Temporomandibular Joint Involvement in Mice with Proteoglycan Induced Arthritis and the Role of TSG-6 in Proteoglycan Induced Arthritis

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Members of the Examination Committee: Prof. Dr. Pál Géher MD, PhD
Prof. Dr. Ildikó Márton MD, PhD, DSc

The Examination takes place at the Department of Obstetrics and Gynaecology (Library), Faculty of Medicine, University of Debrecen) on Monday, 17th June, 2019, at 11a.m.

Head of the Defense Committee: Prof. Dr. Zoltán Hernádi MD, PhD, DSc
Reviewers: Dr. Zoltán Griger MD, PhD
Prof. Dr. György Nagy MD, PhD, DSc

Members of the Defense Committee: Prof. Dr. Pál Géher MD, PhD
Prof. Dr. Ildikó Márton MD, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on Monday 17th June, 2019, at 1 p.m.
INTRODUCTION
The pathology of temporomandibular joint arthritis

The temporomandibular joint (TMJ) is a hinge and gliding, so-called ginglymoarthrodial joint, which is formed by the mandibular condyle and glenoid fossa of the temporal bone. The joint space is divided by a fibrocartilaginous disc (meniscus) which allows the condyle to move easily and it also helps to absorb shock when the mouth opens and closes. TMJ is one of the most frequently used and it is one of the few synovial joints in the body with an articular disc. The frequent use exposes the joint to variety of the disorders.

TMJ syndrome or temporomandibular joint disorder (TMD) can be caused by trauma, such as grinding the teeth (bruxism) and clenching (jaw tightening) or a punch to the jaw or impact in an accident; and also conditions which might affect other joints in the body, such as bony ankylosis, arthritis, neoplasia, developmental anomalies, and pathologic lesions.

Meniscus displacement, such as internal derangement disorder, or the development of OA-like degenerative changes of articular cartilage, covering the mandibular condyle can be most common cause of TMD.

TMD can be associated with headache disorders, facial pain, head and neck pain, locking and difficulty to opening the jaw. In addition, problems with biting and jaw clicking may also appear. Otalgia can be confused with TMJ pain due to the proximity of the TMJ to the ear. Arthritis is one cause of TMJ symptoms. Rheumatoid arthritis (RA), which is an autoimmune disease, affects 0.5-1% of the human population. It causes synovial inflammation in peripheral joints and can also affect the TMJ, especially in children. The progression of the disease may lead to destruction of the articular cartilage, underlying bone erosion and causes joints deformation and complete loss of joint function which can dramatically decrease the living standard of patients.

Degenerative arthritis or degenerative joint disease, also known as osteoarthritis (OA) is metabolically active and it is a dynamic process. The jaw joint can be involved in arthritic changes, which are caused by breakdown of the joint including the articular cartilage and subchondral bone, or by normal aging. Due to a variety of causes including mechanical, metabolic, developmental and hereditary, OA may cause a progressive loss of cartilage which can happen gradually, and the unprotected surface of the exposed bone is prone to damages in the joint.

In this study, a murine model of RA is chosen because it can mimic a number of the characteristics of the human disease. Genetic studies, clinical assessments, X-rays and histopathology of diarthrodial joints and laboratory tests including the presence of serum cytokines, autoantibodies (including rheumatoid factor) and anti-citrullinated protein antibodies indicate the advantageous use of this model.
Cartilage proteoglycan (PG)-induced arthritis (PGIA) in BALB/c mice can be used to examine the TMJ involvement and the pathologic changes during the development and in the late phases of the arthritis. PGIA shares many similarities with RA, for example progressive joint disease, where gradually the number of involved joints will increase and eventually joint deformation due to loss of cartilage and bone erosion will occur.

Depending on methods used for diagnostic evaluation and patient selection, TMJ involvement in patients with RA ranges from 4 to 86%.

The symptoms of arthritic joints and TMD are not similar, but available information about the histopathology of the TMJ in RA is very limited. Complete replacement of cartilage by fibrous tissue may occur in advanced RA. Other studies using arthroscopy revealed degenerative and inflammatory changes and fibrosis in the TMJ of RA and OA patients. Lack of information about cartilage histopathology makes it ambivalent, whether TMJ involvement in RA is inflammatory or degenerative in nature.

In order to study the reaction of the TMJ to different interventions causing inflammatory reactions, animal models have been developed. In these models, the inflammation-inducing compounds, such as antigen or adjuvant, are injected directly into the TMJ. Although the involvement of peripheral synovial joints and the spine has been extensively investigated in PGIA, the TMJ, which is also a synovial joint, has not yet been examined.

Features of TSG-6

TSG-6, (the product of tumor necrosis factor α-stimulated gene-6; also known as TNFAIP6 or TNFIP6, is a hyaluronan (HA) -binding protein, which is secreted by a variety of cells in response to pro-inflammatory mediators and growth factors. This protein has anti-inflammatory and chondroprotective effects in different models of inflammation and arthritis, such as the recombinant murine TSG-6 (rmTSG-6) model. Large amount of TSG-6 have been detected in synovial fluids and synovial tissues of inflamed joints in patients with RA. TSG-6 consists of a “link” module and a “CUB” (complement C1s/C1r-, uEGF-, BMP-1-like) domain. The positively charged link module binds various glycosaminoglycans (GAGs), including HA, chondroitin sulfate, heparin, and heparan sulfate. The CUB domain of TSG-6 is similar to the CUB module found in several developmentally regulated proteins that are thought to be involved in protein-protein interactions. However, to date, only fibronectin has been shown to bind to the CUB domain of TSG-6. TSG-6 is not expressed constitutively but can be induced by pro-inflammatory cytokines or LPS. In contrast, anti-inflammatory cytokines, such as IL-4 or IL-10, suppress TSG-6 expression either directly or via inhibition of LPS/Toll-like receptor-induced cell activation.
TSG-6 can modulate the binding of HA to the cell surface HA receptor, CD44. Treatment of leukocytes with soluble HA·TSG-6 complex has been shown to inhibit the CD44-mediated adhesion of these cells to immobilized HA in vitro. Because the CD44-supported adhesion of leukocytes on HA-covered surfaces of inflamed vascular endothelium is required for the emigration of these cells from the bloodstream into inflamed tissue, inhibition of this adhesion step by the HA·TSG-6 complex could have a negative impact on the extravasation of inflammatory cells. The in vitro observations are consistent with in vivo studies reporting reduced leukocyte influx into the arthritic joints of TSG-6-treated mice and enhanced leukocyte extravasation in the joints of TSG-6-deficient mice. Collectively, the in vivo observations lend support to the concept that TSG-6 has a critical role in the resolution of inflammation, but this function of TSG-6 may rely on more than one mechanism.

Aims of the studies
In the studies included in the present thesis I have tried to find the answers for the following questions:

study 1:
• Are there pathologic changes in the TMJ in mice with PGIA?
• If there are, do these changes resemble RA or OA?

study 2:
• What is the function of TSG-6 in mice with PGIA?
• Is there a way to find a sensitive detection method to measure the concentrations of TSG-6 from BALB/c mice with PGIA?
• Do TSG-6 levels correlate with the severity of arthritis in mice with PGIA?
• Do TSG-6 levels correlate with immune responses?
• Is TSG-6 expressed in any tissues or cells during the development of PGIA?

Materials and methods

study 1

Antigen, animals, immunization and assessment of peripheral arthritis
PG was extracted from OA cartilage and purified by gradient centrifugation, deglycosylated, characterized, and tested for arthritis.

Animals were immunized according to the standard procedure for induction of PGIA, intraperitoneal injection was given with an emulsion of PG and 2 mg dimethyl dioctadecyl-ammonium bromide (DDA) adjuvant on days 0, 21, and 42. Control mice were injected with an emulsion of PBS and DDA under the same schedule.
Immunized mice were examined 2–3 times a week for clinical symptoms of arthritis, and after the second immunization we were able to record abnormalities due to arthritic changes of the joints. The time of onset and incidence of arthritis were recorded, and disease severity was scored in a blinded manner by two different investigators, based upon swelling and redness of each paw on a scale ranging from 0 to 4, yielding a potential maximum severity score of 16 for each animal.

Arthritic mice were divided into an acute and a chronic group, the acute group was sacrificed during the clinically determined acute phase, 6–7 days after the onset of arthritis whilst the chronic group was sacrificed during the chronic phase of the, approximately two weeks after the onset of arthritis. Control mice were sacrificed with the chronic group on day 60.

Measurements of cytokines in serum samples

Blood was collected from control and arthritic mice at different time points of immunization, before and after the onset of arthritis. Concentrations of pro-inflammatory serum cytokines, including interleukin (IL)-1β, TNF-α, IL-6, and IL-17, were measured using capture enzyme-linked immunosorbent assays (ELISA).

Histology, histochemistry and immunohistochemistry

Dissection of hind limbs and heads were done from euthanized mice on day 49 or 50 and from mice with chronic arthritis and control groups on day 60. Heads were cut in half in the sagittal direction, heads and paws were fixed in 10% neutral buffered formalin. Limbs and heads were acid-decalcified and embedded in paraffin. The heads were carefully oriented in the paraffin blocks so that the sectioning blade could run parallel to the posterior mandible and the mandibular head. In order to evaluate structural integrity, staining of the deparaffinized sagittal serial sections of paws and TMJs were done with hematoxylin and eosin, or with safranin O and fast green and assessed cartilage PG aggrecan loss.

For immunohistochemistry (IHC), deparaffinized sections were rehydrated in 50 mMTris-acetate buffer. The sections were digested with 0.5 units/ml chondroitinase ABC dissolved in 50 mM Tris-acetate buffer for 60 min, to unmask the protein epitopes in cartilage.

Non-specific protein binding sites were blocked with 10% normal goat serum in PBS and then stained with rabbit antibodies specific to cartilage PG (aggrecan) neoepitopes. These antibodies were raised against the neoepitopes -VIDIPEN\textsuperscript{341} and -NVTEGE\textsuperscript{373}. Neoepitope -VIDIPEN is generated mostly by stromelysin (matrix metalloproteinase [MMP]-3) by cleaving the interglobular domain (IGD) of the core
protein of cartilage PG aggrecan. NITEGE/NVTEGE is another cleavage product of the IGD by aggrecanase-1, or aggrecanase-2 (ADAMTS-5).

Neoepitopes which are bound to hyaluronan via the G1 domain will be available for the respective antibodies after cleavage of the IGD of the aggrecan by these enzymes. The core protein released by the enzymes, with negatively charged glycosaminoglycan (GAG) side chains, and diffuses out of the cartilage. As a result, the negatively charged GAGs, stained red with safranin O, are lost.

Rabbit antibodies were used at a 1:40 dilution, and the binding of the primary antibodies was detected with peroxidase-conjugated goat anti-rabbit IgG. Digestion with chondroitinase ABC was sufficient for these antibodies to access the epitopes in the paraffin sections, and no antigen retrieval was necessary. Peroxidase reactions were developed with diaminobenzidine (DAB) chromogen and H₂O₂ substrate as described. All slides were exposed to DAB for the same length of time, and the specificity of immunostaining was ensured by replacement of the first antibodies with normal rabbit serum in one set of slides. All tissue sections were viewed under a Nikon Microphot-FXA bright-field microscope. TIF images were generated using MetaView image acquisition software.

**Morphometric analysis of safranin O and immunohistochemical staining**

Morphometric analyses were performed by a blinded investigator on the densities of Safranin O staining and immunohistochemistry, for quantitative image analysis. The data of the image histogram (mean intensity and pixel number of the selected color) were exported to Microsoft Excel. The image was then converted to grayscale, and the gray pixel values of the histogram of the entire field (total pixels) were also exported to Excel.

The percentage of the stained pixels, relative to the total pixels of each area, was calculated by dividing the selected color pixels by the total (gray) pixels of the entire area, and then multiplying by 100. By using the same main color intensity values for all tissue sections stained with the same reagent, quantitative comparison of positively stained areas of control and of arthritic specimens was carried out. Average of data from left-side and right-side joints from the same mouse was handled as a single value from a single animal.

**Quantitative real-time polymerase chain reaction (QRT-PCR)**

Mandibular cartilage and adjacent synovial tissue of wild-type and RA mice were dissected from euthanized animals under a preparative stereo microscope in RNAsae-free conditions to isolate RNA. RNAlater solution is used for collection of the tissue pieces were collected in and homogenized in TRI Reagent. Tissue samples from at least two
animals per group were pooled, and RNA was isolated from three independent pools. RNA was also isolated similarly from normal and inflamed paw homogenates, which contained cartilage and soft tissue dissected from the ankle and mid-foot area, but not bone. The quality of RNA samples was checked using an Agilent 2100 Bioanalyzer. High quality samples were used in reverse transcription reactions. cDNAs were synthesized using an oligo-dT reverse primer and the SuperScript First Strand Synthesis kit. QRT-PCR was carried out with the iQ5 real-time PCR detection system.

According to the manufacturer’s instructions TaqMan gene expression assays were carried out. QRT-PCR data were analyzed with the iQ5 system’s software package. To normalize the data within each pooled sample, expression of the GAPDH housekeeping gene was used. Quantification of gene expression among pooled samples (relative to control) was done employing the $2^{-\Delta\Delta CT}$ method and results were expressed as fold changes relative to the corresponding control samples.

**Statistical analysis**

Statistical analysis was performed using SPSS software. Descriptive statistics were used to determine group means and 95% confidence intervals. Statistically significant differences among the groups were assessed using ANOVA with post-hoc Dunnett’s t test for comparison of multiple groups. Two-tailed Pearson-correlation analysis was used to find correlations between data sets. A p value of <0.05 was considered statistically significant.

**Study 2**

Mice were immunized and assessed for arthritis as described in study 1.

**Preparation of synovial fluid and tissue extracts**

Immediately after euthanasia, synovial fluid was harvested from arthritic ankle joints (with the highest arthritis score of 4 in 20 μl of PBS/joint and centrifuged at 5,000 rpm for 10 min, and the supernatant was stored at −70 °C until use. Skin from paws of normal and arthritic mice was removed for tissue extracts preparation, Ankle and tarso-metatarsal joint soft tissues were dissected and placed in cold radioimmune precipitation assay lysis buffer containing Halt protease inhibitor mixture. Homogenates were prepared by ultrasonication for 90 seconds on ice and centrifuged at 5,000 rpm. Measurement of the protein content of the supernatant of tissue extracts were done by using the bichinconic acid assay.
Histology, histochemistry and IHC

For frozen section IHC, after euthanasia paws with similar degrees of arthritis were embedded in optimal cutting temperature medium, frozen, and sectioned with a tungsten knife at −32 °C in a MICROM HM 550 cryostat. Frozen sections were immunostained with mAbs to TSG-6 or goat Abs to mouse tryptases mMCP-6 and mMCP-7. Non-immune goat or mouse IgG served as a background control.

Reagents and cell culture

Unless noted otherwise, the standard cell culture medium was DMEM, containing 4.5 g/liter glucose and supplemented with 1% non-essential amino acid solution, 1 mM sodium pyruvate, 1% L-glutamine, 100 mg/liter gentamicin sulfate, 0.5 μM β-2-mercaptoethanol, and 10% FBS. All cell cultures were performed in a humidified atmosphere of 5% CO₂ in air at 37 °C.

Production and purification of rmTSG-6 fusion protein

A 753-bp-long cDNA fragment of mouse IgG2a heavy chain was obtained by reverse transcription of RNA purified from a mAb-producing murine B-cell hybridoma. The cDNA fragment was amplified by PCR using primers with linkers for restriction enzyme (EcoRI and BclI) cleavage sites. The 5′-end of the cDNA included the hinge region of the mouse IgG2a heavy chain, and it was inserted into a Lonza pEE14.1 mammalian expression vector. The stop codon of the full-length (828-bp) mouse TSG-6 cDNA in a pBlueScript S/K vector was replaced with a sequence coding for the cleavage site of the endopeptidase factor Xa (Ile-Glu-Gly-Arg) followed with a 9-bp spacer before the EcoRI cleavage site (ATAGAAGGTCGT/GACTCGAGG/GAATTC) at the 3′-end of the TSG-6 cDNA. Purified mTSG-6 cDNA was inserted into the EcoRI site between the Lonza vector and the 5′-end of the mouse IgG2a heavy chain. Insert orientation was determined by PCR, and the construct was sequenced. Semiconfluent CHO-K1 cells were transfected with the mTSG-6-Xa-mFc2aLonza construct using CaCl₂ precipitation according to a standard protocol.

The Lonza expression vector contains a minigene encoding glutamine synthase, an enzyme responsible for the biosynthesis of glutamine (using glutamate and ammonia as substrates). The transfected glutamine synthase gene (a part of the Lonza vector) can act as a selection tool in the presence of methionine sulfoximine, and CHO cells containing the Lonza vector with the glutamine synthase gene can survive in the absence of glutamine and in the presence of 25–50 μM methionine sulfoximine. Approximately 2 weeks after the transfection, individual CHO colonies were transferred into 96-well cell culture plates. Glutamine-free DMEM was replaced with CHO serum-free, and 48 h later, 100 μl of supernatant from each well was transferred to 96-well Maxisorp ELISA plates.
and incubated overnight. Free binding sites of the wells were blocked with 1% BSA, and the clones expressing the fusion protein (TSG-6-mFc2a) were identified with HRP-conjugated goat anti-mouse IgG2a. Positive colonies were retested using affinity-purified and biotinylated RC21 rabbit Ab raised against mouse TSG-6 followed by incubation with HRP-labeled streptavidin and tetramethylbenzidine substrate. Cell lines secreting the highest amounts of fusion protein were cloned using the limiting dilution (0.5 cell/well) method and cultured in the presence of irradiated (70 grays) mouse embryonic fibroblast feeder cell layers. Positive colonies (retested by ELISA as described above) were recloned by limiting dilution, and stable clones, secreting high amounts of fusion protein, were subjected to further testing.

The rmTSG-6-Xa-mFc2a fusion protein from the serum-free supernatant of the CHO transfectant (clone 514) was purified on Protein G-Sepharose 4 Fast Flow according to the manufacturer's instructions. The eluted product was dialyzed against ultrapure H2O, lyophilized, and stored at −20 °C until further use. The purity of the fusion protein was determined using SDS-PAGE with Coomassie Blue staining. Western blots were performed with HRP-labeled goat anti-mouse IgG2a or with affinity-purified and biotinylated rabbit RC21 anti-TSG-6 antibody. Purified rmTSG-6-Xa-mFc2a fusion protein was cleaved with factor Xa (200 units/mg protein) in 2.5 ml of factor Xa cleavage/capture buffer (100 mM NaCl, 50 mM Tris-HCl, 5 mM CaCl2, pH 8.0) overnight at room temperature. The enzyme was removed by XarrestSepharose, and the IgG2a-Fc fragment was absorbed onto Protein G-Sepharose.

After optimization of factor Xa cleavage, we tested the glycosylation level of purified rmTSG-6 (clone 514). Twenty μg of purified protein was digested using a deglycosylation kit containing a mixture of peptide:N-glycosidase F, O-glycosidase, sialidase, β-galacosidase, and glucosaminidase, which removed all N-linked and most of the O-linked oligosaccharides. The digested product was loaded on 12% SDS-PAGE and transferred onto nitrocellulose membrane for immunostaining as described above.

**Testing of HA binding and enhancement of HA-CD44 interaction by rmTSG-6**

Binding of rmTSG-6 to immobilized HA or heparin was tested using a microplate titration assay. In brief, rooster comb HA was serially diluted in PBS (from 200 μg/ml to 3.1 μg/ml). One hundred μl of each HA solution was dispensed into duplicate wells of 96-well Maxisorp plates and incubated overnight at room temperature. Free binding sites were blocked with 1% BSA. rmTSG-6 (from clone 514, free of IgG-Fc tail) was diluted in PBS (concentration range, 0.031–2.0 μg/100 μl per well) and incubated with the HA-coated wells for 2 h at 37 °C. HA-bound rmTSG-6 was detected with biotinylated RC21 antibody, followed by HRP-streptavidin and TMB substrate. rmTSG-6-mediated enhancement of HA binding to cell surface CD44 was tested using murine CTLL-
2/CD44⁺ cells. Relative amounts of cell surface-bound fluorescein-conjugated HA (FL-HA) in the presence or absence of rmTSG-6 were measured by flow cytometry.

**TSG-6-specific monoclonal antibody (mAb) production and development of capture Enzyme-Linked ImmunoSorbent Assays (ELISA)**

RmTSG-6 has been used to immunize TSG-6-deficient BALB/c mice, in order to generate murine B cells for hybridoma fusion and subsequent TSG-6-specific mAb production. Immunization of TSG-6-deficient BALB/c mice were done by intraperitoneal injection with 50 μg of purified rmTSG-6 emulsified with 2 mg of dimethyldioctadecyl-ammonium bromide (DDA) adjuvant in a total volume of 100 μl of PBS. After the third injection, the peripheral blood samples were collected, and anti-TSG-6 serum titers were determined by ELISA. Following the standard protocol, we fused Spleen cells from positive mice with the Sp2/0-Ag14 myeloma cell line (ATCC) and selection of the mAb-producing hybridomas were done by ELISA. Positive hybridomas were repeatedly Cloned by limiting dilution, and after hybridoma injection, ascites fluid was produced in BALB/c mice. By using a mouse IgG isotype determination kit, we could identify the isotypes and light chains of each mAb. The purification of the Immunoglobulin fractions of five hybridoma clones (designated NG2, NG3, NG4, NG5, and NG8) were done on protein G columns, and by using a standard inhibition ELISA a portion of each was biotinylated and tested for cross-reactivity.

As additional positive controls, rmTsg-6 mouse and human TSG-6 proteins were used and for the in-house development of new murine and human TSG-6 capture ELISA systems employing non-cross-reactive pairs of anti-TSG-6 mAbs. For the capture of either murine or human TSG-6 Purified mAb NG3 (IgG2b κ) was used.

For the detection of murine TSG-6 Biotinylated NG8 mAb (IgG1 κ) was employed, and for the detection of human TSG-6 NG4 (IgG1 κ) was used.

The sensitivity of capture ELISAs ranged from 500 pg/ml to 200 ng/ml TSG-6.

**In vitro detection of interactions of TSG-6 with HA or heparin and mast cell tryptases**

In preliminary experiments, optimal concentrations of rooster comb HA or heparin; rmTSG-6, rmMCP-6, and rmMCP-7; and Abs against TSG-6 and mMCP-6 and mMCP-7 were determined for the detection of HA- or heparin-bound proteins (TSG-6 and the mast cell tryptases mMCP-6 and mMCP-7), by employing ELISA-based systems.

For detection of a tripartite interaction of HA or heparin with TSG-6 and tryptases, a triple layer sandwich microplate assay were developed. Briefly, overnight coating at room temperature is done with 2 μg of HA in 100 μl of PBS (first layer) onto the wells of Maxisorp (Nunc) plates. Overnight coating of Heparin (10 μg) in 1% N-(3-
dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride which was dissolved in H₂O and added onto the wells of CovaLink (Nunc) plates at 37 °C.

Blocking of the free binding sites was done with 5% BSA, then wells were filled with 0.1 μg of rmTSG-6 (second layer) and incubated for 1 h at 37 °C, followed by incubation with rmMCP-6 or rmMCP-7 (third layer; concentration range, 0.025–0.2 μg/well). Detection of the third layer of bound tryptases was done with anti-mMCP-6 and anti-mMCP-7 Abs.

RmMCP-6 or rmMCP-7 was used as a second layer, and rmTSG-6 was used as the third layer, followed by detection of bound rmTSG-6 with biotinylated NG8 mAb.

Reference wells contained only a single protein (TSG-6 or tryptase) in each assay, and detection of the immobilized proteins was done with the irrelevant Ab (rmTSG-6 with anti-tryptase Abs and vice versa) which served as background controls. Development of the reactions was done with HRP-conjugated secondary reagents and TMB substrate, and values were expressed as the absorbance measured at 450 nm using a Synergy 2 ELISA reader.

Identification of mast cell tryptases by Western blotting and co-immunoprecipitation of tryptases and HCs of IαI with TSG-6

As described earlier tissue extracts were prepared from arthritic and non-arthritic paws, and 50 μg protein of each was loaded onto 12% SDS-PAGE, along with 0.2 μg of rmTSG-6, 0.1 μg of rmMCP-6, and 0.05 μg of rmMCP-7. Under reducing conditions the proteins were resolved, and transferred to a nitrocellulose membrane, and probed with different antibodies: affinity-purified biotinylated rabbit RC21 anti-TSG-6 Ab, anti-MCP-6, or anti-MCP-7.

In most cases, we stripped the membranes and reprobed them with another antibody to detect comigration of the two proteins. With or without immunoprecipitation the supernatants of paw extracts were subjected to Western blotting.

We preabsorbed the supernatants of paw extracts (200 μg of protein of each) with Protein G-Sepharose for co-immunoprecipitation, washed, and then incubated them overnight at 4 °C on a rotary shaker, with 10 μg of TSG-6 mAb mixture (NG3, NG4, NG5, and NG8; 2.5 μg of each) Immune complexes were absorbed to Protein G-Sepharose for 30 min at room temperature, washed, and boiled in reducing buffer. We loaded 1/3 of each sample on 12% SDS-PAGE, and under reducing conditions proteins were separated. Transferring of the Proteins were done onto nitrocellulose membrane and staining was done with biotinylated Abs to TSG-6 (RC21 or NG8) or with goat antibodies to HC1 or HC2 (both at 1:500 dilution) or goat antibodies to mMCP-6 or
mMCP-7 (both at 1:2,000 dilution) followed by HRP-conjugated rabbit antibody to goat IgG.

In order to ensure that equal amounts of cell extracts were loaded HRP-labeled mouse mAb to β-actin was used. All incubations were performed at room temperature for 1–2 h. Detection of the positive protein bands were done by ECL.

Statistical analysis was performed using SPSS as described in study 1.

Results

We examined and evaluated TMJ involvement in PGIA in the acute and subacute/chronic phases of peripheral joint inflammation compared with age-matched, non-arthritic, control animals. Mice with acute and chronic arthritis were sacrificed on days 49–50 and day 60, respectively to monitor the progression of inflammation or cartilage destruction in the TMJs, we compared their TMJs and ankle joints with each other or with the corresponding tissues of non-arthritic control mice. The ankle joints of the control mice did not exhibit any signs of structural damage, but inflammatory cell infiltration of the synovial tissue and massive joint destruction was evident in the ankles of the PG-immunized animals. The extent of inflammation-induced loss of cartilage aggrecan was determined with staining of adjacent sections of the ankle joints of normal and arthritic mice with safranin O and fast green.

Intense red staining with safranin O could be seen on cartilage of the distal tibia of the control mouse, although very little red staining was seen in the tibial cartilage of the mouse with chronic arthritis in the ankle joint. We were investigating to determine whether the aggrecan neoepitopes generated by ADAMTSs or MMP-3 were detectable in the cartilage of arthritic ankles by considering that the loss of GAG-decorated aggrecan fragments from cartilage is the result of cleavage of the core protein by aggrecanases or stromelysin. As compared to the normal cartilage, which showed essentially negligible immunostaining with the anti-NITEGE antibody, staining for this aggrecan neoepitope was very strong in the cartilage of arthritic ankle. Similar differences could be seen when sections of control and arthritic ankle joints were reacted with the anti-VDIPEN antibody.

After comparing hematoxylin and eosin (HE) stained sections of the TMJs from control and arthritic mice, prominent difference was the diminished basophilic staining in the mandibular cartilage of arthritic mice. Structural evidence of cartilage damage of the TMJ, or small collections of inflammatory cells could be seen in the TMJ synovium in the most severe cases of chronic PGIA. Adjacent sections of the corresponding TMJs were stained with safranin O and fast green to be able to determine if in the apparent loss of hematoxylin staining from the TMJ cartilage of the arthritic mouse could reflect a loss of
aggrecan. Indeed, the control TMJ cartilage showed strong and nearly homogenous staining with safranin O whereas significant loss of red staining was observed in the TMJ of the arthritic mouse. In the latter case, safranin O staining could be seen around the chondrocytes mainly and was absent from the inter-territorial matrix. In addition, the chondrocytes appeared to form clusters in the TMJ of the arthritic mouse. Immunostaining for aggrecan neoepitopes showed light staining for both -NITEGE and -VDIPEN around some of the chondrocytes in the healthy TMJ, which is consistent with the normal turnover of aggrecan in the cartilage. In comparison with the control mice, immunostaining for -NITEGE and -VDIPEN was very strong in the TMJ cartilage of the arthritic mouse, which indicated that aggrecan fragments were generated more extensively in the TMJ of the mouse affected with peripheral joint inflammation than in the control mice without arthritis.

While the presence of inflammatory cells in the TMJs was not typical, diminished safranin O staining and enhanced aggrecan neoepitope immunostaining could be seen in the TMJs of most animals at the chronic phase of PGIA and in a few TMJs in the acute arthritic group. Morphometric analyses of safranin O staining and IHC (anti-NITEGE and anti-VDIPEN) reactions were performed to quantitatively assess the extent of aggrecan degradation in the different joints by using tissue sections from the TMJ and ankle joints of control mice and of animals with acute and chronic PGIA. Neither safranin O staining nor immunostaining for the NITEGE neoepitope in the TMJ cartilage was significantly different between normal mice and those with acute proteoglycan induced arthritis, but after the mice reached the chronic phase of PGIA the differences became statistically significant. In the arthritic ankle joints, aggrecan degradation (diminished safranin O staining and generation of NITEGE neoepitope) was significantly greater than in the controls already at the acute phase of PGIA, and progressed further in chronic disease. These results indicated that aggrecan degradation occurred in both the ankles and the TMJs in PGIA, but was more progressive in the ankles than in the TMJ.

The absence of synovial inflammation or presence of very little inflammation coupled with massive loss of aggrecan is one of the typical features of osteoarthritis. Pro-inflammatory cytokines, such as IL-1β, TNFα, IL-6, or IL-17, have been implicated in the initiation of matrix breakdown in cartilage. Among these cytokines, IL-1β, which can also be produced by the chondrocytes themselves in osteoarthritis (OA), is thought to be the main effector of aggrecan breakdown via induction of aggrecanase and MMP expression in the chondrocytes. Evidence of upregulated expression of the genes encoding ADAMTS-4, ADAMTS-5 and MMP-3 in the TMJs of animals with both acute and chronic proteoglycan induced arthritis could be found, but the IL-1β gene was not upregulated in these TMJ samples. As inflammation progressed the expression levels of aggrecanases and MMP-3 in the arthritic peripheral joints increased, but the overall
magnitude of gene expression was similar to the levels found in the TMJ; except for ADAMTS-5, which was expressed at slightly lower levels in the arthritic ankle joints.

In contrast, the IL-1β gene was highly over-expressed in the arthritic limbs at both the acute and the chronic phases of proteoglycan induced arthritis relative to the normal control, although there was a statistically significant decrease in expression as arthritis progressed into the chronic phase. This latter observation suggested that the inflamed limb joints were the major sources of the “catabolic” cytokine IL-1β.

IL-1β needs to be present in the circulation to be able to act on the temporomandibular joint cartilage at an area that is distant from the sites of production. As expected, IL-1β could be detected in the sera of mice with either acute or chronic PGIA although could not be detected in the sera of control animals.

Moreover, additional pro-inflammatory cytokines, such as TNFα, IL-6, and IL-17, all of which could also contribute to cartilage matrix breakdown, were detected in the sera of arthritic animals at both the acute and chronic phases of PGIA.

None of these cytokines were detectable in serum samples of control mice. The differences between mice with acute and chronic PGIA were not statistically significant in the serum levels of any of these cytokines (p>0.05).

Production, purification, and cleavage of recombinant mouse TSG-6 (rmTSG-6) fusion protein

Our first aim of this study was to develop a simple expression system for high yield production of functionally active rmTSG-6.

Positive selection of transfectants, followed by a limiting dilution cloning procedure and a direct ELISA system using either a tag-specific (mouse IgG2a-Fc) or a protein-specific (TSG-6) Ab, allowed us to select clones with the highest yield of the fusion protein. Real-time PCR confirmed the presence of >80 copy numbers using the template of genomic DNA of the CHO transfectant, and ∼0.8–1.2 mg of recombinant fusion protein could be purified from 100 ml of conditioned medium of clone 514.

The purity of the rmTSG-6-Xa-mFc2a fusion protein (∼72 kDa) and rmTSG-6 (∼39 kDa after cleavage with factor Xa and repurification on Protein G-Sepharose), were over 95%, although some degradation occurred during the enzymatic cleavage and repeated purification. Because rmTSG-6 was synthesized by CHO cells in serum-free medium, serum immunoglobulins and IαI could not affect the purity (first and second lanes in or the functionality of secreted rmTSG-6. The molecular mass of purified rmTSG-6 after Xa cleavage was ∼39 kDa (instead of ∼30 kDa), indicating that the CHO cell-secreted rmTSG-6 was glycosylated. Indeed, enzymatic removal of all N-linked and most O-linked oligosaccharides reduced the mass of rmTSG-6 to the expected ∼30 kDa size. The Fc-free rmTSG-6 was largely water-insoluble but could be solubilized by the
addition of 5% BSA and dialysis against serum-free CHO medium. Purified rmTSG-6 bound HA in a concentration-dependent manner, and similar to recombinant human TSG-6, it enhanced the binding of fluorescence-labeled HA to cell surface CD44. We used rmTSG-6 for immunization of TSG-6-deficient mice and subsequent generation of TSG-6-specific mAbs (clones NG2, NG3, NG4, NG5, and NG8). RmTSG-6 also served as a reference standard for the development and use of mouse TSG-6 ELISA systems.

Serum TSG-6 concentrations correlate with arthritis severity and serum levels of pro-inflammatory cytokines but not with immune responses in PGIA

We used purified rmTSG-6 and optimally paired mAbs to be able to measure TSG-6 concentrations in serum and synovial fluid samples and in tissue extracts of inflamed joints of BALB/c mice immunized with cartilage PG on days 0, 21, and 42 to induce PGIA. By day 61 of immunization serum TSG-6 reached the maximum levels, around the time when the acute joint inflammation also reached the maximum. Although, whereas joint inflammation declined slowly, serum TSG-6 concentrations declined more rapidly. Low levels of TSG-6 could also be detected in the serum of PBS/DDA-injected mice, which was probably due to the activation of innate immune cells at the injection site.

Thus, the amount of TSG-6 in serum can be regarded as an indicator of pre inflammatory and inflammatory processes and appears to be a more reliable “biomarker” of the severity of acute disease than the levels of proinflammatory cytokines in PGIA. The correlation between arthritis score and serum TSG-6 was not significant at the early stage of disease (day 54) but became significant at the peak of arthritis (day 61) and remained such during progression toward chronic disease.

Although TSG-6 became undetectable in serum samples harvested from mice at late stages of PGIA (120–150 days after the first immunization), when acute synovial inflammation had given way to pathologic joint remodeling, it lead to deformities and loss of function (data not shown).

Whereas serum TSG-6 concentrations showed a strong positive correlation with arthritis severity from day 61 to 75 after the first immunization, serum levels of other “arthritis signature” pro-inflammatory cytokines correlated with the arthritis scores and serum TSG-6 at the acute or subacute phase (day 61 or 69) of PGIA, whereas serum IL-1β concentrations increased in response to immunization and subsequent arthritis onset but did not seem to correlate with disease severity or serum TSG-6. No correlation between serum TSG-6 levels and the concentrations of anti-PG Abs in serum or the magnitude of PG-specific T-cell responses could be detected.
TSG-6 in synovial fluid and tissue extracts of inflamed joints of mice with PGIA

The involved paws were heavily infiltrated by inflammatory cells and also showed histological evidence of cartilage and bone destruction at an advanced stage of acute arthritis. At this stage of PGIA, we harvested synovial fluid from arthritic ankle joints and prepared tissue extracts from the inflamed paws for measurement of TSG-6 content by ELISA and Western blotting. The amount of TSG-6 in pooled samples of synovial fluid was 110 ± 7 pg/mg protein, and it ranged from 20 to 550 pg/mg protein in the extracts of inflamed paws and from 0 to 60 pg/mg protein in non-arthritic control paws, as determined by ELISA. Western blotting demonstrated the presence of TSG-6 in both synovial fluid and tissue samples. In both cases, TSG-6 was found mostly in the form of high molecular weight; only small amounts of the free form could be detected.

Interestingly, synovial fluid contained a single TSG-6-positive band at ~120–125 kDa, whereas the extracts of inflamed paws showed multiple bands below that size. A Western blot of paw extracts revealed two TSG-6 bands at ~125 and ~80 kDa, respectively. Both of these bands were also recognized by an anti-mouse HC1 Ab, but only the upper band was recognized by anti-HC2 Ab.

Cellular localization of TSG-6 in the inflamed paws of mice with PGIA

TSG-6 could be detected in most of the cells of the inflamed tissue, by using immunohistochemistry on frozen sections of arthritic hind limbs. Unexpectedly, the strongest immunostaining appeared to be localized within large, granulated cells. Such cells also showed metachromatic staining with toluidine blue and red staining with safranin O, indicating that they could be connective tissue mast cells.

Antibody against the mast cell-restricted tryptase mMCP-6 for immunostaining of tissue sections was used to confirm the identity of this cell type. Anti-TSG-6 and anti-mMCP-6 Abs both stained the same type of cell. Co-localization of TSG-6 and mMCP-6 was further confirmed by the overlay of images of sections simultaneously stained with fluorescent Abs against the respective molecules.

HA is a stronger competitor than heparin for TSG-6 binding

RmTSG-6 bound to either HA- or heparin-coated plates, although the amount of TSG-6 bound to heparin appeared to be less than the amount that bound to HA. This difference might be due to the different molecular mass of HA versus heparin or different affinity of TSG-6 with HA or heparin.

Although TSG-6 binding sites to HA and heparin are different, and an octasaccharide of HA is sufficient to bind TSG-6, these two GAGs may compete for and/or interfere with TSG-6 binding either in vivo or in vitro.
In a competitive inhibition assay, we compared how HA or heparin could compete for TSG-6 binding. Indeed, whereas the in vitro preformed TSG-6-heparin complex could not inhibit TSG-6 binding to HA, the in vitro preformed TSG-6-HA complex inhibited TSG-6 binding to heparin. The controls confirmed that the TSG-6-HA complex can inhibit binding to HA, and the TSG-6-heparin complex can inhibit the TSG-6 binding to heparin.

TSG-6 binds mast cell tryptases in the presence of heparin or HA

Co-localization of TSG-6 and mMCP-6 in mast cells in inflamed joint tissue suggested that TSG-6 might be stored in mast cell granules, which are known to contain tryptases (mMCP-6 and mMCP-7 and heparin. Among other constituents, TSG-6 has been shown to bind either HA or heparin. In addition, TSG-6 can form a ternary complex with IαI in the presence of HA or heparin. Then, it was of interest to determine whether TSG-6 could interact with mast cell tryptases in a similar manner in vitro. RmTSG-6, prebound to HA, could bind rmMCP-6 or (to a lesser extent) rmMCP-7, but this was not the case with TSG-6 prebound to heparin. In a reciprocal system, rmMCP-6 and rmMCP-7, bound to either HA or heparin, could bind TSG-6.

TSG-6 can be co-immunoprecipitated with mast cell tryptases from tissue extracts of arthritic joints

TSG-6, mMCP-6, and mMCP-7 could be detected in relatively high amounts in tissue extracts of inflamed paws but were detected in lower amounts in tissue extracts of non-inflamed paws. TSG-6 could be co-immunoprecipitated with both, which can suggest that complex formation between TSG-6 and mast cell tryptases did occur in vivo under inflammatory conditions.

TSG-6 is a hyaluronan (HA)-binding protein, secreted by a variety of cells in response to proinflammatory stimuli. TSG-6 protein has been detected in large quantities in the synovial fluids and synovial tissues of inflamed joints of patients with rheumatoid arthritis. Recombinant mouse TSG-6 (rmTSG-6) has demonstrated anti-inflammatory and chondroprotective effects in mouse models of rheumatoid arthritis. TSG-6 forms a stable complex with a heavy chain (HC) of inter-α-trypsin inhibitor (IαI), a major serine protease inhibitor in serum. Because IαI exhibits increased inhibitory activity against plasmin, a key activator of matrix metalloproteinases after encountering TSG-6, it has been postulated that TSG-6 exerts its anti-inflammatory and chondroprotective effects primarily through inhibition of the protease network.

TSG-6 consists of a “Link” module and a “CUB” (complement C1s/C1r-, uEGF-, BMP-1-like) domain. The positively charged Link module binds various glycosaminoglycans (GAGs), including HA, chondroitin sulfate, heparin, and heparan
sulfate. The CUB domain of TSG-6 is similar to the CUB module found in several developmentally regulated proteins that are thought to be involved in protein-protein interactions. However, to date, only fibronectin has been shown to bind to the CUB domain of TSG-6. TSG-6 is not expressed constitutively but can be induced by proinflammatory cytokines or LPS. In contrast, anti-inflammatory cytokines, such as IL-4 or IL-10, suppress TSG-6 expression either directly or via inhibition of LPS/Toll-like receptor-induced cell activation.

TSG-6 can modulate the binding of HA to the cell surface HA receptor, CD44. Treatment of leukocytes with soluble HA·TSG-6 complex has been shown to inhibit the CD44-mediated adhesion of these cells to immobilized HA in vitro. Because the CD44-supported adhesion of leukocytes on HA-covered surfaces of inflamed vascular endothelium is required for the emigration of these cells from the bloodstream into inflamed tissue, inhibition of this adhesion step by the HA·TSG-6 complex could have a negative impact on the extravasation of inflammatory cells.

The in vitro observations are consistent with in vivo studies reporting reduced leukocyte influx into the arthritic joints of TSG-6-treated mice and enhanced leukocyte extravasation in the joints of TSG-6-deficient mice. Collectively, the in vivo observations lend support to the concept that TSG-6 has a critical role in the resolution of inflammation, but this function of TSG-6 may rely on more than one mechanism.

One of the initial goals of the present study was to develop a sensitive detection method for measuring the concentrations of TSG-6 in serum and synovial fluid samples of mice with arthritis. Using cartilage proteoglycan (PG)-induced arthritis (PGIA) in BALB/c mice, we monitored serum levels of TSG-6 in correlation with the onset and progression of arthritis and identified TSG-6-positive cells in the joints. Although many connective tissue cells were TSG-6-positive in the arthritic joints, unexpectedly, the strongest immunostaining of TSG-6 was detected in the granules of mast cells that accumulated in inflamed paws of mice. In vitro, rmTSG-6 bound to both heparin and the mast cell-restricted tryptases, murine mast cell protease-6 (mMCP-6) and mMCP-7, two major serine proteases present in mast cell secretory granules. Further, TSG-6 could be co-immunoprecipitated with both mMCP-6 and mMCP-7 from tissue extracts of arthritic paws, in which the heavy chains of IαI were also present. These data suggest that TSG-6 may modulate mast cell function via its interactions with key components of secretory granules.

Discussion
To our best knowledge, this is the first study to demonstrate that degenerative changes develop in the mandibular cartilage of the TMJs of mice during the progression
of autoimmune inflammatory joint disease in PGIA; an animal model of RA. The lack of previous investigations in RA models is surprising because the TMJ is a synovial joint, which may become a target of autoimmune reactions in both RA and animal models of the human disease. A significant proportion of RA patients experience pain in their TMJs, and analyses of synovial fluid samples clearly indicates the involvement of this joint not only in OA, but also in RA. In a recent study, up to 72 % of newly diagnosed RA patients were found to have TMJ involvement. This percentage is close to those found in studies of RA patients with longer duration or in pediatric patients with juvenile idiopathic arthritis, a disease with RA-like feature.

We found evidence of substantial loss of PG (aggrecan) in the mandibular cartilage of TMJs, with minimal or no local synovial inflammation in mice that developed severe inflammatory arthritis in their limb joints. This finding was consistent in animals that entered the chronic phase of arthritis. Aggrecan loss was occasionally associated with structural damage in the TMJ cartilage. In the ankle joints, severe synovitis was accompanied by chondrocyte loss from the superficial zone. Although pitting was mainly restricted to sites where the cartilage was in contact with inflammatory cells, GAG-containing aggrecan fragments were lost from the entire cartilage. Whereas the TMJ of mice with severe PGIA showed evidence of structural impairment, aggrecan degradation in mandibular cartilage was not greater, and appeared to be less progressive, than in the inflamed ankle joints. This suggests that the insults that triggered aggrecan loss and/or the response of the joints to those stimuli could be different at these anatomically distinct sites. Histopathologic abnormalities were not found in the joints of age- and sex-matched control (PBS/DDA-injected) mice, which allowed us to exclude the possibility of any age- or gender-related association.

Loss of PG from the TMJ cartilage of arthritic animals was reminiscent of the changes described for cartilage in joints affected with OA in humans or in animal models of cartilage degeneration.

Aggrecan fragmentation could be attributed to upregulation of catabolic enzymes, and we found that mRNA levels of aggrecanases and stromelysin were upregulated, and the neoeptitopes generated by these enzymes (-NITEGE and -VDIPEN) accumulated in the TMJs in animals with PGIA. These results were similar to those in the inflamed ankle joints of the same animals.

Pro-inflammatory cytokines have a central role in aggrecanase and MMP upregulation, and the key pro-inflammatory cytokine appears to be IL-1β, although TNFα and IL-6, either directly or via the activation of the IL-1β pathway, may also be involved in the upregulation of aggrecanases and MMPs. Chondrocytes have been shown to produce IL-1β in response to various stimuli, and this cytokine can activate the catabolic pathway in cartilage, which ultimately leads to local matrix breakdown.
In support of this, induction of chondrocyte-specific expression of the IL-1β gene resulted in OA-like histopathological changes and dysfunction in the TMJs of transgenic mice. Moreover, a positive correlation was found between the incidence of TMJ dysfunction and serum levels of the IL-1 receptor in RA patients. We were surprised to find that the chondrocytes of the damaged mandibular cartilage did not upregulate IL-1β expression in mice with PGIA. However, this cytokine was markedly over-expressed in the joint tissues of arthritic limbs of the same animals, suggesting that exogenous IL-1β could be responsible for the catabolic activity of mandibular cartilage chondrocytes and/or surrounding synovial cells. Indeed, serum samples from arthritic mice contained high amounts of IL-1β and other pro-inflammatory cytokines, including TNFα, IL-6, and IL-17. Notably, the serum levels of these cytokines in our arthritic mice were similar to concentrations found in TMJ synovial fluid samples from patients with RA or OA.

We conclude that unlike the peripheral joints, the TMJ is not a target of the autoimmune attack in PGIA. The OA-like cartilage damage in the TMJs of arthritic mice is likely due to the effects of systemic factors, such as circulating cytokines, released from inflamed joints. Sustained production of these pro-inflammatory cytokines appeared to be necessary to induce TMJ cartilage degradation in a consistent manner. Serum concentrations of pro-inflammatory cytokines decline at a late phase of chronic PGIA. Therefore, it is possible that the process of TMJ cartilage degradation slows down after the “burn-out” of limb joint inflammation.

In summary, we show evidence of cartilage matrix degradation in the TMJs of mice having autoimmune inflammatory arthritis in their limb joints, and propose that systemic pro-inflammatory cytokines could be involved in TMJ pathology. Our results suggest that early treatment of RA, especially with biologics, such as IL-1 or TNF antagonist, holds promise for the prevention of irreversible tissue damage in the TMJs of RA patients.

On the other hand, the beneficial effects of TSG-6 treatment have been described in different murine models of RA, and the protective function of this protein is consistent with the development of more severe arthritis in TSG-6-deficient mice than in wild type mice upon immunization with cartilage PG. In adoptively transferred PGIA, TSG-6 gene expression was detected in synovial tissue of recipient mice before the clinical symptoms of arthritis developed, and TSG-6 was also described as a signature gene of early inflammatory events in a number of other diseases. However, no systemic studies have been carried out to investigate the kinetics of TSG-6 production during the development and progression of any inflammatory joint disease, including RA.

As a first step of generating a highly sensitive assay, we constructed an expression vector that contained both TSG-6 and mouse IgG2a-Fc fusion partner cDNA, separated by an endoprotease cleavage site (Ile-Glu-Gly-Arg) specific for factor Xa (which
converts prothrombin to thrombin). We also developed a stepwise cloning procedure for rapid selection of CHO transfectants with the highest yield of rmTSG-6 fusion protein released into serum-free medium. RmTSG-6 protein was then used to generate B cell hybridomas from TSG-6-deficient mice and to select pairs of mAbs for capture ELISAs, where rmTSG-6 could also be employed as a reference standard. Some of the anti-TSG-6 mAbs were also suitable for immunoprecipitation, Western blotting, and immunohistochemistry. With this repertoire of detection tools in our hand, we immunized BALB/c mice with cartilage PG to induce PGIA, with the goals to determine the kinetics of serum TSG-6 levels in correlation with arthritis severity and other disease markers and to detect the accumulation of this protein at the site of inflammation.

Serum TSG-6 concentrations showed significant positive correlation with the progression of arthritis. In this regard, TSG-6 appeared to be a better biomarker of joint inflammation than the arthritis-related pro-inflammatory cytokines IL-6, IL-17, TNF-α, and IL-1β, which showed correlation with the disease development only at certain time points or not at all. Serum TSG-6 levels declined when arthritis progressed toward the chronic phase, during which massive tissue destruction was followed by synovial fibrosis and bone remodeling. We could accurately measure TSG-6 levels in synovial fluid samples and tissue extracts of severely inflamed paws of mice. Western blots carried out on these samples revealed that the majority of TSG-6 was present as a high molecular weight species, indicating that at these inflammatory sites, TSG-6 was probably complexed with HCs from lαI, as described before. The inflammatory paw extracts consistently contained both a ∼125-kDa species (probably a TSG-6-HC1 and a TSG-6-HC2 complex) and an ∼80-kDa species. The latter band could be a degradation product of the TSG-6-HC1 complex and was not detected in synovial fluids of arthritic joints.

For the first time, we were able to immunolocalize TSG-6-producing cells in acutely inflamed joint tissues of mice using the newly generated mAbs. Whereas most cells in the arthritic joints stained positively for TSG-6 at low to moderate levels, very high amounts of TSG-6 protein were detected in the secretory granules of mast cells, most of which cells showed evidence of activation (degranulation) in the inflamed tissue. Beyond metachromatic and positive safranin O staining, we sought to identify these cells more precisely by employing Abs to mouse mast cell-specific proteins. An Ab against the mast cell-specific tryptase mMCP-6 (also a component of secretory granules) gave a signal that overlapped with TSG-6-specific staining, indicating colocalization of the two proteins in mast cell granules.

The fact that both mMCP-6 and TSG-6 can bind heparin prompted us to investigate whether TSG-6 could associate with mMCP-6 via heparin. Indeed, our in vitro studies revealed a tripartite association of rmTSG-6 with rmMCP-6 (and, to a lesser extent, with rmMCP-7) through heparin as well as through HA. MMCP-6 and, to a lesser
extent, mMCP-7 could be pulled down by TSG-6 immunoprecipitates of arthritic paw extracts, suggesting that these proteins were associated (or formed a complex) in vivo under the conditions of joint inflammation.

Mast cells constitute the first line of defense in various IgE-mediated allergic reactions and helminth infections, and they are strategically positioned near blood vessels, which enables them to capture allergen-specific IgE, IgG, or other substances from both blood and tissue. Mast cell secretory granules contain proteases, GAGs, and proinflammatory cytokines that are released into extracellular matrix upon degranulation. Tryptases, such as mMCP-6 and mMCP-7 (murine orthologs of human β-tryptases), are the predominant serine proteases stored in mast cell secretory granule. These enzymes are tightly packaged in complexes with serglycin PGs through electrostatic interactions between their positively charged amino acid residues and the negatively charged GAG side chains of serglycin. In addition to packaging, heparin may play a role in the maintenance of tryptases in an active conformation at low pH inside the mast cell granules, but proteolytic activity increases at neutral pH after release of the tryptase-heparin complexes into the extracellular environment.

The presence of TSG-6 in mast cells has not been reported earlier. Here we have found that the tryptases mMCP-6 and mMCP-7, upon binding to either HA or heparin, associate with TSG-6. As noted above, HA-bound TSG-6 has a stronger association with both tryptases than heparin-bound TSG-6. Some of the differences between HA- and heparin-bound complexes might be explained with the better adsorption of high molecular mass HA than low molecular mass heparin to plastic surfaces. However, in a competitive inhibition assay, we found that HA inhibited the binding of rmTSG-6 to heparin, but heparin did not inhibit the binding of rmTSG-6 to HA. Nonetheless, enhancement of TSG-6-tryptase interactions on the GAG-coated surfaces is obvious under the conditions of our in vitro assays. Moreover, co-immunoprecipitation of TSG-6 and tryptases suggests that complex formation may also occur in vivo. However, more extensive studies are required to specify the type of molecular interactions among HA, heparin, TSG-6, and tryptases.

What could be the relevance of these tripartite interactions to pathophysiological events involving mast cells? As mentioned before, TSG-6 readily forms a covalent complex with the HC1 or HC2 subunit of the serine protease inhibitor lαI (composed of two HCs bound to the chondroitin sulfate chain on bikunin, a Kunitz type protease inhibitor) and augments the inhibitory capacity of this molecule.

Recent studies indicate that potentiation of lαI activity is achieved by the TSG-6-mediated transfer of a HC from the single chondroitin sulfate side chain of bikunin onto HA. This is followed by the release of bikunin with a single HC or without HC. Bikunin, which harbors the protease-inhibitory activity of lαI, is more active in the absence than
in the presence of the HC s on its GAG chain. Intriguingly, the tryptase inhibitor present in mast cells has been identified as bikunin. Because only bikunin protein but not mRNA could be detected in mast cells, the authors concluded that bikunin was trapped by these cells from serum of circulating blood. It is tempting to speculate that TSG-6 has a role in liberating bikunin from IαI and trapping it inside the mast cell granules or on the surface of granule membranes. Upon mast cell exocytosis, the tryptases would meet bikunin and thus be inactivated. Whether or not TSG-6 plays a regulatory role in these tryptase-bikunin interactions in mast cell granules remains to be determined.

The role of mast cells in non-IgE-mediated diseases, such as arthritis, is being increasingly recognized. Mast cells are abundant in inflamed synovial tissues of both humans and mice. Mast cell-restricted tryptases can convert matrix metalloproteinase-3 from latent to active form, which cleaves cartilage aggrecan and also activates synoviocyte procollagenase. Mice lacking mast cells are less susceptible to inflammatory arthritis than the wild type counterparts. Moreover, mice deficient in both mMCP-6 and mMCP-7 or lacking the enzyme responsible for heparin biosynthesis are remarkably protected from cartilage degradation after induction of destructive arthritis. In our earlier studies, intra-articularly injected rmTSG-6 and cartilage-specific expression of a TSG-6 transgene were both found to exhibit substantial chondroprotective effects in murine models of arthritis. These observations are consistent with the involvement of TSG-6 in the negative regulation of the activity of matrix-degrading proteases, probably including mast cell-restricted tryptases.

Mast cell granule components, specifically mMCP-6-heparin complexes, also stimulate neutrophil extravasation via induction of chemokines. Influx of neutrophils into the joints is a prominent feature of arthritis in both humans and experimental animals. Systemic administration of rmTSG-6 to mice with PGIA significantly suppressed leukocyte infiltration into the joints. TSG-6 also inhibited neutrophil influx into zymosan-stimulated air pouches, apparently due to reduced amounts of neutrophil-attracting chemokines at the site of inflammation. Conversely, the most distinctive phenotype of TSG-6-deficient mice is the exaggerated recruitment of neutrophils in the joints after the development of PGIA and in the peritoneal cavity after peritonitis induction. Collectively, these data argue for a role of TSG-6 in the resolution of inflammation, in part due to a negative influence on mast cell protease-induced chemokine production and subsequent leukocyte extravasation. These novel functions of TSG-6, proposed here in inflammatory joint destruction, warrant further investigation.

Summary
In this present study, I was investigating the involvement of the TMJ in mice with PGIA and the role of TSG-6 in mice with PGIA. My new findings are as follows:

- There are pathologic changes in the temporomandibular joint in mice with PGIA and these changes resemble OA rather than RA.
- I described a simple expression system for high yield production of functionally active rmTSG-6.
- Serum TSG-6 concentrations correlate with arthritis severity and serum levels of proinflammatory cytokines.
- but not with immune responses in PGIA.
- The strongest expression of TSG-6 is in mast cells in the inflamed tissues.
- The likely role of TSG-6 is that it modulates mast cell function via its interactions with key components of secretory granules.
List of other publications

4. Kobezda, T., Ghassemi-Nejad, S., Mikecz, K., Glant, T. T., Szekanecz, Z.: Of mice and men: how animal models advance our understanding of T-cell function in RA.
   DOI: http://dx.doi.org/10.1038/nrrheum.2013.205
   IF: 9.845

   DOI: http://dx.doi.org/10.1016/j.cellimm.2012.08.003
   IF: 1.743

   DOI: http://dx.doi.org/10.1016/j.ajpath.2010.12.053
   IF: 4.89

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List of publications related to the dissertation

   DOI: http://dx.doi.org/10.1016/j.jbspin.2016.05.016 
   IF: 3.329 (2016)

2. Ghassemi-Nejad, S., Kobezda, T., Rauch, T. A., Matesz, K., Glant, T. T., Mikecz, K.: 
   Osteoarthritis-like damage of cartilage in the temporomandibular joints in mice with autoimmune inflammatory arthritis. 
   DOI: http://dx.doi.org/10.1016/j.joca.2011.01.012 
   IF: 3.904

3. Nagyeri, G., Radacs, M., Ghassemi-Nejad, S., Tryniszewska, B., Olasz, K., Hutas, G., Győrfy, Z., 
   Hascall, V. C., Glant, T. T., Mikecz, K.: TSG-6 Protein, a Negative Regulator of Inflammatory Arthritis, 
   Forms a Ternary Complex with Murine Mast Cell Tryptases and Heparin. 
   DOI: http://dx.doi.org/10.1074/jbc.M111.222026 
   IF: 4.773