PhD Dissertation

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

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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. A putative figure of human RA summarizes the hypothetical immune mechanisms suggested to be involved in this disease (Fig.1).

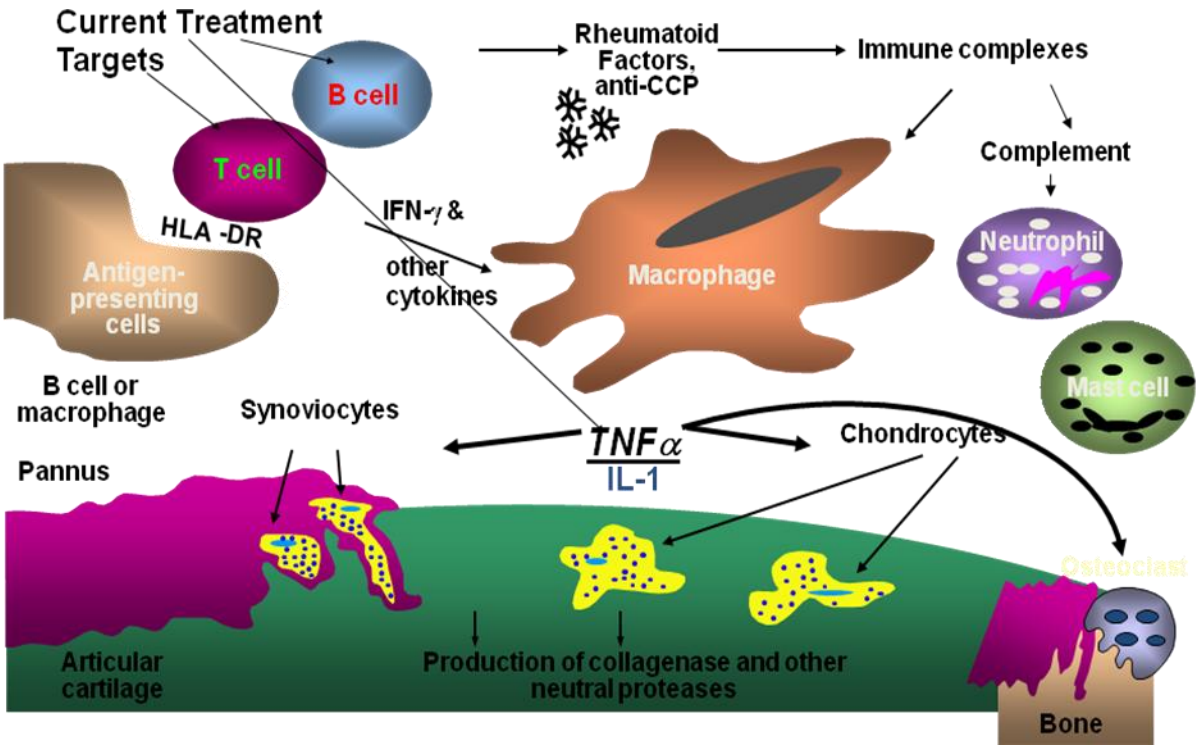

Figure 1. : Immunopathogenesis of rheumatoid arthritis [schematic figure is adapted with minor modifications from Arend WP, Dayer JM. *Arthritis Rheum*. 1990;33:305–15 (8)]

Animal models are remarkably aids in the research of human autoimmune diseases, and there are numerous systemic animal models, mostly genetically altered rodents, which involve joint pathology. One of them is the adjuvant-induced arthritis, which is an experimental model of polyarthritis in rats. Induction of adjuvant disease can be done with either Freund's complete (FCA) supplemented with mycobacterium or by injection of the synthetic adjuvant N,N-dioctylddecyl-N', N-bis (2-hydroxy-ethyl) propanediamine (LA) (9). The injection can be administered i.v. or directly into the footpad.

Collagen-induced arthritis

In another animal model the Collagen-induced arthritis (CIA), inflammation occurs when susceptible strains of rats (10), mice (11) or monkeys (12) are immunized with native type II collagen in complete or incomplete Freund's adjuvant. CIA can be induced in susceptible DBA/1 strain of mice by native heterologous type II collagen (11). In the initial phase of CIA, fibrin deposition occurs and anti-collagen antibodies bind to the joint cartilage resulting in activation of 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 and characterized as a pannus associated cartilage destruction. Autoreactive T cells, as well as B cells, which produce antibodies to type II collagen, are suggested to play critical roles in disease progression.

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.2).

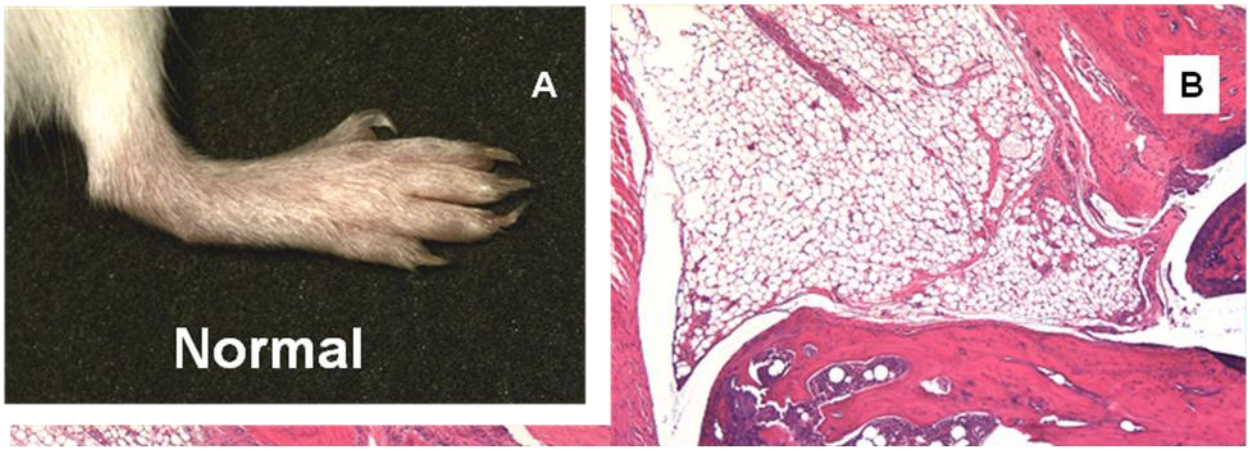

Figure 2. : Photo image of a naïve control BALB/c mice's hind paw (A), and the Hematoxylin – eosin (HE) stained histology picture of a normal ankle-joint (B), comparing with an inflamed paw, which is swollen, red and very painful (D). HE- histology slide shows (C) massive inflammatory cell invasion resulting 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.

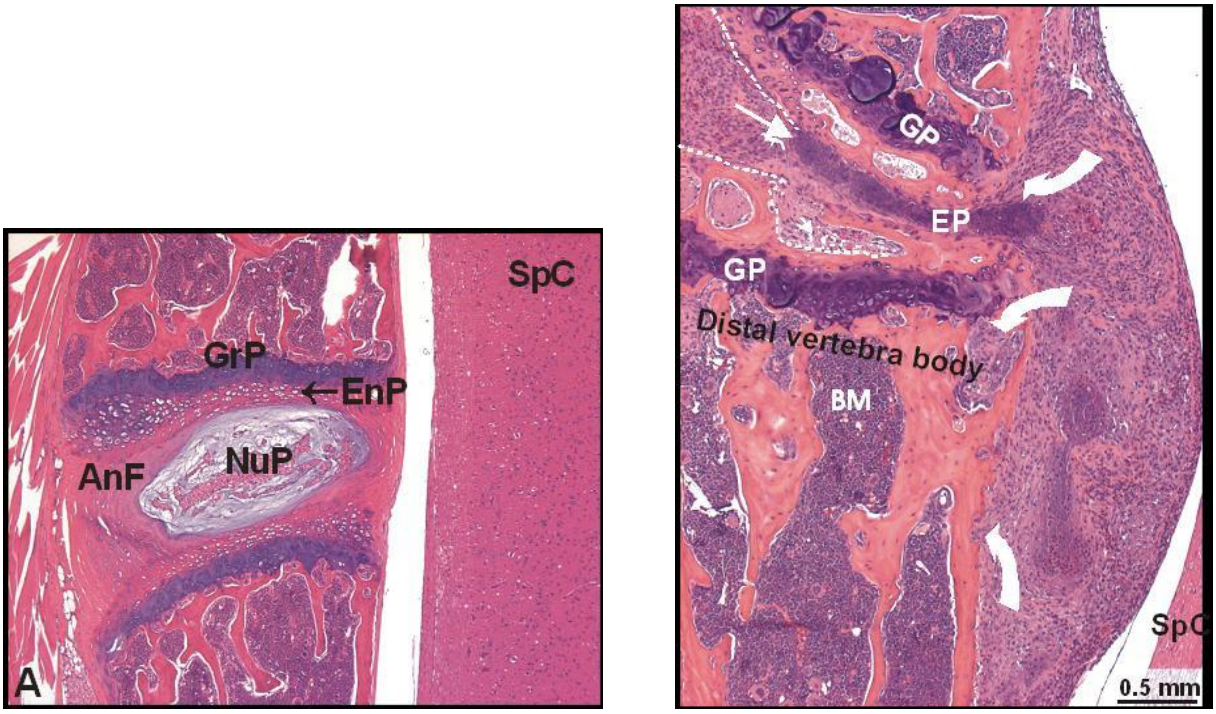

Figure 3. On the left, high resolution image of a normal intervertebral disc (IVD) with HE staining. Between the vertebral bodies the nucleus pulposus (NuP) can be found surrounded by the annulus fibrosus (AnF). The growth plate (GrP) is located at the top and the bottom of the vertebral bodies and covered with a single layer of cartilageous cells on them, called the end plate (EnP). On the right, an inflamed IVD is displayed where an aggressive mononuclear cell invasion mounted up against the disc and the cartilages

surfaces, resulting bony ankylosis and the classic “bamboo-spine” appearance. The inflamed disc often pressures the spinal cord (SpC).

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.

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Chapter 1

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

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Abstract

Introduction: The MHC (H-2d) and non-MHC genetic background make the BALB/c strain highly susceptible to inflammatory arthritis and spondylitis. Although different BALB/c colonies develop proteoglycan (PG)-induced arthritis (PGIA) and spondylitis (PGIS) in response to immunization with human cartilage PG, they show significant differences in disease penetrance, despite being maintained by the same vendor either at the same or different location.

Methods: BALB/c female mice (24-26 weeks old after 4 weeks acclimatization), were immunized with a suboptimal dose of cartilage PG to explore even minute differences among 11 subcolonies purchased from five different vendors. Serum cytokines and (auto)antibodies, *in vitro* measured T cell responses were correlated with arthritis (and spondylitis) phenotypic scores. cDNA microarrays were also performed using spleen cells of naïve and immunized BALB/cJ and BALB/cByJ mice (both colonies from The Jackson Laboratories) which represent the two major BALB/c sublines.

Results: The 11 BALB/c colonies could be separated to high (n=3), average (n=6) and low (n=2) responder groups based upon their arthritis scores. While the clinical phenotypes showed significant differences, only a few immune parameters correlated with clinical or histopathologic abnormalities, seemingly none of them affected differences found in altered clinical phenotypes (onset time, severity or incidence of arthritis, or severity and progression of spondylitis). Affymetrix assay explored 77 differentially expressed genes (at $p<0.05$ significant level) between the Jackson's BALB/cJ (original) and BALB/cByJ (transferred from the NIH). Fourteen of the 77 differentially expressed genes had unknown function; in which 24 of 77 genes showed over two-fold differences, and only 8 genes were induced by immunization, some in both colonies.

Conclusions: Using different sub-colonies of the BALB/c strain, we can detect significant differences in arthritis phenotypes, single nucleotide polymorphisms (SNPs), and a large number of differentially expressed genes, even in non-immunized animals. A number of the known genes (and SNPs) is associated with immune responses and/or arthritis in this genetically arthritis-prone murine strain, and a number of genes of yet unknown function may affect or modify clinical phenotypes of arthritis and/or spondylitis.

List of abbreviations: AS = ankylosing spondylitis; ELISA = enzyme-linked immunosorbent assay; DDA = dimethyldioctadecyl-ammonium bromide; IFN-γ = interferon-gamma; IL-1β, IL-2, etc. = interleukin-1β, interleukin-2, etc; IVD = intervertebral disc; MHC = major histocompatibility complex; NCI = National Cancer Institute; PG = cartilage proteoglycan aggrecan; PGIA = PG-induced arthritis; PGIS = PG-induced spondylitis; QTL = quantitative trait locus; RA = rheumatoid arthritis; SNP = single nucleotide polymorphism; TNF-α = tumor necrosis factor alpha.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease which leads to inflammatory cartilage destruction and bone erosion in synovial joints. Although the pathologic mechanism of RA is unknown, both environmental and genetic factors are thought to be involved in the etiology and pathogenesis of the disease [1]. Animal models are invaluable aids in the research of human autoimmune diseases, especially those that involve joint pathology in genetically altered rodents [2-6]. Among the systemic animal models of RA, cartilage proteoglycan (PG) aggrecan-induced arthritis (PGIA) is a T cell-dependent and autoantibody/B cell-mediated disease in BALB/c mice, which is frequently accompanied with spondylitis [7-10]. In addition to the major histocompatibility complex (MHC), PGIA and PG-induced spondylitis (PGIS) are controlled by multiple genetic loci [9,11]. Although various non-MHC genetic loci (quantitative trait loci, QTLs) may contribute to disease, different combinations of these QTLs may result in a remarkably uniform clinical phenotype of arthritis [12]. Due to a specific genetic background, the BALB/c strain shows strong predisposition toward arthritis. In addition to PGIA, immunization with cartilage link protein [13] or human cartilage glycoprotein-39 (HC-gp39) [14] can also induce arthritis but only in BALB/c mice. Moreover, interleukin-1 (IL-1) receptor antagonist protein-deficient mice [15], and SKG mice, in which a spontaneous point mutation occurred in ZAP-70 develop spontaneous arthritis [16], both only in BALB/c background.

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 (http://www.informatics.jax.org), 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.

Since we have "simplified" the model by replacing the highly purified human fetal cartilage PG [7,17] with PG isolated from human osteoarthritic cartilage [18,19] and changed the Freund's adjuvants to a synthetic adjuvant [20], the PGIA/PGIS model became available to a wide range of applications including the testing of new pharmacological agents. 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 [21], 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 (onset time, susceptibility, severity and progression) simultaneously in various colonies of BALB/c mice in the same experimental setup.

Because the BALB/c strain is highly susceptible to PGIA (and PGIS), and sooner or later all immunized animals develop arthritis independently of the colony source, we used a suboptimal dose of PG antigen to be able to monitor even minute differences among the colonies.

MATERIALS AND METHODS

Chemicals, antigen, animals and immunization of mice with cartilage PG aggrecan

All chemicals, unless otherwise indicated, were purchased from Sigma Chemical Co. (St. Louis, MO) or Fischer Scientific (Chicago, IL). Mouse-specific cytokine enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN) or BD Biosciences (San Jose,CA). Cartilage specimens from knee joints were obtained from osteoarthritic patients undergoing joint replacement surgery. The use of human cartilage for PG isolation was approved by the Institutional Review Board of Rush University Medical Center (RUMC), Chicago, IL. PG isolation has been described earlier in detail [18,19]. All animal experiments were approved by the Institutional Animal Care and Use Committee (RUMC). Animals were maintained in a pathogen-free environment in the same room and rack. A total number of 178 (retired breeder) female BALB/c mice of 11 colonies (Table 1) were ear-tagged, and registered mice (all 24-26 weeks old) were randomly mixed, left for acclimatization to the local environment for 4 weeks prior to the first immunization. Mice were injected intraperitoneally with a "suboptimal" dose of human cartilage PG aggrecan (equivalent to 75 μg, instead of the “standard” dose of 100 μg core protein of PG) emulsified with 2 mg of dimethyldioctadecyl-ammonium bromide (DDA) adjuvant in 200 μl of phosphate-buffered saline (pH 7.4). DDA is a synthetic adjuvant with positively-charged micelle-forming hydrophobichydrophilic (detergent-like) properties, and does not contain mineral oil or mycobacterial components as Freund’s adjuvants do [20]. Intraperitoneal injections were given on days 0, 21, and 42 of the experiment, and mice were sacrificed on days 63-64. The goal of using a suboptimal dose of cartilage PG aggrecan was necessary, otherwise all animals develop arthritis with high arthritis score after the third PG aggrecan (100 μg) injection in DDA [19,20]. Approximately half a year later, these experiments were repeated using Jackson's BALB/cJ and BALB/cByJ strains, Charles River's Portage P08 and Hollister H42 as well as NCI (Kingston) BALB/c colonies (10-15 animals per group).

Clinical and histological assessment of arthritis and spondylitis

Arthritis severity was determined using a visual scoring system based on the degree of swelling and redness of the front and hind paws [7,17,19]. Animals were examined at least three times a week and inflammation was scored from 0 to 4 for each paw, thus resulting in a cumulative arthritis score ranging from 0 to 16 for each animal [7,19]. To monitor early inflammatory reactions as well, in this particular study, an acute arthritis score of 0.5 was given if at least two interphalangeal, metacarpophalangeal or metatarso-phalangeal joints were swollen, but the paw inflammation (swelling and redness) did not reach the "standard" level of arthritis score 1 [19]. Animals were scored alternating by two investigators in a blind approach. Incidence of the disease was expressed as percent of arthritic to the total number of PG-immunized mice per colony. Acute arthritis (severity) score was applied only to arthritic animals. In addition, an arbitrary score (from 5 to 0) from the earliest onset of arthritis (onset score 5) to negative (onset score 0) was established for each mouse [22,23]. This onset score represents how quickly a mouse developed arthritis. Upon sacrifice, limbs and spines were removed, fixed in 10% formalin, acid decalcified and processed according to standard histological procedures [7-9,19]. The total number of 2,298 intervertebral discs (IVDs) of 127 spine (7-15 per colony) were examined and scored. A modified histological scoring system of the spine [10] was established by assessment of the severity of spine involvement, and given a score for each IVD ranging from 0 to 8. No inflammation was scored as 0, inflammatory (leukocyte) cell accumulation (peridiscitis and enthesitis) was scored 1-2, progression of IVD resorption was scored 3-6, fibrotic or fibro-cartilaginous ankylosis (with complete disc resorption) received a score of 7, and complete ankylosis due to chondrophyte/osteophyte formation was scored 8. A cumulative spodyloarthropathy score (the sum of spondylitis scores per spine) was calculated for each animal.

Measurements of serum cytokines and anti-PG antibodies, and lymphocyte responses

Serum cytokines IL-1β, IL-4, IL-6, IFN-γ and TNF-α were measured by ELISA. Antigen-specific lymphocyte responses were measured in spleen cell cultures in the presence or absence of 50 μg/ml human PG antigen. Antigen-specific IL-2 production was measured as a proliferative response of CTLL-2 cells to IL-2 in 48-hour spleen cell supernatants (CTLL-2 bioassay) [19]. Lymphocyte proliferation was assessed on day 5 of culture by measuring [$_3$H]-thymidine incorporation [17,19], and antigen-specific T cell proliferation was expressed as stimulation index [7,17,19]. *In vitro* production of the above-listed cytokines was also measured in supernatants of antigen (PG)- stimulated (50 μg/ml) spleen cell cultures on day 5 using ELISA. Secreted cytokine concentrations were normalized to ng/million cells [9,11]. PG-specific serum antibodies were measured by ELISA using at least three different serum dilutions. Highly purified human or mouse cartilage PG [24] was immobilized onto the surface of Nunc-Maxisorp 96-well plates (Nunc International, Hanover Park, IL) [19]. For PG-specific IgG isotype assays, peroxidase-labeled goat anti-mouse IgG1 (Zymed Laboratories, San Francisco, CA) and IgG2a (BD Biosciences) were employed. Serum PG-specific antibody levels were calculated using serial dilutions of pooled and standardized sera of mice with PGIA [19].

Affymetrix hybridization and related statistical analysis

RNA samples from spleen cells of naïve and immunized (12 days after the i.p. PG injection) were extracted with TriReagent (Sigma) according to the manufacturer's instructions. Affymetrix hybridization was performed using "Mouse Genome 430 2.0" gene chips. Biotinylation of cRNA, labeling and hybridization were processed by the Genomic Core Facility of University of Illinois at Chicago. Data were analyzed using GeneSpring GX 10.0 (Agilent Technologies, Santa Clara, CA) software package. Robust Multi-array Average [25] summarization algorithm (with Quantile Normalization and Median Polish probe summarization procedures) and baseline transformation

(i.e., per gene normalization; baseline to median of all 12 samples) was run on data using logarithmic scale. All sample replications passed quality control. For pairwise comparisons, Student- Newman-Keuls Post Hoc test was performed after Oneway ANOVA on 4 groups (naïve BALB/cJ, naive BALB/cByJ, immunized BALB/cJ and immunized BALB/cByJ) to identify statistically significant ($p < 0.05$) differentially expressed transcripts, and statistical differences between naïve and immunized mice of the two colonies. Asymptotic p-value computation and Benjamini Hochberg False Discovery Rate Multiple Testing Correction were applied [26]. Hierarchical clustering was applied to significantly differentially expressed genes, based on Pearson centered distance metric and centroid linkage rule. Differentially expressed transcripts were annotated with the GeneSpring software. Bivariate linear correlation (Pearson) test was performed to identify statistical correlations among spine and arthritis parameters. Fisher's Exact Chi-square test was applied when normal and diseased animals were compared. These statistical analyses were performed using SPSS version 16.0 statistical software (SPSS, Chicago, IL).

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 (Table 1 and Fig. 1A). Overall, arthritis scores ranged from 2.4 ± 0.7 to 11.0 ± 1.1 and the onset score of arthritis from 1.0 ± 0.2 to 2.8 ± 0.5 (Table 1). Most of the BALB/c colonies (colony numbers 4-9, henceforth called group II) developed arthritis at an average of 7.2 ± 0.5 severity and at 1.8 ± 0.1 onset scores (Table1 and Fig. 1A). 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 (Table 1 and Fig. 1A). In arthritic animals the histopathological abnormalities (cellular infiltration, synovitis, pannus formation, cartilage and bone erosions) were similar to those (data not shown) as described earlier in numerous papers [7-9,19,27], and there were no differences in the histopathology when peripheral joints of any subcolonies with the same clinical scores [19] were compared (data not shown).

Table 1. : Arthritis susceptibility, severity, and onset of different BALB/c colonies

Colony[a]		Vendors	Arthritic/total number of animals		Arthritis score (acute)[b]	Onset score
Number	Symbol		Incidence	Percentage		
1.	●	Portage P08; Charles River Laboratories, Inc. (Wilmington, MA, USA)	16/16	100%	11.0 ± 1.1	2.3 ± 0.3
2.	●	Canada II; Charles River Laboratories, Inc.	15/15	100%	10.3 ± 1.3	2.5 ± 0.4
3.	●	Harlan Laboratories, Inc. (Indianapolis, IN, USA)	12/13	92%	9.7 ± 1.5	2.8 ± 0.5
4.	■	Kingston K72; Charles River Laboratories, Inc.	13/14	93%	8.8 ± 1.2	2.1 ± 0.4
5.	■	Raleigh R02; Charles River Laboratories, Inc.	15/15	100%	7.6 ± 1.0	1.9 ± 0.3
6.	■	Taconic Farms, Inc. (Hudson, NY, USA) Charles River Laboratories, Inc	15/18	83%	7.5 ± 1.4	1.6 ± 0.3
7.	■	NCI/Kingston (Charles River Laboratories, Inc.)	17/20	85%	6.8 ± 0.9	1.5 ± 0.2
8.	■	BALB/cJ; The Jackson Laboratory (Bar Harbor, ME, USA)	16/19	84%	6.6 ± 1.1	2.1 ± 0.3
9.	■	Raleigh R12; Charles River Laboratories, Inc.	10/13	77%	6.4 ± 1.5	1.2 ± 0.2
10.	▲	Hollister H42; Charles River Laboratories, Inc.	10/15	67%	3.8 ± 0.8	1.1 ± 0.2
11.	▲	Bailey's BALB/c ByJ; The Jackson Laboratory	15/20	75%	2.4 ± 0.7	1.0 ± 0.2

All animals were immunized with human cartilage proteoglycan aggrecan in dimethyldioctadecyl-ammonium bromide. Values represent mean ± standard error of the mean. aColony numbers indicate the different BALB/c colonies. bHighest arthritis score measured at any time point of the experiment. cOnset score was calculated at the end of the experiment (days 63 and 64) and ranged from the earliest onset of inflammation (5) to no arthritis (0). Circles (●) represent the most arthritis-prone colonies. Designation was based on the statistical analysis showing no significant differences between these three colonies (1 to 3) comparing two major clinical variables: onset and severity. Therefore, these three colonies were combined and designated as group I (Figure 1a). Squares (■) indicate the average clinical phenotype of arthritis (colonies 4 to 9) with no significant differences using onset and severity scores as clinical phenotype

markers. This combined group is designated as group II (Figure 1a). Triangles (▲) represent the two least arthritic or least susceptible colonies, 10 and 11. Data of these two colonies were combined, and the two colonies together were designated as group III (Figure 1a). NCI, National Cancer Institute (Bethesda, MD, USA).

Histopathology of the spine

A total of 127 spines were formalin-fixed, X-ray imaged and then processed for histological analysis. Following the earlier scoring system [10], IVD involvement was analyzed using three parameters: (i) the cumulative spondyloarthropathy score of each animal (Fig. 1C), (ii) the mean (IVD) inflammatory score per animal (Fig. 1D), and (iii) the ratio of the number of the inflamed per total number of IVDs expressed as percentage (Fig. 1E). Scoring of the 127 spine sections, 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 [9,28]. However, the most susceptible, and most severely arthritic BALB/c colonies (Table 1) showed the most extensive spine involvement (Figs 1C-E) as assessed by using any of the three spondylitis parameters listed above. Similarly, animals that developed arthritis sooner exhibited more progressive spondylitis (Fig. 1B). 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.

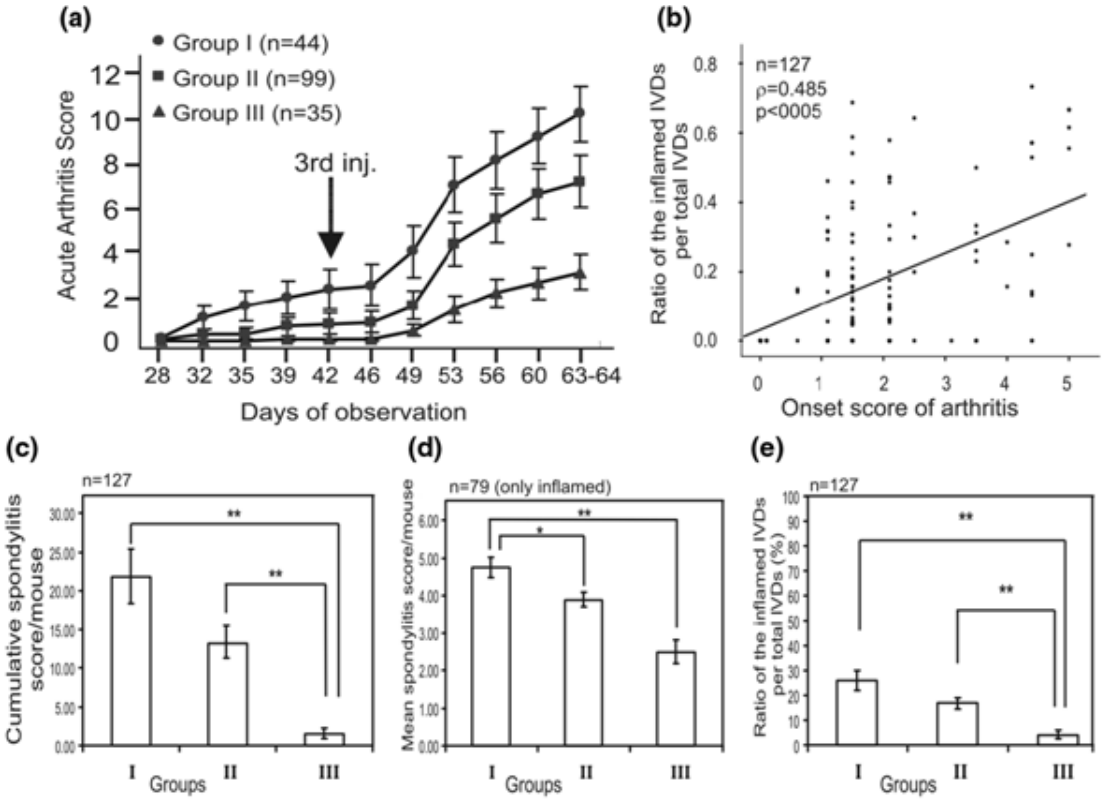

Figure 1. : Progression and severity of arthritis in 11 BALB/c colonies sorted into three different groups (listed in Table 1), correlation between the onset of arthritis and spine involvement, and comparison of the three arthritic groups with different spine inflammation scores. **(a)** Each animal was scored for arthritis three times a week, and scores are shown as mean ± standard error of the mean. Arrow indicates the third injection, administrated on day 42. Significant differences ($P < 0.01$), calculated by one-way analysis of variance, were found from day 32 between groups I, II, and III. **(b)** The ratio of the number of inflamed intervertebral discs (IVDs) per the total number of IVDs showed positive significant correlation (Pearson correlation coefficient $\rho = 0.485$; $P < 0.0005$) with the onset of arthritis. **(c-e)** Significant differences were found among the three arthritic groups when compared with three spine representative scores: cumulative spondylitis score (c), the mean spondylitis score (d), and the ratio of the number of inflamed IVDs per total number of IVDs (e). Asterisks indicate the level of significance between groups (*$P < 0.05$ and **$P < 0.01$) using Tamhane (c, e) (n = 127) and least significant difference (d) (n = 79) *post hoc* tests.

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. Interestingly, female BALB/c mice of the Hollister and ByJ colonies (Table 1, colonies 10-11, group III), which were the animals least susceptible to PGIA and PGIS, produced the highest levels of anti-inflammatory IL-4, and proinflammatory IL-6 and IFN-γ cytokines when assayed in spleen cultures (data not shown). We hypothesized that BALB/c mice of these two "low susceptibility" colonies (with delayed onset and less severe arthritis) still might be in the initiative (pro-inflammatory) phase of arthritis at the end of experiments (days 63-64). This was supported by the serum levels of autoantibodies to mouse PG (either IgG1 or IgG2a), which were significantly lower in animals of group III than in any other colonies (data not shown). Indeed, in supplemental experiments using age-matched females of Jackson's BALB/cJ and BALB/cByJ colonies, or Kingston and Hollister colonies of Charles River Laboratories (average versus low susceptibility animals) injected with the standard dose of 100 µg PG protein on day 42 (3rd immunization), these particular mid-term differences disappeared (data not shown).

Relationship between immune responses and clinical features

Next, we compared arthritis- and spondylitis-"specific" immune markers between the three groups of the clinical phenotypes (Table 2). 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 (Table 2).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 (Table 2).

We also compared the *in vitro* antigen (PG)-specific T cell responses and serum antibody levels in mice having (n=80) or not having (n=47) spondyloarthropathy. PG-stimulated spleen cell cultures expressed significantly more IFN-γ in mice without spondyloarthropathy than those that already had spine inflammation (Table 2). In contrast, all antibody isotypes (either to the immunizing human or autoantibodies to mouse cartilage PG) were significantly higher in the spondyloarthopathic animals than in those having no spondylitis (Table 2).

Table 2.: Immunological differences between arthritic and non-arthritic animals as well as mice with or without spondylitis

Measured parameters	Arthritic animals (n = 154)	Non-arthritic animals (n = 24)	Mice with spondylitis (n = 80)	Mice without spondylitis (n = 47)
In vitro T-cell proliferation, SI	2.96 ± 0.07	3.25 ± 0.13	3.00 ± 0.09	3.00 ± 0.14
In vitro IL-2 production (CTLL-2),	SI 2.67 ± 0.07	2.73 ± 0.15	2.68 ± 0.10	2.66 ± 0.11
In vitro IL-4 production, ng/106 cells	2.65 ± 0.14	2.91 ± 0.34	2.79 ± 0.22	2.39 ± 0.18
In vitro IL-6 production, ng/106 cells	1.83 ± 0.01	2.17 ± 0.33	1.88 ± 0.14	1.86 ± 0.19
In vitro IFN-γ production, ng/106 cells	8.06 ± 0.28	8.50 ± 1.07	7.61 ± 0.37	9.56 ± 0.65a
In vitro TNF-α production, ng/106 cells	0.74 ± 0.01	0.77 ± 0.02	0.74 ± 0.01	0.75 ± 0.02
Serum IL-4, pg/mL	15.29 ± 2.41	22.80 ± 5.92	15.52 ± 3.32	18.49 ± 4.46
Serum IL-6, pg/mL	166.45 ± 20.21	106.18 ± 26.97	97.59 ± 14.94	65.05 ± 20.61
Serum IL-1β, pg/mL	96.36 ± 11.55	41.70 ± 11.29	163.78 ± 27.72	143.10 ± 1.16
Serum IFN-γ, pg/Ml	26.14 ± 2.62	34.59 ± 6.27	25.05 ± 3.65	33.49 ± 4.55
IgG1 antibodies to human PG, mg/mL	12.75 ± 0.57b	8.30 ± 0.94	23.33 ± 0.78b	8.85 ± 0.86
IgG2a antibodies to human PG, mg/mL	1.36 ± 0.14a	0.72 ± 0.29	1.44 ± 0.21a	0.82 ± 0.17
IgG1 antibodies to PG, µg/mL	172.55 ± 13.47b	76.41 ± 10.56	174.99 ± 18.95a	116.68 ± 17.7
IgG2a antibodies to PG, µg/mL	68.01 ± 5.68b	24.79 ± 6.76	72.10 ± 8.18b	34.57 ± 6.16

All animals were immunized simultaneously with human cartilage proteoglycan in dimethyldioctadecyl-ammonium bromide. A total of three injections were given at 3-week intervals, and mice were sacrificed 3 weeks after the third injection (on days 63 and 64) when all *in vitro* assays were performed. Values represent mean ± standard error of the mean. Histological analysis was performed, and each inflamed intervertebral disc (IVD) was scored from '0' to '8' as described in Materials and methods. Positive (spondyloarthopathic) animals were combined if at least one IVD was affected with inflammation. Superscript letters indicate the level of significance (a$P < 0.05$ and b$P < 0.01$) between arthritic and non-arthritic animals or between mice with or without spondylitis. IFN-γ, interferon-gamma; IL, interleukin; PG, proteoglycan; SI, stimulation index; TNF-α, tumor necrosis factor-alpha.

Microarray results

Certain genetic differences between colonies of the same murine strain have already been analyzed (e.g., Jackson Laboratory detected 492 SNPs between BALB/cJ and BALB/cByJ colonies, which two sublines were separated approximately 73 years ago; http://www.informatics.jax.org). Therefore, in one of our "prototype" experiments, 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. Figure 2 shows the results of the analysis of 12 microarrays using three animals in each group. All samples passed over all quality control tests and 36,816 probe sets were analyzed. As shown on the hierarchical clusterization panel, 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 (Fig. 2), and 11 of the 77 genes were described as immune response genes or associated with arthritis (Supplemental Table 1 and attached reference list). When we compared the 77 genes expressed significantly in naïve and immunized mice, 70 were specific for naïve, and only eight genes associated with the immunization (Fig. 2).

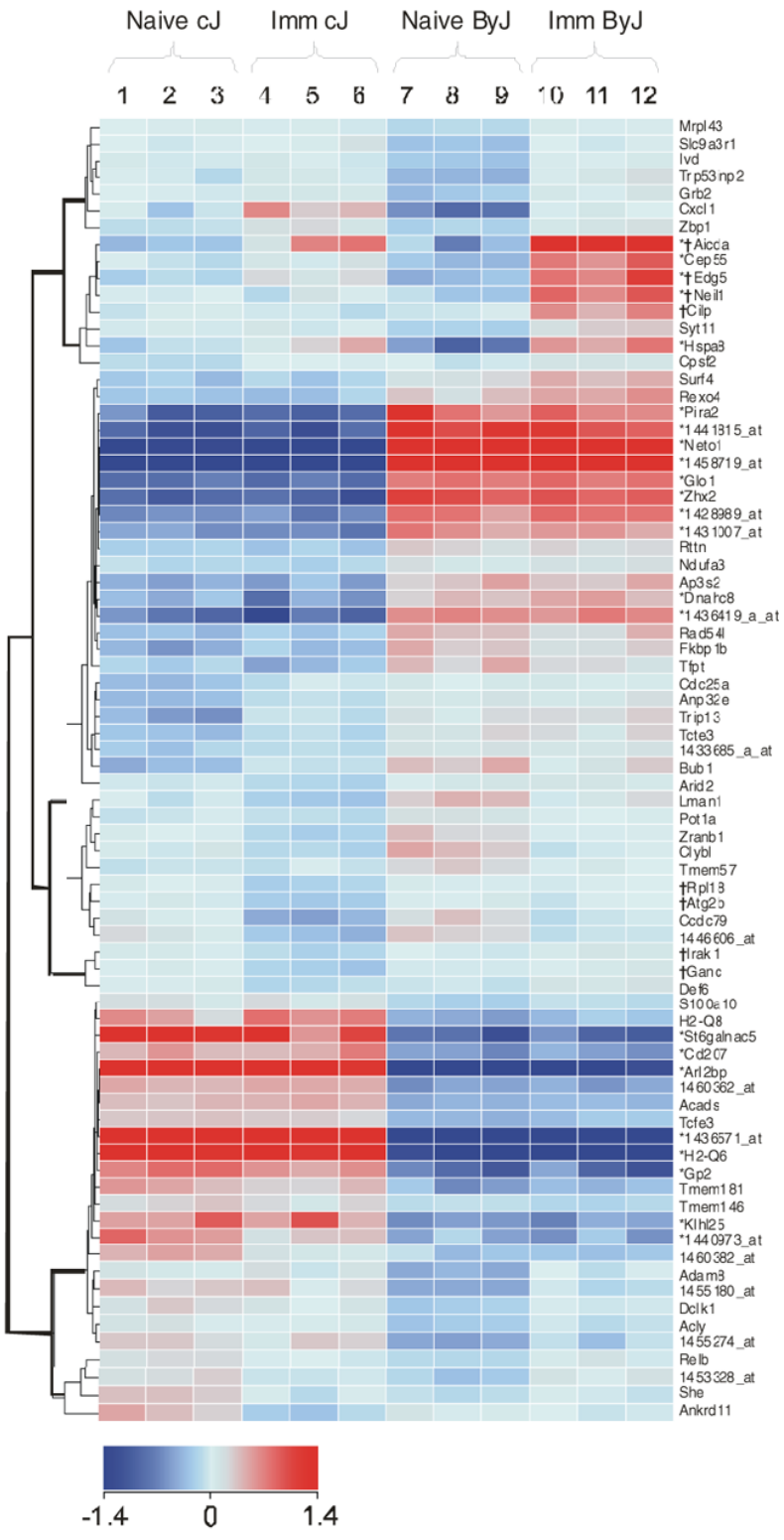

Figure 2.: Hierarchical clusterization comparing the 77 genes expressed differently at significant levels in spleen cells of naïve and proteoglycan-immunized (not-yet-arthritic) mice of BALB/cJ and BALB/cByJ

colonies (n = 3 of each, four-group cross-comparison: naïve BALB/cJ versus immunized BALB/ cJ, naïve BALB/cByJ versus immunized BALB/cByJ, naïve BALB/cJ versus naïve BALB/cByJ, and immunized BALB/cJ versus immunized BALB/cByJ). Color code indicates the normalized intensity expression values (with baseline transformation) on a logarithmic scale. Twenty-three genes showing over twofold differences in any of the four comparisons are labeled with asterisks. Whenever a gene name was not identified (n = 14), the original probe set ID (number_at), the Riken ID (numberRik), or the expressed sequence tag clone number is used. Those genes that showed significant differences only in response to immunization (n = 8) are labeled with the '†' symbol. Original data files are available via Gene Expression Omnibus (accession number [GEO:GSE13730] and National Center for Biotechnology Information tracking system number 15549466).

DISCUSSION

Although female BALB/c mice are almost 100% susceptible to PG-induced arthritis, following three consecutive immunizations with human cartilage PG aggrecan [7,17,29], we found significant differences in arthritis severity, onset and progression among the inbred colonies. Our findings, however, do not indicate that animals in group III acquired resistance, rather, these mice showed a tendency to develop arthritis, but they needed either a longer period of time, a higher dose of antigen or an additional (fourth) injection of human PG. Similar results were found when F2 hybrid mice of susceptible BALB/c and resistant strains were immunized and tested for arthritis- or spondylo-arthropathy-associated QTLs using the same antigen, immunization protocol, and scoring system (visual and histology), and when MHC- and age-matched animals were housed in the same room, occasionally for over half a year [9,11,22,23,28,29]. These genome-wide screening studies explored overlapping QTLs in different genetic combinations between high- or low-susceptibility F2 hybrids, indicating that different combination of genes may affect disease onset and/or severity [9,23,28]. In this comparative study, differences among different colonies suggest that either yet unknown genetic factors or transforming environmental

effects different at the site of origin and/or our animal facility (although both pathogen-free) caused these unexpected findings. One of the most important environmental factor is the normal intestinal microbial flora, and various bacterial cell wall components, for example peptidoglycans, which have proven to contribute to the induction of arthritis [30]. Although a variation in the composition of normal bacterial flora can explain some of our findings, we have not had a chance yet to investigate intestinal flora-related differences in detail in the 11 BALB/c colonies. Certain BALB/c substrains are known for the production of plasmacytoma in response to mineral oil injection [31], which generated a myeloma cell line (Sp2/0.Ag.14), a fusion partner with lymphoblasts routinely used in monoclonal antibody technology [32]. Moreover, although not frequently (less than in 2% of retired breeder female BALB/c mice, and found only in NCI/Kingston colony), we also observed spontaneous arthritis with less or more extensive synovitis, inflammation (Dr.Glant/Dr. Mikecz unpublished observation), occasionally associated with cartilage erosion in small peripheral joints, which histology features were indistinguishable from those seen in PGIA (unpublished observation). Although the dominant genetic factor is the MHC in both RA and in PGIA, the MHC alone is insufficient to affect arthritis susceptibility and severity (e.g., in H2-d DBA/2 mice) [9]. Two “Q” subloci (Q6 and Q8) were expressed at a significantly higher level in BALB/cJ than in BALB/cByJ mice (Fig. 2 and Supplemental Table 1), which might contribute to the earlier onset or more severe arthritis in BALB/cJ mice, but none of these subloci was associated with the immunized state (Fig. 2, see naïve vs. immunized pair-wise comparisons of the two subcolonies). Another critical factor in the pathogenesis of PGIA is the non-MHC genetic component (reviewed in [9,33]). The first albino mouse was found by a pet dealer in Ohio in 1913 [34]. Brothers and sisters were systematically mated and an inbred colony was established in 1920 [34]. The original BALB/c colony was separated in 1935. One of them was maintained by G. Snell at The Jackson Laboratory (Bar Harbor, ME) [BALB/c J], and the other was maintained by H. B. Andervont (BALB/c AnN) and then transferred to the National

Institute of Health (NIH) in 1951 [35] All other BALB/c colonies are derived from these two ancient ancestors. Charles River Laboratories (Wilmington, MN) started breeding BALB/c mice from 1974 (mice from NIH), Harlan from 1986 (mice from NIH) and Taconic Farm Inc., from 1988 (mice were purchased from the NIH). It is also relevant to note that except for one BALB/c colony (Hollister, CA) all of the distributors are located on the East Coast or in the Midwest regions of the United States. During their 88-year history, inbred BALB/c colonies have been exposed to various environmental effects (moving to other location, repopulation from other colonies due to fire, etc.). Although the companies ensure the genetic homogeneity of the colonies by applying strict breeding and maintenance rules, differences still occur among them. Historically, the BALB/cJ colony represents the original Jackson's BALB/c mice (maintained since 1935), whereas the BALB/cByJ mice were inherited from the NIH and the breeding stock was transferred to the Jackson Laboratory in 1967, when Dr. D.W. Bailey joined the company. The two colonies have been maintained separately and represent the pedigree of the two original BALB/c lines (Jackson vs. NIH). Therefore, the 492 SNPs (http://www.informatics.jax.org) and the 77 differentially expressed genes in (Fig. 2) of the two colonies attest to the dynamic flexibility of the mammalian (mouse) genome, which keeps changing despite being exposed to comparable environmental conditions.

Thirty-three of the 492 SNPs and 11 genes of the 77 differentially expressed genes in BALB/cJ and BALB/cByJ mice are related to immune regulatory functions, which, in turn, may affect arthritis onset, severity and susceptibility. Although most of the SNPs are present in intron sequences, some of them may have an effect on exon-splicing. However, the differences in the phenotypes can hardly be explained by the SNPs and the few immunoregulatory genes that are expressed differentially in both naïve and immunized BALB/cJ and BALB/cByJ mice. An important observation was the inflammation around the IVD in arthritic BALB/c mice, which is

found in up to 60 % of patients with ankylosing spondylitis, when examined by magnetic resonance imaging [36]. The nucleus pulposus of IVDs is mostly composed of hyaluronan and "cartilage-specific" PG aggrecan, and the core protein of the human aggrecan molecule has over 100 predicted and at least 27 confirmed T cell epitopes in BALB/c mice [9,37]. A number of these epitopes have been reported as dominant/arthritogenic in wild-type or humanized BALB/c mice [37- 41], and possibly involved in immune reactions to IVD components. The immune attack, characterized by a predominantly lymphocytic infiltration around the IVD in the early phase of the spondylitis [7-9], is most likely elicited by cross recognition of IVD PG in mice immunized with human PG. The spondyloarthropathy has a progressive character, and shows correlation with the onset and progression of peripheral arthritis, although we have shown that PGIA and PGIS are two independent diseases [11], and different genes in different QTLs control PGIA [9] and PGIS [28]. Interestingly, although inflammatory (autoimmune) spondyloarthropathy occurs only in BALB/c and C3H mice [7,8,11], spontaneous or experimentally induced disc degeneration has been reported in numerous animal models [42-45]. Autoimmune mechanisms are thought to play a major role only in HLA-B27 transgenic rodents [46-48] and in PGIA [7,9].

We expected to find robust T and B cell responses *in vitro* in antigen (PG)-stimulated spleen cell cultures of arthritic mice, because RA is thought to be a T cell-dependent and B cell-mediated disease [49]. Due to the intense involvement of various lymphoid organs (spleen, lymph nodes) in the regulation of immune responses, the serum cytokine levels may represent a momentary status rather than a general level of *in vivo* activation [50]. In this respect, it is not surprising that we could not find significant correlations between clinical or histological findings and serum cytokine levels. On the other hand, when we analyzed and compared the results of individual animals with or without arthritis or spondylitis at the end of the observation period, i.e., without pooling animals within a group (colony), significant correlations were found (Table 2). For example, significantly higher levels of hetero- and autoantibodies to cartilage PG were measured

in the sera of arthritic and spondyloarthopathic animals than in yet non-affected cage-mates (Table 2). An example of negative correlation was found when we compared PG-specific *in vitro* IFN-γ production by spleen cells in animals with and without spondylitis (Table 2), perhaps suggesting that Th1 T cell activation was still restricted to the lymphoid organs before the immune attack against the spine occurred. Similar differences and/or negative-positive correlations, although at lower levels, were found when other markers were compared to the clinical phenotype. Both RA and PGIA require T cells and B cells (or autoantibodies), where the autoimmune attack culminates in the inflammatory destruction of peripheral joints. A number of similarities between RA and PGIA suggests that certain yet unknown alterations of the immune system exists in both humans and mice. As a continuation of the experiments presented in this study, we are comparing gene expression in various lymphoid organs, and joint tissues of representative colonies at different time points after immunization, and correlating these results with clinical phenotypes of arthritis and spondylitis as well as with the results of our earlier genome-wide studies [9].

CONCLUSIONS

The MHC (H-2d) and non-MHC components of the genetic background make the BALB/c strain highly susceptible to inflammatory arthritis and spondylitis. Although BALB/c colonies uniformly develop PGIA (>95%) and PGIS (>80%) in response to immunization with human cartilage PG aggrecan, even in the absence of mycobacterial components (i.e., without the use of Freund's complete adjuvant), there are significant differences among BALB/c colonies maintained even by the same vendor at different locations, or when the "sub-colonies" were separated several decades ago. Technically, among the BALB/c colonies tested so far, we have not found a PGIA- or PGIS-resistant colony, but the "level of susceptibility" is different among them. This may be a critical question when laboratories use different colonies to induce other diseases, PGIA or PGIS,

or when transgenic/gene-deficient mice in "different" BALB/c backgrounds are compared with control wild-type BALB/c animals. Relatively small or yet unidentified mutations in the genome may significantly affect disease susceptibility, preferentially leading to incorrect conclusions. Genetic components are major players in the development of PGIA, and our genome-wide studies explored close to 30 different loci (twelve corresponding to human RA susceptibility loci identified in familial studies) [9]. Here we present the results of a systemic, age- and gender-matched comparative study using 11 substrains/colonies of BALB/c mice. Using a sub-optimal dose of arthritogenic cartilage PG, significant differences were found in arthritis susceptibility among colonies. Although no single gene or “biomarker” has been identified that could account for these differences, the large number of SNPs in two sister colonies (Jackson's BALB/cJ and BALB/cByJ) separated approximately 70 years ago, and the corresponding microarray results indicate that, indeed, a single or a limited number of mutations may dramatically affect the clinical phenotype of arthritis in BALB/c mice. The differences identified among colonies may help us to target disease-affecting gene(s) and may become nearly as a valuable tool as subcongenic approaches. The results of our study may serve as a direction toward a more accurate selection of disease-controlling genes from previously identified QTLs, especially from those that are shared in RA and corresponding animal models.

Authors' contribution: HLR made the first note for the differences between colonies and involved in an extensive preliminary results, using different colonies (NCI, Hollister and Jackson in Portland (OR), simultaneously with the preliminary studies performed in Chicago. These preliminary results led to the current study. BF carried out the most significant part of the research in Chicago, and was involved in manuscript writing. FB performed T cell separation and tissue culture, and OT isolated, purified and prepared RNA for microarray hybridization. BF, FB, AL and SML performed the statistical analysis. BT controlled and supervised animals on a daily basis, while GH and BF scored animals. GN and AM measured serum cytokines and antibodies. BF and GH collected and pulverized human cartilage samples, and TTG isolated and purified PG antigen for immunization. AF and KM coordinated and supervised the immunizations, and they contributed to data selection, interpretation of results and manuscript preparation. TTG designed and coordinated all experiments and prepared the final version of the manuscript.

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Chapter 2

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

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Objectives: To prove the hypothesis that arthritis-resistant DBA/2 mice can develop spondylitis in response to cartilage proteoglycan (PG) immunization (PG-induced spondylitis, PGIS), and confirm the feasibility of microarray assays using RNA samples from mouse intervertebral discs (IVDs).

Methods: Major histocompatibility complex (MHC)-matched BALB/c and DBA/2 mice, and both arthritis- and spondylitis-resistant C57BL/6 (B6) mice were immunized with cartilage PG to induce spondylitis (in DBA/2 mice), or both arthritis and spondylitis (in BALB/c mice). Animals were monitored for clinical phenotype of arthritis and spondylitis, X-ray analysis and near-infrared (NIR) fluorescence up to 186 days, and sequentially sacrificed (7-10 mice at 5 time points) for histological studies to correlate *in vivo* and *in vitro* results. RNA samples were isolated from normal and most/first involved IVDs for Affymetrix microarray analysis.

Results: DBA/2 mice developed only spondylitis (approximately 10-12 days after the fourth PG antigen injection), which was delayed and less extensive than in BALB/c mice. Osteoblast activity was detected in spine of DBA/2 mice using NIR fluorescence, in spine and peripheral joints of BALB/c mice. Affymetrix hybridization explored 58 differentially expressed genes at significant levels, of which 9 genes have yet unknown or only expected function.

Conclusions: A new murine model of spondyloarthropathy (PG-immunized DBA/2 mice) can be used to identify *in situ* “spondylitis-specific” signature genes. In an MHC-matched system, when compared to BALB/c strain, these genes can be distinguished from those which are either only arthritis-, or both arthritis- and spondylitis-associated.

INTRODUCTION

Ankylosing spondylitis (AS), a systemic polygenic autoimmune disease of the axial skeleton, affects approximately 0.5 % of the adult male Caucasian population.[1 2] AS is frequently associated with the inflammation of large peripheral joints, and the disease has extra-articular manifestations such as inflammation of ligament and tendon attachments (enthesitis), anterior uveitis, intestinal bowel disease, and cardiac and lung manifestations.[3-5]

Although the etiology of the disease is unknown, environmental and genetic components have been implicated as predisposing factors. The dominant genetic component is the class I major histocompatibility complex (MHC) encoded human leukocyte antigen (HLA)-B27,[6] but the presence of HLA-B27 alone is insufficient for disease development.[7-9] Although a number of non-MHC candidate genes were listed,[9-14] disease-causing genes have not been identified so far. There are two major hypotheses which explain the association of HLA-B27 with AS. The receptor theory assumes that certain T cell receptors can recognize a complex of foreign and MHC self peptides when together, but this putative pathogenic peptide is unknown.[15] The molecular mimicry hypothesizes that microorganisms which partially resemble or cross-react with HLA molecules are the source of antigenic components. This hypothesis of molecular mimicry targeted mostly *Klebsiella*[15-18] and *Yersinia*[19-21] antigens, but no appropriate microorganisms have yet been identified in patients with AS.[22] Therefore, extensive studies have been undertaken to identify other, non-MHC genetic factors [10,23-26] and, indeed, approximately a dozen chromosome regions or gene clusters have been linked to AS.[9-12,24-27]

Animal models are invaluable aids for the research of human (autoimmune) disorders. The *ank/ank* mouse has a loss-of-function mutation in the *ank* gene and develops a progressive spondyloarthropathy, similar to human AS,[28,29] but the *ank* gene, either in humans or mice, is not

involved in autoimmune processes.[29,30] Other models of spondyloarthropathy are described in HLA-B27 transgenic rodents,[31,32] or in transgenic mice expressing a mutant type IX collagen[33] or a truncated form of TNF-α.[34,35]

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,[36-39] which is similar to human AS (PG-induced spondylitis; PGIS), but most likely different genes control arthritis and spondylitis.[39,40] Genome-wide studies identified two major non-MHC genetic loci regulating PGIS in mice.[39,41,42] 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.[41,42] 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.

MATERIALS AND METHODS

Chemicals, antigen, animals, and immunization

All chemicals, unless indicated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO) or Fischer Scientific (Chicago, IL). Mouse recombinant cytokines and ELISA kits were purchased from R&D Systems (Minneapolis, MN) or BD Biosciences (San Jose, CA). Human cartilage was collected from patients undergoing knee-joint replacement surgery. The collection of cartilage from consenting patients was approved by the Institutional Review Board of Rush University Medical Center (RUMC), Chicago. Cartilage PG (aggrecan) was extracted and prepared for immunization as described.[43,44] All animal procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee of RUMC. A total of 40 PGIA- and PGIS-susceptible BALB/c, 46 PGIA-resistant DBA/2, and 46 both PGIA and PGIS-resistant C57B6 (B6) retired breeder (20-22 weeks old) female mice were purchased from the National Cancer Institute (Fredrick, MD). Although AS is a more frequent disease among males than females regardless we used age-matched female mice of the three strains. This was a compromised solution for a longitudinal study because aging BALB/c males have unusually aggressive behavior, and paw and proximal tail inflammation may be the consequence of fighting. In support of this solution, in earlier studies, using F2 (BALB/c x DBA/2) hybrids, the incidence of PGIS was comparable in males and females.[39]

Mice were immunized via intraperitoneal injection with an emulsion of cartilage PG (100 µg protein) and 2 mg dimethyldioctadecyl-ammonium bromide adjuvant on days 0, 21, 42, 63 and 84. This was an unusually long-term immunization because 50% of BALB/c mice developed arthritis after the second, and 100% after the third, PG injection.[38,44] However, in this longitudinal study focusing on the progression of spondyloarthropathy, mice received 5 injections, and the total observation period was 186 days.

Clinical assessment of arthritis and *in vivo* monitoring of spondylitis

PG-immunized BALB/c, DBA/2, and B6 mice were examined 3-4 times a week for clinical symptoms of arthritis.[36,44] X-ray images were carried out with KODAK Image Station 4000 Digital Imaging Systems (Chicago, IL). Both antero-posterior and lateral X-ray images were taken under ketamine/xylazine-anesthesia *in vivo,* and then *ex vivo*, when mice were sacrificed. Osteoblast activity was assessed *in vivo* using OsteoSense^{TM} 750 probe, a biphosphonate derivative labeled with NIR fluorescent agent (Visen Medical, Woburn, MA, USA). This compound binds to newly synthesized hydroxyapatite by osteoblasts,[45] thus allowing visualization of areas of microcalcifications and bone remodeling. The compound (10 ng/mouse) was injected intravenously, and mice were imaged 24 hours later using KODAK Image Station 4000, same as described above for X-ray analysis, with excitation filter of 720 nm and emission filter of 820 nm wavelength for 30 sec exposure time.

At each time point (on days 74, 87, 101 and 129 after the first PG injection) eight animals of each group were scanned *in vivo* by X-ray and OsteoSense^{TM} 750 probe, and then sacrificed; the remaining animals were sacrificed on day 186. Sera were collected and spleen cells used for *in vitro* assays. Hind limbs and spines were dissected, fixed, decalcified, and processed by standard histological procedures.[36-38,44] For preparing sagittal spine sections, a paramedian orientation of the spine was used.[40] A detailed spondylitis scoring system was established to quantify the severity and progression of spine involvement (ranging from 0 to 8) as described in figure 1 legend. A cumulative spondyloarthropathy score was calculated for each animal, which was a sum of the spondylitis scores in a given animal.

Measurements of serum cytokines and immune responses

Serum cytokines IL-1β, IL-4, IL-6, IL-17, IFN-γ and TNF-α were quantified by enzyme-linked immunosorbent assay (ELISA). Antigen-specific lymphocyte responses were measured in spleen cell cultures in the presence of 50 µg/ml human PG antigen. Antigen-specific IL-2 production was determined as a proliferation response of CTLL-2 cells to IL-2 in 48-hour spleen cell supernatants (CTLL-2 bioassay).[44] The *in vitro* secretions of IL-4, IL-6, IFN-γ and TNF-α were measured in 5-day supernatants of antigen (PG)-stimulated spleen cell cultures using ELISA. Secreted cytokine concentrations were normalized and expressed as ng/million cells.[39,41] Lymphocyte proliferation was assessed on day 5 by incorporation of [^{3}H]-thymidine.[39,41,44] Antigen-specific lymphocyte proliferation was expressed as stimulation index.[36,39,41,44]

Serum antibodies were measured by ELISA using at least three different serum dilutions. Highly purified human or mouse cartilage PG aggrecan[46] were immobilized onto the surface of Nunc-Maxisorp 96-well plates (Nunc International, Hanover Park, IL).[39,41,44] For PG-specific isotype assays, peroxidase-labeled goat anti-mouse IgG1 (Zymed Laboratories, San Francisco, CA) or IgG2a (BD Biosciences) were employed. Serum PG-specific antibody levels were calculated in µg/ml or mg/ml comparing serial dilutions of standardized sera of mice with PGIA.[44]

Gene expression profiling in intervertebral discs (IVDs) of mice

RNA samples were extracted with TriReagent from lumbar-1 (L1) to L6 IVDs of naïve and immunized BALB/c mice. Note, most of the murine strains, including BALB/c and DBA/2, have 13 thoracic and 6 lumbar vertebra bodies.[47] RNA samples of 6 pooled IVDs were prepared. The quality and quantity were determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Finally, RNA samples of pooled IVDs of three naïve and five PG-immunized mice were used for Affymetrix hybridization (Mouse Genome 430 2.0 gene chips) in accordance with

Affymetrix protocols as described.[14,48,49] Data were analyzed using GeneSpring GX 10.0 (Agilent Technologies) software package. Robust Multi-array Average[50] summarization algorithm (with Quantile Normalization and Median Polish probe summarization procedures) and baseline transformation (i.e., per gene normalization; baseline to median of all 8 samples) was run on data using logarithmic scale. All sample replications passed quality control. To identify statistically significant ($p < 0.05$) differentially expressed transcripts in naïve (control) versus immunized mice with spondylitis unpaired t-test was used. Asymptotic p-value computation and Benjamini Hochberg False Discovery Rate Multiple Testing Correction were applied [51]. Hierarchical clustering was applied to significantly differentially expressed genes, based on Pearson centered distance metric and centroid linkage rule. Differentially expressed transcripts were annotated with the GeneSpring software. A total of four significantly up-regulated and two down-regulated genes were confirmed by real-time polymerase chain reaction (PCR) using Applied Biosystems (Foster City, CA) probes in an iQ5 real-time PCR machine (BioRad, Hercules, CA).

Statistical analysis

In addition to the analysis of Affymetrix data sets, *in vitro* and *in vivo* results were analyzed using Student's *t*-tests to compare means, and Oneway ANOVA method by using Least Significant Difference post hoc tests for multiple comparisons. All statistical analyses were performed using SPSS (version 16.0) statistical software package (SPSS, Chicago, IL).

RESULTS

Incidence and severity of PGIA and PGIS in inbred mice

Mice were immunized and sacrificed following a protocol described in methods. 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 (fig 1).

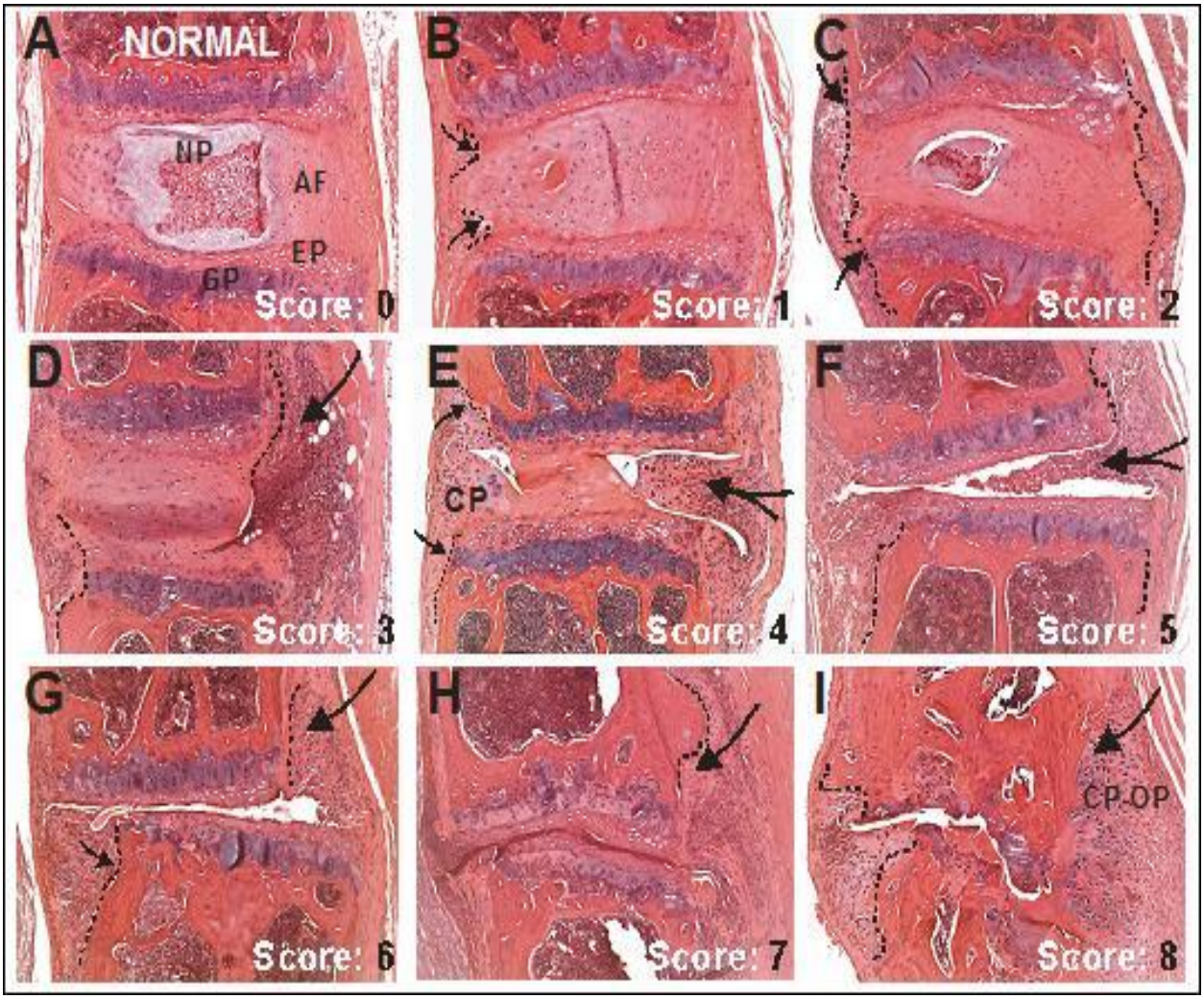

Figure 1 Histopathological scoring system of intervertebral discs (IVDs). Histology images of hematoxylin and eosin-stained sections (original magnification: 10x) of normal and inflamed IVDs of the distal cervical (earliest involved) region after immunization of BALB/c mice with cartilage PG aggrecan. Broken lines

indicate the borderline between the intact tissue and the inflammatory cell invasion, and arrows indicate bone erosion. The nine histology panel represent a normal IVD (A), and those affected or ankylotized (Panels B - I; histopathology scores: 1-8). Panel A shows the normal structure of the IVD (scored as 0): end plates (EP), growth plates (GP), nucleus pulposus (NP) and annulus fibrosus (AF); panels B-G represent the progression of inflammatory disc resorption from a mild stage of peridiscitis (B) to the complete resorption of the IVD (G). Inflammatory (leukocyte) cell accumulation (peridiscitis) around the IVD and/or enthesitis was scored 1-2, progression of IVD resorption was scored 3-6, fibrotic or fibro-cartilaginous ankylosis (with complete disc resorption) received score 7, and complete ankylosis with chondrophytes and/or osteophytes was scored 8. Occasionally, a pannus-like structure erodes the IVD (Panels E and F, arrows with double-tails). The last two panels show mostly fibrotic-fibrocartilaginous ankylosis (H) and completely destroyed IVD with bony ankylosis (I). In these panels, osteophytes (OP) and chondrophytes (CP) led to ankylosis, and bony fusions are indicated.

During the course of the spine involvement, the lumbar and later the distal cervical and proximal thoracic segments became involved, but not all IVDs were equally affected at any given time point (fig 2). Some IVDs seemed to be intact or mildly damaged even when the majority or the neighboring discs were resorbed and the vertebra bodies underwent ossification or fusion.[40] Throughout the 186-day-long observation period, 35 of 46 (76.1%) DBA/2, and 38 of 40 (95%) BALB/c mice developed spondylitis, but no sign of inflammation was found either in the peripheral joints or axial skeleton of B6 mice.

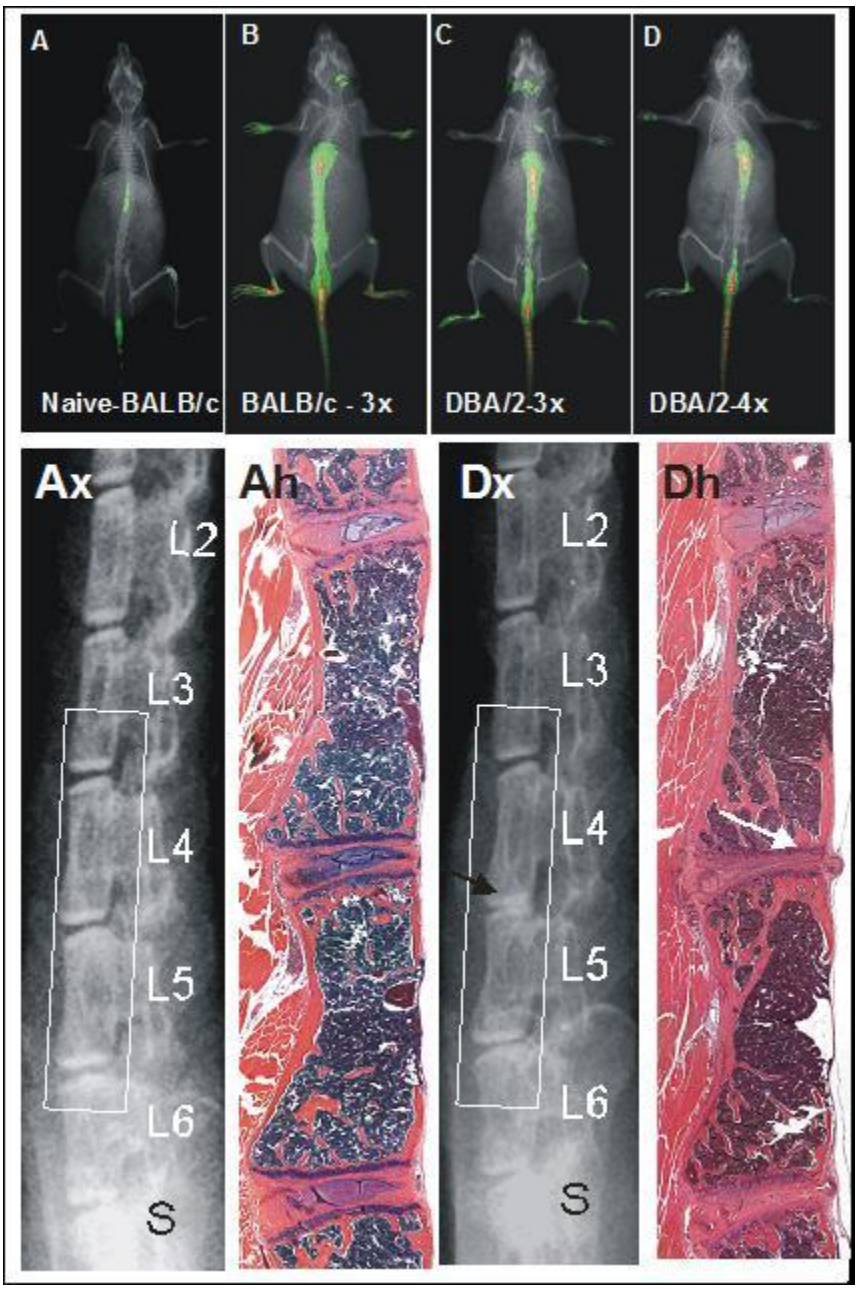

Figure 2 Near-infrared (NIR) fluorescence and x-ray images with corresponding histology sections. Panels A-D**:** OsteoSense^{TM} 750 was administered intravenously and mice were imaged 24 hours later under Ketamin/Xylazine anesthesia. The NIR fluorochrome-labeled biphoshonate binds to newly formed bony structure, and fluorescent imaging agent visualizes *in vivo* osteoblast activity (red and yellow are the most active areas). In an age-matched naïve mouse (A) specific signal is not detectable. Arthritis and spondylitis in BALB/c mice (panel B) show signs of massive bone remodeling in both spine and peripheral joints. A

relatively early spondyloarthropathy (after the third [C] or fourth [D] PG injection) is shown in DBA/2 mice. Representative images comparing *in vivo*-generated X-ray (x) and corresponding histopathology (h) pictures of the lower lumbar spine of naïve (Ax and Ah) and a DBA/2 mouse 17 days after the fourth PG/DDA injection (Dx and Dh, same animal shown on Panel D). Arrows indicate collapsed intervertebral discs (IVD) between L4 and L5 vertebral bodies (Dx and Dh). Squared areas on the X-ray images are displayed on the hematoxylin and eosin-stained montage images (original magnification: 10x).

PGIS was progressive in both BALB/c and DBA/2 strains, although spondylitis started earlier and affected more IVDs (figs 3A-B) and more IVDs ankylotized (indicated by higher scores) in BALB/c than in DBA/2 mice (data not shown). *In vivo* bone remodeling was detected with NIR fluorescence images using OsteoSense^{TM} 750 probe as early as after the third intraperitoneal PG injection (fig 2A-D), and found to be more progressive after the fourth immunization in DBA/2 mice (fig 2D). We were able to visualize *in vivo* osteoblast activities in both the peripheral joints and spine of PG-immunized BALB/c mice (fig 2B).

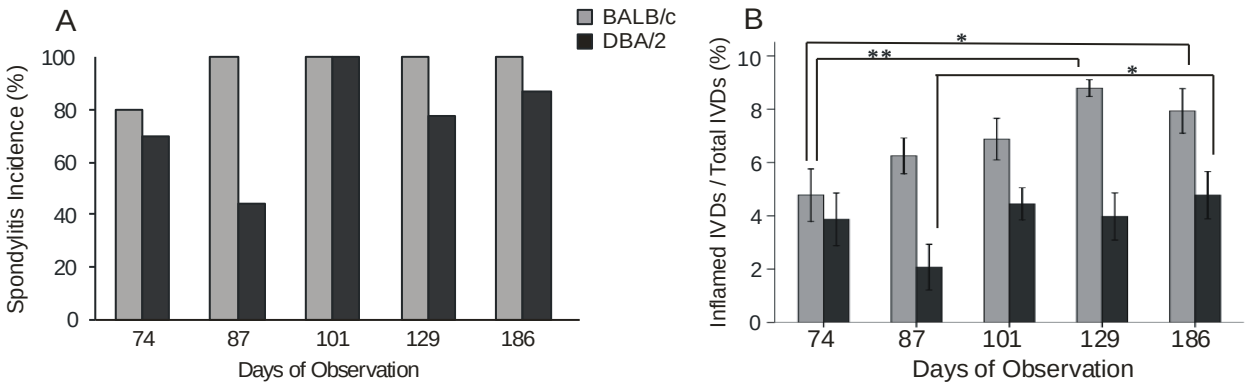

Figure 3. : Spondylitis scores, based upon histopathology scoring system (fig 1), measured at different time points during the immunization period in BALB/c and DBA/2 mice. Due to the absolute negative results, C57BL/6 mice are not shown. Seven-ten animals were sacrificed at each time point throughout the 186-day experimental period. Spondylitis incidence (either per animal number: panel A, or the ratio of

inflamed and total IVDs in Panel B) expressed in percent, even if only one or a few IVDs were involved at the given time point. Levels of significance are indicated (*$p<0.05$ and **$p<0.01$) using Least Significance Difference post hoc test for multiple comparison (Panel B).

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. There were strong B and T cell responses, which, in general, were higher in BALB/c than in DBA/2 mice, except in *in vitro*-measured antigen (PG)-specific T cell proliferation (Table 1). 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), even less (0.68) in B6 mice. All BALB/c, but none of the DBA/2, mice were affected with peripheral arthritis, a disease condition which naturally contributed to the higher levels of measured biomarkers of immune responses. Remarkably, three-four times higher autoantibody (either IgG1 or IgG2a) levels were measured in arthritis-prone BALB/c than only spondylitis-susceptible DBA/2 mice. Together, a significantly higher level of *in vitro* T-cell proliferation was measured in DBA/2 mice, which associated with low IFN-γ/IL-4 ratio. This suggests that PGIS is a more T cell-dependent (possible Th2) than antibody-mediated disease (Table 1). If we compare immune parameters determined in DBA/2 and C57BL/6 mice, the most evident difference, in addition to the T-cell proliferation, is the significantly higher levels of TNF-α measured either in serum or in *in vitro*-stimulated T cell cultures of DBA/2 mice (Table 1).

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. While *in vitro*-produced TNF-α was 2.04 ± 0.29 (ng/million cells) (mean ± SEM) in DBA/2 mice with spondylitis, it was only 1.41 ± 0.17 ng/million cells in animals without spine involvement ($p<0.05$). 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).

Table 1. **:** Summary of *in vivo* and *in vitro* T and B cell responses to cartilage PG aggrecan in BALB/c, DBA/2 and C57B6 strains.

Immune parameters	**BALB/c** n=40	**DBA/2** n=46	**C57BL/6** n=46
Serum IL-4 (pg/ml)	15.36 ± 4.80	50.26 ± 16.14	170.16 ± 41.61
Serum IL-6 (pg/ml)	134.56 ± 39.58	88.13 ± 13.83	55.33 ± 22.81
Serum IL17 (pg/ml)	****51.38 ± 12.06**	**12.37 ± 6.00**	24.39 ± 21.92
Serum IL-1β (pg/ml)	***64.38 ± 14.91**	**25.27 ± 6.00**	36.74 ± 21.38
Serum TNF-α (pg/ml)	27.09 ± 9.60	****17.95 ± 3.71**	**0.41 ± 0.41**
Serum IFN-γ (pg/ml)	88.12 ± 12.40	105.58 ± 22.02	115.09 ± 32.28
Serum IgG1 to human PG (mg/ml)	10.89 ± 1.11	11.54 ± 1.06	8.93 ± 0.77
Serum IgG2a to human PG (mg/ml)	****1.73 ± 0.23**	**0.15 ± 0.04**	0.33 ± 0.07
Serum IgG1 to mouse PG (:g/ml)	****173.38 ± 15.22**	**77.97 ± 5.75**	76.72 ± 7.59
Serum IgG2a to mouse PG (:g/ml)	****193.35 ± 25.02**	**39.23 ± 7.32**	52.63 ± 7.37
In vitro IL-2 (CTLL-2) (SI)	4.98 ± 0.42	5.66 ± 0.43	4.82 ± 0.26
In vitro T cell proliferation (SI)	**2.98 ± 0.19**	****4.87 ± 0.40**	**1.27 ± 0.18**
In vitro IL-4 (ng/10^b cells)	****2.39 ± 0.34**	**0.98 ± 0.19**	0.45 ± 0.11
In vitro IL-6 (ng/10^b cells)	****1.57 ± 0.18**	**0.59 ± 0.06**	1.14 ± 0.13
In vitro TNF-α (ng/10^6 cells)	1.52 ± 0.43	***1.54 ± 0.15**	**0.72 ± 0.08**
In vitro IFN-γ (ng/10^6 cells)	****3.47 ± 0.13**	**1.29 ± 0.17**	1.52 ± 0.20

Animals were immunized with human cartilage PG in DDA as described in Methods. Data represents the cumulative results of all mice. Values are mean ± SEM. SI=stimulation index. Asterisks indicate the level of

significance (*p<0.05 and **p<0.01) between the spondylitis- and arthritis-susceptible BALB/c strain and only PGIS-susceptible DBA/2 mice (compared values of these two strains are in bold-face). Mice of the C57BL/6 (B6) strain, although responding well to the immunization, remained asymptomatic, and no inflammation could be detected in the peripheral joints or axial skeleton. These C57BL/6 mice served as negative controls for both arthritis and spondylitis.

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 (fig. 4). The complete set of differentially expressed 58 genes is listed in supplemental Table 1, and original data files are submitted to, and available via, Gene Expression Omnibus (GEO No: GSE13782; NCBI tracking system #15551885). 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 (Supplemental Table 1)].

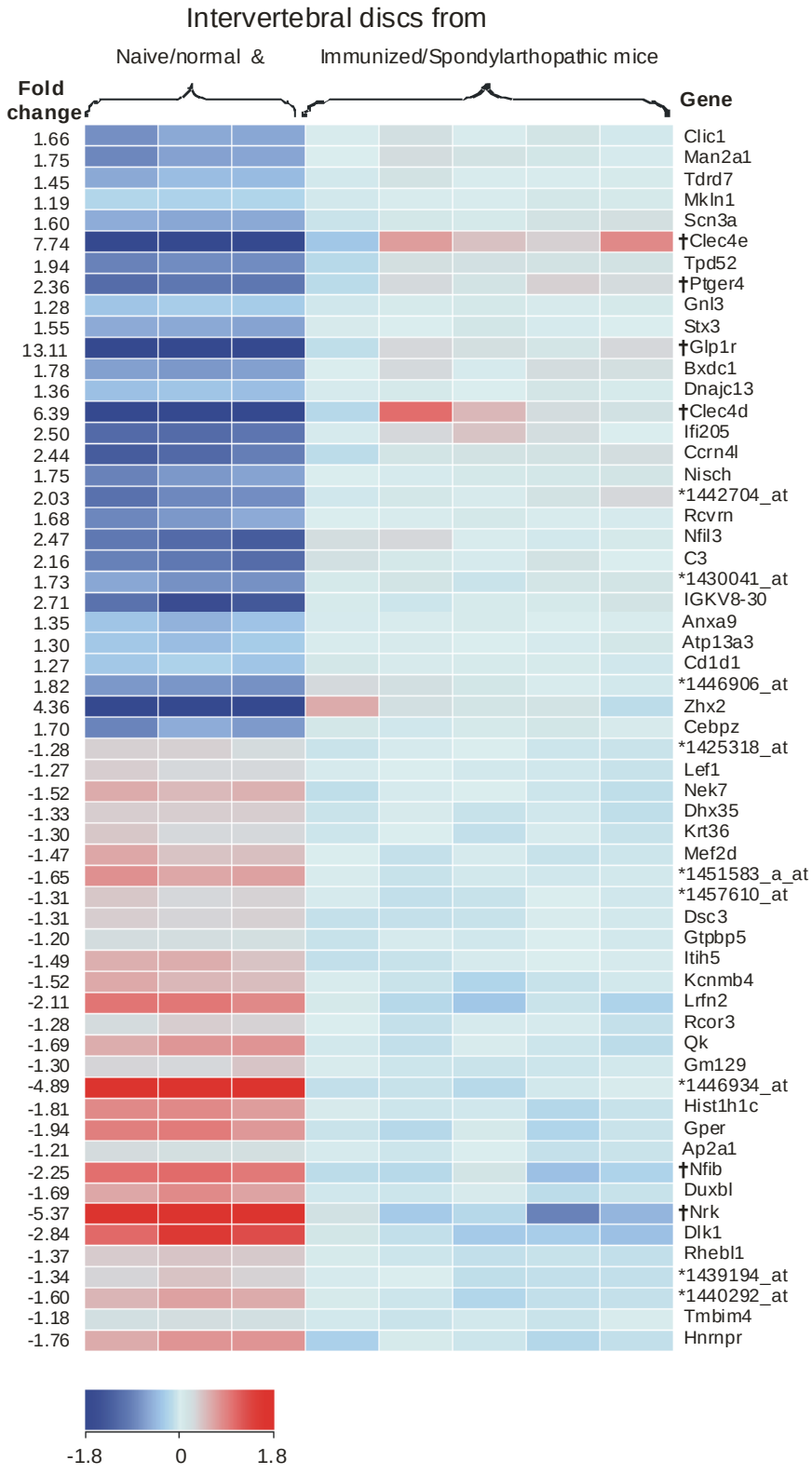
Intervertebral discs from
Naive/normal &
Immunized/Spondylarthopathic mice
Fold change
Gene
1.66 Clic1
1.75 Man2a1
1.45 Tdrd7
1.19 Mkln1
1.60 Scn3a
7.74 †Clec4e
1.94 Tpd52
2.36 †Ptger4
1.28 Gnl3
1.55 Stx3
13.11 †Glp1r
1.78 Bxdc1
1.36 Dnajc13
6.39 †Clec4d
2.50 Ifi205
2.44 Ccrn4l
1.75 Nisch
2.03 *1442704_at
1.68 Rcvrn
2.47 Nfil3
2.16 C3
1.73 *1430041_at
2.71 IGKV8-30
1.35 Anxa9
1.30 Atp13a3
1.27 Cd1d1
1.82 *1446906_at
4.36 Zhx2
1.70 Cebpz
-1.28 *1425318_at
-1.27 Lef1
-1.52 Nek7
-1.33 Dhx35
-1.30 Krt36
-1.47 Mef2d
-1.65 *1451583_a_at
-1.31 *1457610_at
-1.31 Dsc3
-1.20 Gtpbp5
-1.49 Itih5
-1.52 Kcnmb4
-2.11 Lrfn2
-1.28 Rcor3
-1.69 Qk
-1.30 Gm129
-4.89 *1446934_at
-1.81 Hist1h1c
-1.94 Gper
-1.21 Ap2a1
-2.25 †Nfib
-1.69 Duxbl
-5.37 †Nrk
-2.84 Dlk1
-1.37 Rhebl1
-1.34 *1439194_at
-1.60 *1440292_at
-1.18 Tmbim4
-1.76 Hnrnpr
-1.8
0
1.8

Figure 4 Hierarchical clusterization comparing the 58 genes expressed differently at significant levels ($p<0.05$) in intervertebral discs (IVDs) of naïve (n=3) and PG-immunized BALB/c mice with spondyloarthropathy (n=5) identified by X-ray images. RNA samples were isolated from six IVDs of the lumbar region. Color code indicates the normalized intensity expression values (with baseline transformation) on logarithmic scale. Whenever a gene name was not identified, the original probe set ID number ("number_at") was used, and genes with unknown or not fully confirmed function are indicated by asterisks (n=9) (see fig 4 and supplemental Table 1). indicated by asterisks. Fold-change values between the two groups (inflamed vs. normal) are listed at the left side. A detailed list of genes with their known and/or expected functions, corresponding references, their chromosome localizations, and the level of significance are listed in Supplemental Table 1.

DISCUSSION

In the current study we demonstrated a new autoimmune murine model of human AS in an otherwise arthritis-resistant mouse strain: DBA/2. While the axial involvement was achieved after three-four consecutive injections of human cartilage PG, peripheral arthritis was not detected even at day 186 of the observation period. Therefore, the PGIS in DBA/2 mice can be a unique model for investigation of the pathogenesis of human AS, because the chronology of pathological events and the primary localization of spine inflammation appears to be exactly the same in this model as in human AS. The inflammation starts simultaneously in the sacroiliac joints[40] and at the sites of entheses, where mononuclear influx together with pannus-like tissue formation were found (fig. 1F). However, these early events might not be seen on X-rays, not even when OsteoSense probe was used, and therefore animals needed to be sacrificed. Recently, a more sensitive ProSense test (detecting proteolytic cleavages, thus the earliest tissue damage) is under development in our laboratory. Nonetheless, once the cartilage of the sacroiliac joint was eroded,[40] and the IVDs affected, the inflammation and tissue damage appeared to be more

aggressive until the whole disc resorbed resulting in ankylotized vertebral bodies (figs 1H-I and 2). Because the spine instability has to be reconstituted, first, a fibro-cartilaginous tissue had overgrown from the edge of the vertebral bodies. Later, this fibrocartilaginous tissue was replaced with chondrophytes and osteophytes leading to the classic "bamboo-spine",[40] which can be diagnosed on X-ray images. Mice lost weight, and exhibited limited cage activity. NIR fluorescence and X-ray analyses (fig. 2) showed the same progression of the disease as described for the histopathologic abnormalities of the spine. At the end of the 186-day experiment, the spine of all BALB/c and most of the DBA/2 mice were completely ankylotized as described and demonstrated in earlier studies.[36,38,40] The major difference between the two strains, however, was that BALB/c mice developed peripheral arthritis as well, which resulted in ankylotized and deformed diarthrodial joints. DBA/2 mice developed only extensive enthesitis and later spine ankylosis, but the skeletal structure of the B6 mice was unaffected. In fact, the DBA/2 model offers us a useful tool to identify spondylitis-associated genes, which appear to be independent from those identified in synovial joints, and may represent a disease-specific pool.

The combination of X-ray and NIR fluorescence (ProSense and OsteoSense) with high resolution contrast-enhanced micro-CT or MRI, supported by histopathologic analysis, may offer a new and highly specific diagnostic approach to identify very early inflammatory events in, or around, IVDs in mice. Thus, individual IVDs then can be dissected[52] and identified from early-to-late stage of the disease, and then can be used for microarray analysis to detect signature genes of early inflammatory events. This is a compelling hypothesis, and we believe that there are spondylitis-specific genes, and if so, these genes are not involved in peripheral arthritis in DBA/2 mice. These spondylitis-associated or -specific genes are supposed to be located on mouse chromosome 2 (*Pgis1* locus) in DBA/2 mice, while genes of chromosome 18 (within the *Pgis2* locus, and a few other loci) appear to be more general and probably more dominant in BALB/c

strain.[39,41] However, many of the genes (gene products) associated with arthritis may affect the severity of spondylitis.

Although there are a number of open questions (serum and local levels of cytokines and chemokines, the expression of cytokine receptors (e.g., IL-1R, IL-23R), the role of TNF-α, IL-17, IL-1β, Arts1, RANK/RANKL, etc.,) that need to be answered, we believe that this unique model of spondyloarthropathy, without association with peripheral arthritis, may open a new direction towards identifying “spondylitis-specific” genes. Moreover, this is the first murine spondylitis model which is induced by systemic autoimmunity. Notable, the major *Pgis2* locus, inherited from DBA/2 strain carry the entire IL-1 gene cluster and Arts1. A specific value of this "spondylitis only" model is that it can be paired with more complex, however, MHC-matched, models of PGIA and PGIS of the BALB/c strain, thus spondylitis-specific and/or arthritis-specific genes can be identified.

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Discussion

In Chapter 1 we explored the differences of the clinical manifestation and the immune response in PGIA and PGIS in a multiple comparison of colonies of the same inbred strain of mice, the universally studied BALB/c, which is the most susceptible to PGIA and PGIS. The goal of the study was to compare the incidence and severity of PGIA and proteoglycan-induced spondylitis and to determine expression patterns of a panel of relevant genes in a large number of distinct colonies of the susceptible mouse strain BALB/c. Although induction of disease by the usual immunisation regime did not reveal any differences in incidence and severity, the sub-lines of BALB/c mice showed modest but significant differences in immune parameters such as cytokine and antibody responses and clinical scores when disease was induced with suboptimal doses of aggrecan, clearly revealed a genetic drift in the colonies that which influenced the immune response and disease pathology.

In conclusion, firstly my report strengthened the validity of previous studies of the PGIA model in different laboratories and secondly, provided a challenge research groups in regard to use of inbred strains of mice as models for other human diseases, particularly in cases when groups have published contradictory data.

Confidence in data derived from studying the mechanisms of immunopathology in patients with rheumatoid arthritis (RA) or ankylosing spondylitis is undermined by many variables, for instance the genetic diversity of patients in a single cohort or diversity between cohorts studied in different laboratories.

Despite a strong association between the expression of certain HLA DR alleles and RA, many additional major histocompatibility (MHC) and non-MHC loci differ between individuals and are thought to contribute to RA pathogenesis [1]. As a consequence, RA cohorts in studies to

date have included patients with a spectrum of disease. Therefore, it is doubtful that a single aetiology will account for disease in all these patients and most likely future studies will segregate patients into distinct disease subsets [2].

To avoid these issues, the other approach is to study the mechanisms of disease in genetically defined animal models. A variety of models of autoimmunity have been developed, each providing a window on some, but not all, aspects of the immunopathology [3]. Of course, animal models have their own drawbacks, such as whether the immune system of the mouse parallels that in human, and how similar the disease processes observed in experimental arthritis are to RA [4]. Hopefully, appreciation of the information extracted from several different animal models combined with studies possible in RA patients is likely to lead us forward in the application of improved treatment modalities.

In particular, the reductionist approach has been taken by exploiting the genetic homogeneity of inbred mice in which autoimmune arthritis can be induced by synchronized immunisation of groups of animals with single candidate joint autoantigens in adjuvants. The reliable kinetics of the consequent immune response can then be studied in relation to the ensuing RA-like disease pathology. This approach offers the opportunity to observe the sequence of events during disease onset and provides clear answers to questions such as the composition of cellular infiltrates into the synovium and the mechanisms of cartilage and bone destruction.

The most widely studied animal models are collagen-induced arthritis [5] and proteoglycan-induced arthritis (PGIA) [6] in which inflammatory arthritis is induced by immunizing genetically susceptible strains of inbred mice, sometimes transgenic for disease-associated HLA alleles or T cell antigen receptors specific for arthritogenic peptides from joint autoantigens. The best studied autoantigens are the two major cartilage components, type II collagen and proteoglycan aggrecan, both joint autoantigens that induce RA-like disease in mice as well as recall T cell response in a proportion of RA patients [7,8].

Although the manifestations of late phases of both RA and Ankylosing Spondylitis are quite evident and possible to visualize, diagnose and monitor progression with radiology(x-ray, MRI, CT), the initial stages are less obvious and still remain a challenge to asses. To-date, no non-invasive method of diagnosing or scoring early inflammatory events around the IVDs exists. We attempted to identify serum immune elements which would correlate with disease severity or incidence which could later be used as rheumatic markers, yet despite our numerous efforts, we were unsuccessful.

The fact that a massive immune response is mounted upon administration of human and mouse PG, we ideated that behind this reaction there might be a specific marker which would correlate with bone reactions, but unfortunately it was not among the parameters we examined.

Fortunately we can now monitor early inflammatory events by using near infrared fluorescence dyes such as Prosense 750 and Osteosense 750 as previously it was impossible to visualize early stages *in vivo*. ProSense 750, a tissue destruction marker was activated and detected during initial step of inflammation by cleavage of tissue damaging proteases and upon this cleavage allows detection of early phase of inflammation. Osteosense 750 probe is a bone remodeling marker and consists of hydroxyapatite crystals which are incorporated into bone and thereby allowing visualization of early osteoblast activity [9].

These probes enabled us to isolate specific RNA from the IVDs which will bestow upon us identification of genes that are up- and down-regulated during the initial phases of tissue destruction, thereby allowing us to modify and specify treatment in the hope tailor made therapy may be offered to patients suffering from these crippling diseases.

During our half-year experiment, in a longitudinal study we discovered that the DBA/2 murine strain which was deemed to be resistant to PGIA, proved to be susceptible to PGIS after consecutive intraperitoneal injections of human PG and adjuvant. The Ankylosing spondylitis-like

inflammation seen in DBA/2 mice has several overlapping symptoms to the human disease, although in comparison to the BALB/c mice it was not found as rapidly progressive.

The inflammatory events began with inflammatory cells appearing around the IVDs, followed by a massive neutrophil cells invasion mounted up against the disc, which started to digest the annulus fibrosus and the nucleus pulposus. Later, erosion of the bone surfaces led to bony ankylosis and within 1-3 months, the classic “bamboo-spine” appearance was observed. The disease itself is has a progressive autoimmune nature, although it is in the hyperactive form, that results in rapid ankylosis.

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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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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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