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Liposomal treatment of experimental arthritis can be monitored non-invasively with radiolabeled anti-FAP antibodies

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disorder resulting in synovial inflammation. Fibroblast activation protein (FAP) is over-expressed by fibroblast-like synoviocytes in arthritic joints. Radioimmunoimaging with anti-FAP antibodies might be used to monitor therapy response, thus enabling tailored therapy strategies and therapeutic outcomes. The aim of this study was to assess whether radiolabeled anti-FAP antibodies can be used to monitor the efficacy of treatment with prednisolone phosphate containing long-circulating liposomes (PLP-LCL) in a mouse model of arthritis. Collagen-induced arthritis (CIA) was induced in male DBA/1J mice. Mice were treated with a single injection of 10 mg/kg PLP-LCL or empty LCL as control. Single photon emission computed tomography (SPECT) and computed tomography (CT) images were acquired 24 h after injection of ^{99m}Tc-labeled succinimidyl-hydrazinonicotinamide (S-HYNIC)-conjugated anti-FAP antibody, 28H1, at 2, 5 and 9 days after treatment. Uptake of ^{99m}Tc-HYNIC-28H1 in all joints was quantified and correlated with macroscopic arthritis scores. Treatment of CIA with PLP-LCL significantly suppressed joint swelling. Already at one day after treatment the macroscopic arthritis scores showed a 50% decrease. Scores further decreased to only 10% of the initial scores at 5 and 9 days post treatment. In contrast, macroscopic scores increased up to 600% in untreated mice at 9 days post injection of empty LCL. ^{99m}Tc-HYNIC-28H1 uptake ranged from 1.5 %ID/g in non-inflamed joints to 22.6 %ID/g in severely inflamed joints. Uptake of radiolabeled 28H1 in inflamed joints (%ID) correlated with arthritis score (Spearman's ρ 0.77, $p<0.0001$). Moreover, uptake of ^{99m}Tc-HYNIC-28H1 slightly increased 9 days after therapy, which was not seen macroscopically, indicating that SPECT/CT imaging might be more sensitive compared to the macroscopic arthritis scoring method. SPECT/CT imaging with ^{99m}Tc-HYNIC-28H1 specifically monitored response to therapy and tracer accumulation correlated with the severity of inflammation. In addition, imaging is potentially more sensitive than the macroscopic scoring method. This study showed that SPECT/CT with ^{99m}Tc-HYNIC-28H1 can be used to noninvasively monitor the course of CIA in mice.

Keywords: Fibroblast activation protein, SPECT/CT imaging, collagen-induced arthritis, prednisolone phosphate-encapsulating PEG liposomes, therapy monitoring

INTRODUCTION

RA is an autoimmune disorder which affects 0.5% - 1 % of the European population (1). It is characterized by inflammation of synovial joints, eventually leading to cartilage and bone destruction. Currently, RA is mostly treated systemically (oral, intramuscular, intravenous) by combinations of non-steroidal anti-inflammatory drug, disease-modifying anti-rheumatic drugs, biologicals or corticosteroids or sometimes locally (intra-articular corticosteroid injections) (2,3). All these therapeutics aim to relieve pain, reduce inflammation, and prevent irreversible damage. Unfortunately, it may take several months to show a therapeutic effect, especially after treatment with disease-modifying anti-rheumatic drugs, while in a subpopulation of patients unresponsiveness to the drugs is seen. Therefore, there is a need for a non-invasive imaging method to objectively determine the severity of the symptoms, to monitor therapeutic efficacy and to predict the occurrence of exacerbations during the course of the disease. At an early stage after treatment, specific and sensitive therapy monitoring could allow for alteration of the therapeutic strategy when therapeutic effects are shown to be insufficient. This potentially decreases the unwanted side effects of the ineffective drugs and an early switch to an effective drug could protect the patient from irreversible damage due to RA.

The synovium of RA patients contains high levels of inflammatory cells, including macrophages and fibroblast-like synoviocytes (4,5). These cells are prominently present in areas with high inflammatory activity and have an aggressive invasive character. When activated, macrophages produce pro-inflammatory cytokines, such as interleukin-1 and tumor necrosis factor-α, triggering the production of pro-inflammatory cytokines (6). After stimulation by interleukin-1 and tumor necrosis factor-α, fibroblast-like synoviocytes produce pro-inflammatory cytokines and proteolytic enzymes, such as interleukin-6 and matrix metalloproteinases (7,8). These mediators stimulate and amplify the inflammation and contribute to degradation of the extracellular matrix and exacerbate joint damage. Patients are often treated with corticosteroids, such as PLP, to suppress inflammation and reduce pro-

inflammatory cytokines. PLP reduces accumulation of macrophages and irreversible cartilage damage (9,10). To increase efficacy and reduce toxicity to the drug, PLP can be given locally or encapsulated in drug delivery systems. In experimental arthritis models, the use of LCL to target all inflamed joints at once by one intravenous injections, created local persisting depots of steroids and increasing efficacy over safety ratio (11,12).

Molecular imaging has already been proven successful in non-invasive and early response monitoring, but is in most cases not specific for arthritis at a molecular level (11,13). FAP is a cell surface-bound, type II transmembrane glycoprotein, belonging to the family of serine prolyl oligopeptidases. In arthritic joints, FAP is dominantly expressed by activated FLS, and is found in human arthritis as well as experimental arthritis, such as the CIA model (14). FAP plays a role in pannus formation and progression of disease in all stages (15). The 28H1 antibody is a non-internalizing antibody with high affinity for human and murine FAP (16). Specificity of 28H1 was shown in an vitro binding assay and by immunohistochemistry in arthritic joints. SPECT/CT imaging with ^{111}In-labeled 28H1 as a tracer showed a significant correlation with uptake of ^{111}In-DTPA-28H1 in inflamed joints and the severity of arthritis (16). To further improve this tracer, we examined the use of ^{99m}Tc for radiolabeling. Due to the shorter half-life of ^{99m}Tc (6 h), compared to that of ^{111}In (67 h), it is a better match with the relatively short circulation time of 28H1. Additionally, radiation dose per scan will be lower, ^{99m}Tc is less expensive and image resolution will be even higher compared to that of ^{111}In-labeled tracers.

In this study, we focus on the potential role of the 28H1 antibody as a specific and sensitive tool for early response monitoring after treatment with PLP-LCL in murine CIA. The CIA model is considered a suitable model to study disease progression and the therapeutic effects of anti-rheumatic drugs.

MATERIALS AND METHODS

Preparation of the Liposomes

LCL containing 2.1 mg/ml PLP were prepared by injection of an ethanolic lipid solution into an aqueous dispersion medium, followed by extrusion, as described earlier (11,17). Briefly, dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (mPEG2000-DSPE) (both from Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (BUFA, Uitgeest, The Netherlands) (1.85: 0.15: 1 molar ratio) were dissolved in ethanol. The aqueous drug solution contained 100 mg/ml PLP (BUFA, Uitgeest, The Netherlands) in water for injection. The ethanolic lipid solution was injected in the aqueous solution and the resulting coarse dispersion was downsized by multiple extrusion steps (4-5 per pore size) through polycarbonate filter membranes with decreasing pore sizes of 200, 100 and 50 nm, respectively (Nuclepore™ Track-Etch Membrane, Whatman®, Sigma-Aldrich, Zwijndrecht, The Netherlands). Size (111 nm) and mean polydispersity (0.07) were determined by dynamic light scattering using a Malvern 4700 system (Malvern Ltd, Malvern, UK). Unencapsulated PLP was removed by dialysis against saline using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10 kD. Dialysis medium was refreshed 4 times after at least 8 h.

Antibody Conjugation

The monoclonal anti-FAP antibody 28H1 (3.7 mg/ml) was conjugated with S-HYNIC (Solulink, Inc. San Diego, CA, USA) in 1 M $NaHCO_3$, pH 8.2, using a 10-fold molar excess of S-HYNIC for 1 h at room temperature (RT). Unconjugated S-HYNIC was removed by dialysis against ammonium acetate (0.25M NH_4Ac, pH5.5) using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10 kD. Dialysis medium was refreshed 4 times after at least 8 h.

Radiolabeling Procedure

$SnSO_4$ (1mg/ml) was freshly prepared by adding 5 mg $SnSO_4$ to 5 ml 0.1 M HCL. This solution was flushed with N_2 (g) for 10 min to prevent oxidation of $SnSO_4$ during the labeling procedure. Tricine (100 mg per ml PBS, pH 7, co-ligand), 0.02 mg $SnSO_4$ and 240 MBq $^{99m}TcO_4^-$ was added to 300 µg HYNIC-28H1 and was incubated at RT for 30 min. Radiolabeling efficiency was determined by instant thin-layer chromatography (ITLC) on ITLC-SG strips (Agilent Technologies, Santa Clara, CA), with 0.1 M citrate, pH 6.0, as the mobile phase. Labeling efficiency exceeded 95% or, when >95%, the labeled product was purified by gel filtration chromatography on a PD-10 column (GE Healthcare Life Sciences**,** Buckinghamshire, United Kingdom) to provide a preparation with a radiochemical purity of > 95%.

Animals

Male DBA/1J mice, 10-12 weeks of age, were purchased from Janvier Labs (le Genest-Saint-Isle, France). All mice were housed in filter-top cages under pathogen-free conditions. Food (SNIFF Voer, Soest, The Netherlands) and water were available *ad libitum*. All animals were accustomed to the environment for at least one week before experiments were initiated. All *in vivo* experiments were approved by the institutional animal welfare committee of the Radboud University, Nijmegen, The Netherlands, and were conducted in accordance with the principles laid out by the revised Dutch Act on Animal Experimentation (1997).

CIA Model

CIA was induced as described previously (18). Briefly, male DBA/1J mice were immunized with 100 µg of bovine type II collagen (CII) emulsified in Freund complete adjuvant (CFA, Difco Laboratories, Detroit, USA), which was injected intradermally into the tail base. After 3 weeks (day 21) the mice received a booster injection of 100 µg of CII in saline. Onset of CIA occurred a few days after the booster

injection. Onset and development of arthritis was macroscopically monitored by independent observers in a masked fashion on a scale from 0-2 for each paw. Cumulative scoring was based on redness, swelling and ankylosis: 0 = no sign of arthritis; 0.25: 1-2 toes red or swollen; 0.5: 3-5 toes red or swollen; 1.0: swollen ankle; 1.5: swollen footpad; 2.0: severe swelling and ankylosis (16).

Liposomal Treatment With PLP

Liposomal treatment was given one day after the booster injection. The treated mice received a single injection (i.v.) of 10 mg/kg PLP in LCL with a lipid dose of 100 µmol/mouse in a total volume of 200 µl. The control groups received empty LCL at the same lipid dose.

Micro-SPECT/CT Imaging and E*x Vivo* Biodistribution Studies

Treated and untreated DBA/1J mice with CIA were injected i.v. with 50 µg ^{99m}Tc-HYNIC-28H1 (specific activity 800 MBq/mg in 200 µl, n=5 per group) at day 1, 4 or 8 after therapy. At 24 h after tracer injection, mice were euthanized by CO_2/O_2 suffocation. Subsequently, images were acquired with the U-SPECT-II/CT scanner (MILabs, Utrecht, The Netherlands (19)). Images were acquired as 2 frames of 25 minutes using a 1.0 mm-diameter cylindrical pinhole mouse ultra-high sensitivity collimator (UHS-M). SPECT scans were followed by CT scans for anatomical reference (spatial resolution of 160 µm, 65 kV, 615 µA). Scans were reconstructed with MILabs reconstruction software, which uses an ordered-subset expectation maximization algorithm, with a voxel size of 0.2 mm, 16 subsets and 1 iteration. After image acquisition, relevant tissues were dissected to determine accumulation of the tracer in the organs and the joints. Tissues of interest (right wrist joint, left wrist joint, right ankle joint, left ankle joint) were dissected, weighed and radioactivity was measured in a shielded 3''-well-type gamma counter (Perkin-Elmer, Boston, MA, USA). Injection standards (1%) were counted simultaneously with the tissue samples

to correct for physical decay and to allow calculation of percentage of the injected dose per gram tissue (%ID/g).

Quantitative SPECT Analysis

Reconstructed micro-SPECT scans were coregistered with CT images. A Gaussian filter of 0.8 was applied to the SPECT images and a 3-dimensional volume of interest was drawn around the arthritic joints using Inveon Research Workplace 4.1 software (Siemens Preclinical Solutions, Knoxville, TN, USA). Voxel intensity values were converted to %ID using decay correction and a standard curve (voxel intensity vs. kBq) acquired by scanning and reconstructing known ^{99m}Tc activities from 0 to 370 kBq under identical conditions as during the animal scans.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism version 5.03 for Windows (Graphpad Software, San Diego, CA, USA). Mean values are given ± standard deviations. The data was analyzed using nonparametric Mann-Whitney *U* tests. All tests were two-sided, considering $p < 0.05$ as significant p values.

RESULTS

PLP-LCL Therapy

Treatment with a single injection of 10 mg/kg PLP-LCL was started after onset of arthritis and was effective as macroscopic arthritis scores were lower in treated mice than in the control group (Fig. 1). Visual arthritis scores were measured on a scale of 0-2 per paw and 0-8 per mouse until 9 days after

therapy. Arthritis scores were significantly lower in the treated group as compared to the control group at all time points. At day 2 after treatment arthritis scores decreased in treated mice, while it increased in the control group ($p = 0.01$). This effect continued at 5 days post treatment ($p = 0.01$) and at 9 days post treatment ($p = 0.02$).

SPECT/CT Imaging

Inflamed joints were specifically visualized using SPECT/CT imaging with ^{99m}Tc-HYNIC-28H1 at 2, 5 and 9 days after treatment (Fig. 2). Images clearly showed preferential uptake of the anti-FAP antibodies in inflamed paws, with low background accumulation in other tissues and even joints with minimal arthritis scores (0.25 on a scale of 0-2) were delineated. Increased uptake of radiolabeled anti-FAP antibodies in inflamed joints of control mice was shown in time (Figs. 2A-C), while the joints of the treated mice did not show enhanced uptake (Figs. 2D-F).

Quantitative SPECT Analysis

Tracer accumulation in each paw in SPECT/CT images was analyzed quantitatively. Analysis showed that tracer accumulation was decreased in treated mice and was significantly lower at day 5 and day 9 after treatment than in the control group (* = $p < 0.02$, fig. 3A). Joint accumulation in the treated group was 4.1 ± 3.5 %ID/g, 1.9 ± 1.0 %ID/g and 3.2 ± 1.1 %ID/g 2, 5 and 9 days after treatment, respectively, and for the untreated group 3.5 ± 3.0 %ID/g, 3.1 ± 2.8 %ID/g and 6.9 ± 4.1 %ID/g at 2, 5 and 9 days after injection of empty LCL, respectively. A good correlation was found between values obtained for ^{99m}Tc-HYNIC-28H1 uptake by quantitative SPECT analysis and arthritis score (Fig. 3B, Spearman's $r = 0.6830$, $p < 0.0001$).

Biodistribution Studies

Measurement of the activity concentration in dissected organs showed that joint accumulation of ^{99m}Tc-HYNIC-28H1 was lower in arthritic mice treated with PLP-LCL as compared to the control group ($p = 0.01$, Fig. 4A). Joint accumulation was 5.9 ± 3.1 %ID/g, 3.3 ± 1.4 %ID/g and 4.1 ± 1.2 %ID/g in treated mice 2, 5 and 9 days after treatment, respectively. These values were lower than the values found in the control group, where joint accumulation was 7.2 ± 5.2 %ID/g, 5.1 ± 4.2 %ID/g and 11.7 ± 5.6 %ID/g at 2, 5 and 9 days after receiving empty LCL, respectively. Whole-body distribution of the radioactivity in treated mice did not significantly differ from distribution in the control group at all time points (data not shown). Additionally, in line with the quantitative SPECT analysis, a correlation was found between the values for ^{99m}Tc-HYNIC-28H1 uptake in the biodistribution studies and the arthritis scores (Fig. 4B, Spearman's $r = 0.6699$, $p < 0.0001$). Additionally, the uptake values for ^{99m}Tc-HYNIC-28H1 in the biodistribution studies correlated with the uptake values found in the quantitative SPECT analysis ($r = 6.072$, $p < 0.0001$).

DISCUSSION

The present study demonstrated that SPECT images 24 h post injection of ^{99m}Tc-labeled anti-FAP antibodies reflected severity of arthritis and therapeutic efficacy (6,11,12,20). This shows that ^{99m}Tc-HYNIC-28H1 can be used for non-invasive monitoring of the therapeutic effects of PLP-LCL in this model. Our previous study showed that uptake of ^{111}In-diethylenetriamine pentaacetate (DTPA)-28H1 in inflamed joints was specific and correlated with severity of arthritis and ^{111}In-DTPA-28H1 SPECT/CT resulted in high quality images (16). This indicated that molecular imaging with anti-FAP antibodies might be of added value for early and sensitive response monitoring.

Ideally, multiple images would preferably be acquired within a short period of time, just before therapy and early after therapy. To allow for this, repeated injections of the tracer are necessary. High

quality images were obtained using ^{111}In-DTPA-28H1 and the half-life (2.8 d) of ^{111}In is suitable for antibody imaging, its half-life is too long to perform repeated imaging within the same patient within a short period of time. Therefore, we radiolabeled 28H1 with a radionuclide with a relative short physical half-life. With a half-life of 6 hours, ^{99m}Tc is a potentially suitable radionuclide for repeated imaging at multiple time points shortly after each other. To allow efficient and stable labeling of 28H1 with ^{99m}Tc, the antibody was conjugated with HYNIC and labeled in the presence of tricine as coligand.

Since 28H1 is an humanized antibody that would induce a severe immune response upon repeated exposure in mice, we were not able to image these mice repeatedly and follow therapeutic effects in the same animal. However, here we show that ^{99m}Tc-HYNIC-28H1 might be used to monitor therapeutic effects in RA patients at multiple time points. The potential ability of our construct to monitor therapeutic efficacy was examined using PLP-LCL therapy, since this has shown to result in a rapid, strong therapeutic benefit in experimental RA models (6,11,12,20). The therapeutic effects were monitored by visual arthritis scoring of the mice, which already decreased two days after administration of PLP-LCL and showed almost complete remission of macroscopic signs of arthritis at least until 9 days post treatment (Fig. 1). Upon PLP treatment, less TNF-α and IL-1 is produced in the inflamed joints, which leads to decreased activation of FLS, resulting in reduced production of FAP (9,10). We hypothesized that this effect can be visualized non-invasively with ^{99m}Tc-HYNIC-28H1 SPECT/CT. High image quality was obtained, with low activity of the radiolabeled antibody in background tissue (Fig. 2). In time, images show increasing uptake in the joints of mice in the control group and decreasing uptake in the joints of mice that received PLP-LCL, demonstrating that the therapeutic effect of PLP-LCL can be visualized non-invasively using ^{99m}Tc-HYNIC-28H1 SPECT/CT. These findings were confirmed after quantification of the SPECT images (Fig. 3A) and the *ex vivo* biodistribution studies (Fig. 4A). Of both analyses, values correlated with the macroscopic arthritis scores (Figs. 3B and 4B), as was also found with the ^{111}In-

labeled compound (16). Although to a lesser extent compared the macroscopic score, also a correlation was found between quantitative imaging *ex vivo* biodistribution data.

Significant uptake of ^{99m}Tc-HYNIC-28H1 was shown in multiple joints in individual mice, while these joints were not taken into account in our macroscopic scoring system, resulting in a more complete representation of the severity of disease. Even paws with low arthritis scores were already visualized by ^{99m}Tc-HYNIC-28H1 SPECT, while these joints did not show obvious macroscopical symptoms, indicating the potential high sensitivity of this imaging method to monitor onset of disease. This suggests that this imaging method outperforms the macroscopic arthritis scoring method in identifying mildly involved joints and by being able to measure inflammation in joints that cannot be scored macroscopically.

Quantitative SPECT analysis at day 9 after therapy can also support the hypothesis of high sensitivity of imaging with ^{99m}Tc-HYNIC-28H1. The arthritis scores did not increase in the treated group, while the SPECT values increased, though not significantly. This can indicate an early decrease of PLP effect, that could not yet been detected macroscopically, emphasizing that molecular imaging might detect flare-ups at earlier time points as compared to macroscopic arthritis scoring. However, we cannot rule out that the increase of tissue uptake is a result of variations between de groups. The mice that were imaged at day 5 were not the same mice as imaged at day 9. The mice were divided in groups based on the macroscopic scores at the start of therapy, but due to the systemic and progressive character of the model, the groups were not equally affected at time of imaging. Though no significant differences were found in macroscopic scores between the mice in day 5 and day 9 groups at the start of therapy, the differences in tissue uptake could be explained by slightly more severely affected mice in the day 9 group.

In conclusion, ^{99m}Tc-HYNIC-28H1 SPECT is more specific and potentially a more sensitive method for early detection of exacerbations in RA and for therapy monitoring, as compared with the arbitrary macroscopic scoring system. Therefore, this reliable, non-invasive method can be valuable for immediate therapy monitoring and further therapy planning in RA patients to improve personal treatment strategies.

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FIGURE LEGENDS

FIGURE 1

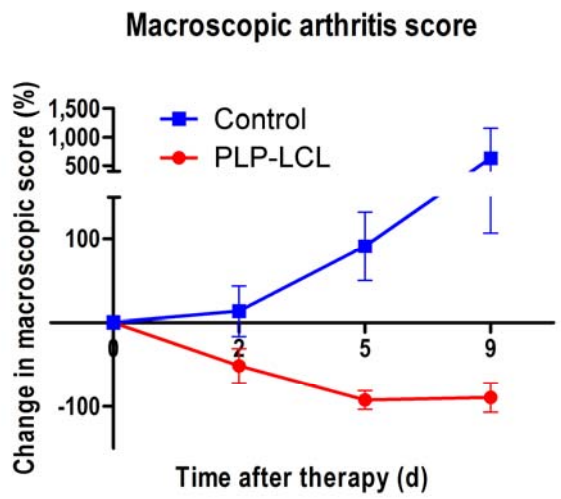

Fig. 1. Change in macroscopic arthritis scores in time after a single treatment with 10 mg/kg PLP-LCL (red circles) compared to empty LCL (blue squares). Values are mean ± SD of 5 mice per group. Mann-Whitney *U* test showed that the difference between control and treated animals is significantly different at all time points (p = 0.01, p = 0.01 and p = 0.02 at 2, 5 and 9 days after treatment, respectively).

FIGURE 2

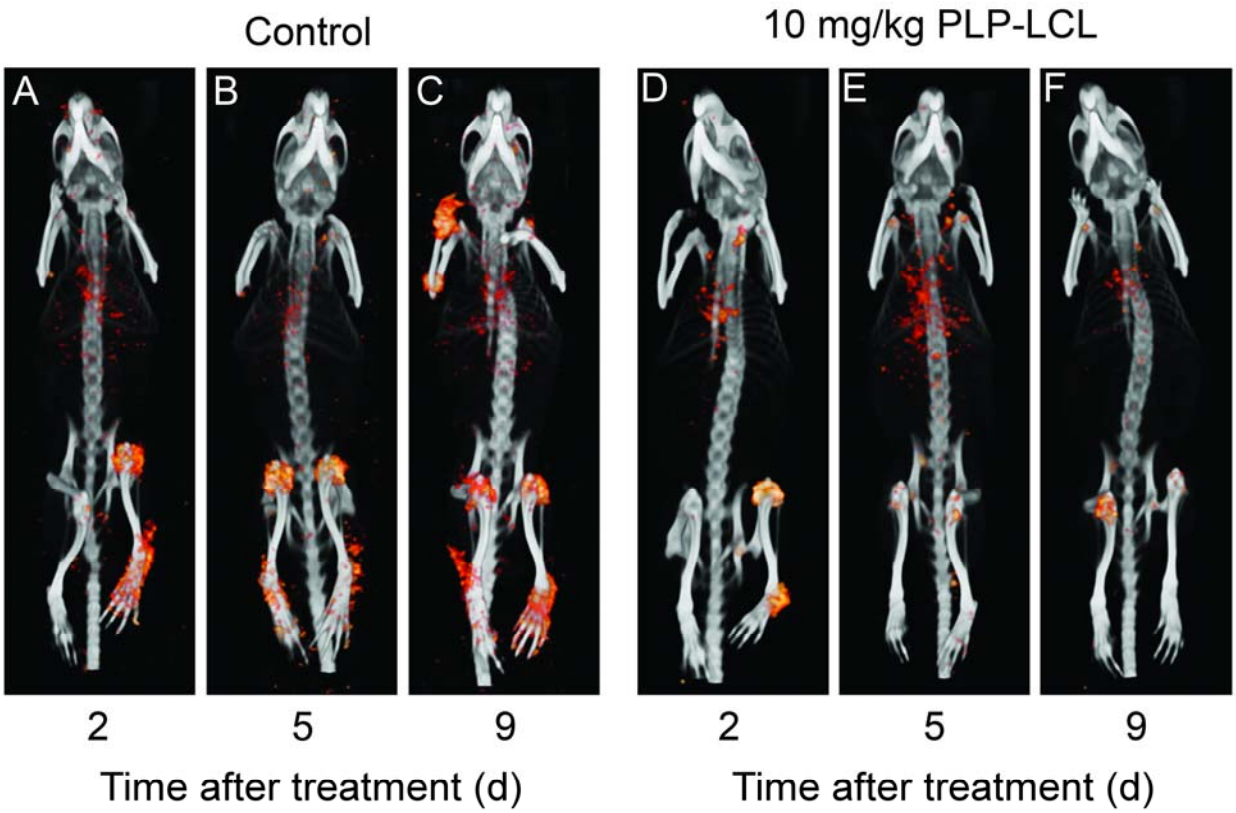

Fig. 2. 3D SPECT/CT scans of control (A-C) and PLP-LCL-treated (D-F) CIA mice 24 h p.i. of ^{99m}TC-28H1 (50 µg). Image acquisition was performed 2 (A and D), 5 (B and E) and 9 (C and F) days after therapy. All images are scaled equally.

FIGURE 3

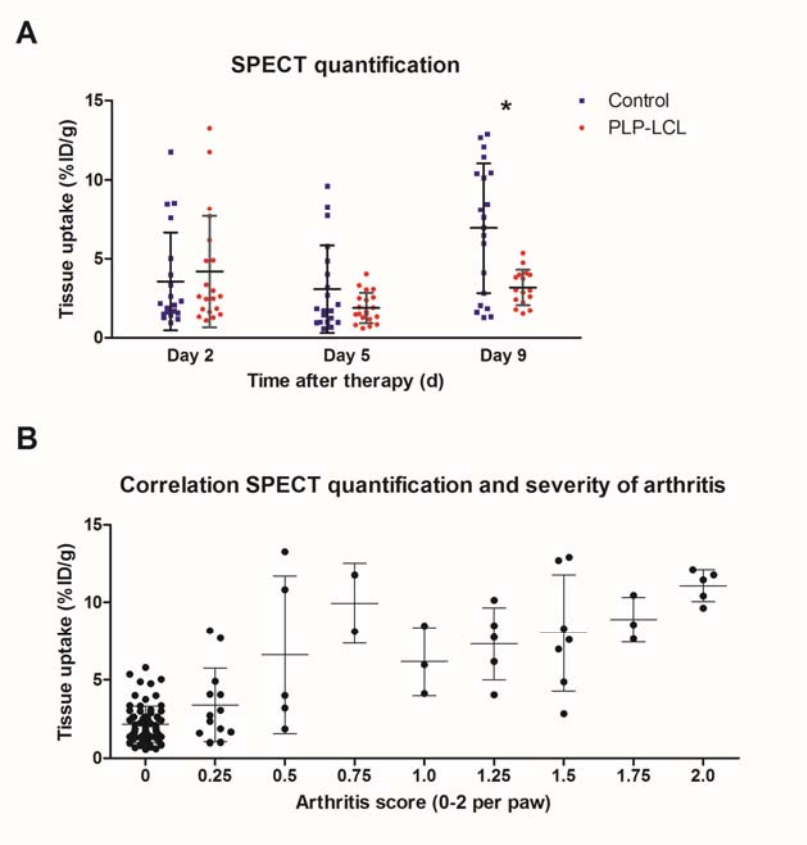

Fig. 3. A.Tissue uptake based quantitative SPECT analysis (A) at 2, 5 and 9 days after treatment with 10 mg/kg PLP-LCL. Mann-Whitney *U* tests show significant lower tissue uptake after treatment with PLP-LCL compared to control groups ($p = 0.02$). B.Correlation between tissue uptake of ^{99m}Tc-HYNIC-28H1 24 h p.i. and arthritis score (Spearman $r = 0.6830$, $p < 0.0001$). Tissue uptake (%ID/g) per individual paw (n = 4-5 mice/group, 4 joints/mouse) is shown.

FIGURE 4

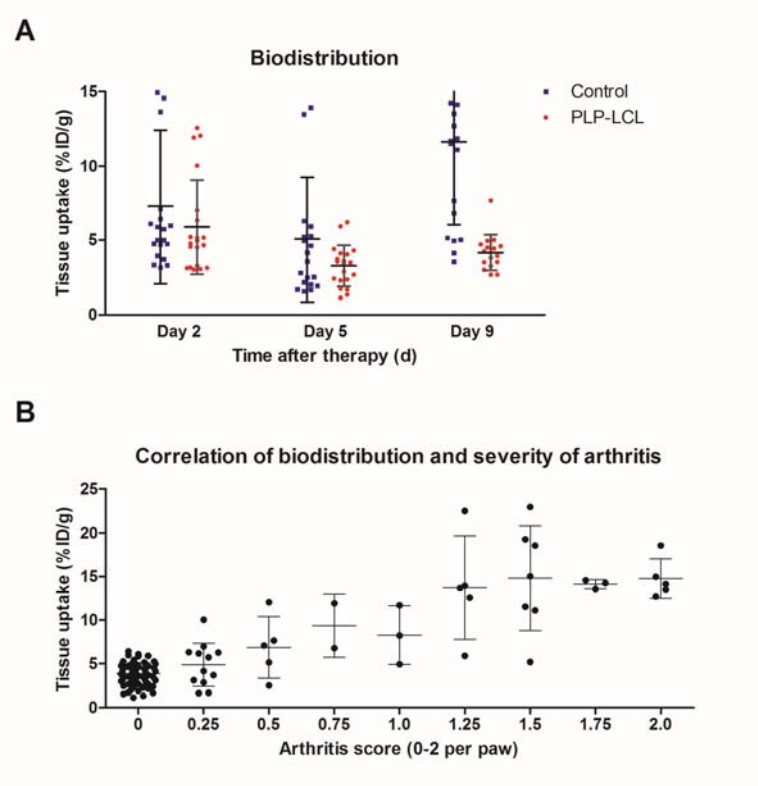

Fig. 4. A.Tissue uptake based biodistribution studies (A) at 2, 5 and 9 days after treatment with 10 mg/kg PLP-LCL. Mann-Whitney *U* tests show significant lower tissue uptake after treatment with PLP-LCL compared to control groups ($p = 0.01$). B.Correlation between tissue uptake of ^{99m}Tc-HYNIC-28H1 24 h p.i. and arthritis score (Spearman $r = 0.6699$, $p < 0.0001$). Tissue uptake (%ID/g) per individual paw (n = 4-5 mice/group, 4 joints/mouse) is shown.

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