Introduction

Components and function of the cartilage

Articular cartilage covers the ends of long bones and is crucial for the functioning of normal synovial joints. It enables frictionless motion between the articular surfaces, transmits load from one articular surface to the other, distributes loads within the joint and provides shock absorption [1]. In human cartilage chondrocytes make up only 0.4-2% of the tissue but provide the synthetic capacity to produce and thereby to maintain the abundant extracellular matrix with two major protein components: collagens and proteoglycans

Collagens

The major collagen in hyaline cartilage is type II collagen representing 80-95% of all collagens in the cartilage [2]. Of the less abundant collagen species in cartilage, type IX and XI collagen are cartilage-specific, while type IV and VI are also found in other tissues [3]. Type X collagen is restricted to the growth plate and to the articular cartilage[4].

Proteoglycans

Proteoglycans (aggrecan, decorin, biglycan, fibromodulin) are the most abundant non-collagenous components of the extracellular matrix of the cartilage. This diverse family of macromolecules is characterized by a core protein to which glycosaminoglycan (GAG) side chains are attached. Aggrecan (large aggregating proteoglycan) is the most prominent proteoglycan in the cartilage accounting for 90% of the proteoglycan content. The molecule has a peculiar brush-like structure and a molecular weight of approximately 2-3 million Da, 90% of which are sulfated GAGs and O- and N-linked oligosaccharides that are attached to a core protein [5] (Fig.1). Aggrecan binds to hyaluronan through the amino-terminal globular domain (G1) of the core protein, and has a

specifically long middle part (between G2 and G3 globular domains) that bears the GAG side chains.

Aggrecan molecules are found in aggregates wherein approximately 200 molecules are non-covalently anchored to long stretches of hyanluronan [6] stabilized by a third component, link protein. Due to the highly negatively charged GAG side chains these multi molecular complexes associate with hydrated cations and entrap substantial amount of water inside the tissue (65-80% of wet weight[7]). Its configuration with matrix collagen molecules prevents free flow of water within the matrix and thereby provides the visco-elastic properties of the cartilage. Depending on the load on the cartilage, part of the solvent can be released and later regained when the compression discontinues. Nevertheless, the more water is extruded, the harder it gets to compress the tissue as the repulsion among the negatively charged GAG chains becomes more manifest. This compression-dependent change in solvent content provides the tissue its resilience, and its ability to withstand compressive loads.

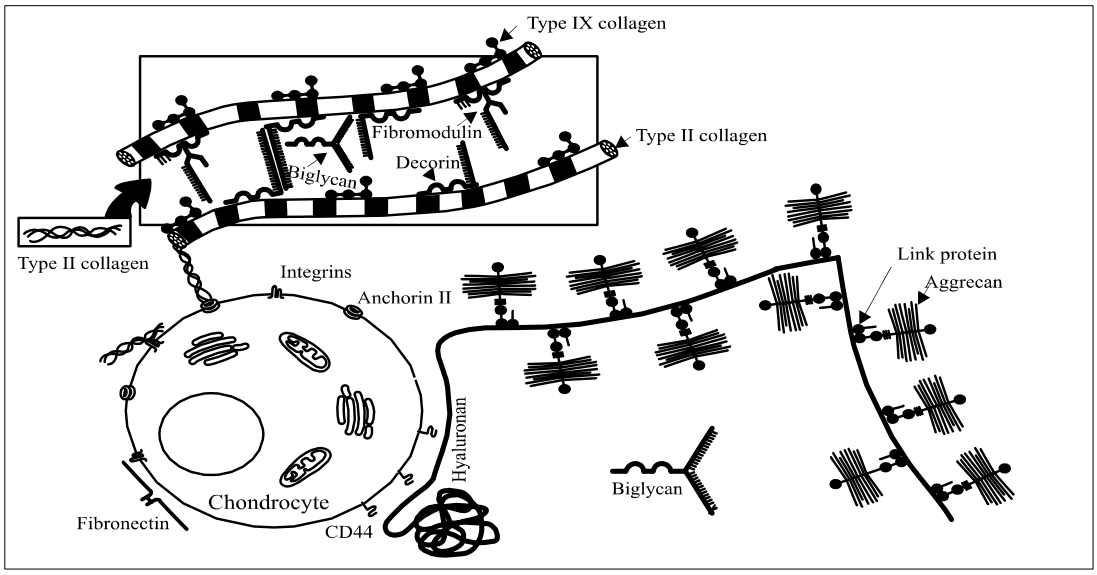

Figure 1. Molecular organization of the extracellular matrix of cartilage. Various matrix components are synthesized by chondrocytes. HA filament, anchored to cartilage via CD 44 receptor, binds high density PG (aggrecan) and link protein forming large multimolecular aggregates. Type II collagen interacts with anchorin II and integrins on the surface of chondrocytes. Type IX collagen, decorin, fibromodulin and biglycan are shown on the surface of type II collagen and possibly involved in collagen fibril formation. (Schematic figure is adapted from Kuettner,KE[8])

Immunogenicity of cartilage components

Cartilage is one of the few immunologically privileged tissues in the body in that it is essentially avascular and therefore not subjected to close 'internal' immunological surveillance. When it gets degraded, however, uniquely antigenic molecules become exposed, released and subsequently recognized by the immune system [9,10]. Thus, articular components may trigger and maintain immune responses to these antigens (immunogenicity). The immune attack on the joints could also be initiated by a cross reactive immune reaction in response to unrelated antigens by the mechanism of 'molecular mimicry'. The net result of such auto-immune reactions could be further destruction of cartilage and release of more autoantigens. This could lead to a chronic, self-perpetuating inflammation in genetically predisposed individuals who are prone to develop these autoimmune reactions [11]. Although autoimmunity to cartilage proteoglycans has been studied less intensively than autoimmunity to type II collagen, cartilage proteoglycan (aggrecan) is considered as causal/contributing factor in rheumatoid joint disease [12].

Immune response to proteoglycans in humans

Patients with rheumatoid arthritis (RA) show cellular immune responses in their peripheral blood to proteoglycan [13,14]. The incidence of proteoglycan reactivity in the patients varied from 5 to 85%, depending upon the study. Cellular immunity to proteoglycan has also been described in patients with juvenile rheumatoid arthritis (JRA) [13]. Furthermore, immunoreactive fragments of proteoglycan have been demonstrated in the synovial fluids of patients with RA [15].

Cellular immunity to proteoglycan has been frequently detected in patients with ankylosing spondylitis (>85% of the patients) [13,14]. Proteoglycan-specific T-cell lines have been isolated from the peripheral blood of three patients with ankylosing spondylitis [16]. These cells were of the T helper phenotype and were highly specific to proteoglycan isolated from human but not bovine or rat origins. These facts all suggest that proteoglycan/aggrecan may play an important role in autoimmune diseases of the peripheral and axial joints.

Rheumatoid arthritis and its experimental animal models

Rheumatoid arthritis is a common chronic inflammatory polyarthritis of worldwide distribution, with a female predominance. Although the cause of RA remains unknown, numbers of evidences are suggestive of an autoimmune etiology. Cell-mediated immune response and autoantibodies to cartilage proteoglycans [13,14] and/or collagen type II [14,17,18] have been detected in RA. These autoimmune reactions to cartilage components are, most likely, a consequence of secondary immune response raised against fragments of macromolecules released by local inflammatory processes. A putative figure of human RA summarizes the hypothetical immune mechanisms involved in this disease (Fig.2).

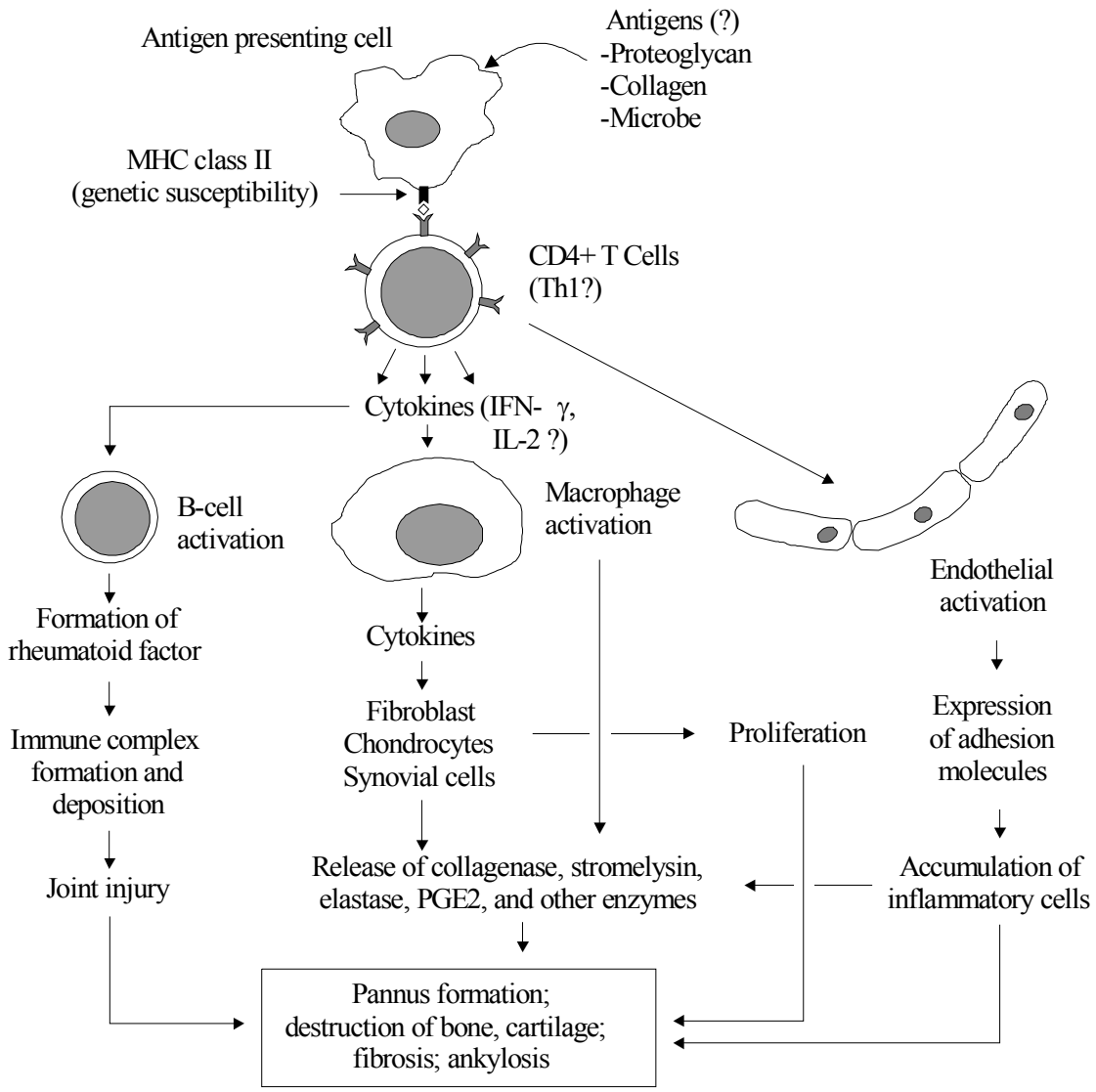

Figure 2. Immunopathogenesis of rheumatoid arthritis (Schematic figure is adapted with minor modifications from Cotran-Kumar-Collins: Pathologic basis of diseases 6th ed.W.B. Saunders Co. 1998.)

Experimental animal models of inflammatory arthritis (e.g., adjuvant arthritis, various forms of antigen-induced arthritis, collagen-induced arthritis and proteoglycan-induced arthritis) have provided important advances in understanding possible mechanisms for human disease and in developing therapeutic agents for treatment in human pathology. The most frequently studied animal models are those induced in rats or mice [19]. Short descriptions of the relevant models of RA are included:

Antigen-induced arthritis (AIA)

AIA is based upon an immune response against a foreign antigen to which the animals are sensitized prior to induction of arthritis. Inflammation is induced by local injection of the antigen into the knee joint of the immunized animals [20]. The severity of local inflammation and subsequent cartilage damage depend on the level of sensitization against the foreign antigen rather that the type of antigen or animal strain [21,22]. The inflammation is characterized by T-cell infiltration, synovial cell proliferation and loss of aggrecan due to the activation of various matrix metallo proteinases (MMPs). These MMPs include stromelysin and aggrecanase, which generate neoepitopes with known sequences of aggrecan (-VDIPEN and –NITEGE). This unique condition allows us to detect early cartilage damage by detecting these neoepitops using specific antibodies [23].

Collagen-induced arthritis (CIA)

Collagen-induced arthritis can be elicited in susceptible strains of rats [24], mice [25] or in monkeys [26] by immunization with native type II collagen in complete or incomplete Freund's adjuvant. CIA is induced in susceptible DBA/1 strain of mice by native heterologous type II collagen [25]. In early stages of CIA, fibrin deposition occurs and anti-collagen antibodies bind to the joint cartilage and

activate complement pathways. Erosive polyarthritis typically develops 10-14 days after the primary immunization or a week after a booster injection given intraperitoneally in FCA on day 21. Autoreactive T cells, as well as B cells, which produce antibodies to type II collagen, play critical roles in disease progression.

Proteoglycan-induced arthritis (PGIA)

Immunization of susceptible mouse strains (BALB/c, C3H/HeJCr) with human cartilage proteoglycan induces progressive polyarthritis [27-29]. The disease develops in all female BALB/c mice when human fetal or newborn proteoglycans are injected intraperitoneally in complete Freund's adjuvant [28]. Although fetal human and calf proteoglycans have many biochemical and immunological similarities, calf proteoglycans treated and administered the same way does not produce arthritis in BALB/c mice [11,27,28]. Thus one may postulate that the arthritogenic structures are present in particular portions of the core protein.

This mouse model shows many similarities to human rheumatoid arthritis and ankylosing spondylitis as indicated by clinical assessments, such as radiographic analysis and scintigraphic bone scans, and by histopathological studies of diarthrodial joints and spine tissue [27]. During early phases perivascular concentration of mononuclear cells occurs, followed by strong proliferation of synovial macrophages and fibroblasts. The arthritis starts as a polyarticular synovitis in bilateral, small peripheral joints and becomes progressive with extensive erosion of cartilage and bone within the joint. The initial, clinical symptoms of joint inflammation (swelling and redness) appear after the third or fourth intraperitoneal injection of antigen (Fig.3).

Figure 3. Macroscopic appearance and histopathology of the mouse paw in normal (left panel) and arthritic mouse (right panel- proteoglycan-induced arthritis). Arrowheads point at bone and cartilage erosions. (Figure is partially adopted from Glant *et. al.* [29])

Antibodies against immunizing proteoglycan (human) appear during the second/third week of immunization [28]. T-cell response to proteoglycan is detectable approximately 5-7 weeks after the start of immunization and along the course of the disease the humoral and cellular immune responses slowly decline as the disease becomes chronic and less active [28]. The development of arthritis in genetically susceptible mice is based upon cross reactive immune responses between the immunizing fetal human and mouse self-PGs [27,28,30]. T cells and autoantibodies react with both native and degraded PGs of mouse cartilage and cross-react with the immunizing human fetal proteoglycan [30,31].

When aggrecans were compared from ten different species the chondroitin-sulfate depleted aggrecans from fetal or newborn human and canine cartilages were the only ones able to induce cross-reactive T-cell response to mouse proteoglycan and subsequently arthritis [28,32].

The important role of GAG side chains (keratan- and chondroitin-sulfate) has recently been discovered. Keratan sulfate side chains can mask dominant arthritogenic T-cell epitopes and their presence may inhibit either antigen presentation or recognition. In contrast, the depletion of the chondroitin sulfate side chains of cartilage aggrecan increases the immunogenicity of the molecule by generating a number of clustered chondroitin sulfate stubs that provoke strong B-cell response and induce professional antigen presenting cells [31].

While the antibody level and T-cell response to mouse aggrecan highly correlate with the onset of arthritis, proteoglycan-immunized [30] non-arthritic animals may also express humoral and cellular immune response against self antigen (mouse aggrecan). T-cell response is linked to MHC (H-2^d in BALB/c and H-2^k in C3H mice), but DBA/2 or NZB mice with the same haplotype (H-2^d) are resistant to PGIA [28,33]. Furthermore, F1 hybrids (CDF1) of BALB/c and DBA/2 parents (both carry H-2^d) are also resistant to PGIA, and the susceptibility returns to 8-28% in F2 hybrids [11,33]. Further genome-wide screening is being performed at present to identify arthritis-associated quantitative trait loci in PGIA.

T cells, especially Th cells seem to be critical for arthritis induction [34,35] and are divided into two major groups based upon the cytokine secretion profile: Th1 and Th2. Th1 cells secrete interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis (TNF)-β, while Th2 cells produce IL-4, IL-5, IL-10 and IL-13 [36,37]. Th1-type cytokines dominate in the flare up of inflammation in arthritis, thus the Th1 response is considered as pro-inflammatory, whereas the activation of Th2 subset seems to be anti-inflammatory [38-40]. While the pathological function of immunoglobulin isotypes in arthritis is unclear, Th1 cells and Th1-type cytokines control IgG2a and Th2 cytokines the IgG1 production [41,42]. A critical balance between Th1 and Th2 responses may control the progression and/or severity of arthritis, as CIA is associated with Th1 dominance and IgG2a isotype switch [43,44].

Aim of the study

The common purpose of studying experimentally induced arthritis is to find an animal model that simulates inflammation, cartilage degradation, and immunological events that are characteristic of human rheumatoid diseases. PGIA shares several features with rheumatoid arthritis and ankylosing spondylitis. The aim of this study was to look into the immunological events behind the pathologic changes in PGIA and to understand the disease in a chronological manner; furthermore, exploiting this experimental model of RA, to work on distinct methods that may influence the disease hoping that these results may contribute to the unravel and treatment of the human autoimmune diseases, i.e. rheumatoid arthritis.

In Chapter 1 a novel adoptive transfer model of arthritis was used to transfer PG-induced arthritis into mice with severe combined immunodeficiency (SCID). The immune dynamics of the disease has been studied during the development of adoptively transferred disease, especially focusing on the two major immune components (T and B cells) and their relative contribution to arthritis.

To modulate/'treat' the experimentally induced arthritis two completely distinct approaches were tested in mice. Firstly, nasal tolerization with proteoglycan, as an antigen specific immune modulation, was studied in the work described in Chapter 2. Additionally, the tolerance has been transferred into SCID recipients and the findings and concerns are discussed. Secondly, the function of tumor necrosis factor-α stimulated gene 6 (TSG-6) and its effect on the inflammation was investigated using two models of arthritis: PG-induced and antigen-induced arthritis. Peculiar anti-inflammatory, and chondroprotective role of TSG-6 is reported in both models in Chapter 3.

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Chapter 1

T- and B-Cell Recovery in Arthritis Adoptively Transferred to SCID Mice. Antigen-specific Activation Is Required for Restoration of Autopathogenic $CD4^+$ Th1 Cells in a Syngeneic System

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Abstract

T-cell homeostasis is a physiological function of the immune system that maintains a balance in the numbers and ratios of T cells at the periphery. A self-MHC/self-peptide ligand can induce weak (covert) signals via the TCR, thus providing an extended life span for naïve T cells. A similar mechanism is responsible for the restoration of immune homeostasis in severe lymphopenic conditions such as those following irradiation or chemotherapy, or upon transfer of lymphocytes to *nu/nu* or SCID mice. To date, the genetic backgrounds of donor and recipient SCID mice were unmatched in all autoimmune arthritis transfer experiments, and the recovery of lymphoid cells in the host has not been followed. Here, we present the adoptive transfer of proteoglycan (PG)-induced arthritis (PGIA) using unseparated, and T or B cell-depleted lymphocytes from arthritic BALB/c donors to genetically matched syngeneic SCID recipient mice. We demonstrate that selectively recovered lymphoid subsets determine the clinical and immunological status of the recipient. We found that when T cells were depleted (>98% depleted), B cells did not produce PG-specific anti-mouse (auto)antibodies unless SCID mice received a second antigen (PG) injection, which promoted the recovery of antigen-specific $CD4^+$ Th1 cells. Reciprocally, as a result of B-cell recovery, high levels of serum anti-PG antibodies were found in SCID mice that received B cell-depleted (>99% depleted) T lymphocytes. Our results indicate a selective and highly effective cooperation between $CD4^+$ T cells and B lymphocytes, that is required for the restoration of pathological homeostasis and development of autoimmune arthritis in SCID mice.

Introduction

Proteoglycan (aggrecan)-induced arthritis (PGIA) is a murine model of rheumatoid arthritis as indicated by clinical assessment, laboratory tests and histopathology of the peripheral joints [1,2]. Arthritis can be induced only in genetically susceptible BALB/c or C3H mice by systemic immunization with human cartilage proteoglycan (PG) [1-4]. The development of the disease is based upon cross-reactive immune responses between the immunizing human and mouse (self) cartilage PGs [3-5].

Several lines of evidence indicate that $CD4^+$ T helper (Th) cells play an important role in PGIA. (i) Susceptibility to PGIA is influenced by MHC ($H\text{-}2^d$ haplotype in BALB/c and $H\text{-}2^k$ in C3H mice) [3,6-9], (ii) immunization of BALB/c mice with PG induces a Th1-dominant T-cell response [10,11], and (iii) PG-specific Th1-type T-cell hybridoma induces arthritis in BALB/c mice [12]. (iv) The disease is prevented when $CD4^+$ T cells are depleted either *in vitro* [13] or *in vivo* [14] and, lastly, (v) treatment of arthritic mice with IL-4 can prevent disease development by inducing a switch from a Th1-type to a Th2-type response [10]. Furthermore, IL-4deficient mice in BALB/c background develop a significantly more severe disease when compared to wild type BALB/c mice, and show a skewed Th1/Th2 profile with Th1 dominance [10,11,15,16]. While the critical function of $CD4^+$ T cells was implicated in arthritis induction [16], the role of antibodies to mouse (self) PG and/or B cells in the pathogenesis of this autoimmune model is not yet fully understood [5,6,13]. Transfer of the disease required both T and B cells, and neither anti-PG antibodies nor PG-specific B cells alone were able to transfer disease [13,17]. On the other hand, PG-specific B cells appeared to play a major role in antigen presentation [5]. Together, a highly specific cooperation between antigen-primed $CD4^+$ Th1 and B cells appears to be critical for the development of the disease [6,11,16]. T-cell homeostasis is a physiological function of the immune system that maintains a balance in the numbers and ratios of T cells at the periphery [18-20]. For long-term survival of naïve T cells, a weak interaction between the TCR and a self-MHC/(self)peptide ligand is necessary [20,21]. Such

”covert” signals provide T cells with an extended life span, and capacity to proliferate and populate the lymphoid organs [18,19,22]. Homeostatic polyclonal T-cell proliferation, i.e., the restoration of the original T-cell pool, occurs in severe lymphopenic conditions such as those following irradiation, chemotherapy, or upon transfer of lymphocytes to *nu/nu* or SCID mice. A low number of T cells can repopulate the lymphoid organs, but only in syngeneic condition which allows for recognition of a wide range of self-MHC-associated/(self)peptide ligands by matched TCRs [23-25]. Thus, the adoptive transfer of PGIA from BALB/c into genetically matched (both MHC and non-MHC genes) SCID mice seems to be an ideal system for studying T-cell restoration in a T cell-mediated autoimmune disease.

Human and experimentally induced autoimmune diseases exhibit complex and polygenic modes of inheritance, dictated by both MHC and non-MHC genes [7,8,26-30]. In previous studies, collagen-induced arthritis (CIA) was transferred to genetically unmatched SCID mice [31-33]. Disparate genetic backgrounds, however, might significantly modulate the cell survival and the outcome of immune reactions, even in an immune-compromised host. Furthermore, anti-collagen antibodies play critical roles in CIA (either in the primary or transferred form of the disease) [34,35], whereas PG-specific antibodies appear to have only a limited function in arthritis induction in PGIA [6,13]. Here, we present adoptive transfer of autoimmune polyarthritis in a syngeneic system to SCID mice. We focus on the recovery and clonal restoration of T and B cells, and demonstrate the role of these selectively recovered lymphoid cells in the regulation of the host’s (recipient) clinical and immunological status.

Materials and Methods

Antigens, animals, and immunization

High-density cartilage PG (aggrecan) was purified from human articular cartilage by CsCl gradient centrifugation, and depleted of glycosaminoglycan side chains as described [2]. Female BALB/c mice (National Cancer Institute, NCI, Friedrich, MD) were immunized i.p. first with cartilage PG (100

μg protein) in CFA and then with the same doses of PG on day 21 and 42 in IFA. BALB/c mice were also immunized with ovalbumin, and lymphocytes from these ovalbumin-immunized control mice were also used for transfer. Female SCID mice of BALB/c background (NCI/NCrC.B-17-s*cid/scid*), 8-12 weeks of age, or young retired breeders, were purchased from NCI and maintained under germ free conditions. SCID mice with a 'leaky' immune system were excluded from the experiments [36].

Cell isolation, depletion of T and B cells, and transfer of arthritis

Single-cell suspensions were prepared in DMEM from spleens of arthritic BALB/c mice. To maintain the uniformity and high reproducibility of transferred arthritis, donor cells were isolated from arthritic BALB/c mice within 2-3 weeks after the onset of the primary arthritis, and the cumulative inflammatory score of arthritic donor mice ranged between 5.0 and 8.0 (Table I). The mononuclear cells were isolated on Lympholyte-M (Zymed, San Francisco, CA) and used either as unseparated lymphocytes, or were depleted of specific subsets (see below). Unseparated lymphocytes were stimulated with cartilage PG, Con A (Amersham, Arlington, IL), or lipopolysaccharide (LPS; Serotype: O26:B6, Sigma Chemical Co., St. Louis, MO). For depletion of T and B cells, respectively, magnetic microbeads conjugated with mAbs to CD90 (Thy1.2) and CD45R/B220 were used, (Miltenyi Biotech Inc., Auburn, CA). Following incubation of lymphocytes with antibodies (10 μl mAb/1x10^7 cells) in 100 μl of PBS (pH: 7.4) containing 2 mM EDTA and 0.5% BSA, the cells were passaged through the magnetic cell sorter according to the manufacturer's instruction (Miltenyi Biotech). The purity of the depleted T- or B-cell populations was determined by staining with anti-CD3 or anti-CD45R/B220, or CD19 mAbs (BD PharMingen, San Diego, CA), using flow cytometry. $CD3^+$ cell contamination in the T cell-depleted population was less than 2%, and the percent of $CD45R/B220^+$ B cells remaining in the B cell-depleted population was less than 1%. Unseparated spleen cells, T or B cell-enriched lymphocytes were injected into SCID mice with 100 μg of cartilage PG. In other experiments, spleen cells (2.5 x 10^6

cells/ml) were cultured *in vitro* in 6-well plates with Con A (1.5 μg/ml), or LPS (25 μg/ml) for 3 days, or with cartilage PG (50 μg/ml) for 4 days in DMEM supplemented with 5 % FBS (Hyclone, Logan, UT). Non-lymphoid and dead cells were removed on Lympholyte. Mononuclear cells (>99 % viable after purification) were injected into recipient SCID mice intraperitoneally.

The optimum cell number, doses of cartilage PG, the route of administration (intravenous vs. intraperitoneal) and the optimum interval between injections, including the potential effect and/or necessity of a second PG injection in T cell-depleted transfers, were determined empirically in preliminary experiments. Finally and uniformly, $2x10^7$ unseparated spleen cells, or $1x10^7$ unseparated, B or T cell-depleted lymphocytes or *in vitro*-stimulated lymphocytes were injected intraperitoneally on day 0 and 7 in all transfer experiments presented in this paper. Cells were injected together with 100 μg PG on day 0, but *in vitro*-stimulated cells were transferred without PG or mitogen.

Clinical assessment of arthritis

Donor BALB/c and recipient SCID mice were examined daily for clinical symptoms of arthritis. A standard scoring system [2,12,37], based upon swelling and redness of each paw (ranging from 0 to 4 of each paw, thus resulting in a possible maximum severity score of 16), was used for the assessment of disease severity. In the primary form of PGIA, typically, BALB/c mice developed swelling and redness in one or more limbs 7-14 days after the third injection with PG and adjuvant [1,2,37]. In the transfer system, recipient SCID mice developed a more uniform disease involving nearly all peripheral joints.

Blood samples without or with 10Units of heparin (Sigma)/ 100 μl blood were collected from the retroorbital venous plexus of recipients at different time-points during cell transfer experiments. Limbs were dissected en block, fixed, decalcified, sectioned, and the sections were stained with hematoxylin and eosin for histopathological examination.

Measurements of antigen-specific antibodies and T-cell responses

During transfer experiments, sera were collected from SCID mice twice a week, and both sera and spleen cells were collected at the end of experiments (usually on week 7). PG-specific antibodies were measured by ELISA. Maxisorp immunoplates (Nunc, Denmark) were coated with human or mouse cartilage PGs (0.1 µg protein/well), and the free binding sites were blocked with 1 % fat-free milk in PBS [4,7,8]. Sera were applied at increasing dilutions, and isotypes of PG-specific antibodies were determined using peroxidase-conjugated rat anti-mouse IgG1, or IgG2a and IgG2b (Zymed) and rat anti-mouse IgG3 (Accurate Chemical & Scientific Corp., Westbury, NY) secondary antibodies as described [8,11]. Serum antibody levels were calculated relative to the corresponding mouse IgG isotype standards (all from Zymed). Antigen-specific T-cell responses were measured in quadruplicate samples of spleen cells (3 x 10^5 cells/well) cultured in the presence of 25 µg PG protein/ml. T-cell proliferation was assessed on day 5 by incorporation of ^{3}[H]-thymidine [5,38]. The antigen-specific T-cell response was expressed as stimulation index (SI), a ratio of incorporated ^{3}[H]-thymidine (cpm) in antigen-stimulated cultures relative to cpm measured in non-stimulated cultures [2,5]. Antigen-specific IFNγ, IL-4 and IL-10 productions were measured in cell culture supernatants (3x 106 cell/ml) on day 4 using capture ELISA methods (BD PharMingen) as described [8].

Flow cytometry

Cell surface markers and intracellular cytokines were analyzed by flow cytometry. Biotinylated, FITC-, PE- or Cy-Chrome-labeled mAbs to cell surface markers (CD3, CD4, CD8, CD45R/B220 and CD19) were purchased from BD PharMingen, and cells were stained according to standard protocols [9,38,39]. Cytokine-producing cells were determined using intracellular cytokine staining kit (BD PharMingen) with minor modifications [40]. Splenocytes or freshly isolated lymphocytes from spleens or joint-draining lymph nodes were stimulated with PMA (50 ng/ml) and ionomycin (10

μM) in DMEM supplemented with 10 % FBS for 8 h. To block the subsequent cytokine release, monensin was added to the cells at 3 μM final concentration 1 h after the addition of PMA and ionomycin [41]. Cells were washed in 10% FBS-containing DMEM, and after initial surface antigen staining with biotinylated-anti-CD4 Ab and streptavidin-Cy-Chrome, cells were fixed and permeabilized with saponin according to the manufacturer's instruction. Intracellular cytokines were stained with FITC-anti-IFN-γ and PE-anti-IL-4 mAbs for 30 min at 4°C. Cells were washed and fixed in 2% formalin. Intracellular cytokine levels were measured using a FACScan instrument (Beckton Dickinson, San Jose, CA), and analyzed using CellQuest software (Becton Dickinson).

Statistical analysis

Statistical analysis was performed using SPSS v7.5 (SPSS, Chicago, IL). The Mann-Whitney and Wilcoxon tests were used for intergroup comparisons. Significance was set at $p < 0.05$.

Results

Adoptive transfer of PGIA to SCID mice using spleen cells

To test whether spleen cells from arthritic animals could transfer arthritis into SCID mice, unseparated spleen cells from arthritic donor BALB/c mice were injected into SCID mice. The number of animals, groups and types of transfer experiments are summarized in Table I. When SCID mice were injected simultaneously with spleen cells and PG, or with *in vitro* PG-stimulated spleen cells, all recipient animals developed arthritis by day 41 (Fig. 1A). The onset of arthritis was delayed and the severity was slightly lower in SCID mice that received *in vitro* PG-stimulated splenocytes than in those injected with cells and PG simultaneously (Fig. 1). However, the clinical appearance of the disease (Fig. 2A) and the histopathology of acutely inflamed joints were similar (Fig. 2C), and indistinguishable from those described in primary PGIA [1,2]. In the primary form of PGIA, BALB/c mice developed arthritis 10-15 days after the third PG injection (approximately on weeks 9-10 after the first injection) and full blown arthritis was reached 4-6 weeks later with a

maximum cumulative score of 7.2±2.1. In comparison with other arthritis models, the disease (primary PGIA) was progressive and, once a joint or paw became inflamed, it progressed with remissions and flare-ups until the cartilage was completely destroyed in the affected joint [1,2]. The clinical picture in BALB/c mice was heterogeneous, as different stages of the disease, from acute inflammation to severe ankylosis and joint deformities, were seen in the same animal. In contrast, SCID mice with transferred arthritis developed acute inflammatory arthritis, which reached the maximum cumulative score of 12.3±3.1within a couple of weeks (Fig. 1B) and, more importantly, essentially all peripheral joints became inflamed almost synchronously (not shown). The earliest onset of arthritis (inflammation) was first seen as massive redness and swelling of the interphalangeal, metacarpo- and metatarso-phalangeal joints (Figs. 2A and 2C).

In contrast to SCID mice injected with either *in vivo* or *in vitro* PG-stimulated spleen cells from arthritic donors, mice that received spleen cells alone (without PG), PG antigen alone (without cells), or spleen cells stimulated *in vitro* with LPS and/or Con A (Fig.1), did not show any symptoms of inflammation. SCID mice injected with PG and spleen cells from BALB/c mice that had been immunized with irrelevant antigen (i.e., ovalbumin), did not develop arthritis either (Table I). These results suggest that antigen-specific lymphocyte stimulation is required for adoptive transfer of PGIA to SCID mice.

Autoantigen (mouse PG) promotes adoptive transfer of PGIA

It is strongly believed that cross-reactive immune responses between the immunizing human and mouse (self) cartilage PGs are involved in the induction of PGIA [3,5]. Thus, mouse PG present in the joint is thought to be an ultimate target of autoimmune attacks in PGIA. Although lymphocytes from inflamed joint-draining lymph nodes exhibit a stronger T-cell response to mouse PG than those from spleen [5,38], no direct evidence supports the role of mouse PG as a target molecule. To investigate the role of mouse PG in PGIA, we performed transfer experiments using mouse PG for challenge. When SCID mice received spleen cells from arthritic BALB/c donors together with

mouse PG, arthritis developed in all recipients with an onset time and severity (cumulative score 11.3±2.9) comparable with those in SCID mice co-injected with human PG (Fig. 1). These results implicate mouse PG to be the target of autoimmune reactions by being recognized as autoantigen in arthritis susceptible mice.

Antigen-specific T-cell response, antibody production, and lymphocyte subsets in recipient SCID mice

We have shown that antigen (PG)-specific T-cell response was detected only in arthritic animals (Fig.3). Interestingly, spleen cells from arthritic SCID mice consistently exhibited higher responses to PG than those isolated from arthritic donor BALB/c mice (Fig. 3, first paired column vs. third to fifth paired columns).. Based on the results of in vitro stimulation with mouse and human PGs (Fig. 3), and the results shown on the side diagram of Figure 4, we estimated that the frequency of PG-specific T cells were approximately four times higher in arthritic SCID mice than in arthritic BALB/c donor mice; suggesting an antigen-specific expansion of these cells in the recipient SCID mice. Remarkably, SCID mice that received spleen cells alone, or spleen cells stimulated *in vitro* with either Con A or LPS, completely lost their antigen (PG)-specificity by the end of the 7-week experimental period.

Serum levels of anti-PG antibodies (against both mouse and human PGs) peaked on days 8-16, usually a few days prior to the onset of arthritis (Figs. 4B and 4C). IgG1 levels remained at the plateau, whereas IgG2a and IgG2b concentrations dramatically declined after the onset of arthritis, and this was consistent in all arthritic animals. Similarly to arthritic donor BALB/c mice [3,5,11], IgG1 antibodies to either mouse or human PG were higher than IgG2a antibodies in recipient SCID mice (Fig. 4). The IgG2b anti-PG antibody levels were lower then the IgG2a, and the kinetics of the anti-PG IgG2b were the same shown for IgG2a (Fig. 4). IgG3 isotype anti-PG antibodies were technically under the detection level in arthritic SCID mice. PG-specific antibodies were detected at very low levels, or not at all, in sera of non-arthritic SCID mice (Figs. 4A, 4D and 4E). In

conclusion, these results (Figs. 3 and 4) demonstrated that PG-specific T-cell responses and serum antibodies together appeared to correlate best with the onset and severity of arthritis, as neither T-cell responses (Fig. 3), nor antibodies (Fig. 4) were detected in the negative (non-arthritic) control groups. Lymphocyte subsets within SCID mice did not discriminate between arthritic and non-arthritic status (see marginal bar graphs in Figs. 4A-4C), except for those mice that received Con A- or LPS-stimulated cells (Figs. 4D-4E). The ratios of $CD4^+$ to $CD8^+$ T cells, or $CD3^+$ T cells to B cells, measured prior to the injection (day 0), were similar to the ratios determined at the end of experiments (day 49) (Fig. 4).

The effects of T- and B-cell depletion upon arthritis transfer

We have shown that, while the transfer of arthritis into lethally irradiated and bone marrow-transplanted syngeneic mice required both T and B cells, antibody alone was insufficient to transfer the disease [13]. To elucidate the roles of different immune cells and antibodies in disease development, T or B cell-depleted donor lymphocyte populations were injected into SCID recipients.

SCID mice injected with B cell-depleted donor T lymphocytes developed arthritis without delay (Fig. 5A) or reduction in severity (Fig. 5B). Although the B cell-depleted donor T lymphocytes used for transfer contained only 0.5-1% $CD45R/B220^+$ cells (Fig.6A, panel 1) (also confirmed by anti-CD19-antibody staining), the percentage of B cells reached 12-15% in arthritic SCID mice by the end (day 49) of the transfer experiment (Fig. 6A, panel 2). In contrast, when T cell-depleted donor lymphocytes were transferred with PG (Fig. 6A, panel 3), no arthritis developed in any SCID mice (n=8) over an extended (10-week) observation period (Fig. 5A, open square symbols). The spleens of these non-arthritic recipients contained less than 2% (original ratio) $CD3^+$ T cells. To explore an arthritogenic potential of this small population of T cells, in the next sets of transfer experiments, an extra 100 μg PG antigen (without cells) was injected i.p. into these non-arthritic SCID mice on day 21 (Fig. 5). All SCID mice (n=18) that received T cell-

depleted lymphocytes developed arthritis within 5 to 12 days after the second antigen challenge (Fig. 5A), although the disease was less severe (Fig. 5B) than in animals receiving unseparated (Fig. 1B) or B cell-depleted lymphocytes (Fig. 5B). The ratio of $CD3^+$ T cells reached 7-10% by week 7 (Fig. 6A, panel 4) in SCID mice that were challenged with an additional PG injection without cells (Fig. 5B, closed squares). Remarkably, 92-96% of the recovered $CD3^+$ cells showed $CD4^+$ phenotype (Fig. 6B, panel 4), and T cells harvested from this group of mice exhibited an unusually high PG-specific response (SI: 6.9±1.2).

Intracellular cytokine staining revealed that the Th1/Th2 ratio (calculated as ratios of $IFN\gamma^+$/IL-4^+ $CD4^+$ T cells) among these recovered T cells was high (Fig.7), and frequently reached more than 8/1 in the arthritic SCID mice. Notably, the Th1 cell proportion within the $CD4^+$ population was higher in all arthritic SCIDs than in arthritic donor BALB/c mice, underscoring the possibility of a selective proliferation of PG-specific Th1 cells in the SCID host. In support of this statement (Fig. 7), the CD4+ IFNγ/IL-4 ratio was 8.2±1.0 in arthritic SCID mice vs. 4.3±3.1 in BALB/c donors with primary arthritis, and the CD4+ IFNγ/IL-10 ratio was 6.0±2.2 in SCID and 2.1 ±1.9 in arthritic BALB/c mice.

PG-specific antibody production is T cell-dependent and correlates with arthritis

The most unexpected observation was that anti-PG antibodies were almost undetectable prior to a second PG antigen injection (Fig. 8A) in SCID mice that received T cell-depleted (i.e., B-cell enriched) lymphocytes. The second PG injection (without cells) strongly promoted the survival and expansion of $CD4^+$ T cells (Fig.9A) without promoting $CD8^+$ cell recovery (Fig. 9B). This was followed by a rise in anti-PG antibody production (either to mouse or human PG, both IgG1 and IgG2a isotypes) (Fig.8A). Soon after the appearance of anti-PG antibodies, mice developed arthritis (Fig. 5). These results imply that B cells require $CD4^+$ (most likely Th1) cells for cooperation in arthritis induction. This finding seems to be supported by an even more intriguing observation: antibody production, although restricted to IgG1 isotype, showed a complete “recovery” by day 8-

12 in SCID mice injected with B cell-depleted T cells (Fig. 8B). As described above (Fig. 6A, panel 1), the contaminating CD45R/B220$^+$ cells were consistently less than 1% in B cell-depleted cell suspensions (i.e., the number of CD3$^+$ T cells was at least 100-150 times higher than the number of B lymphocytes). Based on the kinetics of serum anti-PG antibody production in SCID mice injected with B cell-depleted cells (Fig. 8B), one would expect a rapid B-cell recovery in arthritic SCID mice. However, flow cytometric analysis showed no significant increase in B cell numbers (Fig. 9C).

Discussion

PGIA is a genetically restricted autoimmune disease in which Th1 cells play a dominant role[3,10,11,16], and disease susceptibility is linked to both MHC (H-2^d or H-2^K) and non-MHC genetic components [7,8]. The disease can be transferred by co-injection of B and T cells into irradiated syngeneic mice [13,17]. However, the incidence and severity of the disease are lower than in the donor BALB/c mice and the individual differences are relatively high among the recipients. To simplify the transfer system for studying the functions of T and B cells and/or antibodies, we used BALB/c *scid/scid* mice as recipients, taking advantages of both the immune deficiency and the matching genetic background [42]. To date, in all arthritis transfer studies using either animal [31-33] or human donor cells from patients with rheumatoid arthritis [43-45] to SCID mice the donors and recipients differed in both MHC- and non-MHC-related genetic backgrounds. Under these conditions, the transferred autoimmune disease could be complicated by alloantigen recognition. Therefore, not surprisingly, when lymphoid cells from arthritic DBA/1 x B10.Q F1 mice were transferred into BALB/c-SCID mice, relapsing arthritis was observed over a 250-day period [46], which might be the results of graft versus host disease superimposed on arthritis.

To address the role of T- and B-cell cooperation and the functions of T-cell subsets in arthritis induction, we injected genetically matched SCID mice with different subsets of arthritic BALB/c-derived lymphocytes. In agreement with our previous observations [13], the PG-specificity of

transferred T or B cells, which was initially present, disappeared after a few weeks without antigen stimulation (Fig. 3), and non-specific stimulation (with Con A and/or LPS) of either T or B cells derived from arthritic BALB/c donors failed to transfer the disease (Fig. 1). Therefore, antigen-specific T-cell activation seems to be required for successful transfer of arthritis. This was, however, expected, as all autoimmune disease transfer experiments required an antigen-specific stimulation of donor cells applied either before (*in vitro* stimulation) or at the time (co-injection) of cell transfer [13,31,47]. A single dose (100 μg) of PG co-injected with spleen cells into SCID mice (a dose of PG without adjuvant, which otherwise did not induce detectable immune responses in BALB/c mice) [1,3,6], or *in vitro* stimulation of spleen cells with PG, was absolutely crucial for activation of primed donor lymphocytes and the transfer of a clinically severe disease (Fig. 1). In terms of the source of PG, there were no or little differences in antigen-induced T-cell stimulation in the donor arthritic BALB/c mice, and T-cell recovery and arthritis induction was successful when either human or mouse PG was used. We can postulate that the amount of PG or PG fragments, released during the normal turnover of cartilage in SCID mice at the time of transfer, might be insufficient to support an antigen-specific T-cell proliferation or lymphocyte recruitment to the synovial joints. As the mouse PG was as effective as the human in arthritis induction, this was a strong evidence that mouse PG was a target immunogen *in vivo*, and was critically involved in the progression of autoimmunity and local inflammatory reactions in PGIA.

Restoration of lymphocyte balance can be accomplished using either normal (naïve) or antigen-specific T-cell populations in severe T-cell deficiency syndromes which develop following whole body irradiation or chemotherapy, or in a severe combined immunodeficiency disorder (called 'bare lymphocyte syndrome') [48,49]. In normal T-cell development, as a result of positive and negative selection, the post-thymic repertoire of mature T cells consists of lymphocytes reactive with MHC-associated foreign peptides, but tolerant of self-peptides [21,24]. The long-term survival of mature $CD4^+$ T cells requires continuous, albeit covert, signals through the TCR. These covert (weak) signals that are transmitted by self-MHC/self-peptides [25] acquire a critically important

function when the size of the T-cell pool is dramatically reduced (e.g., after chemotherapy). Similar conditions occur when relatively low numbers of T cells ($1x10^7$) are transferred into syngeneic nude or SCID rodents leading to a massive expansion of donor cells and restoration of T-cell homeostasis [18,19].

A number of studies investigated the function of T and B cells in the development of various autoimmune diseases using transfer of lymphocyte subsets [32,50,51]. We did not expect that the very low proportion of contaminating T or B lymphocytes, within this population even much fewer antigen-specific cells, could modify the outcome of disease transfer. Toward the end of these experiments, it appeared that either T or B cell-depleted lymphocytes from arthritic donors could transfer arthritis into SCID mice, albeit B cells seemed to be less critical, as arthritis was transferred without delay in their absence (Fig. 5).

We found remarkable changes in the recovery of "contaminating" cells during the course of arthritis adoptively transferred to syngeneic SCID mice. It became evident that the very small number of the antigen-specific T or B cells in the 0.5-2.0% transferred contaminating cell population served as precursors of an expanding immune system in recipient SCID mice, but only in the presence of relevant autoantigen (mouse PG). PG-specific B-cell recovery was faster than T-cell recovery, and the T-cell recovery was limited to the $CD4^+$ lymphocytes of primarily Th1 phenotype. Although the initial overall 1-2% T-cell content in T cell-depleted populations did not change for weeks (Fig. 9), these precursor cells gave rise to increasing numbers of antigen-specific Th1 cells (Fig. 6B4), especially after the second PG challenge on day 21 (Figs. 5 and 9A). Concomitantly with the expansion (rescue) of antigen (PG)-specific Th1 cells, B cells were able to differentiate to plasma cells [52] and began to produce PG-specific antibodies (Fig. 8A); this was followed by the development of arthritis in SCID mice. In this mechanism, i.e., arthritis induction in SCID mice with transferred cells, antigen-induced B-cell stimulation and proliferation, the PG-specific antibody production and the antigen presentation by B cells might equally be involved. In previous experiments, we found that B cells from naïve (none immune) BALB/c mice presented PG

antigen several-fold more effectively than e.g., peritoneal macrophages, and B cells from PG-immunized mice proved to be at least 1,000 times better APCs than macrophages or adherent mononuclear spleen cells from the same immunized animal [5]. Therefore, it seems to be very likely that a small number of 'contaminating' B cells in the B cell-depleted cell transfer system were capable of presenting PG antigen to T cells much more effectively than the host's APCs. A second injection of PG antigen (Fig. 5) might amplify this B-cell function accompanied with a concomitant phenomenon: dramatically increasing the PG-specific antibody production (Fig. 8A). While this remains an unsolved question and requires additional studies focusing on the antigen presenting function of B cells, the major hallmark of this observation is that antigen (PG)-specific B cells were unable to produce (auto)antibodies nor induce arthritis or be involved in arthritis induction, in the absence of antigen-specific CD4+ cells.

Expansion of mouse PG-specific syngeneic $CD4^+$ Th1 cells in SCID mice is consistent with the results of other studies that placed the focus on the restoration of the T-cell homeostasis [21-25]. The recovery of pathogenic $CD4^+$ Th1 cells from an extremely small population in an autoimmune transfer system, however, is a novel and unique observation within this study, as restoration of antigen-specific T cells was not monitored during the development of the autoimmune disease in any of the previous transfer experiments. The most unexpected observations were that B cells, when T cells were depleted, did not produce PG-specific antibodies unless SCID mice received a second PG injection (Fig. 8A) and, reciprocally, high levels of serum anti-PG antibodies were found in SCID mice with transferred arthritis that received B cell-depleted T lymphocytes (Fig. 8B).

The critical role of T cells in arthritic processes is unquestionable, although the exact functions of various subsets have not been elucidated. $CD8^+$ cells were generally defined as suppressor cells in autoimmune diseases [33,53], and the ratio of $CD8^+/CD4^+$cells was low at the onset of both CIA [53] and PGIA [38]. $CD8^+$ cells, however, were proposed to function as effector cells in experimental allergic encephalomyelitis [51]. In T cell-depleted cell transfer experiments, antigen-specific T-cell

proliferation resulted in $CD4^+$ lymphocyte recovery without restoration of $CD8^+$ lymphocytes. This might be explained by the observation that $CD4^+$ cells could outlive $CD8^+$ cells in long-term SCID transfer experiments [36]. However, when we injected B cell-depleted (T cell-enriched) mixed ($CD4^+$ and $CD8^+$) T cell populations, or unseparated lymphocytes into SCID mice, the $CD4^+/CD8^+$ ratios did not change for up to 7 weeks (Fig. 4A). As the total number of $CD8^+$ cells did not decrease during the experimental period, (i.e., no selective $CD8^+$ cell death was observed)(Fig. 9B), the expansion of $CD4^+$ cells must be due to a marked increase in the antigen-specific $CD4^+$ cell population. Remarkably, these SCID mice showed high proportion of $CD4^+$ Th1 cells, and SCID mice injected with T cell-depleted lymphocytes exhibited unusually high Th1/Th2 ratios (Fig. 7). These findings clearly indicate that a very low number of PG-responder $CD4^+$ T cells in SCID mice, under the 'pressure' of covert signals from self-MHC/self peptide supported with overt signals from co-injection with PG, underwent positive selection and subsequent expansion recovering the "arthritogenic" phenotype of the immune system in syngeneic SCID-BALB/c background.

Abbreviations used: CIA, collagen-induced arthritis; Con A, concanavalin A; CTLL, cytotoxic T lymphocyte assay; hPG, human cartilage proteoglycan; LPS, lipopolysaccharide; PG, cartilage proteoglycan (aggrecan), PGIA, mPG, mouse cartilage PG; proteoglycan-induced arthritis; SI, stimulation index; Th, T helper

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Table I. Incidence of arthritis in adoptively transferred SCID mice

[1]Donor cells from BALB/c mice	Donor cells depleted of	*In vitro* stimulation	Co-injection of donor cells with	Incidence of arthritis (arthritic/total)	Maximum cumulative score in SCID
Naïve (none immune)	N/A	N/A	hPG	0/10	N/A
[2]OVA-immunized	N/A	N/A	hPG	0/10	N/A
Arthritic	N/A	N/A	N/A	0/24	N/A
Arthritic	N/A	N/A	hPG	36/36	12.1±1.8
Arthritic	N/A	hPG	N/A	30/30	9.7±2.7
Arthritic	N/A	N/A	mPG	16/16	11.3±2.9
Arthritic	N/A	Con A	N/A	0/20	N/A
Arthritic	N/A	LPS	N/A	0/21	N/A
Arthritic	N/A	Con A+LPS	N/A	0/15	N/A
Arthritic	B cells	N/A	hPG	21/21	14.2±2.8
Arthritic	T cells	N/A	hPG (once)	0/8	N/A
Arthritic	T cells	N/A	hPG (twice)	18/18	10.0±3.1

[1]The cumulative arthritis score of donor BALB/c mice was 7.2±2.1 (n=32), and the antigen (PG)-specific T cell proliferation (SI = mean ± S.D) of arthritic donor BALB/c mice was 2.2±0.3 to human and 2.0 ± 0.7 to mouse PG (Figure 3, first paired columns).

[2]OVA-immunized mice exhibited a stimulation index (SI) of 6.8 ±2.1, but no OVA-specific T cells were recovered from SCID mice on week 7.

N/A: none applicable; hPG: human cartilage proteoglycan; mPG: mouse cartilage proteoglycan; OVA: ovalbumin; Con A: concanavalin A; LPS: lipopolysaccharide

Animals used in preliminary experiments are not incorporated in Table I.

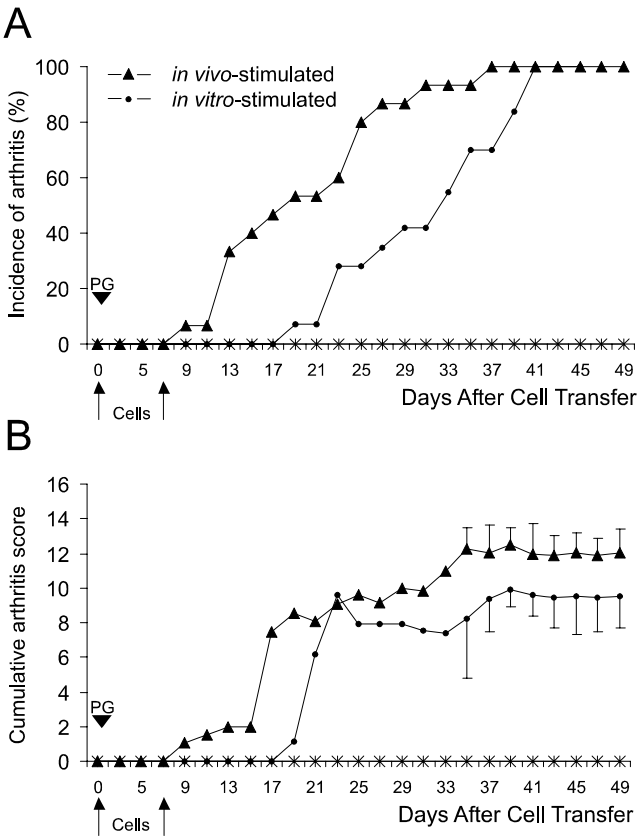

Figure 1. Incidence (A) and severity (B) of adoptively transferred PGIA in SCID mice. Freshly isolated spleen cells from arthritic BALB/c mice along with 100 µg of PG protein or *in vitro* PG-stimulated spleen cells ($1x10^7$ cells/recipient) were injected i.p. into SCID mice on days 1 and 7 (arrows). Arthritis developed in all SCID mice (100% incidence) that received cells stimulated with PG either *in vivo* (-▲-) or *in vitro* (-●-)(**A**). The maximum severity scores of arthritis in SCID recipients (12.4±0.9 and 10.1±0.9) were comparable with those in donor BALB/c mice (10.2±2.5) (**B**). Cells from the same donor mice, injected alone (without PG) or after culture *in vitro* with Con A or LPS, or when these *in vitro*-stimulated cells were mixed, did not provoke joint inflammation in recipient SCID mice. Data from these negative groups are combined and indicated by a shared symbol -✱-. Figure summarizes results of three independent transfer experiments performed on 12 SCID mice in each positive (arthritic), and 8-10 animals in each negative control group. Arrowhead indicates the co-injection of PG in the standard (-▲-) transfer group.

Figure 2. Joint inflammation and paw swelling in SCID mice with adoptively transferred PGIA. All mice that received PG-stimulated spleen cells developed arthritis between days 10 and 40 after the first cell transfer (Fig. 1A). (**A**) An acutely inflamed hind paw (less than two days after onset) is shown with the contralateral (left) paw with initial swelling on one digit only. Inflammation (swelling and redness) was usually first localized in small (interphalangeal and metatarso-phalangeal) joints of paws. Inflammation occasionally caused severe blood congestion and cyanosis (seen as dark phalanges of the digits on the right hind paw). In non-affected groups, all SCID mice exhibited normal joint structures (**B**), whereas arthritic mice showed robust inflammatory cell infiltration accompanied by cartilage erosion (**C**). Histologically, joint inflammation was similar to that described in BALB/c mice with primary PGIA [1;2;13]. For comparison, histology sections from corresponding areas of metatarso-phalangeal joints of control (negative) and arthritic SCID mice were selected for panels B and C.

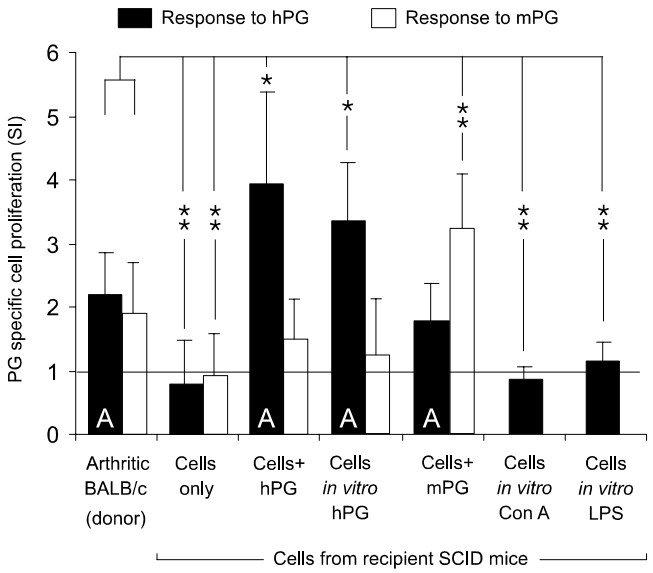

Figure 3. Antigen (PG)-specific T-cell proliferation in SCID mice with adoptively transferred PGIA. Antigen-specific T-cell stimulation in the presence of human PG (hPG; closed columns) and mouse PG (mPG; open columns) was determined by using spleen cells from arthritic donor BALB/c (prior to the transfer) or recipient SCID mice (at the end of experiment on day 49). The PG-specific T cell proliferation in arthritic SCID recipients (except those co-injected with; mPG) was significantly higher than that measured in PG-stimulated spleen cell cultures of arthritic donor BALB/c mice. Arthritic groups are indicated with "A" inside the column. Levels of significances (*$p<0.05$; **$p<0.01$) are shown comparing hPG- or mPG-induced *in vitro* T cell proliferation in arthritic donor BALB/c mice with the response (mean SI ± SD) measured in spleen cell cultures of recipient SCID mice ($n\geq10$ per group).

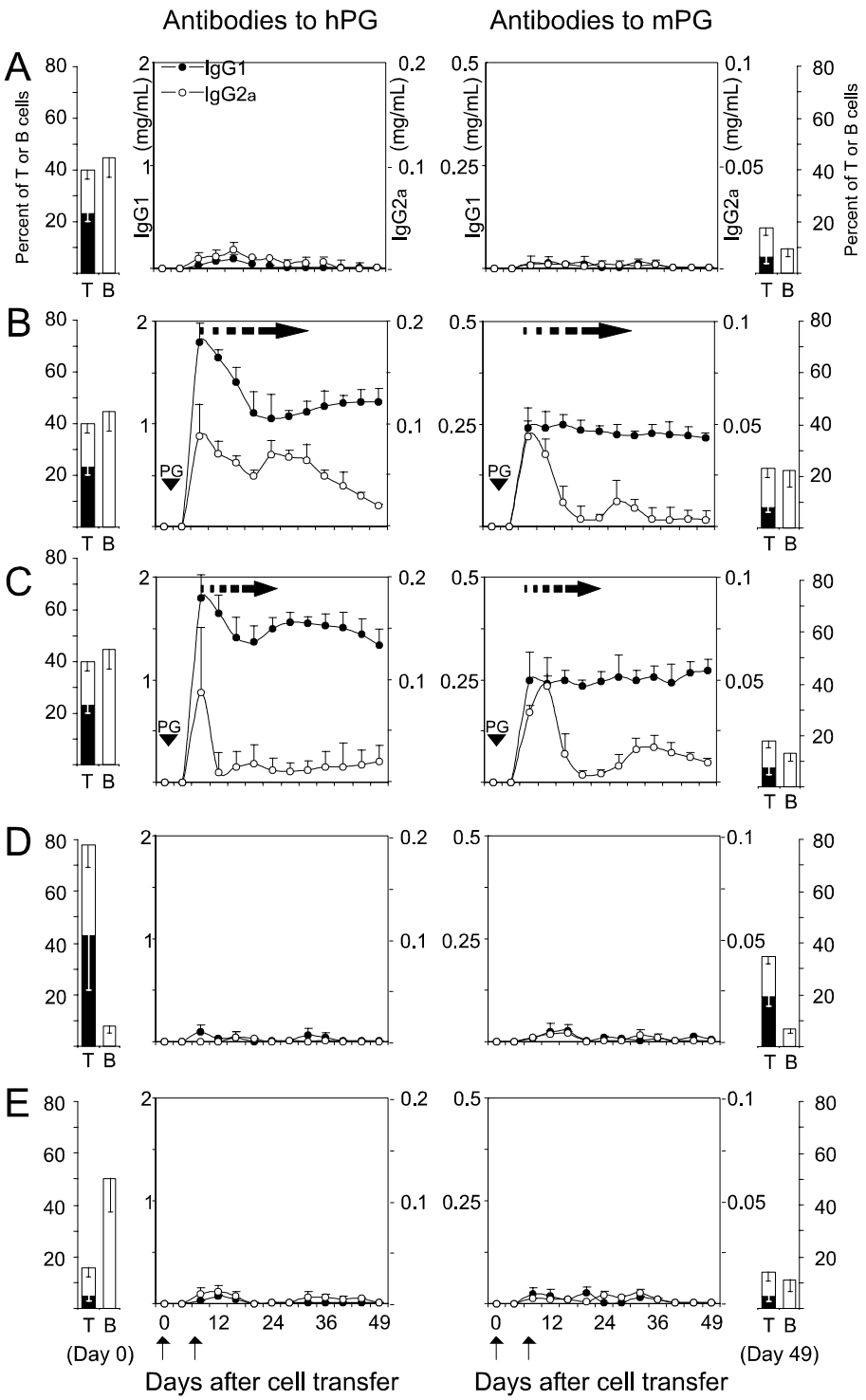

Figure 4. Serum anti-PG antibody levels in SCID mice throughout the transfer experiments, and the percent and ratios of lymphocyte subsets prior to (day 0) and at the end (day 49) of the transfer experiments. Antibodies to human and mouse PGs (both IgG1 and IgG2a) are shown in SCID mice injected with (**A**) spleen cells without PG antigen, (**B**) spleen cells co-injected with

human PG (hPG) or (**C**) mouse PG (mPG), (**D**) spleen cells stimulated *in vitro* with Con A, or (**E**) LPS. *In vitro*-stimulated cells were separated on Lympholyte prior to the i.p. injection. Antibody levels against the immunizing human (hPG) or mouse PG (mPG) were measured by ELISA. Serum levels of IgG1 (-●-) and IgG2a (-○-) isotypes (in mg/ml) are indicated on the left and right *Y*-axes of each panel, respectively. The percent of transferred CD3+ T and CD45R/B220+ B lymphocytes, are shown as bar graphs at both sides of each panel. The left-side bars show the percent of cells prior to transfer, whereas columns at the right-side show cell percentage measured at the end of the transfer experiments. Within the T-cell columns, the open area represents the percent of CD4+ cells, and the closed bars indicate the percent of CD8+ T cells. The proportions of lymphoid subsets after transfer were determined for each animal, and the results represent the means ± SD (n=10-12 animals). Horizontal broken line-arrows on panels B and C illustrate the time frame from the earliest onset to the time point when all mice developed arthritis. Arrows at bottom of the panel show the time of cell transfer (for all groups) whereas arrowheads on panels B and C indicate PG co-administration.

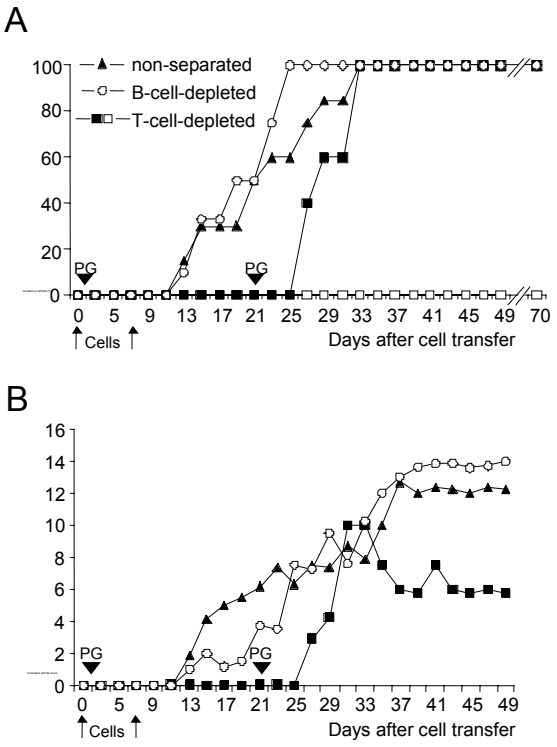

Figure 5. Incidence (**A**) and severity (**B**) of adoptively transferred arthritis in SCID mice using unseparated lymphocytes, or T or B lymphocyte-depleted spleen cells. Experimental conditions were the same as described in Figure 1 (arrows indicate the two cell transfers, and arrowheads the injection of 100 μg human PG) except that Lympholyte-purified spleen cells were used as unseparated lymphocyte populations (-▲-), or these cells were depleted of T (-□-, -■-) or B cells (-○-) prior to the transfer. SCID mice that received T cell-depleted lymphocytes remained non-arthritic (-□-) by week 10 of experiment (n=8). In subsequent experiments, these negative mice were challenged with a second intraperitoneal PG injection without cells (n=18)(-■-) on day 21 (arrowhead). All these mice developed arthritis within a 5-12-day period with an arthritis score of 5.9±1.8 on day 49 after the first cell transfer.

Figure 6. Two-color FACScan analysis of donor BALB/c cells prior to transfer and after retrieval from spleens of SCID mice seven weeks later. Spleen cells were Lympholyte-purified, depleted of either T or B cells, and then injected into SCID mice at days 0 and 7 (first injection was given with 100 μg of PG antigen as shown in Figure 5). Results of FACS analysis in a positive control group which received non-separated lymphocytes with PG antigen, were highly comparable to those that received spleen cells (shown as bar graphs in Figure 4B). Although the B or T cell-depleted lymphocytes contained only 0.5-2 % contaminating B or T cells (flow charts A1 and A3), a selective recovery of these cells was observed in spleens of SCID mice 7 weeks after the initial transfer (flow charts A2 and A4). In SCID mice receiving B cell-

depleted lymphocytes, approximately equal number of $CD4^+$ and $CD8^+$ cells were recovered (flow charts B1 and B2), whereas in mice receiving T cell-depleted spleen cells and then an additional antigen injection on day 21 (group -■- in Fig.5) only $CD4^+$ T cells, but not $CD8^+$ cells, emerged (flow chart B4). Cell debris was gated out based on forward and side scatter properties.

Figure 7. Intracellular cytokine production of $CD4^+$ cells harvested from arthritic donor BALB/c and recipient SCID mice. Three-color flow cytometry charts represent staining for intracellular cytokines (IFN-γ, and IL-4) against cell surface CD4. In general, the Th1 ($CD4^{hi}$-IFN-γ^{hi}) population was abundant in adoptively transferred arthritic SCID mice, and the Th1/Th2 ratio (numbers shown in parentheses above the right top corner of the right-side

panels) was the highest in arthritic SCID mice receiving T cell-depleted donor cells (panels 7-8) and a second PG injection (shown in Fig. 5).

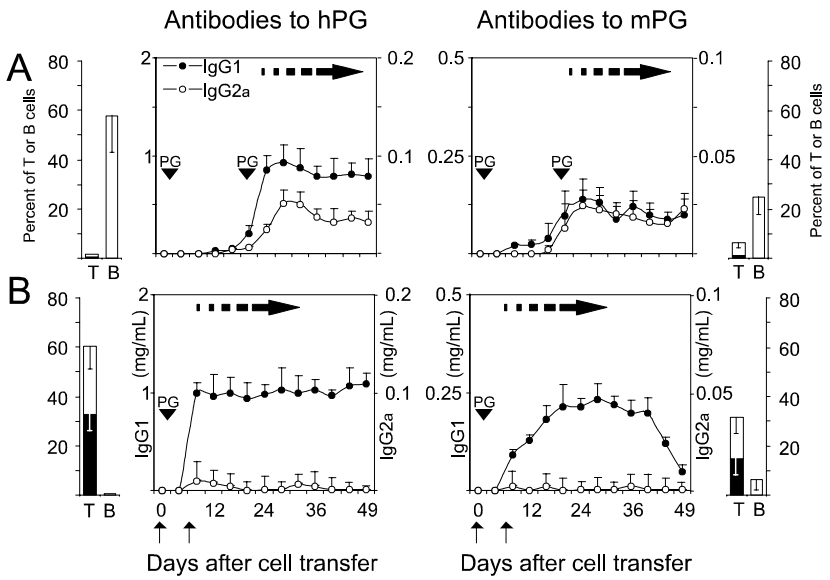

Figure 8. PG-specific serum antibody levels in SCID mice injected with T cell-depleted (**A**) or B cell-depleted (**B**) lymphocytes. T cell-depleted B cells were unable to produce antibodies in transfer system, possibly due to the extremely low number of $CD4^+$ cells (see Fig. 9). This 'blockade', however, was broken after the second injection of PG (on day 21). The second dose of PG induced a highly selective PG-specific $CD4^+$ T-cell proliferation (Figs. 6B, panel 4), and led to the development of arthritis in all SCID mice (Fig. 5) soon after PG-specific antibodies appeared in serum (panel A). In contrast, a very small portion of contaminating B cells (less than 0.5-1.0% $CD45R/B220^+$ cells) present in the transferred cell population, produced significant amounts of PG-specific antibodies, although these antibodies represented only IgG1 isotype (Panel B). In either case, arthritis developed only when antibodies (at least IgG1) and $CD4^+$ cell numbers reached threshold levels. Panels summarize the results of four independent experiments with 12 mice in each group.

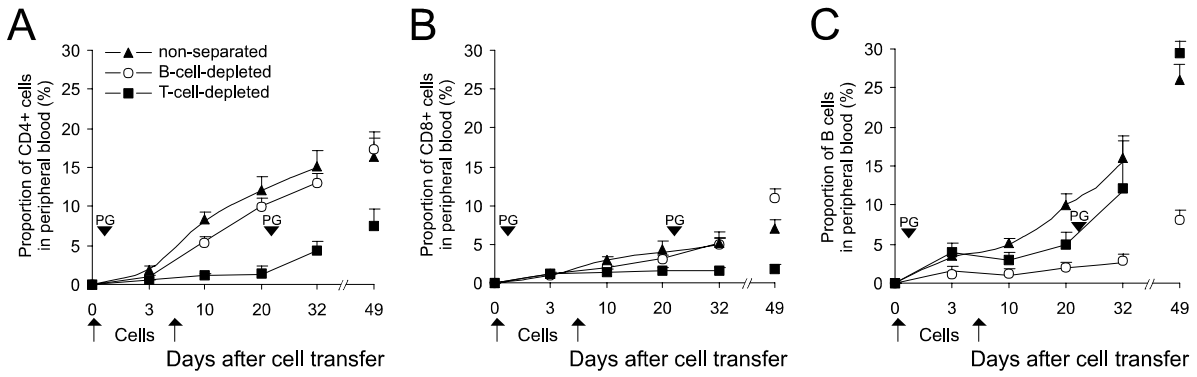

Figure 9. Kinetics of lymphocyte recovery during the development of arthritis in SCID mice. Graphs summarize the flow cytometry data for the recovery of $CD4^+$ (**A**), $CD8^+$ (**B**), and B cells (**C**) determined in peripheral blood of SCID mice up to day 32, and then in spleen on day 49. SCID mice received unseparated lymphocytes from arthritic donor BALB/c mice with 100 µg of human PG (-▲-), B cell-depleted cells and PG (-○-), or T cell-depleted cells and PG (-■-) . Arrows indicate the days of cell transfers and arrowheads the injection of PG (twice only for group -■-). A massive and rapid lymphocyte recovery was observed for all cell types (A-C) in the positive control group (-▲-), whereas the recovery of depleted cell populations in peripheral blood, and then in spleen, was relatively slow.

Chapter 2

Continuous nasal administration of antigen is critical to maintain tolerance in adoptively transferred autoimmune arthritis in SCID mice

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Summary

Mucosal tolerance is a natural mechanism that prevents immunological reactions to antigens by altering the activity of immune cells of pathogenic clones without modulating the entire immune system. This 'natural immune suppression' can be exploited when antigen(s) of the target organ in an autoimmune disease is used for mucosal treatment. Being inspired by the experimental results in animal models, clinical trials using type II collagen for mucosal treatment have been conducted in rheumatoid arthritis. High-density proteoglycan (aggrecan) is another major macromolecular component in articular cartilage, and may be a candidate autoantigen for provoking immune reactions in patients with rheumatoid arthritis. Indeed, like type II collagen, systemic immunization of genetically susceptible mice with proteoglycan (PG) aggrecan induces progressive autoimmune polyarthritis. Here, we investigated whether intranasally applied PG can be effective in suppressing PG-induced arthritis (PGIA) in BALB/c mice. We found that nasal administration of 100 μg PG exerted a strong suppressive effect on both the incidence and severity of the disease, most likely by reducing responsiveness towards the immunizing PG antigen. When we transferred PGIA into genetically matched but immunodeficient SCID mice, we were able to establish a tolerized state, but only if the recipient SCID mice received lymphocytes from tolerized animals and intranasal treatment with PG was continued. Without nasally administered antigen, the transferred anergic cells recovered and arthritis rapidly developed in a severe form. Intranasal PG treatment of recipient SCID mice was ineffective when cells from non-tolerized arthritic donors were transferred, in which case the regular weekly "tolerizing" dose of PG made the disease worse. Our results suggest that mucosal treatment in an already existing disease may result in paradoxical outcomes.

Introduction

One of the most appealing therapeutic approaches in autoimmune diseases is an antigen-specific immune suppression which does not modulate the immune system as a whole, but wherein the activity of antigen-specific T cell clones is significantly altered. Mucosal tolerance is a physiological phenomenon that prevents harmful immunological reactions to 'non-dangerous' antigens such as protein components of food or airborne pollen and dust. In a number of animal models of autoimmune diseases such as diabetes [1], myasthenia gravis [2,3], autoimmune uveoretinitis [4], experimental allergic encephalomyelitis [5,6] or collagen-induced arthritis [7-9], mucosal tolerance has been proven to be effective in suppressing the disease. Based on the success of these animal experiments, several clinical trials have been initiated [10,11], occasionally with promising results [12]. While oral administration of an antigen is the most easily applied treatment, and may down-regulate the disease, relatively large doses are necessary due to gastric and intestinal degradation of the antigen. Delivery of a protein via the respiratory mucosal route has advantages as the antigen is less likely degraded, therefore a significantly smaller quantity may achieve the same efficacy as the dose used for oral tolerance [13].

Systemic immunization with high-density cartilage proteoglycan (PG) aggrecan induces progressive polyarthritis in susceptible mouse strains [14-18]. PG-induced arthritis (PGIA) shows many similarities to human rheumatoid arthritis, such as repeated inflammatory episodes, formation of synovial pannus, which erodes articular cartilage and bone, and female preponderance [14,15,17]. The mechanism of the disease is based upon autoimmune reactions that develop against the mouse (self) PG during systemic immunization with human PG.

To determine whether nasal tolerance can be induced in PGIA, BALB/c mice immunized with PG were treated nasally with human PG throughout the experiment, and incidence and severity of arthritis were monitored. Next, lymphocytes from arthritic donor BALB/c mice (nasally-tolerized or non-tolerized) were adoptively transferred into MHC-matched (H-2^d) severe combined immunodeficient (SCID) [19] mice. This transfer system allowed us to gain

insight into the mechanisms of nasal suppression, and determine the requirements for establishing and maintaining tolerance in mice with PGIA.

Materials and Methods

Antigens, animals, and immunization

High-density PG (aggrecan) was purified from human cartilage by CsCl gradient centrifugation, and depleted of glycosaminoglycan side chains as described [17]. Female BALB/c mice (National Cancer Institute, NCI, Friedrich, MD) were immunized intraperitoneally with cartilage PG [17]. The first antigen injection (100 μg protein) was given in complete Freund's adjuvant, and the same doses of antigen were injected as second and third boosts in incomplete Freund's adjuvant on day 21 and 42. In nasally treated groups of mice the antigen was given intranasally 10 days prior to the first injection, repeated daily for four consecutive days and then once a week during the immunization. Mice received various doses (10 μg and 100 μg) of PG, 100 μg ovalbumin (OVA; Sigma, St. Louis, MO) in 25 μl PBS (phosphate buffered saline, pH 7.4), or 25 μl PBS alone equally distributed among the two nostrils.

The intranasal treatment regimen was optimized in preliminary experiments. The most "optimal" intranasal dose of PG (100 μg) and treatment protocol (intranasal tolerization prior to the immunization, necessary intervals between intranasal treatments) were determined using 5-6 mice in each group. Due to the large number of combinations and variability of results, animals of these preliminary results are not included in this paper. Typically, we used 12 mice in the first experiment and then the appropriate experiment was repeated once or twice using 15 animals in each group.

Female SCID mice with BALB/c background (NCI/NCrC.B-17-*scid/scid*), aged 8-12 weeks, were purchased from NCI and maintained under germ free conditions at our Comparative Research Center. All animal experiments were approved by the Institutional

Animal Care and Use Committee. In order to exclude animals with 'leaky' immune systems, SCID mice were tested for serum immunoglobulins using enzyme-linked immunosorbent assay (ELISA) prior to the experiments [20].

Cell isolation and transfer of arthritis into SCID mice

Single-cell suspensions were prepared in Dulbecco's modified Eagle's medium (Sigma) from spleens of arthritic BALB/c mice by mechanical dissociation. As described above for nasal treatment, the optimal cell numbers for transfer, the route of cell injection (intraperitoneal vs. intravenous), the dose of cartilage PG administered along with cells, and intervals between injections were determined in preliminary experiments. In all transfer experiments $1x10^7$ spleen cells were injected intraperitoneally along with 100 μg of PG into SCID mice. Another group of SCID recipients, in addition to the intraperitoneal injection, also received a weekly dose of 100 μg PG intranasally. Cell transfer was repeated on day 7, whereas the nasal administration of PG antigen was continued (once a week) throughout the entire experiment. Twelve SCID mice were used in each transfer group and experiments were repeated once with 15 mice.

Clinical assessment of arthritis

Immunized BALB/c mice were examined twice a week, and recipient SCID mice daily. The appearance of the first clinical symptoms (swelling and redness) was recorded as the time of onset of arthritis. Joint swelling was scored (from 0 to 4 of each paw) and expressed as the acute arthritis score, which is a summarized score for the four paws of one animal at a given time point [17,21,22]. Typically, in the primary form of PGIA, BALB/c mice developed swelling and redness in one or more limbs 7-14 days after the third injection of PG [14,17,22]. In the transfer system, arthritic SCID mice developed a more "uniform" disease with the involvement of essentially all peripheral joints, beginning 1-2 days after the second cell transfer. Mice were

sacrificed, and limbs were dissected, fixed in neutral formalin, decalcified, and embedded in paraffin. Sections were stained with hematoxylin and eosin for histopathological analysis.

Measurements of PG-specific antibodies, T-cell responses and cytokine production

At the end of experiments, blood samples were collected from the retrobulbar venous plexus. Maxisorp immunoplates (Nalgene Nunc International, Denmark) were coated with human or mouse cartilage PGs (0.1 μg protein/100 μl/well) for ELISA as described [18,23,24]. Sera were applied at increasing dilutions from 1:12,500 to 1:62,500, and the titer of isotypes of PG-specific antibodies were determined using peroxidase-conjugated rat anti-mouse IgG1, IgG2a or IgG2b (Zymed, San Francisco, CA), or rat anti-mouse IgG3 (Accurate Chemical & Scientific Corp., Westbury, NY) secondary antibodies as described [24-26]. The optimal dilutions of isotype-specific second antibodies were determined in preliminary experiments. Serum antibody levels were normalized to mouse isotype standards. The control immunoglobulin isotypes were purified from irrelevant (non-PG specific) monoclonal antibody-containing ascites fluids, and immobilized on the microplate's surface at linear concentrations ranging from 0.2 to 200 ng/well.

Antigen-specific T-cell proliferation was measured in quadruplicate samples of spleen cells ($3x10^5$ cells/well) in the presence of 25 μg human PG protein/ml. Interleukin (IL)-2 secretion was determined by IL-2 bioassay using CTLL-2 cells pulsed with supernatants from 24 hour-cultured spleen cells. Proliferation of CTLL-2 cells and antigen-specific T-cell proliferation were assessed on days 2 and 5, respectively, by measuring incorporation of ^{3}[H]-thymidine [16]. The antigen-specific response was expressed as Δcpm. Antigen (PG)-specific production of interferon-γ (IFN-γ), IL-10, IL-4 and transforming growth factor-β (TGF-β) were determined in media harvested from antigen (PG)-stimulated spleen cells ($2.5x10^6$ cells/ml) on day 4. To detect TGF-β production, spleen cells were cultured in serum free HL-1 medium (Biowhittaker, Walkersville, MD). Cytokine concentrations were measured using capture

ELISA from R&D Systems (Minneapolis, MN). TGF-β was measured after acid treatment of samples by using TGF-β ELISA kit (Promega, Madison, WI) as described [27].

Flow cytometry

The percent of $CD4^+CD25^+$ T cells was determined by staining spleen cells with FITC-labeled anti-CD4 antibody, and a biotin-labeled anti-CD25 antibody followed by CyChrome-labeled streptavidin (BD PharMingen, San Diego, CA), and analyzing double-labeled fluorescent cells on a FacScan flow cytometer (Beckton Dickinson, San Jose, CA). For intracellular CTLA-4 (cytotoxic T lymphocyte antigen-4) analysis, spleen cells were first stained with fluorescence-labeled antibodies, and then fixed, permeabilized using Cytofix/Cytoperm kit (BD PharMingen) and stained with PE-labeled anti-CTLA-4. For flow cytometry, we usually used cells from 3-5 animals of each group.

Statistical analysis

Statistical analysis of data was performed using SPSS v7.5 software package (SPSS, Chicago, IL). The Mann-Whitney and Wilcoxon tests were used for intergroup comparisons. Significance was set at $p < 0.05$.

Results

Nasal tolerance in BALB/c mice with PGIA

BALB/c mice were treated intranasally with cartilage PG (10 or 100 μg), OVA (100 μg), or vehicle (PBS) for four consecutive days beginning 10 days before PG immunization. To maintain tolerance, mice were given the same doses of antigen or PBS once a week throughout the entire experimental period. Nasally PBS-treated control BALB/c mice developed arthritis with 100% incidence and a cumulative arthritis score of 6.8 ± 1.5 after the third intraperitoneal injection of PG (Fig. 1). In contrast, BALB/c mice intranasally-tolerized with 100 μg of PG

developed arthritis much later with reduced incidence (30%) and severity (arthritis score: 2.5±1.1)(Fig.1). The intergroup variance was less than 10% between experimental groups. Higher amounts of intranasally applied antigen (e.g., 200 μg) had no additional suppressive effect on the disease, whereas low doses (1-10 μg) seemed to be insufficient to generate tolerance (data not shown).

The optimum time frame of the intranasal antigen application was tested using 100 μg dose of PG. When the first intranasal treatment with PG was applied at the time of the first or after the second intraperitoneal injection of PG, disease severity was less affected (cumulative scores: 5.5±1.1 (n=12) and 6.3±1.9 (n=12), respectively) and the incidence was reduced by only 30% in both groups (data not shown). Mice (n=12) that received intranasal PG first only after the third intraperitoneal PG injection developed anaphylactic reaction resulting in over 50% mortality. Mice that survived exhibited a poor physical condition and delayed onset of arthritis.

Cellular and humoral immune responses to PG in nasally tolerized mice

PG-immunized BALB/c mice tolerized intranasally with PG had significantly reduced T-cell responses measured *in vitro* either as antigen specific proliferation or IL-2 production (Fig.2). While nasal treatment with a reduced amount of PG (10 μg) appeared to be slightly suppressive on *in vitro* T-cell responses (Fig. 2), the *in vivo* tolerizing effect was very modest (results not shown).

Serum antibody levels to either self (mouse) or immunizing (human) PG showed strong correlation with the clinical appearance of the disease (Fig.3). Both IgG1 and IgG2a isotypes against both mouse and human PG were significantly suppressed in mice that received 100 μg PG intranasally (Fig.3). Serum IgG2b levels were highly comparable to IgG2a, but the PG-specific IgG2b level was only half to two-third of the serum IgG2a levels. IgG3 isotype to either mouse or human PG was hardly detectable, usually 500-1,000 times lower than the IgG1

in the same animal (not shown). Although the BALB/c strain has a genetic predisposition to Th2 type responses [28], PGIA causes a shift to Th1 type response, i.e., an increased production of IgG2a isotype prior to the onset of arthritis [24,29]. However, in the nasally tolerized group, both IgG1 and IgG2a (and IgG2b) isotypes of anti-PG antibodies were suppressed (Fig. 3).

$CD4^+CD25^+$ cells and T-cell cytokines have limited functions in PG-specific tolerance

To investigate whether suppressive $CD4^+CD25^+$ cells play a role in nasal tolerance to PGIA we compared this cell population in control and nasally tolerized mice. We found no difference in the percent of $CD4^+CD25^+$ cells between the two groups, and the intracellular CTLA-4 expression was also identical (Fig. 4). There was no difference in antigen-induced cytokine production between the tolerized and non-tolerized groups, with respect to levels of IL-4, IL-10, IFN-γ and TGF-β (data not shown).

Transfer of arthritis and nasal tolerance into SCID mice

In agreement with our previous observations [30,31], only PG-stimulated spleen cells from arthritic BALB/c mice were able to transfer the disease [26]. SCID mice which received spleen cells alone (without PG) or PG antigen alone (without cells) did not show any symptoms of inflammation, i.e., the transfer was negative [26]. On the other hand, there was no difference in incidence or severity (not shown) whether arthritic cells were co-injected with PG intravenously or intraperitoneally.

When spleen cells from arthritic donor BALB/c mice were transferred into SCID mice along with PG intraperitoneally, recipient mice developed arthritis with 100% incidence (Fig.5). The clinical appearance of the disease and the histopathological abnormalities in affected joints in all positive recipients were identical and indistinguishable from those described in donor BALB/c mice [14,17]. However, arthritic SCID mice developed a more uniform clinical picture of

arthritis (essentially all peripheral joints become inflamed) with a robust involvement of the interphalangeal, metacarpo- and metatarso-phalangeal joints.

To investigate whether the established state of nasally-induced tolerance (Fig.1) can be adoptively transferred into SCID mice, we transferred splenocytes from control (PBS- or OVA-treated) or the PG nasally-tolerized BALB/c donors to SCID mice along with PG injected intraperitoneally. Unexpectedly, SCID mice receiving cells from nasally-tolerized BALB/c mice developed arthritis with similar or even higher severity (Fig.6a) and incidence (not shown) than those inoculated with cells from non-tolerized arthritic donors. In contrast, when SCID mice received spleen cells from nasally-tolerized BALB/c mice and weekly doses of intranasal PG, there was a significant reduction in arthritis severity (Fig.6b). Together, these results indicate that a successful transfer of PGIA and tolerance into SCID mice requires repeated intranasal administration of antigen.

Discussion

Recently, a number of clinical trials have been initiated in autoimmune diseases using oral administration of tolerizing proteins such as type II collagen in rheumatoid arthritis [10,12,32], myelin basic protein in multiple sclerosis [11], and insulin in insulin-dependent diabetes [33]. While the results of these clinical studies showed a promising trend toward clinical improvement, the mechanisms of tolerance are not fully understood and the results are frequently controversial [34,35]. Oral treatment of rheumatoid arthritis is probably in the most advanced stage of these clinical studies. The source of heterologous (bovine or chicken) cartilage type II collagen, the duration of the disease, and previous or ongoing anti-inflammatory medications are important factors that influence the efficacy of the oral administration of type II collagen. Moreover, immune reactions to type II collagen can be detected only in a subset of patients with rheumatoid arthritis, indicating the heterogeneity of cartilage-derived autoantigens in this

disease. Therefore, as proposed by Trentham [36], other non-collagenous cartilage constituents, such as human cartilage glycoprotein gp-39, link protein or PG (aggrecan) should be tested either alone or in combination, for their abilities to induce tolerance.

In addition to type II collagen [7,8,13], recently it has been reported that nasally applied cartilage gp-39 reduced the severity of experimentally induced arthritis [37]. In this study, we suppressed inflammatory reactions in joints and immune responses to PG in BALB/c mice with PGIA by nasal administration of cartilage PG. The incidence was significantly reduced in intranasally treated BALB/c mice and affected animals developed significantly less severe arthritis (Fig. 1). However, effective mucosal tolerization in autoimmune models was achieved only if the treatment began before the systemic immunization of the animal [2,7,8], and the disease suppression was more evident if the nasal administration of the antigen was continued throughout the entire experiment [9]. Tolerance was found effective only in a very rare case when mice were given the antigen intranasally after systemic immunization [37]. In PGIA, the most effective suppression was achieved when the nasal treatment with PG was introduced days before systemic immunization began, and was continued throughout the entire experimental period. If the nasal treatment, for example, was applied first after the second injection of the immunizing PG, i.e., just before the onset of the disease, little or no suppressive effect was observed. Therefore, taken into account that animal models are never identical with human disease, it is unlikely that mucosal tolerance can be induced in the same way in human patients with chronic autoimmune diseases.

By definition, immune cells are tolerized by antigens that are not present in the thymus or bone marrow at the time of their differentiation [38]. A number of studies have suggested that mucosal tolerance can be achieved by either “bystander” suppression or induction of anergy [39,40]. An antigen-specific suppression by Th2 and/or Th3 regulatory cells is thought to be the major mechanism of oral tolerance when multiple low doses of antigen are used [41]. In contrast, anergy can be induced by oral treatment of animals with high doses of antigen [40,42,43]. This may

be the case when high doses of type II collagen are used for oral treatment of patients with rheumatoid arthritis. Anergy induction by mucosal tolerization is antigen-specific, whereby antigen-primed T cells are driven to the state of unresponsiveness. As antigen-specific T cells are present, anergy can clearly be distinguished from clonal deletion. We found that while intranasally administered PG had a strong suppressive effect at the dose of 100 μg, it was ineffective at a low dose (10 μg). Tolerized mice exhibited significantly reduced antigen-specific T-cell proliferation and IL-2 production *in vitro* (Fig.2), which was accompanied by lower levels of PG-specific antibodies in the circulation (Fig.3).

PGIA is a Th1-dominated autoimmune disease [24,29], and Th2-supported cytokines can significantly decrease disease severity [29]. However, we found no changes in PG-induced Th1-(IFN-γ) or Th2 (IL-4, IL-10)-related cytokine productions in nasally PG-tolerized animals, nor was the PG-specific IgG1/IgG2a antibody ratio altered. $CD4^+CD25^+$ cells were recently recognized as naturally anergic and/or suppressive regulatory cells in several autoimmune diseases [44], possibly in the TGF-β-producing Th3 population [45]. However, we found no changes in the number of $CD4^+CD25^+$ T cells or intracellular CTLA-4 expression of these cells in nasally PG-tolerized mice (Fig.4), and the PG-specific TGF-β production was also comparable between the nasally-treated and non-tolerized groups (data not shown). As PG-reactive T cells were present in nasally treated mice (Fig.2), the clonal deletion of these antigen-specific T cells in donor animals could be excluded. This was even more evident when lymphocytes from PG-tolerized mice were transferred to SCID mice without continuation of intranasal treatment (Fig.6a). In summary, the mechanism of nasal tolerance in PGIA appears to be a PG-specific anergy rather than deletion or active suppression of particular T-cell clones.

Cell transfer was used to further characterize once already established tolerance [42,46-51], but nasal treatment in recipients was either not continued or modified in the previous transfer studies [50,51]. We transferred PGIA into SCID mice, and continued the nasal treatment in recipient animals. It is important to note that PGIA could be transferred into SCID mice only if

the splenic T cells were co-injected with PG [26]. As recipient SCID mice had matched genetic backgrounds, both the mucosally and peritoneally administered antigens could be processed by the recipient's antigen presenting cells, thus providing optimal conditions for disease development. The 'transfer of tolerance' into SCID mice was successful, however, only when the nasal administration of the antigen was continued (Fig. 6). Although SCID mice lack functional T and B cells, the mucosal antigen processing and mucosal functions might remain intact, such as in those experiments which investigated oral tolerance toward antibody response [49,51]. Based on the effect of continued nasal administration of PG, transferred lymphocytes might have been able to traffic to the mucosa, and remained anergic, while lymphocytes did not receive an anergizing signal (Fig. 6a), proliferated, and expanded in the host.

The results of disease and tolerance transfer described here suggest that antigen-specific T cells were present in nasally tolerized donor mice, however, these cells were "paralyzed" functionally. As T-cell unresponsiveness is a reversible phenomenon both *in vitro* and *in vivo* [52] thus antigen-specific anergized T cells, after the termination of tolerization following a few cell divisions, might become active again [43]. Homeostatic polyclonal T-cell proliferation, i.e., the restoration of the original T-cell pool, occurs in severe lymphopenic conditions such as those following irradiation, chemotherapy, or upon transfer of a low number of lymphocytes into SCID mice. In such an autologous or syngeneic condition, which allows the recognition of a wide range of self-MHC-associated/(self)peptide ligands by matched TCRs, lymphocytes are signaled to replicate in a rapid manner and repopulate the lymphoid organs [53-55]. If the anergizing mucosal antigen exposure is not sustained/present in the recipient mouse, the low number of antigen-specific transferred T cells may undergo several rounds of division, anergic clones can revert [43,52] and expand [54], thus ultimately contribute to arthritis development in the host animal." We hypothesize that transferred arthritogenic but temporarily anergic clones can revert after a few cell divisions due to the discontinuation of nasal antigen exposure and the presence of antigen co-injected with cells. When non-tolerized cells are transferred, nasal

administration of the antigen has no suppressive effect, rather it challenges the disease (Fig. 6b). Therefore, nasal antigen exposure has opposite effects on activated and anergized T cells. In conclusion, while induction of mucosal tolerance is a logical approach, it might become a "double-edged sword" in treatment of autoimmune diseases, especially in humans where individual responsiveness varies to a great extent.

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Figures and Figure Legends

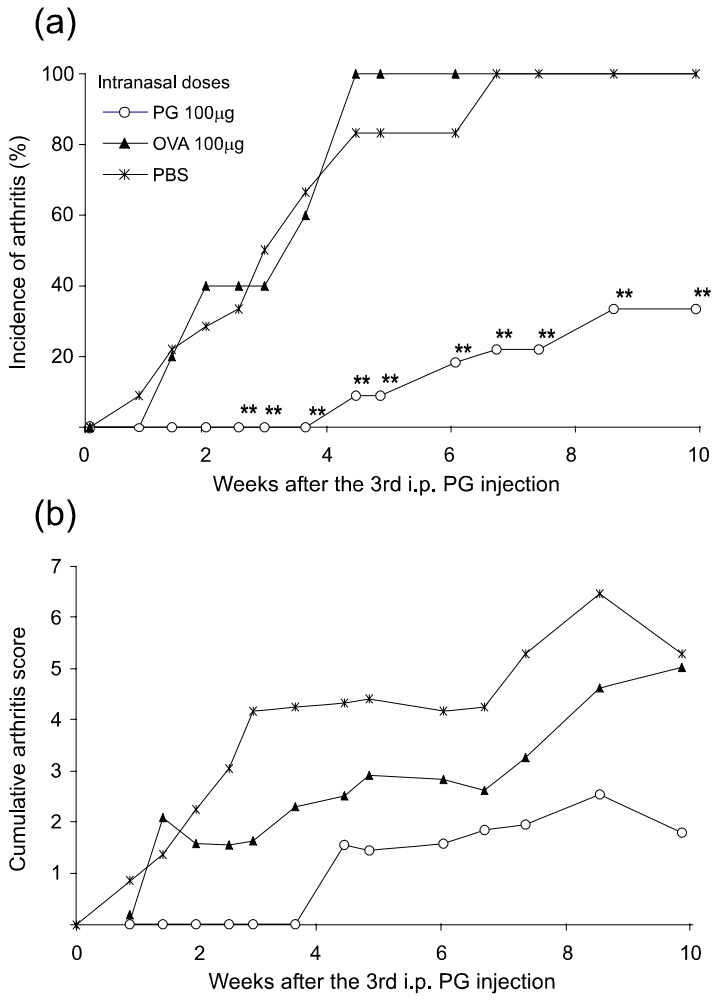

Figure 1. Incidence and severity of arthritis in intranasally treated BALB/c mice immunized with cartilage proteoglycan (PG). Incidence and acute arthritis scores are shown after the third PG injection (indicated as week '0'). All mice were treated intranasally for 4 consecutive days prior to the immunization and then immunized with cartilage PG as described in Methods. Intranasal treatment was repeated weekly. Number of animals in each experimental group is indicated. Arthritis scores (panel **b**) are shown in arthritic mice only. Error bars are omitted for clarity and the level of significance relative to the intranasally PBS-treated group is indicated at each time point (* $p<0.05$; **$p<0.01$; -○-PG 100μg (n=42); -▲-OVA 100μg (n=27); -✱-PBS (n=42)).

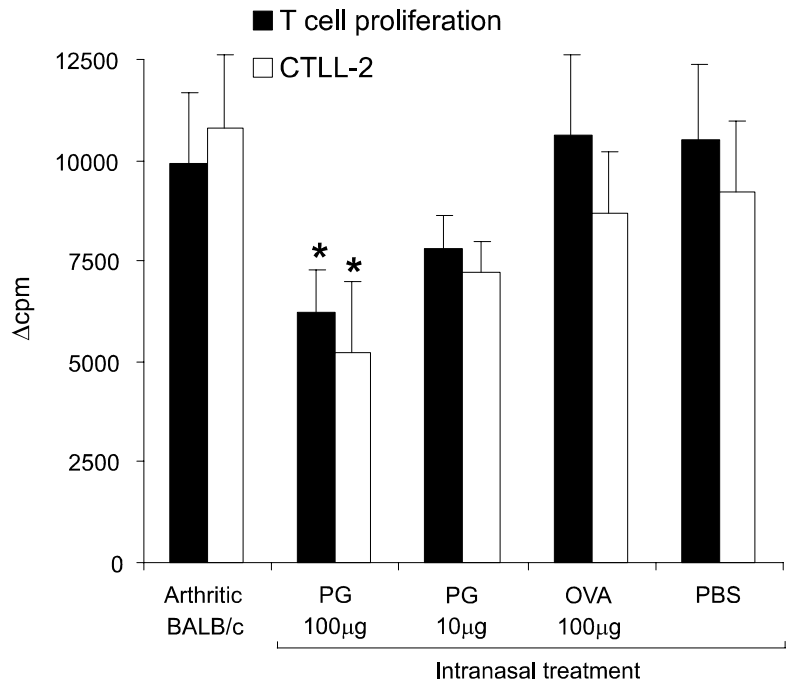

Figure 2. PG-specific *in vitro* T cell proliferation and IL-2 production (CTLL-2 bioassay) of spleen cells from PG-immunized and nasally treated mice. The differences between the PG-stimulated and non-stimulated cells are shown as Δcpm. The same numbers of animals were used as shown in Figure 1, except those were treated nasally with 10 μg of human PG (n=12). (*$p<0.05$)

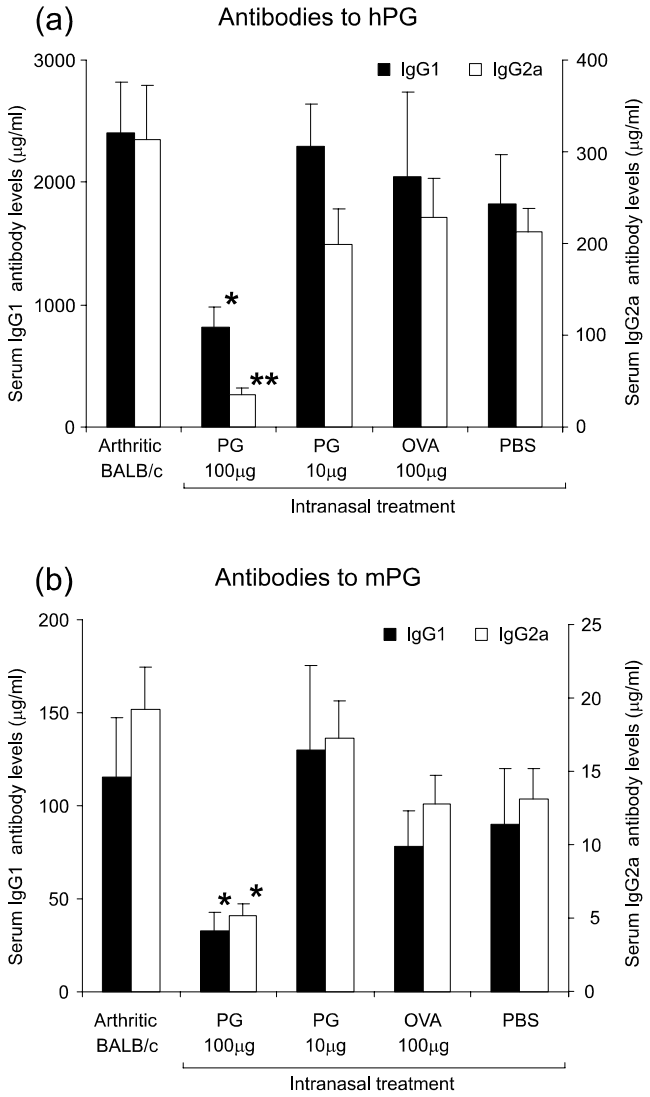

Figure 3. Serum antibody levels in PG-immunized control and nasally treated mice. Antibodies to the immunizing human PG **(a)** and autoantibodies to mouse (self) PG **(b)** were measured by ELISA at the end of the experiment. The group treated with 100 μg PG intranasally showed significantly lower antibody levels compared to other groups (including 10 μg PG-treated mice). Sera of the same animals were used for antibody measurements as shown in Figure 2. (*$p<0.05$, **$p<0.01$)

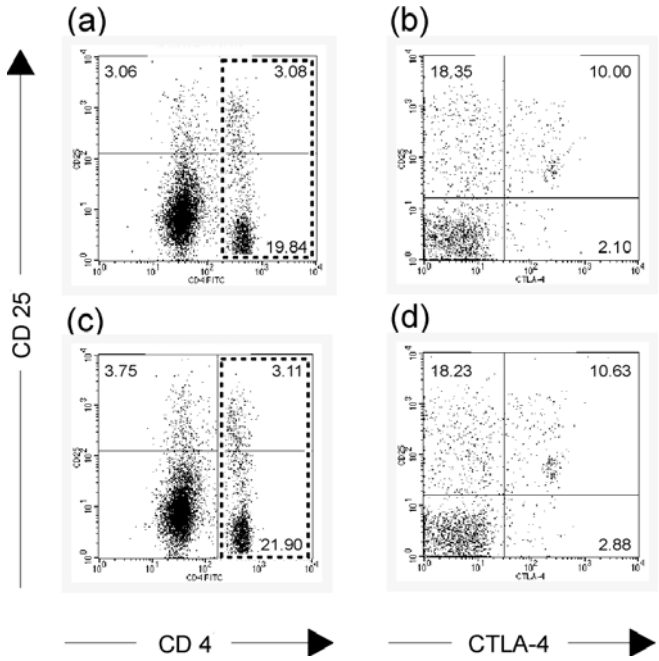

Figure 4. CTLA-4 expression of $CD4^{+}CD25^{+}$ spleen cells harvested from PG-immunized and PBS-treated **(a, b)** or intranasally PG-tolerized BALB/c mice immunized with cartilage PG **(c, d)**. CTLA-4 expression (**b, d**) was measured only in $CD4^{+}$ cells (indicated by broken lines within the right upper and lower quadrants of panels **a** and **c**). Percents of cells are shown in the corner of the corresponding quadrants.

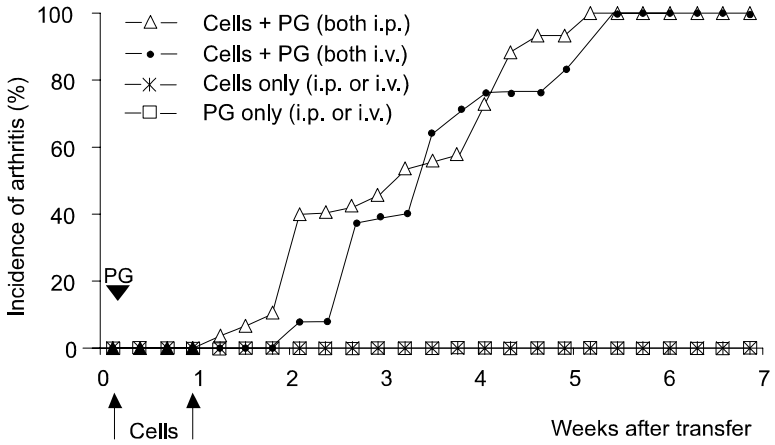

Figure 5. Incidence of PGIA after adoptive transfer into SCID mice. Lymphocytes from arthritic BALB/c mice (non-tolerized) were transferred to SCID mice with or without 100 μg of PG. SCID mice also received PG intraperitoneally or intravenously without cells. Regardless of the route of PG and cell injections, recipient SCID mice developed arthritis (n=27 in each group). Arrows indicate the days of cell transfers (days 0 and 7) and the arrowheads show the PG injection.

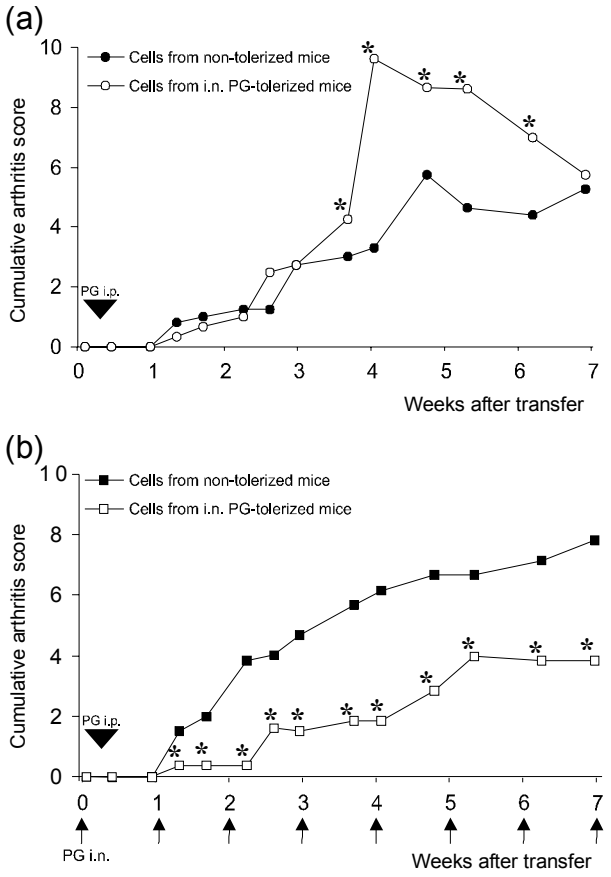

Figure 6. Acute inflammatory arthritis scores in SCID mice that received spleen cells from PG-immunized and nasally PG-treated (open symbols) or nasally untreated (closed symbols) BALB/c mice. Concurrent with the first cell transfer, recipient SCID mice also received PG **(a)** intraperitoneally (i.p.) or intravenously (arrowhead), or **(b)** intraperitoneally and intranasally, latter continued with a weekly dose of 100 μg PG (arrows). Donor cells ($1x10^7$) were injected on days 0 and 7. Significant differences between two groups (*<0.05; n=24 mice in each group) are shown.

Chapter 3

Anti-Inflammatory and Chondroprotective Effect of TSG-6 (TNFα-Stimulated Gene-6) in Murine Models of Experimental Arthritis

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Summary

Tumor necrosis factor-alpha (TNFα)-stimulated gene-6 (TSG-6) is upregulated by various cytokines and growth factors. TSG-6 binds to hyaluronan in inflamed synovial tissue and forms a complex with a serine protease inter-α-trypsin inhibitor (IαI), increasing the protease inhibitory effect of IαI over 100-fold. The TSG-6/IαI complex then blocks serine proteases, including the plasminogen-plasmin activation, probably the most important component in the activation processes of matrix metalloproteinases. To gain insight into the mechanisms of TSG-6 action in arthritis, we have used an autoimmune murine model (proteoglycan-induced arthritis) for systemic, and a monoarticular form of arthritis (antigen-induced arthritis) for local treatment of arthritis with recombinant mouse TSG-6 (rmTSG-6). Intravenous injection of rmTSG-6 induced a dramatic reduction of edema in acutely inflamed joints by immobilizing CD44-bound hyaluronan and, in long-term treatment, protected cartilage from degradation and blocked subchondral and periosteal bone erosion in inflamed joints. The intra-articular injection of a single dose (100 μg) of rmTSG-6 exhibited a strong chondroprotective effect for up to 5-7 days, preventing cartilage proteoglycan from metallo-proteinase-induced degradation. In contrast, rmTSG-6 did not postpone the onset, nor reduce the incidence of arthritis. We were unable to detect any significant differences between control and rmTSG-6-treated animals when various serum markers (including pro- and anti-inflammatory cytokines, auto- and heteroantibody productions) or antigen-specific T-cell responses were compared, nor when the expressions of numerous cell surface receptors or adhesion molecules were measured. TSG-6 seems to play a critical negative regulatory feed-back function in inflammation, especially in arthritic processes.

Introduction

Tumor necrosis factor-alpha (TNFα)-stimulated gene 6 (TSG-6) was first identified by differential screening of a cDNA library of TNFα-stimulated human fibroblasts[1]. While a constitutive expression is low or not detectable, virtually all cell types are able to produce TSG-6 upon stimulation with various components either *in vitro* or *in vivo*[2-5].

TSG-6 is a 35-kDa secreted glycoprotein[2]. The N-terminal half of the amino acid sequence shows homology to the Link module, which is a conservative sequence in hyaluronan (HA)-binding proteins such as CD44, cartilage link protein and G1 domains of aggrecan and versican[2,6]. The C-terminal half of TSG-6 (CUB domain) shares sequence homologies with the A chain of complement component C1s/C1r, uEGF, a protein involved in development of sea urchin embryos, and bone morphogenic protein-1[7,8]. Based on these structural homologies, it is strongly believed that TSG-6 may play a crucial role in extracellular matrix formation, inflammatory cell migration, cell proliferation and developmental processes[4,7].

While TSG-6 can not be detected in synovial fluids or sera of normal individuals, synoviocytes from rheumatoid patients constitutively produce TSG-6 protein and it can be detected in sera of patients with rheumatoid arthritis, or less frequently in osteoarthritis[3,9,10]. Exogenous TNFα or interleukin (IL)-1 further increases in TSG-6 production by rheumatoid synoviocytes[3]. A 120-kDa complex containing TSG-6 was regularly detected in TSG-6 (35-kDa)-producing cultures in the presence of serum, but not in serum-free medium[3,9,11]. The protein that bound TSG-6 was purified from human serum and identified as inter-α-inhibitor (IαI) [9,11]. IαI is a HA-binding serum protein with serine protease inhibitory activity[12]. The target enzymes include trypsin, chymotrypsin, cathepsin G, leucocyte elastase, acrosin and plasmin[8,10,12].

Interestingly, when TSG-6 is complexed with IαI, the complex exhibits an over 100-fold higher protease inhibitory effect against plasmin than the IαI alone[9,11]. This effect of TSG-6 on IαI seems to be specific for plasmin, as no such increase in inhibitory effect was observed on

trypsin or neutrophil elastase[11]. To further test the inhibitory effects of the TSG-6/IαI complex on protease network and inflammation, a murine air-pouch model of acute inflammation was used[11]. Simultaneous injection of human recombinant TSG-6 with carrageenan into the air pouch exhibited a potent anti-inflammatory effect[11]. Recently, it was shown that recombinant TSG-6 had a beneficial effect on collagen-induced arthritis (CIA) reducing both the incidence and severity of arthritis[13]. Taken together, TSG-6 induced by proinflammatory cytokines at the site of inflammation, binds tightly to IαI, and dramatically increasing the IαI's serine protease inhibitory effect. Thus, TSG-6 may be a key component in a negative feedback loop controlling inflammatory response in local arthritic processes.

In this paper, we investigated whether TSG-6 plays a role in prevention of cartilage breakdown in two murine models of experimental arthritis. We studied the *in vivo* role of TSG-6 in a systemic autoimmune disease of proteoglycan-induced arthritis (PGIA), and then in a monoarticular form of inflammatory arthritis by injection of recombinant TSG-6 into the knee joints of animals with antigen-induced arthritis (AIA).

Materials and methods

Recombinant Mouse TSG-6 (rmTSG-6) and Anti-TSG-6 Antibody

Purified plasmid DNA (Lonza pEE14.1 vector, Lonza Biologics plc., Slough, Berkshire, UK) containing a full-length (1654 bp) mouse TSG-6 cDNA clone[4] was used for transfection of Chinese hamster ovary (CHO-K1; American Type Culture Collection, Rockville, MD) cells by standard method of $CaCl_2$ precipitation. Transfected cells were cultured in glutamine-free condition in the presence of 25 μM methionine sulphoximine (Sigma Chemical Co., St-Louis, MO) for the selection of stable transfected cell lines. rmTSG-6 was purified from culture media of stable-transfected cloned cell line using HA-coupled EAH-Sepharose (Pharmacia Biotech, Piscataway, NJ) . HA-bound rmTSG-6 was eluted with 4M guanidine hydrochloride in 0.1 M Na-acetate buffer (pH 5.8), dialyzed against distilled water and then lyophilized. Purified

rmTSG-6 was tested by Western blotting using a rabbit polyclonal antibody (TSG-6-CR21) raised against a synthetic peptide (^{135}CGGVFTDPKRIFKSPG), located at the end-terminal end of the CUB domain.[4] For quantitative measurement of serum TSG-6 (either measuring serum level in arthritic animals or during the treatments) we used a sandwich enzyme-linked immunosorbent assay (ELISA), confirmed by Western blots. In ELISA, the IgG fraction of rabbit serum to mouse TSG-6 synthetic peptide (^{36}GVYHREARAGRYKL) was used as capture antibody and affinity purified and biotinylated TSG-6-CR21 antibody for detection of TSG-6 following a protocol described for other serum markers.[14] For organ/tissue distribution and kinetic studies, rmTSG-6 was labeled with ^{125}I using a chloramine-T method.

Immunization Protocol for PGIA, Scoring System and Treatment with rmTSG-6

High density cartilage proteoglycan aggrecan (PG) was purified by CsCl gradient centrifugation from human cartilage and depleted of glycosaminoglycan side chains as described[15]. All animal experiments were approved by the Institutional Animal Care and Use Committee (Rush University, Chicago). Female BALB/c mice (National Cancer Institute, NCI, Friedrich, MD) were immunized intraperitoneally with cartilage PG[15,16]. First antigen injection (100 μg protein) was given in complete Freund's adjuvant (CFA; Difco, Michigan, MI), and the same doses of antigen were injected as second and third boosters in incomplete Freund's adjuvant (IFA). Typically, BALB/c mice developed swelling and redness in one or more limbs, 7-14 days after the third injection of PG[15,16]. This stage of immunized animals (from 7 days after the third injection) is regarded as "prearthritic" phase of PGIA. A standard scoring system, based upon swelling and redness of paws, was used for the assessment of the severity of arthritis[17,18]. The time of appearance of swelling and redness was recorded as the time of onset of arthritis. Joint swelling was scored (ranging from 0 to 4 of each paw) and expressed as cumulative acute arthritis score resulting in a maximum score of 16. During the treatment period, paw (joint) thicknesses at frontal and sagittal planes of all ankles and wrists were measured daily with a

microcaliper[17]. Arthritic animals were injected intraperitoneally or intravenously via the retro-orbicular venous plexus with 100 µl phosphate-buffered saline (PBS), rmTSG-6 (100 µg in PBS), or rmTSG-6 and Pep-1 simultaneously. Pep-1 is a synthetic peptide (NH_2-GAHWQFNALTVRGGGS) which shows HA-binding properties[19] and can also bind to the Link module of either CD44 or TSG-6 (our unpublished data). The optimal therapeutic dose, serum/tissue distribution, half-life and kinetics of injected rmTSG-6 was determined in preliminary experiments. Sera were collected prior to the injection, or every second hour in kinetic studies, and at the end of the experiment. Spleen cells for *in vitro* tests were isolated at the end of the experiment and paws from all mice were collected and fixed in 10% formalin containing 5% cetylpirimidium chloride (CPC; Sigma). Legs were decalcified in 5% formic acid, paraffin embedded and sections processed for routine histology or immunohistochemistry[15,16,20,21].

Immunization Protocol for AIA and Experimental Groups

C57Bl/6j mice (NCI) were immunized with 100 µg of methylated bovine serum albumin (mBSA; Sigma) in 100 µl of PBS emulsified in 100 µl of CFA. Injections were given subcutaneously in the flanks, and into the proximal tail. After one week, mice were subcutaneously boosted with 100 µg of mBSA emulsified in 100 µl of IFA, and then three weeks later [21]. Two-three weeks after the final boost, knees of immunized mice under Ketamine/Xylazine anesthesia were injected intra-articularly in the following combination: animals of *group 1* received a single dose of 60 µg mBSA in 6 µl PBS into the right knee, and 6 µl PBS into the left knee joint. Animals of *group 2* received a single dose of 60 µg mBSA and 100 µg rmTSG-6 in 6 µl PBS intra-articularly into the right knee, and 60 µg of mBSA in 6 µl PBS into the left knee joint. A therapeutic dose of rmTSG-6 injected intra-articularly with mBSA was found to be 25-50 µg in preliminary experiments. To avoid repeated intra-articular injections, we used a single double dose (100 µg) of rmTSG-6 throughout this study. Mice

(n=6-10 at each time point) were sacrificed on day 1, 3, 5, 7, 12 and 24 after the intra-articular injections. Left and right knees from each mouse were harvested, fixed in 10% formalin containing 5% CPC, decalcified and paraffin embedded. Five-seven µm sections were stained with hematoxylin and eosin, or safranin-O counterstained with fast green. For *immunohistochemical* analysis, sections were deparaffinized, rehydrated in PBS and pretreated with chondroitinase ABC (Seikagaku America of Cape Cod, Falmouth, MA) as described[20]. Sections were stained with polyclonal antibodies to neoepitopes -VDIPEN[341] (a sequence which is generated by stromelysin-1 [MMP-3] cleavage in the interglobular domain of cartilage aggrecan) and -NITEGE[373] (aggrecanase-generated neoepitope) (antibodies were generous gifts from Dr. J. Mort, Shriners Hospital, Montreal, Quebec, Canada and Dr. J. Sandy, Shriners Hospital, Tampa, FL) [21-23]. Affinity-purified peroxidase-labeled anti-rabbit immunoglobulin (Accurate Chemical and Scientific Co., Westbury, NY) was used as a second-step antibody and the peroxidase reaction developed by Fast™ 3, 3'-diaminobenzidine (DAB; Sigma) and H_2O_2 for 5 minutes as described[20]. The DAB reaction was enhanced using DAB Substrate Enhancer (Zymed Laboratories Inc, San Francisco, CA) for 3 minutes. The sections were dehydrated and mounted with Permount.

Measurements of Antigen-specific Antibodies, T-cell Responses and Cytokine Production

MaxiSorp immunoplates (Nunc International, Hanover Park, IL) were coated with human or mouse cartilage PGs (0.1 µg protein/100 µl/well) for ELISAs as described [24,25]. Sera were applied at increasing dilutions and the isotypes of PG-specific antibodies were determined by peroxidase-conjugated rat anti-mouse IgG1 or IgG2a secondary antibodies (Zymed) as described [24,26]. Serum antibody levels were normalized to mouse IgG1 and IgG2a [24,25].

Antigen-specific T-cell responses (i.e., IL-2 production and proliferation), were measured in quadruplicate samples of spleen cells (3 X 10^5 cells/well) cultured in the presence of 50 µg PG protein/ml. IL-2 was measured in supernatants harvested on day two by the

proliferation of the IL-2-dependent CTLL-2 cell line [24,25]. Antigen-specific T-cell proliferation was assessed on day 5 by the incorporation of ^{3}[H]-thymidine. Antigen (PG)-specific interferon-gamma (IFN-γ) and IL-4 production by T cells were determined in identical culture conditions as described for T cell proliferation in 4-day-old conditioned media (2.5 x 10^{6} mononuclear cells/ml) using capture ELISAs from R&D Systems (Minneapolis, MN). Serum levels of TNFα, IL-6 and IL-10 were measured by capture ELISAs (R&D Systems or BD PharMingen) and IL-1 by a bioassay using D10S cell line as described [24,25].

Flow Cytometry

Immunostaining of cell-surface molecules and flow cytometry were carried out as described previously [14]. Spleen and lymph node cells were stained with monoclonal antibodies (mAb) to various cell surface molecules. These mAbs, purchased from BD PharMingen (San Diego, CA), were raised against CD3 (T cell), CD4 (T helper cell), CD8 (T suppressor cell), CD11a/b (leukocyte β2 integrin βsubunits; LFA-1/Mac-1), CD18 (β2 integrin βsubunit; LFA-1), CD19 (B cell), CD29 (β1 integrin subunit), CD25 (IL-2 receptor), CD44 (IM7), CD45/B220 (B cell), CD49d (β1 integrin α4 subunit; VLA-4), CD54 (ICAM-1), CD62L (L-selectin), and CD62P (P-selectin). Isotype-matched irrelevant mAbs were used as controls. Cell surface-bound antibodies were detected by biotinylated goat-anti-rat IgG (Kirkegaard & Perry, Gaithersburg, MD) and streptavidin-phycoerythrin (PE) or streptavidin-FITC (Life Technologies; Gaithersburg, MD).

Mouse synovial fibroblast and small venule endothelial cells (SVEC4-10) were digested with streptomyces hyaluronidase (Sigma) for 30 minutes at 37°C to remove endogenous HA [17]. Mouse synovial fibroblast,[17] SVEC4-10, [19,27] and 5/4 T-cells,[18] each at 1 x 10^{6} in 100 μl, were incubated with fluorescein-labeled HA (5 μg/assay)[14] and rmTSG-6 (0.04 – 5 mg/ml), or unlabeled HA (5 mg/ml), as competitors for 2 hours at 37°C. Cells were washed in PBS, fixed

in 2% paraformaldehyde (Sigma) and analyzed using a FACScan® instrument and CellQuest software (Becton Dickinson, San Jose, CA).

Statistical Analysis

Statistical analysis was performed using SPSS v7.5 (SPSS, Chicago, IL). The Mann-Whitney and Wilcoxon tests were used for intergroup comparisons. For comparison of results of two groups, Student's *t*-test and, for the determination of correlation coefficients, Spearman's ρ test were used. Significance was set at $p < 0.05$.

Results

Short-term Effects of rmTSG-6 Treatment on Joint Swelling in PGIA Mouse Model

PGIA was generated in BALB/c mice by systemic immunization with human cartilage aggrecan. Acutely arthritic animals were injected intraperitoneally with 100 µl PBS (control) or 50-250 µg rmTSG-6 (in 100 µl PBS) for 3 consecutive days. No effect was observed on joint swelling in either the control or the rmTSG-6-treated mice after 3 injections (data not shown). To further test the feasibility of TSG-6 treatment, acutely arthritic mice with PGIA were injected intravenously with 100 µg of rmTSG-6. A dramatic reduction in joint edema of each swollen limb was observed within a few hours (Figure 1A). Subsequent intravenous injections further reduced joint swelling, whereas the effect was less and less spectacular until the edematous swelling virtually disappeared from acutely inflamed paws (Figure 1A and Figure 2B). Control mice injected with PBS showed no change, or only a slight increase, in joint swelling within this short-term experiment (Figure1).

Histopathology of the arthritic joints, except the reduced periarticular edema in TSG-6-treated mice (Figure 2B), however, was indistinguishable from those treated with PBS. Due to the lack of any effect of intraperitoneally injected rmTSG-6 versus the dramatic reduction of edema after the intravenous injection, we hypothesized that a free HA-binding capacity of TSG-

6 should be critical in function without affecting the inflammatory cell infiltration. Indeed, when rmTSG-6 was added together with either large dose of HA (data not shown) or with HA-binding peptide Pep-1, the beneficial effect of TSG-6 disappeared proportionally as the dose of Pep-1 was increased (Figure 1B).

In Vitro and In Vivo Tests of rmTSG-6 Ability to Compete for HA-binding Site

The difference between treated animals and untreated controls was the regression of joint swelling due to the loss of edema in the rmTSG-6-treated group. To test if the loss of edema was due to the ability of rmTSG-6 to compete with cell surface receptor CD44 for HA binding, *in vitro* competition assays were performed using synovial fibroblasts, small venule endothelial cells (SVEC4-10) (both produce high levels of HA), and 5/4 T-cell hybridoma (a non-HA producing cell line). All these cell types express high amounts of CD44, hence can bind HA. Cells were incubated with fluorescein-labeled HA and increasing concentrations of rmTSG-6, and the HA-binding measured by FACS analysis. As shown in Figure 3, rmTSG-6 could indeed compete with CD44 and the effect was dose-dependent. If this was the case *in vivo*, an HA-binding peptide [19] could also reduce joint inflammation and should exhibit an additive or synergistic effect on TSG-6 treatment. As shown in Figure 1B, Pep-1 alone could not reduce joint swelling, even worse, the co-injection of Pep-1 with rmTSG-6 significantly reduced the anti-inflammatory effect of the TSG-6 (Figure 1B). Taken together, TSG-6 had a very strong effect on joint inflammation by reducing tissue edema. This effect was associated with the HA-binding capacity of the TSG-6, which could be competitively inhibited with a synthetic peptide with known HA-binding properties, whereas Pep-1 alone did not exhibit any effect on joint edema.

'Long-term' Effects of rmTSG-6 Treatment on Joint Swelling in PGIA Mouse Model

Next, we have monitored the clinical symptoms and histological abnormalities of inflamed joints during repeated (weekly) treatments with rmTSG-6 in PGIA up to 28 days. Arthritic BALB/c mice (n=14) were injected intravenously with rmTSG-6 (100 μg in PBS) every 24 hours for 7 days and joint/paw swelling measured twice a day (Figure 4). As the cumulative joint score (swelling of all paws) almost returned to the pretreatment level after a 7-day treatment-free period (Figure 4A), mostly due to the newly inflamed paws, a second treatment cycle was repeated from day 14. As found during the first treatment period, although less impressively, the cumulative joint score in rmTSG-6-treated mice showed a statistically significant regression ($p < 0.05$)(Figure 4A). In contrast, the cumulative joint swelling in the PBS-injected (control) mice remained significantly higher ($p < 0.05$) up to three weeks (Figure 4A) which was the consequence of the "normal" progression of the disease.[15,16] Interestingly, additional (new) joints (paws) became inflamed (swollen) in rmTSG-6-treated mice only after weaning of TSG-6 treatment (between days 7 and 14). Therefore, when only the inflamed joint/paw diameters were calculated and shown on Figure 4B, a less impressive effect of the second-cycle treatment was found. While the anti-inflammatory (anti-edematous) effect remained significantly low, relative to the joint diameters measured on the zero day of the experimental group, the second-cycle treatment affected essentially only the freshly (acutely) inflamed paws, thus the anti-inflammatory effect of the second treatment period with rmTSG was almost restricted to these new, acutely inflamed joints/paws (Figure 4A). While the cellular infiltration and/or synovial cell proliferation of inflamed joints were very similar in PBS- and TSG-6-treated joints (Figures 2C and 2D), massive cartilage and bone erosions were found in joints only in PBS-treated animals. In turn, systemic application of rmTSG-6 reduced edema and, more strikingly, prevented articular cartilage from deterioration even in the presence of massive inflammation (Figure 2C vs. Figure 2D).

It is important to note here that, while PGIA is progressive and more and more joints are involved, arthritic limbs/joints exhibit a high variability, i.e., not all limbs (joints) are inflamed simultaneously, or to the same extent, in an acutely arthritic animal at a single time point.[15,16] In view of the clinical picture of PGIA, we reanalyzed the entire data base of all arthritic (PBS- and rmTSG-treated) mice (Figure 4A) and compared only those paws which were inflamed prior to the treatment. The PBS-treated group showed no change during the first two weeks, and then the acute inflammatory symptoms moderately regressed and previously inflamed joints became stiff. In contrast, the joint swelling of rmTSG-6-treated groups showed a statistically significant regression ($p < 0.05$) within a few days (Figure 1A and Figure 4A) which remained unchanged during the four-week experimental period (Figure 4B). In other words, once rmTSG-6 reduced edema and redness, "inflammation" did not return, not even in the absence of treatment (between days 7 and 14 or 21 and 28)(Figure 4B). These results suggest that TSG-6 not only protects arthritic joints from flare-up but also seems to prevent non-arthritic limbs from the clinical symptoms of inflammation determined as redness and swelling.

Effect of rmTSG-6 Treatment on the Development of PGIA

During the intravenous first-cycle of rmTSG-6 treatment (Figure 4), edema waned in arthritic paws and no additional joints became inflamed. In contrast, previously non-arthritic paws became swollen in PBS-injected animals (Figure 4, A and B), and this was also a frequent observation in TSG-6-treated mice after the cessation of the treatment. A significant increase in cumulative paw diameter after day 7 in previously TSG-6-treated animals (Figure 4A) clearly was the consequence of these newly inflamed joints. While conventional histopathology did not reveal differences in cellular infiltration (Figure 2), the anti-inflammatory effect of TSG-6 was found in CIA[13] and in an air-pouch model [11]. All these observations indicated a preventive effect of TSG-6 on arthritis development. To test this preventive potential of TSG-6 in arthritis, PG-immunized mice (n=8) in the prearthritic stage (after the third antigen injection) were

treated intravenously with a daily dose of 100 μg rmTSG-6 (a dose found to be optimal for the suppression of inflammation) or PBS for 3 weeks. While the inflammation (cumulative score: 4.2±2.8) was significantly lower ($p<0.05$) in rmTSG-6-treated mice than in the PBS group (7.6±1.7), neither the incidence nor the time of onset differed in the two groups (data not shown), i.e., TSG-6 had an anti-edematous but not an anti-arthritic effect.

Pathophysiological Markers in rmTSG-6-treated and Untreated Mice with PGIA

As we found differences in severity between TSG-6-treated and untreated animals, and then differences in cartilage and bone erosions, it seemed to be prudent to measure serum levels of pro- and anti-inflammatory cytokines and auto- and hetero-antibodies, as well as monitor the antigen-specific T-cell responses. Serum samples were collected prior to the treatment-cycles and at 24 h after the last (seventh) intravenous injection of rmTSG-6, and T-cell responses were measured at the end of experiment. Treatment protocols were the same as shown in Figure 4. We could not detect any significant differences between PBS- and rmTSG-6-injected mice measuring serum levels of TNFα, IL-1, IL-6, IL-10, and auto- and heteroantibodies (including the ratios of IgG1 and IgG2a), or measuring antigen (PG)-specific T-cell proliferation, IL-2 (CTLL-2 assay), IL-4 or IFN-γ production by spleen cells. Similarly, neither the T-cell subsets nor other expression of cell surface receptors (mAbs listed in Methods) differed when spleen or lymph node cells of the PBS- and rmTSG-6-treated groups were compared (data not shown). Therefore, we concluded that the systemic administration of rmTSG-6 probably has no systemic effect, rather it might be critical at the site of inflammation where tissue destruction occurs.

Histopathology of Knee Joints in AIA under the Protection of TSG-6

To gain insight into the mechanism of the local "chondroprotective" effect of TSG-6 (Figure 2D) in a single joint, a murine model of AIA was used [21]. In this model system, the delayed-type hypersensitivity reaction is localized at a single joint, other joints can be used as controls,

and cartilage degradation can be monitored precisely on a daily basis. Intra-articular PBS injection in the left knee joint of mBSA-immunized animals showed no inflammation (Figure 5A) up to a 24-day period, and no cartilage damage occurred (Figure 5, B-E). The earliest response (lymphocyte infiltration) to the intra-articular injection of mBSA was detected on days 2-3 (data not shown) and became more uniformly extensive by day 5 (Figure 5F). By this time, the loss of cartilage PG from the superficial-middle layers of the articular cartilage became evident (Figure 5, G-J). All mBSA-injected joints exhibited heavy inflammation, independent of whether the mBSA was injected alone (Figure 5F) or with rmTSG-6 (Figure 5K). The loss of PG from cartilage, detected by the loss of safranin-O staining, was moderate on day 3 (not shown), but became extensive by day 5 in mBSA-injected knee joints (Figure 5H). In accordance with the loss of safranin-O staining, an intensive accumulation of immuno-stained -VDIPEN and -NITEGE neoepitopes (the C-terminal cleavage sites of cartilage aggrecan by metalloproteinases and aggrecanase) were detected in the superficial layer of damaged cartilage (Figure 5, I and J). By day 7, inflammation further progressed, pannus-like structures eroded bone and cartilage, safranin-O staining significantly decreased down to the calcifying zone, and the “expression” of -NITEGE neoepitope became slightly reduced (not shown). Knee joints of mBSA-immunized mice were injected with mBSA with or without rmTSG-6. As shown in Figure 5 (F and K), the conventional histopathology of these knee joints (i.e., hematoxylin-eosin stained sections) exhibited no differences at any time point. In contrast, safranin-O staining remained intense (compare the superficial layers of cartilage in Figure 5H with Figure 5M) up to 5-7 days after the intra-articular administration of mBSA with rmTSG-6. Thus, a single dose of 100 μg rmTSG-6 (given together with the mBSA) blocked the degradation and loss of aggrecan from cartilage (Figure 5, M-O). However, this chondroprotective effect of TSG-6 in acutely inflamed joints gradually decreased after day 7 and essentially no differences were found between PBS/mBSA and rmTSG-6/mBSA-injected knee joints after day 12, unless additional doses of TSG-6 were introduced locally or systematically (results are not shown).

Discussion

TSG-6 can be induced by proinflammatory cytokines (TNFα, IL-1), growth factors, and various compounds (including lipopolysaccharides [LPS]),[2,3,5,28] components which in turn activate various transcription factors (NF-κB, NF-IL6 or AP-1) and induce the TSG-6 expression in various cell types [29]. In a complex milieu such as the inflamed rheumatoid synovium, among many other components, TSG-6, HA and CD44 are upregulated simultaneously, and increased serum levels of these compounds correlate directly with the disease activity of rheumatoid arthritis [3,8,30-32]. To study the pathophysiological relevance of TSG-6 in arthritic processes, and gain insight into the function of this cytokine-induced molecule in inflammatory conditions, we used an autoimmune murine model (PGIA) of rheumatoid arthritis.

The intraperitoneal injection of rmTSG-6 had no effect on joint swelling of mice with acute PGIA, whereas the intravenous injection of rmTSG-6 resulted in a dramatic reduction in joint swelling. The lack of any effect of intraperitoneally injected rmTSG-6 is probably due to the entrapment of rmTSG-6 by the excess amount of HA in the peritoneal cavity occupied by adjuvant-induced granuloma, which is the consequence of intraperitoneal immunization. Indeed, we were unable to detect radiolabeled rmTSG-6 in serum after intraperitoneal injection, whereas the intravenously injected rmTSG-6 preferentially accumulated at the site of inflammation. In this kinetic study, the half life of intravenously injected rmTSG-6 was less than 4 hr, but the serum level of TSG-6 measured by ELISA was almost constant 60-90 min after the intravenous injection (unpublished data). Thus, while the exogenous TSG-6 (e.g., radiolabeled) reached the inflamed joint soon after the intravenous injection, and was detectable in the inflamed joint at least 24 hr, a significant amount of TSG-6 remained in, or returned to, the circulation.

The loss of edema in the presence of massive cellular infiltration indicated that TSG-6 affects cell/tissue-bound HA, rather than the migration of inflammatory cells or the proliferation

of synovial cells. The overnight gap in treatment resulted in a plateau in reduction of joint swelling which then further declined after repeated administration of TSG-6 protein (Figure 1A). Therefore, we hypothesized that a relatively high dose of rmTSG-6 can competitively occupy the binding sites on HA and displace CD44 in the inflamed tissue. As a result, the CD44-bound HA (and HA-bound water) became diffusible and receded from the tissue. Using *in vitro* assays with the three different cell lines, SVEC4-10 endothelial cells, mouse synovial fibroblast and 5/4 T-cell hybridoma, we demonstrated that TSG-6 can indeed compete with CD44 in a dose-dependent manner. This was further confirmed *in vivo*, when increasing doses of HA-binding peptide Pep-1[19] reciprocally reduced the effect of rmTSG-6 in short-term experiments. However, while this HA-binding synthetic peptide Pep-1competed effectively with TSG-6 *in vivo*, the peptide by itself had no effect on joint swelling. This seemingly contradictory observation indicates that Pep-1 was able to occupy free binding sites on HA, but it was unable to displace CD44 bound to HA.

The most unexpected observation after noted excessive reduction in joint swelling in a short-term treatment was the undistinguishable histopathological picture in PBS- and TSG-6-treated animals. While the reduction of edema was evident within hours (Figure 1A), and then visible in paw sections of TSG-6-treated animals (Figure 2b), there were no evident differences in cellular infiltration, synovial lining cell proliferation, nor pannus formation for up to four weeks of the experimental period. This comparable picture of synovitis was especially in contrast to the lack of cartilage and bone erosions in TSG-6-treated animals, which otherwise is a typical consequence of joint inflammation in RA and in all corresponding animal models[15,16,20,21,25].

First, we performed experiments in PGIA. We preferentially use this mouse model for genetic, inflammation, cell migration and autoimmune studies, rather than collagen-induced arthritis (CIA), another widely used systemic autoimmune model for human RA. Essentially both inflammatory models show many similarities to RA as indicated by clinical assessment of

arthritis and histopathology of the peripheral joints. However, beyond similarities, there are substantial differences between the two autoimmune inflammatory animal models for RA: PGIA shows higher susceptibility (100% in aging female mice) [15-17], the joint inflammation is more progressive leading to complete deterioration of articular cartilage accompanied with massive bone erosions [15,16,25], the onset of the disease is dictated by a shift from Th2 to Th1 response [26,36], and it has a recessive inheritance [24,37]. Many of these characteristics are comparable to those in rheumatoid arthritis. In contrast to a recently published study for TSG-6 treatment of CIA[13], we were unable to find any significant difference in pathophysiological markers (including the serum level of IL-6) between PBS- and TSG-6-treated animals, synovial cell proliferation, or in cell surface expression of various phenotypic markers. Fluctuation and differences in serum levels of various cytokines clearly associated with the disease state in both PGIA and CIA, rather than the TSG-6 treatment. We could detect the same, occasionally more prominent, differences in serum markers in leflunomide-[38], IL-4-[36,39,40], IL-10-treated[39-41] animals, or during anti-CD44 immunotherapy [14,17] as found in TSG-6-treated arthritic animals. While the TSG-6 treatment reduced the severity (edema and cartilage damage) of arthritis, it did not reduce the incidence or delay the onset of arthritis. Therefore, the anti-inflammatory effect of exogenous (human) recombinant TSG-6 associated with increased serum levels of IL-6 measured at a single and late time-point in the above mentioned study [13] may coincide with, but can hardly be the consequence of, the TSG-6 treatment. Rather, it might be the consequence of immunization of DBA/1 mice with a large dose of human TSG-6, or the LPS or IL-1 injection might be used for "synchronization" of CIA to reach a higher incidence. Although IL-6 locally may stimulate PG synthesis, IL-6 is a key proinflammatory cytokine required for the development of various forms of arthritis, including CIA [24,25,42-44]. In conclusion, it seems to be very likely that a significantly reduced incidence and delayed onset of CIA[13], without suppression of proinflammatory cytokines, antibodies or the Th1 response, should not be related to increased levels of IL-6 in TSG-6-treated animals.

To explore the role of TSG-6 during joint inflammation and understand how the morphologically intact cartilage can be preserved in the presence of massive synovial inflammation we extended our studies and used the AIA model. In this model, cartilage damage can be assessed by loss of safranin-O stained proteoglycan and, simultaneously, we can demonstrate the most characteristic, MMP-generated neoepitopes of cartilage PG (aggrecan), such as the aggrecanase-cleaved '-NITEGE' and various MMP-generated '-VDIPEN' sequences [20,21,23]. A single dose of rmTSG-6 administered simultaneously with the antigen challenge into the knee joints showed protection against the loss of cartilage PG. Therefore, the chondroprotective effect of TSG-6 directly, or indirectly, associated with the inhibition of matrix metalloproteinases, enzymes which are also upregulated by cytokines in inflammatory conditions. It is known that TSG-6 protein can bind to IαI, dramatically increasing the serine protease inhibitory effect of the IαI [8,9,11]. The protease inhibitory effect of IαI/TSG-6 complex was especially evident on plasminogen-plasmin activation, probably the most potent serine protease need for activation of pro-MMPs. Thus, TSG-6 seems to be a key component of a negative feedback loop controlling extensive tissue damage in inflammatory conditions.

A conclusive evidence that the TSG-6/IαI complex mediates the potent antiplasmin activity, will require additional studies in all arthritis models (CIA, PGIA and AIA), and the isolation of the 120 kDa complex [8,11]. While the serine protease inhibitory activity of IαI family members via their common bikunin domain has been known for a long time [47], little is known about their functions. However, disease-associated presence in various tissues and fluctuations seen in the serum levels of IαI and IαI-related protein suggest an involvement in pathologic processes. Daveau *et al* [48]. reported a distinct pattern of changes in serum concentrations of the different members of the IαI family during acute inflammation. Proteins identical with, or closely related to, the bikunin chain of IαI, have been detected in the stroma and the surrounding connective tissue of malignant tumors [49], in the brain tissue of patients with Alzheimer's disease [50] and in the serum and urine of patients with inflammatory diseases,

cancer and leukemia [51,52]. A link between arthritis and IαI was suggested over 20 years ago when Becker and Sandson [53] found IαI associated with HA in the synovial fluid of patients with RA. This finding was confirmed to show that IαI associates *in vitro* with HA isolated from the synovial fluid of healthy patients [54]. In conclusion, TSG-6 stimulated by proinflammatory cytokines may function as an anti-inflammatory component by combining and enhancing the serine protease inhibitory activity of IαI.

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Figures and Figure legends

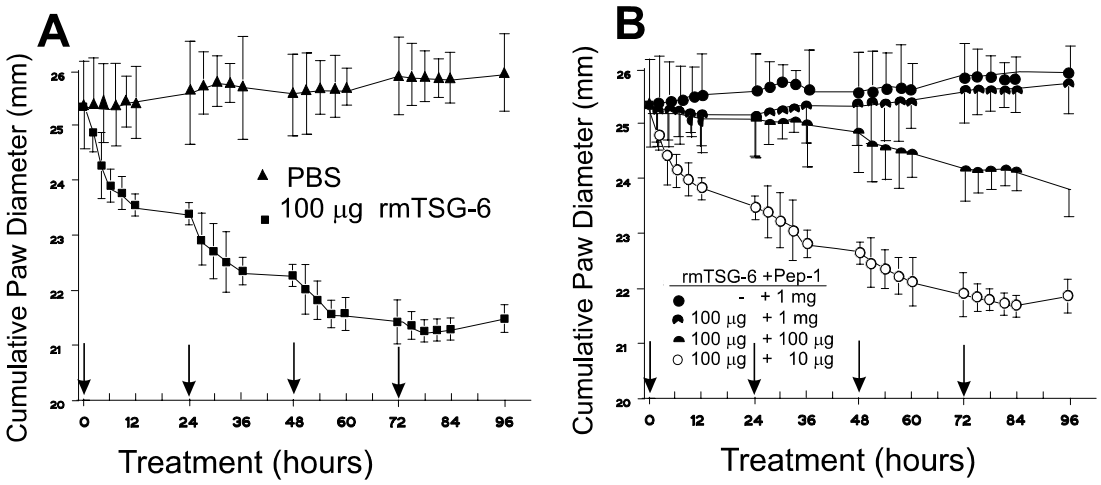

Figure 1. Short term effect of rmTSG-6 on joint swelling in PGIA mice. Animals were randomly grouped 3-7 days after the onset of arthritis (onset of swelling and redness) and each experimental group contained a comparable number of inflamed front and hind paws with approximately the same cumulative clinical scores of 8.2-8.4 ± 1.2-1.4 corresponding to 24.9-25.6 ± 1.1.-1.3 mm cumulative joint diameter. Both PBS- (control; Δ) and 100 µg of rmTSG-6-injected (■) groups contained 16 animals in 4 independent experiments. Treatments with HA-binding Pep-1 (1 mg)(●) or co-treatment of Pep-1 (10µg-1mg) and rmTSG-6 (100µg)(symbols of dose combinations are shown in figure) were repeated twice (a total of seven mice in each group). All injections were given intravenously in 100 µl PBS (arrows). Joints were measured prior to each injection (early morning) and then every 2-3 h after injections. Ankles and wrists were measured in frontal and sagittal planes, and the cumulative joint thickness[17] of the four paws (8 measurements per animal) are shown.

Figure 2. Histopathology of tarsal joints of mice with PGIA. Panels represent sagittal sections of tarsal joints of a non-inflamed paw (A), or sections of arthritic tarsal joints from PGIA mice four days (B) or four weeks after the onset of arthritis (C-D). Arthritic mice were treated with PBS (C) or with rmTSG-6 (B and D). Massive inflammation and synovial cell proliferation were characteristic of both PBS- (C) and TSG-6 (D)-treated mice, but the articular cartilage remained histologically intact after four weeks in TSG-6-treated mice (D) (animals of Figure 3). In contrast, articular cartilage was essentially lost (arrows) from the joint surface and massive bone erosion was also present in non-treated (PBS-injected) mice (C). Hematoxylin and eosin staining. Original magnification: x25.

Figure 3. Flow cytometry (FACS) analysis of competition assays for determining the ability of rmTSG-6 to compete with CD44 for HA binding. Mouse synovial fibroblasts and SVEC4-10 endothelial cells were digested with streptomyces hyaluronidase for 30 min at 37° C to remove endogenous HA and then cells were washed with PBS. All three cell lines (1 X 10^6) were incubated with fluorescein-labeled HA (20 µg/ml; 100µl final volume) and rmTSG-6 (0.04 – 5 mg/ml) or 100 µl unlabeled HA (5mg/ml) for 2 h at 37°C. Cells were washed in PBS and analyzed by FACS. The *y*-axis shows the cell number and the *x*-axis the fluorescence intensity.

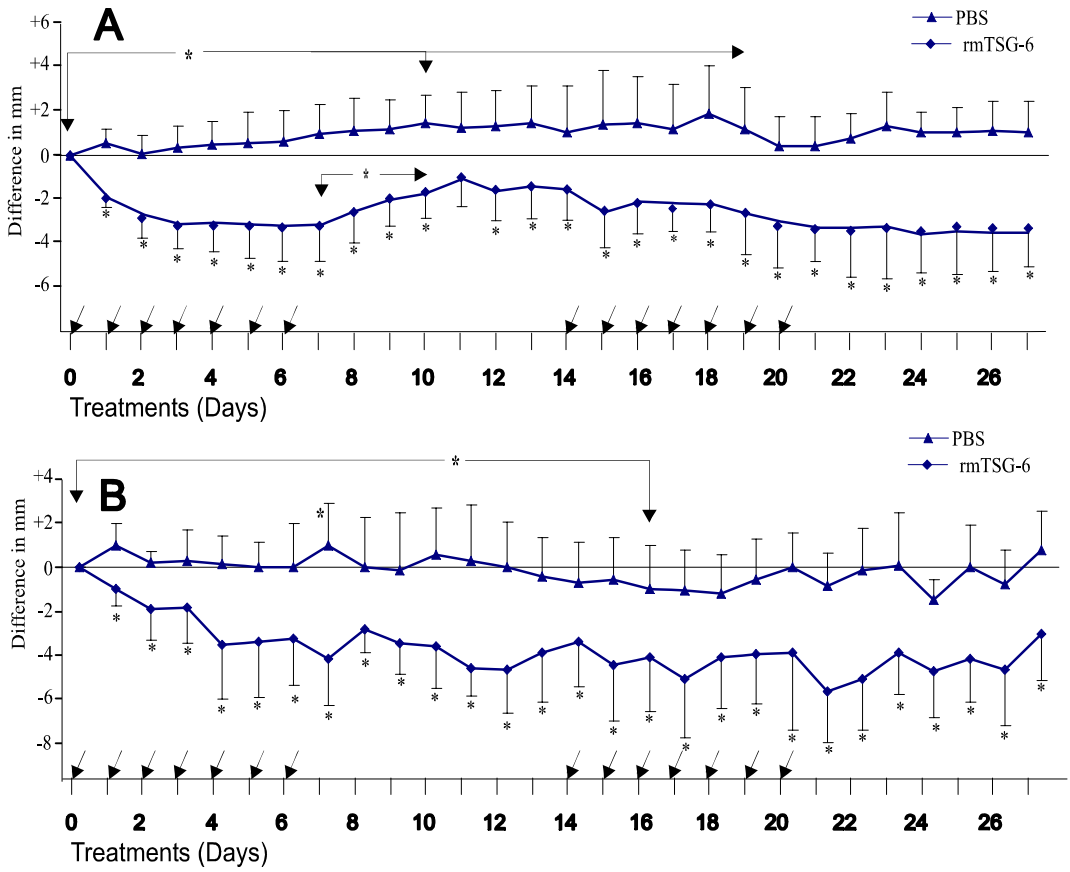

Figure 4. 'Long-term' effect of repeated treatment with rmTSG-6 on PGIA mice. Arthritic animals were injected intravenously with 100 µl of PBS (n=14) or 100 µg of rmTSG-6 (n=15). Each mouse was injected every 24 h for 7 days as indicated by arrows. Joints were measured prior to the injections and then twice per day. The treatment and cessation of therapy was repeated for another 14 days. Ankles and wrists were measured in frontal and sagittal planes and the change in cumulative joint thickness of the four paws (8 measurements) are shown. Panel **A** shows the difference of cumulative joint thickness in mm between the PBS-injected and rmTSG-6-treated group (all paws), while panel **B** shows the differences of the inflamed joint thickness only (i.e., paws were inflamed at the beginning of the TSG-6 treatment). The decrease of joint swelling in the rmTSG-6-treated group was significant within 24 h (as shown in Figure 1), and the increased joint swelling in the PBS-injected group became significant

(*p< 0.05) over the first 10-12 days. Changes in joint swelling between PBS-injected and rmTSG-6-treated groups was statistically significant (*p < 0.05 or smaller) from day 1 of the treatment and remained significant (comparing to day 0) throughout the entire experimental period.

Figure 5. Histology and immunohistochemistry of knee sections from C57Bl/6j mice on day 5 of AIA. Panels A-E represent sections from PBS-injected knees, panels F-J shows sections of knee joints injected intra-articularly with mBSA, and K-O panels are sections from knee joints injected with mBSA and rmTSG-6 together. Panels A, B, F, G, K and L are hematoxylin- and eosin-stained sections. White arrows in panels F and K show the cellular infiltration. Sections of panels C, H and M are stained with safranin-O. Panels D, I and N are sections stained with antibody

to -VDIPEN neoepitope (stromelysin-cleaved product of cartilage aggrecan). Panels E, J and O are sections stained with antibody to aggrecanase-generated -NITEGE neoepitope. Patella (P) and femoral condyle (F) are indicated. Bar on panel K indicates the scale for panels A, F and K, while bar in panel O indicates scale for all other panels.

Discussion

In our arthritis transfer system in Chapter 1. we studied the function of T and B cells and antibodies in the development of arthritis from a novel aspect of cell recovery using immunodeficient SCID mice with BALB/c background as recipients [1], (i.e. genetically matching the donor BALB/c mice) providing physiologic environment for transferred cells and their expansion in the host. We collected information about the immuno components of the disease and their functional chronology. In addition to the more explanatory discussion in Chapter 1 there are some imaginative thoughts regarding this peculiar immuno phenomenon that yet may be noteworthy.

Seldom emphasized that homeostatic repopulation takes place in many cell transfer experiments if animals are administered subnormal number of T cells, as a balancing mechanism to establish normal peripheral T cell population [2,3]. In such wise, our model represents a unique situation, showing some similarities to RA, where homeostasis and repopulation of the T cells are believed to play significant role in the development of the autoimmune imbalance. Patients in RA exhibit age-inappropriate erosion of telomeric reserves of the chromosomes in T cells in patients 20-30 years of age and marked contraction of the T cell repertoire probably due to prematurely compromised thymic cell output. The consumption of telomeric DNA indicates a higher rate of replication in the peripheral T cell compartment [4]. Abnormal T cell population dynamics i.e. impaired thymic function with increased turnover of peripheral T cells, could lead to clonal expansion of cells with higher affinity to self at the expense of those that are weakly self reactive [5]. In our case, when in addition to the cells also antigen (PG) was given to the recipient mice we accelerated the repopulation of the clones recognizing the 'self' and thus augmented the development of the arthritis. Thus we created an

RA-like pathological state with low thymic output (SCID host) and intensive peripheral proliferation (suboptimal transferred cell number – homeostatic cell replication).

The following schematic diagram illustrates how repertoire selection might be abnormal in patients with RA.

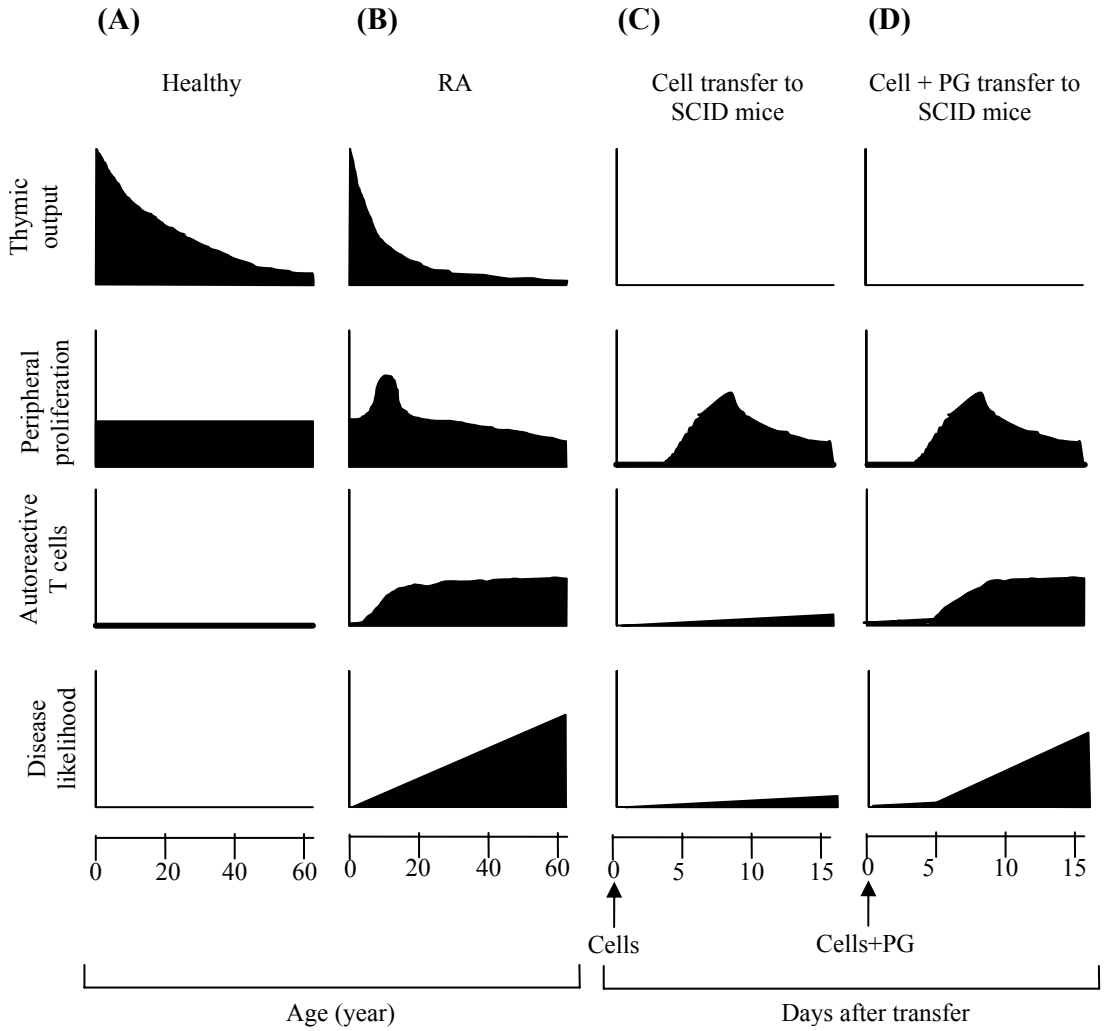

Figure 1. T cell population dynamics along the human life span in normal (healthy) (A) and abnormal-(RA) (B) conditions. Similarly, the hypothetical homeostatic T-cell repopulation is shown in adoptively transferred SCID mice (C and D). (The first two columns of the diagram are adapted with minor modifications from Reference 5.)

Using this transfer model our group is presently investigating further details in B cell recovery and antibody production, as well as immunodominant peptide epitopes of human PG and their arthritogenity. The model appears to be useful in these studies and may help the arthritis research in the future as well.

In Chapter 2 our attempt was to modify the disease and 'treat' the arthritis by intranasal PG administration. Mucosal tolerance was first described in 1911 when Wells fed hen egg proteins to guinea-pigs and found them resistant to anaphylaxis when challenged[6]. Since then it has been extensively investigated in several autoimmune diseases. The sufficiently compelling information acquired in animal models has initiated several human trials, such as in both multiple sclerosis and RA[7,8]. Currently, the basic science immunology community is quite accepting the phenomenon of mucosal tolerance induction in animals despite the unanswered questions regarding the pathways by which it occurs. In contrast, the clinical community, not surprisingly, is some agnostic regarding the mucosal tolerance, since in clinical trials progress has not been definitive and the outcomes are modest at best. In RA one way to amplify the effect of mucosal tolerance is to utilize new cartilage constituents. We found intranasally applied PG effective in suppressing PGIA. Nasally PG-treated mice developed less severe disease with lower incidence. Several more aspects and concerns of this approach, however, are still to be answered. Further studies are required to establish the ideal timing and dose of the intranasally administered protein and i.n. PG treatment should be investigated in other non PG-related animal models of RA (i.e., collagen-induced or GP39-induced arthritis). Since no major or exclusive autoantigen is identified in RA, combination of PG and type II collagen or GP39 may have additional immunomodulant effect on arthritis. In this primary study PG seemed to be effective in suppressing the autoimmune arthritis in mice and we propose PG as a potential candidate in the mucosal tolerance studies in the future arthritis research, although strong conclusions should not be made.

In our second attempt to modify arthritis in Chapter 3 we found that intravenous injection of recombinant mouse TSG-6 protein (rmTSG-6) into mice with established proteoglycan-induced arthritis dramatically reduced tissue edema within hours. Long-term systemic treatment with rmTSG-6 protected cartilage from degradation and thus blocked subchondral and periosteal bone erosion in inflamed joints. In addition, intra-articular injection

of a single dose of rmTSG-6 exhibited a strong chondroprotective effect in antigen-induced arthritis. The collected data may also suggest that TSG-6 protein, induced by either proinflammatory cytokines or injected intra-articularly, is retained by HA at the site of inflammation, and can form a complex with IαI and this process could dramatically increase the serine protease inhibitory effect of IαI [9]. As a result, serine proteases complexed with IαI, including for example plasmin [10-12], are unable to activate (latent) pro-matrix metalloproteinases (MMPs). Thus, TSG-6 could be a key component in a negative feedback loop, which reduces matrix degradation in arthritis. Figure 2. summarizes the hypothetical role of TSG-6 in the inflamed tissues.

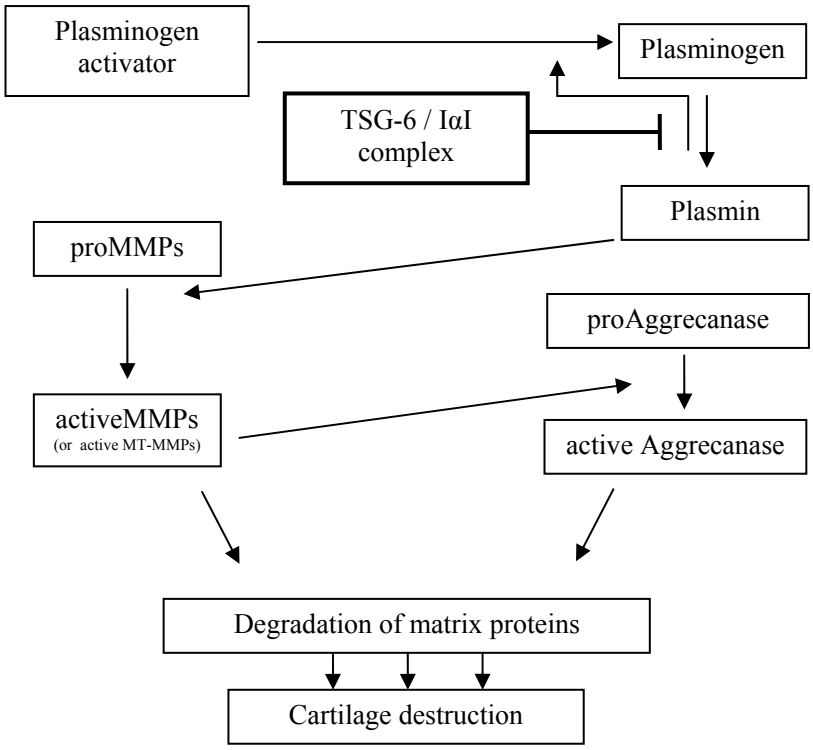

Figure 2. Schematic figure of the functional mechanism of TSG-6 in the chondroprotecion. (MMP: matrix metalloproteinase, MT: membrane type, IαI: Inter-α-inhibitor)

As a continuation of this study our group generated transgenic mice constitutively expressing TSG-6 protein in cartilage. These transgenic mice with antigen-induced arthritis were used to test the endogenous effect of constitutively synthesized TSG-6 protein in

inflammatory cartilage breakdown. We found essentially complete protection against cartilage matrix degradation for at least 5 days upon induction of AIA and 4-5 weeks after the initial challenge the cartilage was almost completely repaired in TSG-6 transgenic mice, while in the control animals the repair was only partial[13].

Based on structural homologies with certain molecules (e.g., CD44, Link protein, complement component C1s/C1r, sea urchin development protein-uEGF, or bone morphogenetic protein-BMP1), it is strongly suggested that TSG-6 protein would play a crucial role in extracellular matrix formation, inflammatory cell migration, cell proliferation, and developmental processes [14,15].

At present, our lab is intensively investigating the genetically manipulated TSG-6 knock out mice (also generated in our lab). Thus far, the lack of TSG-6 seems to induce infertility in female mice (Glant et al. unpublished data), which is in accordance with previous studies [15-17] and emphasizes the broad and vital function of TSG-6 in life.

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Significance

RA is a systemic autoimmune disease of unknown etiology with the main manifestation of acute and chronic inflammation in multiple joints. The major goals in arthritis research are to explore the pathogenesis of rheumatoid arthritis and define targeted therapies, which can selectively inhibit cartilage degradation.

In this thesis, firstly the development of autoimmune arthritis was studied from a new aspect. We established an adoptive transfer of PGIA into SCID mice, used purified (depleted) cells for the transfer and investigated cell recovery in the host along the course of the disease. We found T helper (especially Th1) cells essential in the development of the disease. Furthermore, we showed that T helper cells play crucial role in the facilitation of antibody production (highly correlating with the arthritis). We demonstrated that mouse PG (autoantigen) can induce arthritis in mice and thus it could be the suspected ultimate target molecule in PGIA.

The clinical trials of mucosal tolerance to type II collagen in RA have achieved only moderate progress, and different ways to amplify the effect of mucosal tolerance should be investigated (e.g., use of new cartilage constituents). We found intranasally applied PG effective in suppressing PGIA, since nasally PG-treated mice developed less severe disease with lower incidence. Using genetically-matched recipient SCID mice we were able to transfer mucosal tolerance, but continuation of nasal administration of PG was inevitable. In contrast, we could not induce unresponsiveness to PG in the already established arthritides. Our results make it clear that the treatment of already existing autoimmune arthritis may result in contradictory outcomes.

In our third study using TSG-6 locally (intraarticularly) and systemically (intravenously) as a therapeutical agent we found that it has a definite protective effect against cartilage degradation in heavily inflamed joints. In addition, systemically administered TSG-6 reduced the edema in the acute phase of arthritis as demonstrated in PGIA. TSG-6 in both AIA and PGIA had no effect on the migration of inflammatory cells in the joint space. These appear to be important observations, providing new insights into a novel therapeutical approach to cartilage protection in RA.

I hope and believe that my work on the animal model of human rheumatoid arthritis has helped to get closer to the answer why and how autoimmune arthritis really develops and so maybe one day we can offer the real solution to this still fairly unknown disease utterly wrecking so many lives.

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2. Bárdos T, Kranicz J, Czipri M: Experience with 900 operatively treated club feet. 51st Annual Meeting of the Hungarian Orthopaedic Association, 1999, Kaposvár, Hungary

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PhD Dissertation
2002

Distinct Immunological Factors in Proteoglycan-Induced Arthritis

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