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Induction of arthritis in rats with mBSA

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Disclaimer

The procedures described in this animal experimentation protocol were drawn up in accordance with current rules and regulations on animal ethics and welfare. All animal experimentation practices carried out in this study were approved by the Animal Use Ethics Committee (CEUA) and follow the guidelines established by the National Council for the Control of Animal Experimentation (CONCEA). The procedures described in this protocol must be carried out by trained professionals and in appropriate facilities, under the supervision of qualified personnel. We assume no responsibility for damages or losses arising from the improper application or use of the information contained in this document.

Abstract

Protocol for inducing arthritis in rats, immunofluorescence and immunohistochemistry of the synovium, ELISA for detecting antibodies and interleukin dosage, lymphoproliferation assay and PET/CT imaging.

Materials

Male Lewis rats; Tramadol hydrochloride; Ketamine hydrochloride; Xylazine hydrochloride; mBSA (Sigma Chemical, St. Louis, MO); Freund's complete adjuvant; Saline solution; acetic acid (0.01 N); hematoxylin and eosin (H&E); Isoflurane; Radiotracer (18-37 MBq); PET scanner (Triumph® II Trimodality System, CA, USA); Hydrogen peroxide solution; Methanol; Anti-CD3 mouse monoclonal antibody (1 mg/mL; Dako); anti-CD4 (0.2 mg/mL; Santa Cruz Biotechnology Inc.); anti-CD8 antibody (0.2 mg/mL; Santa Cruz Biotechnology Inc.); anti-CD20 antibody (0.2 mg/mL; Santa Cruz Biotechnology Inc.); anti-CD68 antibody (0.5 mg/mL; Santa Cruz Biotechnology Inc.); anti-FoxP3 antibody (0.2 mg/mL; Santa Cruz Biotechnology Inc.); anti-IL10 antibody (0.2 mg/mL; Santa Cruz Biotechnology Inc.); citrate buffer solution; Plates for ELISA (Immunolon II, Thermo Fisher Scientific); Col II; Col V; bicarbonate buffer; PBS and PBS in Tween 20 (Sigma–Aldrich); BSA in PBS (Sigma–Aldrich); alkaline phosphatase-conjugated goat anti-rat IgG antibody (Sigma–Aldrich); p-nitrophenyl phosphate (pNPP); diethanolamine; MgCl2 buffer; ELISA reader (Labsystem Multiskan MS); Col I, II, III and V antibodies; bovine pepsin (Sigma Chemical Co., St. Louis, MO, USA; 250 units/mg); BSA; Alexa 488-conjugated goat anti-rabbit IgG antibodies (Invitrogen, Life Technologies); 0.006% Evans blue; glycerin solution; fluorescence microscope (Olympus BX-51, Olympus Co., Tokyo, Japan); mouse monoclonal anti-IL10 antibody (0.2 mg/mL; Santa Cruz Biotechnology Inc.); Rabbit polyclonal anti-FoxP3 antibody (0.2 mg/mL; Santa Cruz Biotechnology Inc.); Alexa 546-conjugated (red) anti-rabbit/mouse IgG antibodies (Invitrogen, Life Technology); DAPI (Abcam, Cambridge, UK); RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin; Ficoll-Paque; Trypan blue; Neubauer chamber; 96-well culture plate (R&D Systems, Minneapolis, MN, USA); Concanavalin A; humified chamber; ELISA kit for IL-10 (R&D Systems, Minneapolis, MN, USA).

Troubleshooting

Materials and Methods

1 **Experimental protocol**

Twenty-five three-month-old male Lewis rats with an average body mass of 360g were provided by the animal facility of our institution. All of the experimental procedures were approved by The Committee on Ethical Use of Laboratory Animals of the Faculty of Medicine at the University of São Paulo (Process code: 1294/2019). The experimental study followed relevant guidelines, such as ARRIVE, and global regulations.

For preventive analgesia, tramadol hydrochloride injections (40 mg/kg body weight) were administered subcutaneously one hour before anesthesia and every 8 hours for 48 hours after the experimental procedure. Intraperitoneal anesthesia (ketamine hydrochloride at a dose of 100 mg/kg body mass and xylazine hydrochloride at a dose of 10 mg/kg body mass) was administered. Then, arthritis (IA, n=20) was induced on the right knee with 20 µL of a 500 µg emulsion of mBSA (Sigma Chemical, St. Louis, MO), diluted in 10 µL of saline and 500 µL of Freund's complete adjuvant, delivered intra-articularly, followed by an intra-articular booster of 20 µL of an emulsion of 50 µg of mBSA in saline on days 7 and 14 of the experimental protocol. After the intra-articular injections, some animals (n=10) received Col V (500 µg/300 µL) (ATAYDE et al., 2018) diluted in acetic acid (0.01 N) by gavage 3 times a week from days 15 to 30 (IA-Col V). The control group (CT-Col V, n=5) was given saline (20 µL) and Col V by gavage using the same protocol. The animals were euthanized by intraperitoneal injection of an anesthetic overdose (ketamine hydrochloride at a dose of 300 mg/kg body mass and xylazine hydrochloride at a dose of 30 mg/kg body mass).

2 **Histological and synovial score analysis**

The synovial tissue samples were stained with hematoxylin and eosin (H&E) for morphometric analysis of the synovial architecture and inflammatory cells. Synovial tissue was analyzed at 200x magnification by two observers who did not have prior knowledge of the treatments. Krenn´s score was used, which consists of scoring the synovial lining cell layer, the density of the resident cells and inflammatory infiltrate (0-3 points), after which the points are added together to determine the degree of synovitis (0-9 points) (KRENN et al., 2006).

3 **Evaluation of the inflammatory response using [^{18}F]FDG PET imaging**

The animals underwent a PET scan at baseline (one day before the start of the protocol), 14 days (one day before the start of Col V administration) and 30 days (at the end of the protocol). The animals were then anesthetized with 1.5%-3% isoflurane in 100% oxygen and received an intravenous injection of the [^{18}F]FDG radiotracer (18-37 MBq) via the penile vein. After injection, the animals were allowed to wake up for better radiotracer distribution. After 45 minutes, the animals were again anesthetized and positioned with their knees in the center of the field of view on a PET scanner

(Triumph® II Trimodality System, CA, USA). The images were reconstructed using the 3D-OSEM algorithm using 20 iterations and 4 subsets and quantified using PMOD software version 4.0. [^{18}F]FDG uptake is expressed as the standardized uptake value (SUV), calculated as SUV = radioactivity concentration (Bq/mL)/[injected activity (Bq)/animal weight (g)].

4 **Immunohistochemical analysis of inflammatory cells**

Sections were deparaffinized and blocked in a 0.3% hydrogen peroxide solution to inhibit endogenous peroxidase activity (CD4, CD8 and CD20). For immunostaining of CD3 and FoxP3, the sections were blocked with 0.3% hydrogen peroxide with methanol (v/v), with a cycle of 2 washes for 10 minutes each. For immunostaining of IL-10, the sections were blocked with 0.3% hydrogen peroxide and subjected to additional protein blocking. For immunostaining of CD68, the sections were blocked with 0.3% hydrogen peroxide in methanol (v/v) and subjected to additional protein blocking.

The primary antibodies used were mouse monoclonal anti-CD3 (1:1000; 1 mg/mL; Dako), anti-CD4 (1:50; 0.2 mg/mL; Santa Cruz Biotechnology Inc.), anti-CD8 (1:50; 0.2mg/mL; Santa Cruz Biotechnology Inc.), anti-CD20 (1:600; 0.2 mg/mL; Santa Cruz Biotechnology Inc.) and anti-CD68 (1:3200; 0.5 mg/mL; Santa Cruz Biotechnology Inc.) antibodies and rabbit polyclonal anti-FoxP3 (1:100; 0.2 mg/mL; Santa Cruz Biotechnology Inc.) and anti-IL10 (1:50; 0.2 mg/mL; Santa Cruz Biotechnology Inc.) antibodies. Antigen retrieval was performed with citrate buffer solution, pH 6.0 (for CD3, CD20, FoxP3 and CD68) or pH 9.0 (for CD4, CD8 and IL-10), at 125°C for 1 min in a pressure cooker (Pascal), and the samples were incubated with the primary antibody overnight at 4°C. According to the manufacturer's instructions, the reaction was visualized using a biotin-streptavidin peroxidase kit (Vector). 3,3'-Diaminobenzidine (Sigma Chemical, St. Louis, MO) was used as the chromogen. The sections were counterstained with Harris hematoxylin (H&E; Merck, Darmstadt, Germany).

5 **ELISA for anti-Col II and anti-Col V antibodies**

The plates (Immunolon II, Thermo Fisher Scientific)were sensitized with Col II and Col V (1 µg/well), diluted in bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were washed with 0.05% PBS in Tween 20 (Sigma–Aldrich) and blocked with 1% BSA in PBS (Sigma–Aldrich) for 1 hour at room temperature. Serum samples were diluted 1:50 in 1% BSA in PBS with 0.05% Tween 20 (Sigma–Aldrich), added to duplicate wells, and incubated for 1 hour at room temperature. After a wash cycle, an alkaline phosphatase-conjugated goat anti-rat IgG antibody (Sigma–Aldrich) was diluted 1:1000 in 1% BSA in PBS with 0.05% Tween 20 and added to each well for 1 hour at room temperature. The reaction was visualized by adding 50 µl/well of 1 mg/mL p-nitrophenyl phosphate (pNPP) diluted in 1 M diethanolamine and 0.5 mM MgCl2 buffer, pH 9.8. Optical density was determined with an ELISA reader (Labsystem Multiskan MS) at 405 nm. Control serum was obtained from the rats before arthritis induction. The cutoff values for Col II and Col V antibodies were

determined by adding the mean of the serum negative controls three times the standard deviation.

6 **Immunofluorescence analysis of collagen fibers**

For Col I immunostaining, exposure and recovery of antigenic sites were performed in citrate buffer, pH 6 (Diagnostic BioSystems, Pleasanton, CA, USA), for 10 min at 95°C in a steam cooker. For immunostaining of Col III and V, digestion with bovine pepsin (8 mg/500 µl 0.5 N acetic acid) (Sigma Chemical Co., St. Louis, MO, USA; 250 units/mg) was performed for 30 minutes at 37°C. Nonspecific binding sites were blocked by incubating the sections in 5% BSA in PBS for 30 minutes. Then, the slides were incubated overnight at 4°C with rabbit polyclonal anti-Col I (1:100; 1.15 mg/mL; Rockland), anti-Col III (1:100; 1.16 mg/mL; Rockland) and anti-Col V (1:600) (TEODORO et al., 2004) antibodies diluted in PBS. After this period, the sections were incubated for 1 hour with Alexa 488-conjugated goat anti-rabbit IgG antibodies (Invitrogen, Life Technologies) diluted 1:200 in 0.006% Evans blue. Finally, the slides were mounted on coverslips with buffered glycerin solution and analyzed under a fluorescence microscope (Olympus BX-51, Olympus Co., Tokyo, Japan).

Briefly, for colocalization assays, slides were incubated overnight at 4°C with a mouse monoclonal anti-IL10 antibody (1:50; 0.2 mg/mL; Santa Cruz Biotechnology Inc.) and a rabbit polyclonal anti-FoxP3 antibody (1:100; 0.2 mg/mL; Santa Cruz Biotechnology Inc.). After this period, the sections were incubated for 1 hour at room temperature with Alexa Fluor 488-conjugated (green) and Alexa 546-conjugated (red) anti-rabbit/mouse IgG antibodies (Invitrogen, Life Technology) diluted 1:200 in PBS. The cell nuclei were labeled by incubation with DAPI (Abcam, Cambridge, UK) for 5 minutes. The slides were analyzed under a fluorescence microscope (Olympus BX-51, Olympus Co., Tokyo, Japan).

7 **Cellular and collagen fiber quantification**

Morphometric analysis was performed using Image-Pro Plus 6.0 software. The stereological point counting method (GUNDERSEN et al., 1988) was used to quantify the immunostained cells and cytokines; a reticular grid with 100 points distributed orthogonally on the acquired image was used. Ten fields of synovial tissue were evaluated at a magnification of 1000×, and cell expression was determined according to the number of positive cells coinciding with the crosshair grid in each field and is expressed as the percentage of positive cells relative to the total number of cells. To quantify the area of synovial tissue occupied by collagen fibers, images were acquired at a magnification of 400×, and 10 fields of view were evaluated. The collagen fibers were evaluated by selecting the fluorescent green hue corresponding to each type of collagen stained. The immunostained area was divided by the total area of the analyzed tissue, and the result was expressed as a percentage.

8 **Cell culture and IL-10 production analysis**

The spleens of the IA and IA-Col V groups were collected aseptically and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin for

maceration through a metal mesh for cell separation. The single-cell suspension was maintained for 2 minutes to separate the particulate material. The supernatant was washed in RPMI 1640 by centrifugation at 800 ×g for 5 minutes at 4°C. The mononuclear cells were separated by density gradient with Ficoll-Paque in sterile PBS for 30 minutes at 500 ×g and 20°C. The layer of mononuclear cells was washed in RPMI 1640. After the cells were stained with Trypan blue, the viable cells were counted in a Neubauer chamber. Then, the cells were placed in a 96-well culture plate (R&D Systems, Minneapolis, MN, USA) with 5×10^5 cells in 300 µL of RPMI 1640 per well and stimulated with Col V (100 µg/mL), concanavalin A (5µg/mL) or without stimulus. The cells were maintained in a humified chamber with 5% CO2 at 37°C for 48 hours. The conditioned culture media were collected and maintained at -70°C until analysis. Next, we measured the concentration of IL-10 by a capture ELISA kit (R&D Systems, Minneapolis, MN, USA) using duplicate samples according to the manufacturer's instructions.

9 **Statistical analysis**

For statistical comparisons of the means/medians of the groups, one-way ANOVA and two-way ANOVA or the Kruskal–Wallis test were used, in addition to the Dunn or Sidak posttest. For correlations between variables, Spearman's test was used. When analyzing the differences between means/medians, a *p* value of less than 5% ($p<0.05$) was considered to indicate statistical significance.

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