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Effect of tryptase inhibition on joint inflammation: a pharmacological and lentivirus-mediated gene transfer study

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Abstract

Background: Increasing evidences indicate that an unbalance between tryptases and their endogenous inhibitors, leading to an increased proteolytic activity, is implicated in the pathophysiology of rheumatoid arthritis. The aim of the present study was to evaluate the impact of tryptase inhibition on experimental arthritis.

Methods: Analysis of gene expression and regulation in the mouse knee joint was performed by RT-qPCR and in situ hybridization. Arthritis was induced in male C57BL/6 mice with mBSA/IL-1β. Tryptase was inhibited by two approaches: a lentivirus-mediated heterologous expression of the human endogenous tryptase inhibitor, sperm-associated antigen 11B isoform C (hSPAG11B/C), or a chronic treatment with the synthetic tryptase inhibitor APC366. Several inflammatory parameters were evaluated, such as oedema formation, histopathology, production of IL-1β, -6, -17A and CXCL1/KC, myeloperoxidase and tryptase-like activities.

Results: Spag11c was constitutively expressed in chondrocytes and cells from the synovial membrane in mice, but its expression did not change 7 days after the induction of arthritis, while tryptase expression and activity were upregulated. The intra-articular transduction of animals with the lentivirus phSPAG11B/C or the treatment with APC366 inhibited the increase of tryptase-like activity, the late phase of oedema formation, the production of IL-6 and CXCL1/KC. In contrast, neutrophil infiltration, degeneration of hyaline cartilage and erosion of subchondral bone were not affected.

Conclusions: Tryptase inhibition was effective in inhibiting some inflammatory parameters associated to mBSA/IL-1β-induced arthritis, notably late phase oedema formation and IL-6 production, but not neutrophil infiltration and joint degeneration. These results suggest that the therapeutic application of tryptase inhibitors to rheumatoid arthritis would be restrained to palliative care, but not as disease-modifying drugs. Finally, this study highlighted lentivirus-based gene delivery as an instrumental tool to study the relevance of target genes in synovial joint physiology and disease.

Keywords: Tryptase, β-defensin, SPAG11B, APC366, Protease inhibitor, Inflammation, Synovial joint, Rheumatoid arthritis
Rheumatoid arthritis (RA) is a multifactorial autoimmune disease affecting nearly 1% of the world population, whose pathophysiology involves multiple cellular and molecular processes underlying synovial inflammation, joint swelling and pain, and ultimately destruction of articular cartilage and subchondral bone [1]. One of the histopathological hallmarks of RA is a marked increase of mast cell infiltration within the synovium [2–4]. Initial indications on how mast cells could be implicated in the pathophysiology of RA came from studies demonstrating that mast cell-deficient mice, bearing mutations in the c-Kit signalling pathway, developed less severe forms of antigen- or autoantibody-induced arthritis [5–7]. Recent studies, based on more selective strategies for mast cell deficiency in mice, supported a role for these cells in this disease, notably in the pre-clinical phase of T cell-driven antigen-induced arthritis models [8, 9].

The trypsin-like serine protease isoenzymes tryptase-α/β1 (TPSAB1) and -β2 (TPSB2), commonly referred as tryptases, are amongst the most abundant proteases stored within mast cell secretory granules and are pivotal pro-inflammatory mediators in the pathophysiology of allergic and inflammatory diseases [10, 11]. For instance, genetically modified mice lacking mast cell protease-6 and -7 (Mcpt-6 and Mcpt-7), have reduced inflammatory and degenerative parameters associated to experimental arthritis [12, 13]. Indeed, tryptases are emerging as potential therapeutic targets to treat chronic inflammatory diseases. However, these enzymes are stored and secreted as tetramers, wherein the active site is sheltered within the oligomeric catalytic pocket. While this tetrameric assembly conveys substrate specificity, it renders this enzymatic complex resistant to traditional inhibitors, such as α1-anti-trypsin and α2-macroglobulin [14, 15]. Additionally, this structural arrangement challenges the design of highly selective and orally bioavailable inhibitors [16].

Interestingly, the N-terminal region of the recombinant human β-defensin sperm-associated antigen 11B isoform D (hSPAG11B/D), which is conserved in hSPAG11B/C, has been reported in vitro as a potent inhibitor of tryptase-β1 [17]. β-defensins are anti-microbial proteins primarily associated with host defense [18, 19]. Moreover, increasing evidences suggest that β-defensins have immunomodulatory properties and a variety of other non-immunological activities [20, 21]. Indeed, we recently reported that the rat β-defensin SPAG11C, the ortholog of the human SPAG11B isoform C, hSPAG11B/C, is expressed in articular chondrocytes during the male rat embryonic development [22]. However, their potential contribution in controlling inflammatory conditions such as RA remains elusive.

Herein, we hypothesized that an unbalance between tryptases and their endogenous inhibitors, leading to an increased proteolytic activity, is implicated in the pathophysiology of RA. Therefore, we sought to investigate whether this novel endogenous tryptase inhibitor, SPAG11C, is expressed and under regulation during experimentally induced arthritis in the adult mouse knee joint. Considering evidence from studies employing genetically engineered tryptase-deficient mice [12, 13], we also evaluated the potential of tryptase inhibition as a therapeutic alternative for the management of RA.

We assessed the impact of two distinct approaches for tryptase inhibition on several inflammatory parameters associated to methylated bovine serum albumin/interleukin-1β (mBSA/IL-1β)-induced arthritis: the lentivirus-mediated heterologous expression of hSPAG11B/C gene product within the adult mouse knee joint, as well as the mouse intra-articular treatment with the synthetic tryptase inhibitor APC366 [23].

### Methods

#### Cloning of the hSPAG11B/C coding sequence into pWPXld-IG

The lentivirus vector pWPXld (Addgene, Cambridge, MA, USA) was modified by the replacement of the enhanced green fluorescent protein (eGFP) coding sequence by a PCR fragment harboring the internal ribosomal entry site (IRES)-eGFP cassette from pIRE-eGFP vector (Clontech, Mountain View, CA, USA), thus generating the bicistronic vector pWPXld-IG. Additionally, an adaptor oligonucleotide was inserted between PmeI and BamHI in order to increase the restriction endonuclease repertoire in the multiple cloning site (MluI, SglI, PvuII, RsrII and BstSAI).

The coding sequence of hSPAG11B/C was obtained from human testis and epididymis total ribonucleic acid (RNA) reverse transcribed using ThermoScript RT-PCR System (Thermo Fisher Scientific, Waltham, MA, USA) followed by a PCR performed with the Phusion High-Fidelity DNA Polymerase Kit (NEB, Ipswich, MA, USA) and respective oligonucleotide pair as follows: forward 5’-AGTTTTAACGCCACCATGAGGCAACGA-3’; reverse 5’-CTATGGATCCTTAAATGTAAACAGCAGGCGTC-3’. QIAquick purified (Qiagen, GmbH, Hilden, Germany) PCR products were A+ tailed (Thermo Fisher Scientific) and ligated into the transfer plasmid pGEM-T Easy System I (Promega, Madison, WI, USA), transformed into TOP10 competent cells (Thermo Fisher Scientific), amplified and purified using QIAprep Spin Miniprep Kit (Qiagen). Inserts of interest were released from positive pGEM-T plasmids by double digestion with the endonucleases PmeI and BamHI (NEB), fractioned by preparative agarose gel electrophoresis, purified with the QIAquick Gel Extraction Kit (Qiagen) and ligated into...
linearized and dephosphorylated (NEB) pWPXLD-IG lentivirus vectors using a 1:3 vector to insert ratio with the T4 DNA Ligase Kit (Thermo Fisher Scientific). For simplification reasons, the resulting vector pWPXLD-hSPAG11B/C-IG will be referred as phSPAG11B/C. NEB 5-alpha electrocompetent E. coli were transformed with the ligation reaction mix using a MicroPulser Electroporator (Bio-Rad, Hercules, CA, USA). Clones were amplified and purified and subcloning efficiency was confirmed by automatic DNA sequencing. Lentivirus transfer and the structural vectors pMD2.G and psPAX.2 (Addgene plasmids #12260 and #12259, both provided by D. Trono) were amplified and purified using the NucleoBond® Xtra Maxi Plus EF Kit (Macherey-Nagel, Düren, Germany).

**Lentivirus production and titration**

HEK293T/17 cells were cultured according to supplier’s recommendations (ATCC, Manassas, VA, USA). Cells (1.7 × 10^7 per plate) were seeded into 10-cell culture flasks (175 cm^2) containing 30 mL of DMEM (Gibco, Carlsbad, CA, USA) and then incubated at 37 °C 5% CO_2_. The next day, cells were transfected with a mixture of structural (146 μg of psPAX2 and 79 μg of pMD2.G) and transfer vectors (225 μg of pWPXLD-IG or phSPAG11B/C), by using the transfection reagent GeneJuice (EMD Millipore, Billerica, MA, USA). Cells were incubated overnight at 37 °C 5% CO_2_, then the medium was replaced by 18 mL of OptiMEM (Gibco). Cell culture supernatants were harvested 24 and 48 h later. Each supernatant was cleared by centrifugation and filtration with a 0.45 μm syringe filter and stored at 4 °C. The virus harvests from 24 and 48 h were pooled and layered onto 5 mL of a 20% sucrose solution in Dulbecco’s phosphate-buffered saline (DPBS) containing Ca^{2+} and Mg^{2+} and then centrifuged at 106,750 × g for 2 h. The pellets were solubilized in DPBS, the samples were fractioned into 20 μL aliquots and stored at -80 °C until use. For the biological titration of the lentiviruses, HEK293T/17 cells (4 × 10^4 per well) were seeded into a 24-well plate containing coverslips. The next day, cells were transduced with a serial dilution of lentivirus (10^{-5} to 10^{-10}) and cultivated for an additional 72 to 92 h. Cells were fixed with 4% buffered formalin and processed for immunofluorescence, as described below. The protocol presented above is the final standardization of several attempts to optimize the production of recombinant lentivirus at high titers for in vivo use.

**Animals**

Male C57BL/6 mice (N = 127, 8–10 weeks old, weighting from 22.5–27.3 g), which naturally lack functional Mcpt-7, were from Institute of Pharmacology and Molecular Biology (INFAR) animal facility (Universidade Federal de São Paulo – Escola Paulista de Medicina, UNIFESP-EPM, São Paulo, Brazil) and from Janvier Labs (Le Genest-Saint-Ise, France). Mice were housed in groups of up to five animals per cage with access to standard food and water ad libitum, and maintained in level 2 biosafety installations under a 12/12 h light/dark cycle and controlled room temperature (22 ± 1 °C). All invasive or stressful procedures were performed in animals under anaesthesia, either inhalatory isoflurane 1.5% v/v in oxygen or intraperitoneal ketamine (80 mg/kg and xylazine 20 mg/kg, respectively).

**Induction and analysis of experimental arthritis**

Animals were submitted to methylated bovine serum albumin/interleukin-1β (mBSA/IL-1β)-induced arthritis, as previously described [12]. Briefly, arthritis was induced by one intra-articular injection in the right knee joint with 10 μL of mBSA (Sigma-Aldrich, St. Louis, MO, USA) at 20 mg/mL in vehicle (PBS; phosphate-buffered saline), followed by three subcutaneous daily injections in the ipsilateral rear footpad with 250 ng of recombinant human interleukin 1β (rhIL-1β; Peprotech, Rocky Hill, NJ, USA) diluted in 20 μL of vehicle (0.9% sodium hydrochloride (NaCl) solution with 0.5% normal C57BL/6 mouse serum). Animals from the control group were submitted to the same procedures, but injected with the respective vehicle solutions. Oedema formation was monitored by measurements (triplicates) of the medio-lateral knee joint diameter by using a specially designed spring-loaded calliper (model #C1X018; Kroepelin, GmbH, Schluchtern, Germany) on days 0, 1, 2, 5 and 7. Animals were sacrificed at the peak of disease, 7 days after the induction of arthritis [24]. The knee joint samples were harvested and processed accordingly to the downstream applications to be performed. For histopathological analysis, the knee joints were fixed in 4% buffered formaldehyde, decalcified and embedded in paraffin. Medial sections with 5 μm thickness were obtained and submitted to haematoxylin and eosin (H&E) or safranin O staining. The severity of arthritis was scored by observers, single blinded to the experimental groups, by using a semi-quantitative scale based on a previously established method [12, 24, 25]. Briefly, the severity of arthritis in coded slides was graded from 0 (normal) to 5 (severe) for five components that comprised joint space exudate, synovitis, pannus formation, cartilage degradation and bone erosion. Likewise, joint space exudate was identified as leukocytes, either scattered or in aggregates within the joint space. Synovitis was defined as hyperplasia of the synovial membrane due to the proliferation of lining layer fibroblast-like synoviocytes as well as to the infiltration of polymorph and mononuclear leukocytes. Pannus formation was defined as hypertrophic synovial tissue forming tight junctions with articular surfaces. The extent of cartilage and
bone erosion was evaluated separately on both condylar surfaces. Scoring was based on the loss of cartilage matrix, disruption and loss of cartilage surface, and the extent and depth of subchondral bone erosion. The average score for two sections analysed from each joint was calculated for each component, then the mean values from the five components was summed, giving an overall mean histopathological severity score for each joint (maximum possible score of 25).

**Lentivirus-mediated heterologous expression of hSPAG11B/C**

Animals were submitted to an intra-articular injection into the right knee joint with 20 μL of 2 × 10^6 transduction units (TU) per joint of pWPXLd-IG or phSPAG11B/C. Arthritis was induced 7 days later, as described above. Seven days after the induction of arthritis, the animals were sacrificed; the knee joints were harvested and processed for histopathological analysis as described above. In other sets of experiments, 7 days after the induction of arthritis, the knee joint was washed twice with 25 μL of sterile saline, which was pooled and then centrifuged at 3000 × g for 5 min at 4 °C. The resulting cell pellets and supernatants were stored separately at -80 °C until required for downstream experiments. Additionally, the knee joint was harvested and stored at -80 °C. As a control procedure for intra-articular lentivirus injection, in every experimental set, one group of animals was injected with the same volume of lentivirus vehicle (PBS), 7 days prior to the induction of arthritis.

**Intra-articular administration of APC366**

Mice were treated by intra-articular injections with the synthetic tryptase inhibitor APC366 (10 μL of 10 or 100 μM; Ki = 7.1 μM) or its vehicle (DMSO 0.1%) 1 h before the induction of arthritis, which were repeated every other day (days 2, 4 and 6). Oedema formation was monitored as described above. The animals were killed 7 days later and both the synovial fluid and knee joints were harvested for downstream assays, as described above.

**Reverse transcription, end-point and semi-quantitative PCR**

Total RNA was extracted from knee joints using the RNaseasy Mini Plus Kit (Qiagen) and 1 μg was reverse transcribed with the ThermoScript RT-PCR System and Oligo d(T), according to manufacturer’s instructions (Thermo Fisher Scientific). For end-point PCR, complementary deoxyribonucleic acid (cDNA) was amplified with Taq DNA polymerase Kit (Thermo Fisher Scientific) and 800 nM of each forward and reverse oligonucleotide (Table 1). Semi-quantitative PCR (qPCR) was performed with the SYBR Fast q-PCR Kit (Kapa Biosystems, Cape Town, South Africa) and 300 nM of each forward and reverse oligonucleotide (Table 1). The relative expression of the target gene was normalized to the endogenous control Hprt1, using the method 2^(-ΔΔCt) [26]. The products from end-point, and in some cases, from semi-quantitative PCR, were loaded onto 2% agarose gels containing ethidium bromide, visualized under UV illumination and photographed.

**Immunofluorescence**

Briefly, after permeabilization in a solution containing PBS, 0.1% Triton X-100 and 1% BSA (Sigma-Aldrich), cells were incubated with a primary antibody anti-GFP raised in rabbit (1:1000; Invitrogen, Carlsbad, CA, USA). Slides were mounted with Prolong Gold containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). The number of GFP-positive colonies was counted by epifluorescence microscopy and expressed as transduction units (TU) per mL. Knee joint sections with 10 μm thickness were obtained in a cryostat and mounted onto Superfrost slides (Thermo Fisher Scientific). After permeabilization, the sections were incubated with a primary antibody anti-GFP raised in rabbit (1:1000; Invitrogen) overnight at 4 °C, and then processed as described above. Images were acquired using a Zeiss LSM-710 confocal microscope (Carl Zeiss MicroImaging, GmbH, Jena, Germany).

**In situ hybridization assays**

In situ hybridization was conducted as described previously [22]. Briefly, formalin-fixed paraffin-embedded tissue sections (4 μm) from knee joints were deparaffinized, rehydrated and treated with proteinase K (10 μg/mL for 10 min, room temperature). Sections were then incubated for 16 h at 37 °C with hybridization buffer (30% formamide, 50 mM Tris-HCl, 5 mM EDTA, 618 mM NaCl, and 10% dextran sulfate) containing 200 nM of locked nucleic acid (LNA)-modified antisense oligonucleotide probes labelled with digoxigenin at their 3’- and 5’-ends (Exiqon, Vedbaek, Denmark). The antisense probe targeting an exon–exon junction of the Spag11c transcript sequence was 5'-TGGTGCCAGCT-CATGGTAAAGG-3'. A scrambled LNA probe was used as a negative control. Sections were washed in series of graded saline-sodium citrate buffer (SSC) solutions (5, 1, and 0.2 × SSC) at 37 °C. Hybridized mRNA was detected using sheep antibody anti-digoxigenin conjugated with horseradish peroxidase (Roche, Indianapolis, IN, USA; 1:50 v/v) and peroxidase activity was revealed by 3,3’-diaminobenzidine (DAB) reaction. Sections were counterstained with Toluidine blue and mounted with

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**Table 1.** Semi-quantitative PCR (qPCR) was performed using sheep antibody anti-digoxigenin conjugated with horseradish peroxidase (Roche, Indianapolis, IN, USA; 1:50 v/v) and peroxidase activity was revealed by 3,3’-diaminobenzidine (DAB) reaction. Sections were counterstained with Toluidine blue and mounted with.
Myeloperoxidase (MPO) activity

MPO activity was measured as an index of granulocyte infiltration, as previously described [27]. Briefly, cell pellets from knee joint lavages were homogenized in a potassium phosphate-buffered (pH = 6) solution containing 0.5% hexadecyltrimethylammonium bromide. MPO activity was measured in the presence of o-dianisidine dihydrochloride (Sigma-Aldrich) by optical density readings at 450 nm in a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Sample results were interpolated into a linear regression generated with a standard curve of MPO (0.05–0.8 U/mL; Sigma-Aldrich). Data were expressed as units of MPO/mL.

Tryptase-like activity assay

Tryptase-like activity was measured in the supernatant from knee joint lavages in the presence of 200 μM benzyloxy carbonyl-glycine-proline-arginine-7-amino-4-methylcoumarin (Z-GPR-AMC) (Enzo Life Sciences, GmbH, Lörrach, Germany) buffered in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 120 mM NaCl (pH = 8.0) with 365/440 nm (excitation/emission) in a Varioskan Flash microplate reader (Thermo Fisher Scientific). Sample results were interpolated into a linear regression generated with a standard curve of recombinant human tryptase-β1 (0.5–4.0 mU/mL; Promega). Data was expressed as mU of tryptase-like activity per mL.

Cytokine and chemokine quantification

The concentration of IL-1β, -6, -17A and (C-X-C) ligand (CXCL)1/KC in the supernatant of knee joint washes was determined by using CBA Flex Kits, according to the manufacturer’s instructions (BD, Franklin Lakes, NJ, USA). Data were acquired in a FACSCanto II flow cytometer (BD) and analyzed with the program FCAP Array v3.0 (Soft Flow Inc., Pecs, Hungary). Detection limits for IL-1β, IL-6, IL-17A and CXCL1/KC were 4.32, 9.01, 8.72 and 10.48 pg/mL, respectively.

Statistical analysis

Data were analysed by using the software Prism version 6 (GraphPad Software, San Diego, CA, USA). Data comparison between the two groups was performed by unpaired Student’s t test. The data from the histopathological scores were analysed by Kruskal-Wallis and Dunn’s multiple comparisons test. The time course of oedema formation was analysed by two-way ANOVA and Tukey’s multiple comparisons test. The remaining data was analysed by ordinary one-way ANOVA and Tukey’s multiple comparisons test. Data are expressed as mean ± SEM and values of P < 0.05 were considered as statistically significant.

Results

The proteolytic balance is impaired in mBSA/IL-1β-induced arthritis

Transcripts for Spag11c were constitutively expressed in the knee joint from control mice, whereas transcripts for Spag11a were not detected. On the other hand, both transcripts were detected in positive control samples prepared with RNA extracted from adult mice epididymis (Fig. 1a). Additionally, the relative expression of Spag11c did not change 7 days after induction of arthritis (Fig. 1b). In situ hybridization revealed a positive signal for Spag11c mRNA in chondrocytes of the hyaline cartilage and in cells from the synovial membrane of the knee joint from control (Fig. 1c and d) and mice submitted to mBSA/IL-1β-induced arthritis (Fig. 1e and f). No hybridization signal was detected in slices processed with a negative control scrambled probe (Fig. 1g and h). By qualitative analysis of knee joint sections stained with
Toluidine blue, a few scattered mast cells were observed in the synovia of control mice, whereas 7 days after induction of arthritis, the number of mast cells seemed to increase (Fig. 1h). Accordingly, the relative expression of Mcpt-6 in the mouse knee joint increased up to twofold 7 days after induction of arthritis (Fig. 1i). Additionally, tryptase-like activity was upregulated in the synovial fluid from the knee joint of mice submitted to mBSA/IL-1β-induced arthritis (Fig. 1j).

The lentivirus system promotes a stable heterologous expression of hSPAG11B/C

The protocol for lentivirus production presented in this study provided consistent titers ranging from $1.1 \times 10^9$ up to $2.0 \times 10^{10}$ TU/mL. We then evaluated if transgene expression was still detected in the knee joint 7 days after induction of arthritis (Fig. 1). Additionally, tryptase-like activity was upregulated in the synovial fluid from the knee joint of mice submitted to mBSA/IL-1β-induced arthritis (Fig. 1).

Lentivirus-mediated heterologous expression of hSPAG11B/C or treatment with APC366 re-establishes the proteolytic balance

The Mcpt-6 transcript level and tryptase-like activity in the synovial fluid were both increased in the mouse knee joint 7 days after the induction of arthritis (Fig. 3). The intra-articular injection of the tryptase inhibitor APC366 (10–100 μM) or the transduction of knee joints with phSPAG11B/C reduced the tryptase-like activity in animals with arthritis. In contrast, transduction with the control lentivirus pWPXld-IG or injection with vehicle had no effect (Fig. 3a and b). Neither APC366 nor transduction with phSPAG11B/C, however, reverted the increase of Mcpt-6 gene expression observed 7 days after induction of arthritis (Fig. 3c and d).
**Lentivirus-mediated heterologous expression of hSPAG11B/C or treatment with APC366 has marginal impact on mBSA/IL-β-induced arthritis**

Animals submitted to mBSA/IL-1β-induced arthritis presented a long-lasting and biphasic oedema, which peaked on days 1 and 7. The intra-articular treatment with APC366 (10–100 μM) reduced the late phase of oedema formation, 7 days after the induction of arthritis (Fig. 4a). Similarly, animals with arthritis previously transduced with phSPAG11B/C, but not with pWPXLd-IG, presented reduced oedema at day 7 (Fig. 4b).

Histopathological analysis evidenced that while knee joints from control animals presented no signs of joint inflammation (Fig. 4c), mBSA/IL-1β-treated mice developed a severe arthritis 7 days after its induction, with an intense influx of leukocytes to the knee joint cavity and synovial membrane (synovitis), pannus formation, subchondral bone erosion (Fig. 4d) and degradation of the extracellular matrix of hyaline cartilage (Additional file 1). Similarly, animals previously transduced with pWPXLd-IG developed severe arthritis (Fig. 4e). The knee joints of mice transduced with phSPAG11B/C presented a trend for a lower degree of inflammatory arthritis, particularly regarding synovitis and joint cavity exudate (Fig. 4f). Although none of the histopathological parameters was significantly reduced in comparison to animals submitted to mBSA/IL-1β-induced arthritis, either pre-injected with vehicle or transduced with the control lentivirus pWPXLd-IG (Fig. 4g).

MPO activity, a biochemical index of granulocyte infiltration, increased in the joint cavity exudate 7 days after induction of arthritis, when compared to control animals. The treatment of animals with APC366 or the transduction with phSPAG11B/C had no major effect on the increased MPO activity (Fig. 5a and b).

**Lentivirus-mediated heterologous expression of hSPAG11B/C or treatment with APC366 inhibits the production of inflammatory mediators**

The release of IL-6 and CXCL1/KC to the synovial fluid increased 7 days after induction of arthritis, albeit CXCL1/KC concentration was just slightly above the detection limit (Fig. 6). This increase of IL-6 associated to mBSA/IL-1β-induced arthritis was dose-dependently reduced by the treatment with APC366 (Fig. 6a), or the previous transduction with phSPAG11B/C (Fig. 6b).
control lentivirus pWPXLd-IG had no effect on IL-6 release. Similarly, the production of CXCL1/KC was reduced by the treatment with APC366 (Fig. 6c), or the previous transduction with phSPAG11B/C (Fig. 6d). However, the transduction with pWPXLd-IG also reduced the production of CXCL1/KC. Neither IL-1β nor IL-17A was detected in any of the experimental conditions tested.

**Discussion**

Previous studies have shown that rheumatoid arthritis and experimental models of this disease are associated with an intense mastocytosis and mast cell degranulation in affected joints, suggesting that an increased release of active tryptase occurs in this disease [2–4, 12]. Since proteolytic activity can be counterbalanced by the release of endogenous inhibitors, we initially evaluated if an actual increased proteolytic activity due to an unbalance between mast cell-restricted tryptase and endogenous inhibitors takes place during joint inflammation. Indeed, this hypothesis was supported by our results, wherein Mcpt-6 gene expression as well as tryptase-like activity were up-regulated 7 days after mBSA/IL-1β-induced arthritis to levels seemingly not counterbalanced by endogenous tryptase inhibitors, such as SPAG11B/C. However, in animals subjected to lentivirus-mediated heterologous expression of SPAG11B/C, as well as to the treatment with APC366, the proteolytic balance was re-established to a significant extent, since both strategies inhibited the increase of tryptase-like activity in the synovial fluid, 7 days after induction of arthritis. In contrast, neither the recombinant expression of SPAG11B/C nor the treatment with APC366 impacted the upregulation of Mcpt-6 gene expression, thus indicating that the reduced tryptase-like activity was due to enzymatic inhibition. Hence, in view of the upregulated tryptase-like activity,
we investigated whether the re-establishment of the proteolytic balance by tryptase inhibition would be beneficial.

Initially, we assessed the feasibility of using a lentivirus-mediated expression system to promote the stable expression of the β-defensin SPAG11B/C in synovial joints. It is worthwhile mentioning that the protocol for lentivirus production presented herein yielded the production of constant high titers of functional lentiviral particles. Likewise, in transduced animals, both GFP and SPAG11B/C were still detected 14 days after mice knee joint transduction, indicating a stable expression of transgenes, even after the induction of arthritis, wherein an extensive hyperplasia of fibroblast-like synoviocytes occurred. Additionally, GFP-positive cells were restricted to the synovial membrane, whereas no positive cell was observed in chondrocytes, in accordance with previous studies using VSV-G pseudotyped HIV-1 derived lentivirus in knee joints of rodents [28, 29]. Furthermore, a previous study reported the occurrence of only negligible levels of transduction of off-target organs after an intra-articular injection of lentiviral particles in synovial joints [28].

Fig. 4 Effect of lentivirus-mediated heterologous expression of hSPAG11B/C and APC366 in mBSA/IL-1β-induced arthritis. a and b. Time course of oedema formation (increase in knee joint medio-lateral diameter) after induction of arthritis with mBSA/IL-1β in animals treated with the synthetic tryptase inhibitor APC366 at 10–100 μM (N = 6–8 mice per group), or previously transduced with pWPXLDIG or phSPAG11B/C (N = 13–14 mice per group). ***P < 0.001, arthritis + vehicle groups vs. control; #P < 0.05, arthritis + APC366 (10 μM) or arthritis + pWPXLDIG groups vs. control; @@P < 0.01, arthritis + APC366 (100 μM) or arthritis + phSPAG11B/C groups vs. control; ***P < 0.001, arthritis + APC366 (100 μM) or arthritis + phSPAG11B/C groups vs. arthritis + vehicle. c-f. Representative light photomicrographs of knee joint slices stained by the method of eosin and haematoxylin from a control animal (c), or animals submitted to mBSA/IL-1β-induced arthritis, previously injected with vehicle (d), or transduced with 2 × 10^6 TU/joint of pWPXLDIG (e) or phSPAG11B/C (f). The arrows indicate areas of intense synovitis. The arrowheads show areas with moderate synovitis. g. Total and individual histopathological scores of inflammatory parameters (N = 4–6 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001, vs. control group.

Abbreviations: JC joint cavity, SM synovial membrane, Ctrl control, n.s. not significant
Fig. 5 Effect of lentivirus-mediated heterologous expression of hSPAG11B/C and APC366 on MPO activity. a and b. MPO activity in the synovial fluid of knee joint 7 days after induction of arthritis in animals treated with APC366 at 10–100 μM (N = 6–8 mice per group) or previously transduced with pWPXLd-IG or phSPAG11B/C (N = 14–15 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001 vs. control groups. Abbreviation: n.s. not significant.

Fig. 6 Effect of lentivirus-mediated heterologous expression of hSPAG11B/C and APC366 on cytokines and chemokine production. a and b. Effect of the synthetic tryptase inhibitor APC366 (10–100 μM) or the knee joint transduction (1 × 10^7 TU/joint) with phSPAG11B/C in the release of IL-6 (a and b) and CXCL1/KC (c and d) 7 days after the induction of arthritis with mBSA/IL-1β. (N = 6 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001 vs. control groups; #P < 0.05; ##P < 0.01; ###P < 0.001 vs. arthritis + vehicle groups. Abbreviation: n.s. not significant.
whole, this indicates that lentivirus-based gene delivery is an instrumental tool to study the relevance of target genes in synovial joint physiology and disease.

We then screened the anti-inflammatory potential of SPAG11B/C isoform by using the lentivirus-mediated expression system, in parallel to the treatment with the synthetic tryptase inhibitor APC366. Mast cell-restricted tryptases are well-established oedematogenic mediators, by a mechanism involving activation of PAR2 receptors [30–32]. For instance, an intra-articular injection of synthetic hexapeptides corresponding to the tethered ligand sequence revealed after PAR2 cleavage by trypsin-like serine proteases, such as tryptase, induced swelling in synovial joints from normal rats [33–35]. Besides, a recent study shown that an intra-articular injection of mast cell tryptase into the mouse knee joint induces hyperaemia, oedema and pain [36]. This suggests that an oedematogenic pathway triggered by PAR2 activation is ready to take place in normal tissue conditions. Herein, both strategies of tryptase inhibition reduced oedema formation, but only in the late phase of mBSA/IL-1β-induced arthritis, thus indicating that mast cell accumulation and/or Mcpt-6 upregulation occur late in this model. Accordingly, the relative level of Mcpt-6 mRNA was upregulated 7 days after induction of arthritis. As a whole, while previous studies developed by us and others have shown that PAR2 pro-inflammatory and nociceptive signalling pathways are ready to be pharmacologically triggered even in synovial joints from healthy animals, the present study indicates that its endogenous proteolytic activator (i.e. mast cell-restricted tryptase), only becomes quantitatively relevant on the late phase of mBSA/IL-1β-induced arthritis.

Earlier studies based on mast cell-depleted mice due to c-kit signalling deficiency reported a central role of these cells in neutrophil infiltration and joint degeneration [5–7]. Later on it was shown that c-kit deficiency was associated with important hematopoietic impairments, including a high degree of neutropenia, indicating that the resistance of these mice to experimental arthritis was mostly associated to this hematopoietic disorder [37], since neutrophils are pivotal cellular effectors in joint degeneration [38]. Considering that mast cells produce a large repertoire of inflammatory mediators, the role of tryptase in experimental arthritis was investigated in mice lacking Mcpt-6 and -7. This study reported a reduced neutrophil infiltration and joint degeneration associated to mBSA/IL-1β-induced arthritis [12]. Later on, the same group demonstrated that the in vitro stimulation of mouse or human fibroblast-like synoviocytes with tryptase upregulated the expression of the neutrophil chemokines CXCL1/KC, CXCL5/LIX and CXCL8/IL-8 [13, 39]. Similarly, our study demonstrated that the concentration of CXCL1/KC in the knee joint synovial fluid was reduced after tryptase inhibition.

In view of the findings reported with Mcpt-6 and -7 knockout mice [12, 13], we then assessed whether tryptase inhibition would suffice to impact neutrophil infiltration and joint degeneration. As revealed by histopathological analysis, animals submitted to mBSA/IL-1β-induced arthritis, either pre-injected with vehicle or transduced with the control lentivirus pWPXLd-IG, developed a severe inflammatory arthritis, notably with an intense infiltration of leukocytes within the joint cavity and synovial membrane. Surprisingly, although the transduction with pSPAG11B/C or the treatment with APC366 clearly inhibited tryptase-like activity by a significant extent, both inhibitory strategies had only marginal, if any effect on neutrophil infiltration, as depicted by histopathological analysis and MPO activity. This suggests that residual tryptase activity over the baseline level may be sufficient to support neutrophil influx, by a mechanism yet to be identified, but seemingly independent of CXCL1/KC, since the concentration level of this chemokine barely surpassed the detection limits 7 days after the induction of arthritis.

The production of IL-6 is increased in RA [40] and its signalling plays a central role in joint inflammation and degeneration [41]. Indeed, studies with monoclonal antibodies designed to target IL-6 receptors (IL-6R) reported an attenuation of collagen-induced arthritis in mice [42], as well as in human RA [43]. In our study, IL-6 production was upregulated in the synovial fluid of mice 7 days after induction of arthritis. Additionally, IL-6 overproduction was consistently reversed by the recombinant expression of SPAG11B/C or the treatment with APC366. Similarly, a recent study based on collagen-induced arthritis reported a reduction of IL-6 serum levels in mast cell-deficient mice [9]. In this way, our data clearly indicates that tryptase must be the major mast cell mediator regulating IL-6 production in arthritis. Intriguingly, while some inflammatory parameters associated to mBSA/IL-1β-induced arthritis were attenuated by both strategies of tryptase inhibition, the degenerative parameters investigated were not affected. Similarly, a previous study demonstrated that IL-6 knockout mice were only mildly protected from mBSA/IL-1β-induced arthritis [44]. This dichotomy seems to be due to the fact that while IL-6 exert pro-inflammatory actions through activation of soluble IL-6 receptors (sIL-6R), the activation of membrane-bound IL-6 receptors (mIL-6R) is rather protective [41]. In this way, sIL-6R blockade is beneficial, the global inhibition of IL-6 seems to be detrimental. This may explain the limited anti-inflammatory effect of tryptase inhibition on mBSA/IL-1β-induced arthritis observed in the present study.

**Conclusions**

Our study demonstrated that the lentivirus-mediated heterologous expression of an endogenous tryptase
inhibitor (hSPAG11B/C) as well as the intra-articular administration of APC366 presented closely overlapping inhibitory effects on tryptase-like activity, oedema formation, IL-6 and CXCL1/KC production, whereas leukocyte infiltration, cartilage degradation and subchondral bone erosion were not affected. These results show that tryptase-β inhibition offers limited effect on mBSA/IL-β-induced arthritis, thus suggesting that the therapeutic application of these inhibitors to rheumatoid arthritis would be restrained to palliative care, but not as disease-modifying drugs, unless the development of far more potent molecules in the future uncovers a broader potential, as previously highlighted by studies based on transgenic animals. Furthermore, clinical relevance may also lay in their potential effectiveness as RA adjuvant therapy if associated with drugs encompassing other inflammatory pathways implicated in the pathophysiology of this disease, a topic that will require future investigation.

Additional file

**Additional file 1:** Effect of lentivirus-mediated heterologous expression of hSPAG11B/C and APC366 in mBSA/IL-β-induced arthritis. A-D. Representative light photomicrographs of knee joint sections stained by the method of safranin O from a control animal (A), or animals submitted to mBSA/IL-β-induced arthritis, previously injected with vehicle (B), transduced with 2 x 10^6 TU/joint of pWPXLD-IG (C) or pHSPAG11B/C (D). The arrows indicate areas of intense extracellular matrix degradation in the hyaline cartilage. The arrowheads show areas of normal hyaline cartilage. Abbreviations: JC, joint cavity. (TIF 2265 kb)

**Abbreviations**
cDNA: Complementary deoxyribonucleic acid; CXCL: (C-X-C) ligand; DAB: Diaminobenzidine; DAPI: 4',6-diamidino-2-phenylindole; DPBS: Dulbecco’s phosphate-buffered saline; eGFP: Enhanced green fluorescent protein; HEPEs: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL: Interleukin; IRES: Internal ribosomal entry site; LNA: Locked nucleic acid; mBSA: Methylated bovine serum albumin; Mcpt-6: Mouse mast cell protease-6; MPO: Myeloperoxidase; n.s: Not significant; NaCl: Sodium hydrochloride; PBS: Phosphate-buffered saline; RA: Rheumatoid arthritis; RNA: Ribonucleic acid; RT-PCR: Reverse transcription-polymerase chain reaction; SPAG11: Sperm-associated antigen 11; SSC: Saline-sodium citrate

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**References**
1. Lee DM, Weinblatt ME. Rheumatoid arthritis. Lancet. 2001;358(9285):903–11.
2. Crisp AJ, Chapman CM, Kirkham SE, Schiller AL, Krane SM. Articular mastocytosis in rheumatoid arthritis. Arthritis Rheum. 1984;27(8):845–51.
3. Godfrey HP, Illardi C, Engber W, Graziano FM. Quantitation of human synovial mast cells in rheumatoid arthritis and other rheumatic diseases. Arthritis Rheum. 1984;27(8):852–6.
4. Gregorov PA, Lee DM. Synovial mast cells: role in acute and chronic arthritis. Immunol Res. 2007;20:17:19–37.
5. van den Broek MF, van den Berg WB, van de Putte LB. The role of mast cells in antigen induced arthritis in mice. J Rheumatol. 1988;15(4):544–51.
6. Cerr M, Crain B. The role of FcgammaR signaling in the K/B x N serum transfer model of arthritis. J Immunol. 2002;169(1):6604–9.

**Availability of data and materials**
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**
AD-S performed RT-PCR, RT-qPCR and molecular cloning assays, enzyme kinetics assays, lentivirus production, in vivo experiments, cytokine measurement, data analysis and wrote the manuscript. CMR performed in situ hybridization/immunohistochemistry studies and data analysis. AT inserted the IRES-eGFP cassette into pWPXLD and established the protocol for lentivirus production and supervised its production and titration. CR improved the multiple cloning site of the lentivirus vector pWPXLD-IG and assisted the cloning of hSPAG11B/C into pGEM-T. CS participated in the conception of the study. CD developed the protocol for the tryptase-like activity assay and assisted on data analysis and interpretation. DG-D supervised the optimization of lentivirus production and conception of experiments. NV participated in the conception of experiments, data analysis and interpretation. MCWA participated in the conception of the study, data interpretation and wrote the manuscript. All authors read and approved the final manuscript.

**Competing interests**
The authors declare that they have no competing interests.

**Consent for publication**
Not applicable.

**Ethics approval**
The procedures in mice were conducted in compliance with the guidelines for the care and use of laboratory animal and were approved by the institutional research ethics committees (UNIFESP-EPM, CEUA #1564/09, Toulouse, CEEA-122, #13-U1043NV-A2-04). Human tests and epididymis total RNA samples were extracted from tissues provided by Dr. James L. Mohler, Department of Urology/Surgery, and by the Tissue Procurement Core Facility of the Ligneburger Comprehensive Cancer Center, University of North Carolina (Chapel Hill, NC, USA). Human tissues were not accompanied by identifying information and cannot be traced to the donor. Tissues were obtained from prostate cancer patients ranging in age from 56 to 83 years after informed consent and institutional human research committee approval.

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7. Lee DM, Friend DS, Gurish MF, Bencos M, Mathis D, Brenner MB. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. Science. 2002;297(5587):1689–92.

8. Schubert N, Dudeck J, Liu P, Karutz A, Speier S, Maurer M, Tuckermann J, Dudeck A. Mast cell promotion of T cell-driven antigen-induced arthritis despite being dispensable for antibody-induced arthritis in which T cells are bypassed. Arthritis Rheumatol. 2015;67(4):903–13.

9. van der Velden D, Lagrauw HM, Wezel A, Launay P, Kuiper J, Huzinga TW, Toes RE, Bot I, Stoop JN. Mast cell depletion in the preclinical phase of collagen-induced arthritis reduces clinical outcome by lowering the inflammatory cytokine profile. Arthritis Rheum. 2016;68(1):138.

10. Stevens RL, Adachi R. Protease-proteoglycan complexes of mouse and human mast cells and importance of their beta-tryptase-heparin complexes in inflammation and innate immunity. Immunol Rev. 2011;247(1):155–67.

11. Peijl G, Ronnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. Blood. 2010;115(24):4981–90.

12. McNeil HP, Shin K, Campbell IK, Wicks IP, Adachi R, Lee DM, Stevens RL. The mouse mast cell-restricted tetramer-forming tryptase mouse mast cell protease 6 and mouse mast cell protease 7 are critical mediators in inflammatory arthritis. Arthritis Rheum. 2008;58(8):2338–46.

13. Shin K, Nigrovic PA, Crish J, Boilard E, McNeil HP, Larabee KS, Adachi R, Gurish MF, Gobeze R, Stevens RL, et al. Mast cells contribute to autoimmune inflammatory arthritis via their tryptase/heparin complexes. J Immunol. 2009;182(1):647–56.

14. Pereira PJ, Bergner A, Macedo-Ribeiro S, Huber R, Matsoncher G, Fritz H, Sommerhoff CP, Bode W. Human beta-tryptase is a ring-like tetramer with active sites facing a central pore. Nature. 1998;392(6673):306–11.

15. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. Immunol Rev. 2007;217:141–54.

16. Cairns JA. Inhibitors of mast cell tryptase beta as therapeutics for the treatment of asthma and inflammatory disorders. Pulm Pharmacol Ther. 2005;18(1):55–66.

17. Radhakrishnan Y, Hamil KG, Tan JA, Grossman G, Petrusz P, Hall SH, French FS. Novel partners of SPAG11B isoform D in the human male reproductive tract. Biol Reprod. 2009;81(4):467–54.

18. Seldsted ME, Hanvig SS, Ganz T, Schilling JW, Lehrer RI. Primary structures of human neutrophil defensins. J Clin Invest. 1985;76(4):1436–9.

19. Taylor K, Clarke DJ, McCullough B, Chin W, Seo E, Yang D, Oppenheim J, Uhnri D, Govan JR, Campopiano DJ, et al. Analysis and separation of residues important for the chemoattractant and antimicrobial activities of beta-defensin 3. J Biol Chem. 2008;283(11):6631–9.

20. Patil AA, Cai Y, Sang Y, Blecha F, Zhang G. Residues important for the chemoattractant and antimicrobial activities of three human neutrophil defensins. J Exp Med. 2016;204(12):2797–802.

21. Calabrese LH, Rose-John S. IL-6 biology: implications for clinical targeting in inflammatory disease. Trends Pharmacol Sci. 2007;28(9):441–51.

22. Nakanoo S, Mishiro T, Takahara S, Yoki H, Hamada D, Yutaka K, Takata Y, Goto T, Egawa H, Yaseoka S, et al. Distinct expression of mast cell tryptase and protease activated receptor-2 in synovia of rheumatoid arthritis and osteoarthritis. Clin Rheumatol. 2007;26(8):1284–92.

23. Hirano T, Matsuda T, Turner M, Miyazaki S, Fujiwara G, Sato K, Shimizu M, Marini R, Feldmann M, et al. Excessive production of interleukin 6 in vivo stimulates factor 2 in rheumatoid arthritis. Eur J Immunol. 1988;18(11):1797–801.

24. Calabrese LH, Rose-John S. IL-6 biology: implications for clinical targeting in rheumatic disease. Nat Rev Rheumatol. 2014;10(12):720–7.

25. Fujimoto M, Serada S, Mihara M, Uchiyama Y, Yoshiha K, Koike N, Ohtsugi U, Nishikawa T, Ripley B, Kimura S, et al. Interleukin-6 blockade suppresses autoimmune arthritis in mice by the inhibition of inflammatory Th17 responses. Arthritis Rheum. 2008;58(12):3710–9.

26. Murakami M, Nishimoto N. The value of blocking IL-6 outside of rheumatoid arthritis: current perspective. Curr Opin Rheumatol. 2011;23(3):273–7.

27. Lawlor KE, Wong PK, Campbell IK, van Rooijen N, Wicks IP. Acute CD4+ T lymphocyte-dependent interleukin-1-driven arthritis selectively requires interleukin-2 and interleukin-4, joint macrophages, granulocyte-macrophage colony-stimulating factor, interleukin-6, and leukemia inhibitory factor. Arthritis Rheum. 2005;52(12):3749–54.