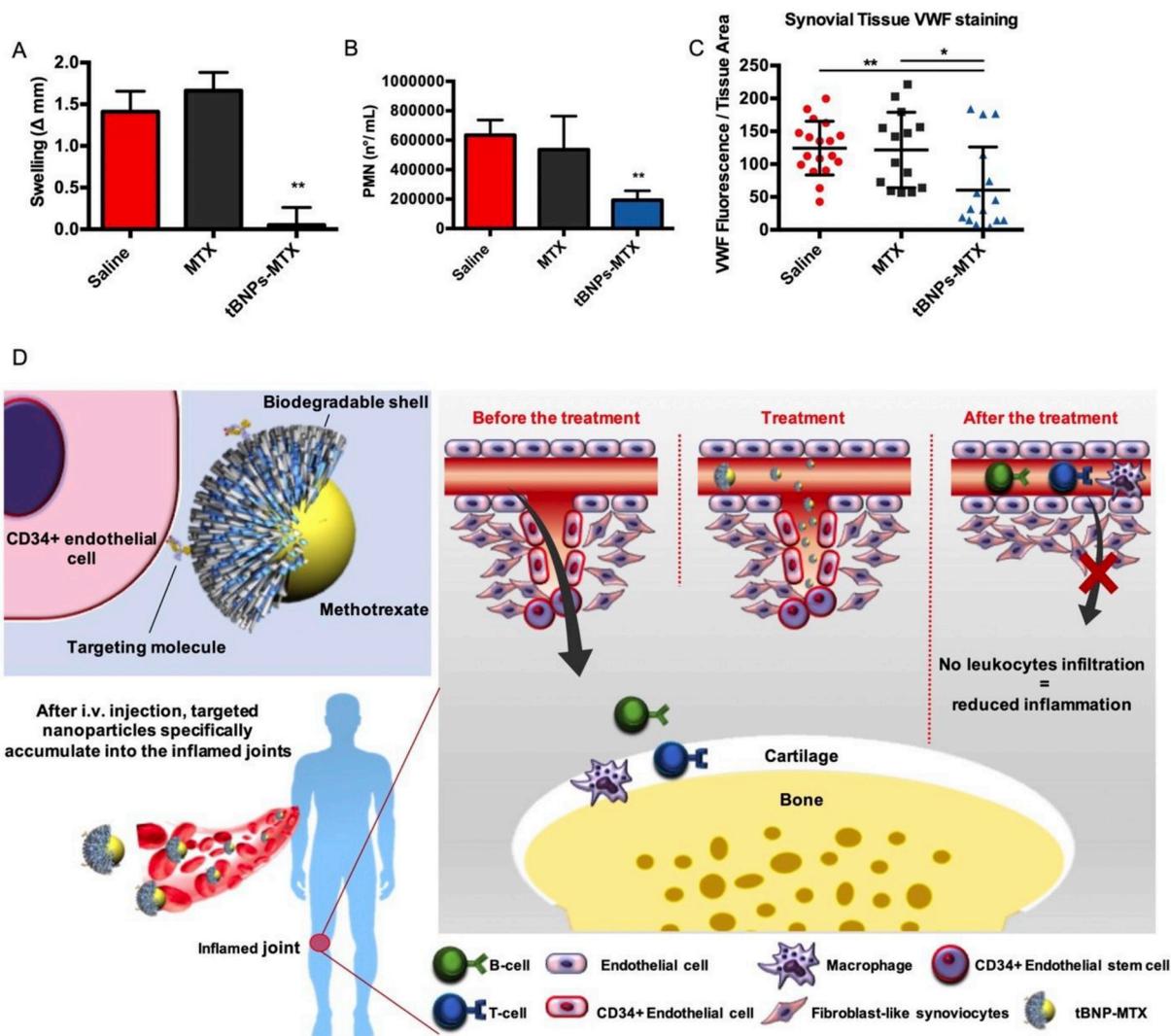


Fig. 8. Effect of tBNP-MTX in AIA and mechanism of action. (A) swelling (diameter day 3 –diameter day 0 in mm) of the inflamed knees and (B) quantification of polymorph nucleated cells detected into synovial fluids from joints of rats treated with saline solution (n = 5), 1 mg/kg/day of free MTX (n = 4) and 1 mg/kg/day of tBNP-MTX (n = 5). A parametric unpaired t-test has been used to assess the statistical significance. (C) vWF staining (Mean fluorescence intensity normalized for the tissue area \pm SD) has been determined by ImageJ software. For each animal at least 2 regions of interest (ROIs - showed in [Supplementary Fig. 9](#)) have been analyzed. Number of region of interests (ROIs) analyzed for each group: inflamed = 18; tBNPs-MTX = 16; MTX = 14; negative CTRL = 8. Statistical significance has been assessed using a parametric t-test. * = $P \leq 0.05$; ** = $P \leq 0.01$. (D) Schematic representation showing that tBNPs-MTX target endothelial progenitor CD34⁺ cells. The cytotoxic effect of MTX kills CD34⁺ cells and prevents the formation of new vessels, which are essential for the whole inflammatory process.

unresponsive to free MTX [40,41] due to the insufficient time that MTX has to inhibit the production of antibodies that initiate the inflammatory process. MTX-tBNPs demonstrated their capacity to completely prevent inflammation in this acute model of arthritis where free MTX was ineffective. This result was not dependent on immune-complexes deposition or complement activation but to the capacity to target CD34⁺ endothelial cell precursor, preventing neo-angiogenesis specifically at synovial sites and, as a consequence, inflammatory cell migration from the circulation.

5. Conclusions

We present data in support of a novel approach for the treatment of RA through the use of polymeric nanoparticles that block neoangiogenesis only in an inflamed synovial microenvironment. The MTX-loaded targeted nanoparticles generated in this study have the great advantage of delivering the drug to inflamed synovia using the synovium-homing peptide as a targeting molecule. This approach allows the local release of the smallest amount of MTX sufficient to be

therapeutically effective, while minimizing side effects by reducing systemic toxicity.

Declaration of interests

Luis Núñez working in Biotarget Inc. and LNK Chemsolutions LLC has commercial interests in the particle systems described in this work. The production of the structure was included in the patent US20070296099 A1.

No conflicts of interest for other authors.

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Author contributions

F.C. designed and performed the experiments and wrote the manuscript; P.D. performed and developed and study particles in AIA model; L.D.M. produced targeting agent; S.B. performed optical imaging experiment in rat model; B.B. and C.T. performed and analyzed tissues by histology and immunohistochemistry; R.O. performed in silico study of targeting agent structure and interaction with BNP; P.B., G.M.M. and E.T. performed and analyzed particle distribution in mouse model; G.P. analyzed mouse blood for toxicological studies; E.R. performed characterized BNPs' structure in vitro; J.B. provided human samples; B.F. performed nanotrack analysis; J.J. and J.A. obtained and analyzed STEM images; L.N. produced BNPs; C.P. contributed with critical revision of the manuscript for important intellectual contents; P.L.M. and F.T. contributed in the design of the study and in the preparation of the manuscript; D.S. designed and contributed to produce the targeting agent; P.M. Designed the study, organized the experiments and the collaborations, wrote the manuscript.

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Appendix A . Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2019.05.016>.

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