Investigating the Genetic Background of Proteoglycan-Induced Arthritis and Proteoglycan-Induced Ankylosing Spondylitis

PhD dissertation thesis

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Introduction

Rheumatoid arthritis and its experimental animal models

Rheumatoid arthritis (RA) is one of the most common human autoimmune diseases, characterized by the chronic inflammation of the synovium of diarthrodial joints with a female predominance. Although the etiology of RA is still unknown, accumulating evidence indicates that it is a T cell-mediated and autoantibody-dependent disease in which both genetic and environmental factors play crucial roles (1-3). Cell-mediated immune response and autoantibodies to cartilage proteoglycans (PG) (4-5) and/or collagen type II (5-7) 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.

Antigen-induced arthritis

Among the animal models of RA, the third category is the antigen-induced arthritis. However many species (rabbits or Guinea pigs) can be used in the conduct of antigen arthritis studies, the most common are immunizing susceptible mice (BALB/c and some C3H substrains) with human cartilage PG or with the G1 domain of aggrecan or versican, causing progressive polyarthritis (13-15) called Proteoglycan-Induced Arthritis (PGIA), which is frequently associated with spondylitis resembling human ankylosing spondylitis (AS). This mouse model shares many features with the 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 (13). In the beginning, perivascular concentration of mononuclear cells accumulates, followed by intensive proliferation of synovial macrophages and fibroblasts. The arthritis starts as a polyarticular synovitis in bilateral, small peripheral joints and progrediates with extensive destruction of cartilage and bone within the joint. Initially, the clinical

signs of joint inflammation (swelling and redness) appear after the third or fourth intraperitoneal injection of antigen (Fig.1).

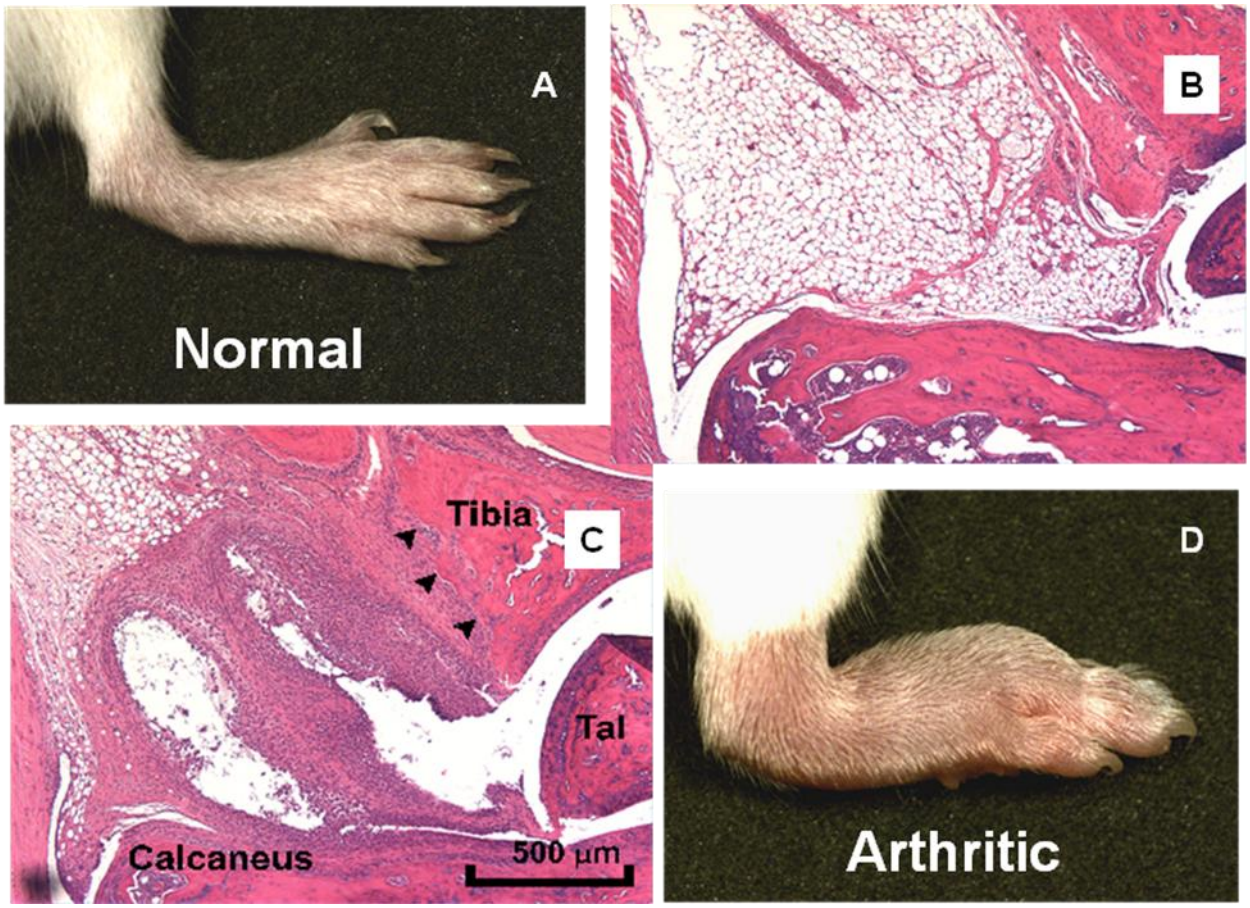


Figure 1. Photo image of a naïve control BALB/c mice's hind paw (A), and the Hematoxylin – eosin (HE) stained histology picture of a normal ankle-joint (B), comparing with an inflamed paw, which is swollen, red and very painful (D). HE- histology slide shows (C) massive inflammatory cell invasion resulting cartilage destruction and bone destructions on the surface of the tibia (arrowheads).

Spine involvement in PGIA

One of the most remarkable “side-effect” of the PGIA is that arthritis susceptible BALB/c mice also specifically develop spondylitis, called proteoglycan-induced spondylitis (PGIS). Similarly just like the human autoimmune disease Ankylosing Spondylitis (AS), both PGIS and AS are progressive in nature and involvement typically begins in the sacroiliac joints; initially with erosion of the cartilage surfaces than later the pannus like inflammatory cell invasion, which result in complete bony ankylosis. In genetically susceptible mice, obvious signs of inflammation can be observed and confirmed with histopathology as early as the 1st to 3rd week post-antigen injection. Development of bony ankylosis occurs during 1st and 3rd month and results in the classic bamboo spine appearance (Fig 3.). In an earlier study of the F2-hybrid population of BALB/c and DBA/2 intercrosses, our group managed to identify a murine spondylitis severity and susceptibility loci through a genome-wide screening [Vegvari et al 2005 (16)]. One loci in particular, found in DBA/2 murine strain on chr. 18 appeared to be resistant peripheral arthritis even after massive consecutive i.p. injections of antigens.

Aim of the study

The antigen induced murine model of rheumatoid arthritis, PGIA, is a useful way to investigate experimentally induced arthritis due to the fact it diverse capabilities to simulate several characteristics of the human disease, such as cartilage destruction or invoke the immune responses. Our goal was; #1 ascertain an explanation of the clinical-pathological changes which we observed after the consecutive immunization of genetically susceptible and resistant mice, #2 to find non-invasive methods to assess and diagnose early inflammatory reactions, and derived from that, to explore which genes are suppose to be controlling the inflammatory reactions around the peripheral joints and the IVDs.

In Chapter 1 by using the PGIA model we performed a multiple comparison of the inbred BALB/c colonies of North-America by investigating the (i) susceptibility, progression, and the severity of peripheral arthritis, and the (ii) involvement of the spine, described by the IVD affectivity of eleven BALB/c colonies, all purchased from different vendors. Moreover (iii) to find immune markers that correlate with the clinical status and (iv) to identify the genes that control the immune system and which can explain our findings of the dramatic clinical diversity during the identically match twin comparisons.

In Chapter 2 the purpose of the spondylitis study was (i) to investigate the PGIS susceptibility of the PGIA resistant DBA/2 mice in a six month long longitudinal study, (ii) to find immune markers corresponding with AS severity and/or progression. Beyond that (iii) to identify genes responsible for controlling the inflammation around the IVD and (iv) to asses early inflammatory events around the disc with a non-invasive method by using *in vivo* immuneflourescent agents.

Chapter 1

BALB/c mice genetically susceptible to proteoglycan-induced arthritis and spondylitis show colony-dependent differences in disease penetrance

Arthritis Research And Therapy 2009 Feb;11(1):R21.

Introduction

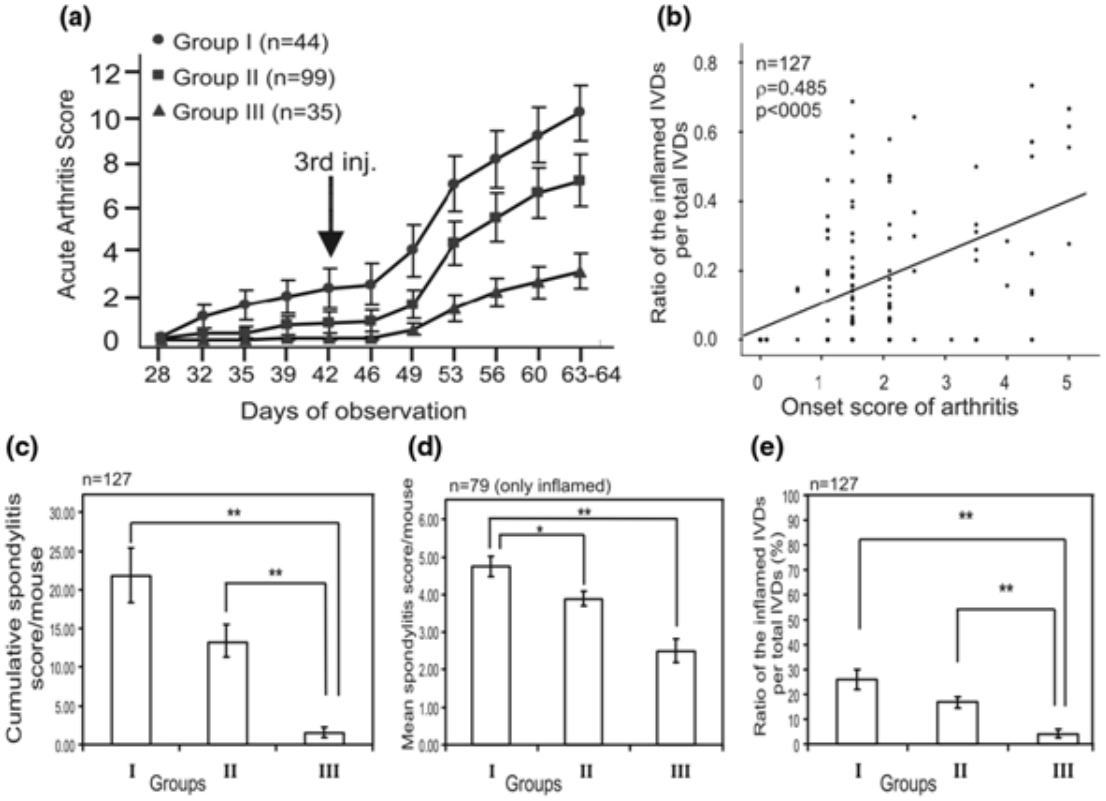
Due to a specific genetic background, the BALB/c strain shows strong predisposition toward arthritis. Despite the efforts of companies to maintain genetically homogenous inbred colonies, there are differences among BALB/c colonies/substrains (e.g., in body weight, size of littermates, the composition of intestinal bacterial flora) maintained at different locations by the same vendor. According to The Jackson Laboratories' online public database, there are at least 492 single nucleotide polymorphism (SNP) differences between their two inbred BALB/cJ and BALB/cByJ colonies, of which at least 59 SNPs are present in 33 immune-regulatory genes in the mouse genome (authors' unpublished *in silico* analysis data). Some of these known, or yet unknown, mutations may significantly influence the pathogenesis and progression of PGIA or PGIS.

However, we and others observed differences in the onset, incidence and severity of arthritis, even when the source of antigen (e.g., prepared in our laboratory) and immunization protocols were the same. Therefore, either local environmental components or the source of BALB/c colony might account for the different levels of susceptibility to, or severity of, PGIA. Because environmental factors also play critical roles in RA susceptibility, and different BALB/c colonies may have different panels of spontaneous mutations, it has become necessary to test these components under uniform conditions. In the present study, we investigated the disease parameters simultaneously in various colonies of BALB/c mice in the same experimental setup.

Results

Susceptibility, severity and onset of arthritis in different BALB/c colonies

Based on the visual scoring system [19], and later confirmed by histology, we could sort the 11 BALB/c colonies into three major groups. There were no statistical differences in arthritis severity and onset time within any of these three groups. Compared to group II, group I (colonies 1-3, Table 1) comprised the most susceptible substrains, which developed arthritis earlier and with greater severity than any other colonies. In contrast, group III (colonies 10- 11, Table 1) showed the least severe arthritis (mean arthritis score 3.0 ± 0.6) with delayed onset time (1.0 ± 0.2), and, approximately 30% of the immunized animals did not have arthritis at the end of the experiment.



Progression and severity of arthritis in 11 BALB/c colonies sorted into three different groups (listed in Table 1), correlation between the onset of arthritis and spine involvement, and comparison of the three arthritic groups with different spine inflammation scores.

Histopathology of the spine

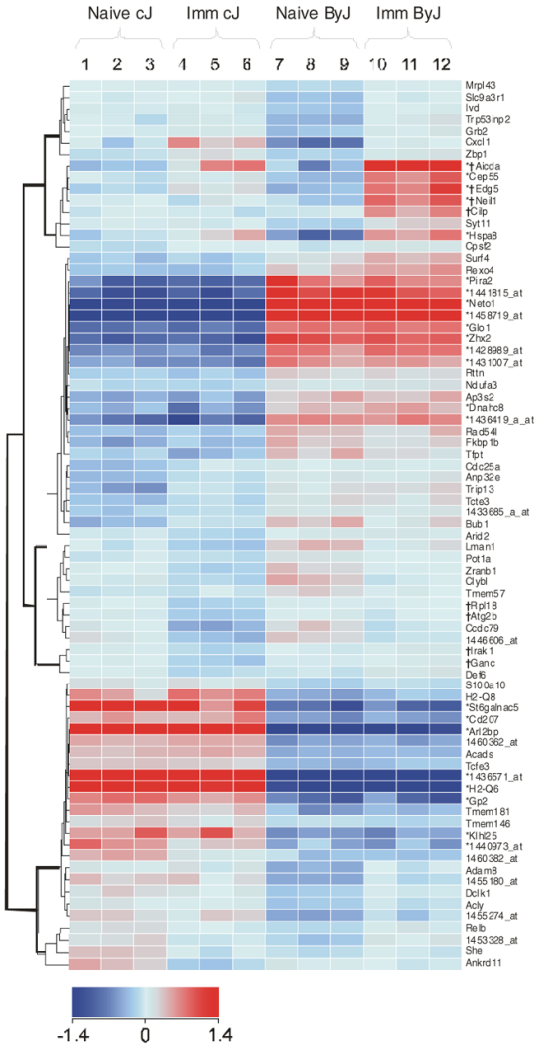
A total of 127 spines were formalin-fixed, X-ray imaged and then processed for histological analysis, spondylitis was diagnosed in 62.2% of BALB/c mice, which was significantly lower ($p<0.05$) than the mean of arthritis incidence (86.5%; n=178). This observation confirmed that arthritis and spondylitis could occur either together or separately in BALB/c mice [9], and most likely different genes of different QTLs control PGIA and PGIS. Although no PGIS-resistant BALB/c colony was found, there were large individual variations in the spine involvement. In addition, neighboring IVDs of the same animal frequently showed different stages of inflammation. Typically, the most affected spine segments were the distal lumbar and distal cervical regions, whereas the IVDs in the thoracic and proximal lumbar regions remained less affected.

T and B cell-mediated immune responses

Despite screening a wide spectrum of immunological parameters we could not identify "colony specific" cytokine, T- or B-cell responses. *In vitro* tests (T cell proliferation and cytokine production) showed evidence of T cell activation in response to PG-stimulation, but it did not correlate significantly with either arthritis or spondylitis scores. Next, we compared arthritis- and spondylitis-"specific" immune markers between the three groups of the clinical phenotypes. We compared serum antibody, cytokine, and antigen-specific *in vitro* T cell responses of 154 arthritic animals with 24 immunized, but yet non-arthritic mice.The incidence of PGIA in the three major groups was as follows: 98% in group I, 85% in group II, and 40% in group III. Although there was a trend, we found that none of the *in vitro*-measured T cell activation markers (antigen-specific T cell proliferation and cytokine production) correlated significantly with the clinical phenotype (severity) or histological results of arthritis. In contrast, IgG1 and IgG2a (auto)antibodies were significantly higher in arthritic than in non-arthritic animals

Microarray results

We compared the gene expression profile of splenocytes of these two colonies prior to, and then 12 days after the first PG injection, when the initial immune responses are detectable, but there is no arthritis. Hierarchical clusterization panel showed, a total of 77 genes were expressed at significantly different levels between naïve and immunized BALB/cJ and BALB/cByJ age-matched female mice. Twenty-three genes showed higher than two-fold differences, and 11 of the 77 genes were described as immune response genes or associated with arthritis (Hierarchical clusterization is shown below, comparing the 77 genes expressed differently at significant levels).



Chapter 2

A New Model of Spondyloarthropathy: Arthritis-Resistant DBA/2 Mice Develop Autoimmune Ankylosing Spondylitis

Submitted to Annals of Rheumatic Diseases

Introduction

Immunization of genetically susceptible BALB/c mice with human cartilage proteoglycan (PG) induced autoimmune progressive polyarthritis (designated PG-induced arthritis; PGIA). PGIA is frequently accompanied with spondyloarthropathy, which is similar to human AS (PG-induced spondylitis; PGIS), but most likely different genes control arthritis and spondylitis. Genome-wide studies identified two major non-MHC genetic loci regulating PGIS in mice. One of the most dominant loci (*Pgis2* on chromosome 18) derived from BALB/c, whereas the other dominant locus (*Pgis1*, chromosome 2) was inherited from DBA/2 strain. This observation raised the question whether the arthritis-resistant DBA/2 strain, having the same and appropriate MHC (H-2d) as the PGIA- and PGIS-susceptible BALB/c strain, was susceptible to PGIS. During this longitudinal study we applied a detailed histopathological grading system of the progression of spondyloarthropathy, and a combination of high resolution x-ray with near-infrared (NIR) fluorescence to monitor disease progression *in vivo*, and performed an initial DNA microarray study which may guide the direction of future studies.

Results

Incidence and severity of PGIA and PGIS in inbred mice

Whole-body radiographs were carried out approximately every second week, but the axial involvement (narrowing of the sacroiliac joint or intervertebral space) was detected *in vivo* as early as on day 74-78 after the first PG-injection, which was then confirmed by histology (Figure

1). Throughout the 186-day-long observation period, 76.1% DBA/2, and 95% BALB/c mice developed spondylitis, but no sign of inflammation was found either in the peripheral joints or axial skeleton of B6 mice (negative control). *In vivo* bone remodeling was detected with NIR fluorescence images using OsteoSense^{TM} 750 probe as early as after the third intraperitoneal PG injection, and found to be more progressive after the fourth immunization in DBA/2 mice, allow us to visualize *in vivo* osteoblast activities.

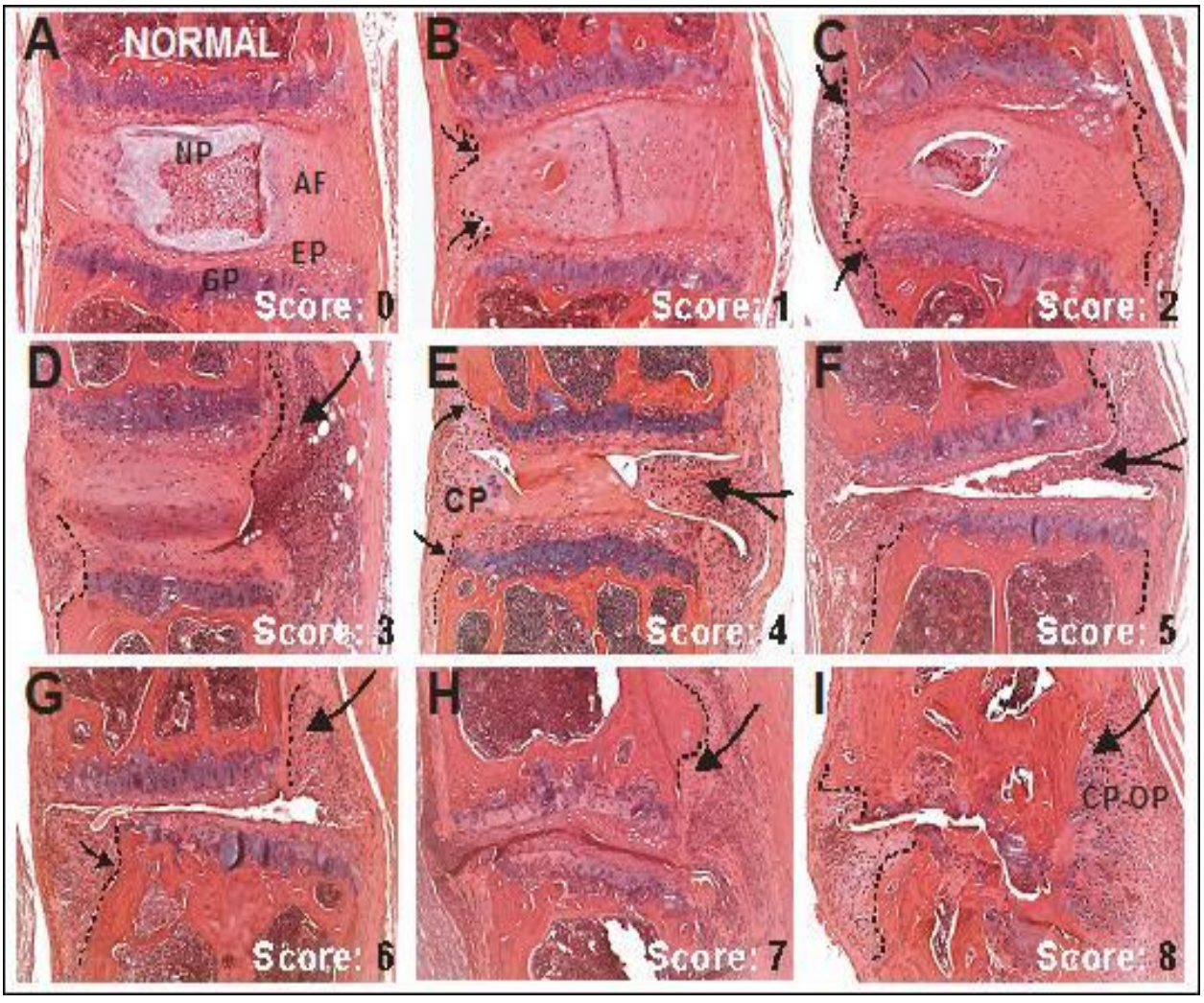


Figure 1 Histopathological scoring system of intervertebral discs (IVDs). Histology images of hematoxylin and eosin-stained sections (original magnification: 10x) of normal and inflamed IVDs.

T and B cell responses

Despite a wide spectrum of immune parameters (Table 1) measured at each time point throughout the study, neither *in vivo* (serum) nor *in vitro* (PG-stimulated spleen cells) cytokine productions, or serum antibody levels, showed correlation with the severity of spine involvement in DBA/2 mice. Although the serum pro-inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-4 levels were higher in DBA/2 than in BALB/c mice, the Th1 dominance, expressed as IFN-γ/IL-4 ratio, was significantly higher ($p<0.01$) in BALB/c than in DBA/2 mice (5.74 vs. 2.10). Based on the extended analysis of the data, our result suggests that PGIS is more like a T cell-dependent (possible Th2) than antibody-mediated disease. To find a strain-specific immune marker of PGIS we compared the immune parameters of the 35 DBA/2 mice having spondylitis with those (n=11) that did not develop spondylitis by the end of the experimental period. The serum levels of IL-17 in spondyloarthropathic DBA/2 mice were significantly higher (14.52 ± 2.41 pg/ml) ($p<0.05$) than in asymptomatic DBA/2 animals (3.54 ± 3.13 pg/ml).

Microarray analysis of IVDs from mice with spondyloarthropathy

To identify genes potentially involved in PGIS, we performed microarrays using RNA samples isolated from IVDs in the earliest and most affected areas (L1-L6). Comparing all differentially expressed genes in these IVDs of naïve versus spondyloarthropathic BALB/c mice, a total of 58 genes showed significant differences at expression levels, of which 16 genes were at least two-fold up- or down-regulated, and 9 genes had no known or ONLY expected function. Functional gene classification identified several major clusters of biological activity. They were genes which encode immune and/or inflammation-associated proteins, differentiation markers, cell surface receptors (including cytokine/chemokine receptors, transcription factors, and adhesion/cell migration molecules).

Significance

RA and AS are systemic autoimmune diseases with unknown etiology, resulting acute and chronic inflammation of multiple joints. The major aim of research in arthritis and spondylitis is to achieve understanding of the pathogenesis and identify which genes are responsible for the clinical manifestation of the diseases and therefore create the possibility for thus enables targeted therapies, which can selectively inhibit cartilage degradation.

In the current thesis, firstly a multiple comparison was carried out within the same RA and AS susceptible BALB/c mouse strain collected from all the available certified vendors from North-America. Based on the clinical manifestation of RA and the histopathological analysis of the IVDs we categorized these theoretically genetically identical twin mice into three significantly different groups of susceptibility, severity and progression. We also investigated the possible differences within their immune responses by analyzing a wide spectrum of serum antibodies and levels of cytokines, furthermore we performed a microarray analysis of spleen cell culture cells in the high and the less susceptible group's samples and than compared them to demonstrate the presence of immune markers in correlation of disease progression and severity

Then we focused on the DBA/2 mice which are MHC matching with the RA and AS susceptible BALB/c mice. Previous studies carried out by our group implicated that one of the PGIS severity loci is derived from the arthritis resistant DBA/2 mouse strain by using F2 hybrid intercrosses. Based on that information a longitudinal, prospective multiple comparison study was carried out, proving that despite DBA/2 mice are resistant to peripheral arthritis, they are definitely susceptible to PGIS, even if the involvement was not as rapidly progressive like in the BALB/c mice. Using *in vivo* flourescent agents we isolated inflamed and normal IVDs, and performed a microarray analysis and identified several genes which are at the initiation phase of disease are up or down regulated.

Across the word countless number of people are afflicted with RA and AS as the complex puzzle of these autoimmune diseases still remains to be solved. I firmly believe my research conducted on the animal model of RA and AS has contributed towards revealing this perplexing mystery of autoimmune disease in the hope that one day in the future a solution will be achieved and we may envisage a decline in intense pain and disability of sufferers across the globe.

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Peer-reviewed

1. B Farkas, F Boldizsar, O Tarjanyi, A Laszlo, K Mikecz and T T Glant: A New Model of Spondyloarthropathy: Arthritis-Resistant DBA/2 Mice Develop Autoimmune Ankylosing Spondylitis.

Additional podium and poster presentations:

1. Katalin Mikecz, Eva Bajnok, Istvan Gal, Balint Farkas, Tibor T. Glant, Gabor Hutas). Drastic inhibition of leukocyte recruitment and synovitis by anti-CD44 or anti-granulocyte treatment in a murine model of rheumatoid arthritis. Poster presentation at the ACR/AHRP Scientific Meeting, 2007, Boston, MA

2. Keith M. Hamel, Paul D. Doodes, Yanxia Cao, Yumei Wang, Rachel Rodgero, Balint Farkas, Lieping Chen, Alison Finnegan: Non-lymphoid Expression of B7-H1 Regulates Severity of Proteoglycan-Induced Arthritis. Poster presentation at the ACR/AHRP Scientific Meeting, 2008, San Francisco, CA
3. Ferenc Boldizsar, Oktavia Tarjanyi, Balint Farkas, Katalin Mikecz, Tibor T. Glant: Th1 and Th17 Cells Dominate in The Peritoneal Cavity of BALB/c Mice During The Initiation Phase of Proteoglycan –Induced Arthritis (PGIA). Poster presentation at the ACR/AHRP Scientific Meeting, 2008, San Francisco, CA
4. Paul D. Doodes, Keith M. Hamel, Yanxia Cao, Yumei Wang, Rachel Rodgero, Balint Farkas, Alison Finnegan: IL-17 Promotes Proteoglycan-Induced Arthritis When IFN-γ is Impaired or Ablated. Poster presentation at the ACR/AHRP Scientific Meeting, 2008, San Francisco, CA

5. Balint Farkas, Ferenc Boldizsar, Oktavia Tarjanyi, Aaron Mangold, Anna Laszlo, Katalin Mikecz, Tibor T. Glant: A New Model of Spondyloarthropathy: Arthritis-Resistant DBA/2 Mice Develop Autoimmune Ankylosing Spondylitis. Oral presentation at the ACR/AHRP Scientific Meeting, 2008, San Francisco, CA

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