THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

INVESTIGATION OF THE GENETIC BACKGROUND OF SPONDYLARTHROPATHY IN MURINE MODEL OF SPONDYLITIS

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1. Introduction

Ankylosing spondylitis (AS) is the prototype of spondyloarthropathies (SpA), a group of inflammatory rheumatic diseases with shared genetic background, as well as common clinical features. Family clustering is an important feature of AS that suggests the role of genetic factors in susceptibility to AS. For example, in families of SpA patients, additional SpA cases occur mostly among HLA-B27$^+$ relatives. Regarding twin studies in AS, in a Finnish study the concordance was 50% between monozygotic twins, 15% overall among dizygotic twins and 20% among HLA-B27$^+$ dizygotic twins. In a study carried out in Britain, these values were 75%, 12.5% and 27%, respectively. Differences in concordance rates between monozygotic and dizygotic twins indicate the crucial role of genetic factors in susceptibility to AS.

Considering the role of genes, the major histocompatibility complex (MHC) alone is not sufficient to explain the heritability of AS. While more than 90% of Caucasian AS patients are HLA-B27 positive, only <5% of HLA-B27$^+$ members of the general population develops AS. Thus, HLA-B27 has been accounted for only approximately 20-50% of the overall genetic susceptibility to AS.

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 MHC encoded human leukocyte antigen HLA-B27, but the presence of HLA-B27 alone is insufficient for disease development. 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. 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* and *Yersinia* antigens, but no appropriate microorganisms have yet been identified in patients with AS. Therefore, extensive studies have been undertaken to identify other, non-MHC genetic factors and, indeed, approximately a dozen chromosome regions or gene clusters have been linked to AS.

Linkage analysis, genome-wide screening and candidate gene association studies have led to the identification of several non-MHC chromosome regions possibly linked to AS. Some of these loci, such as the interleukin-1 (IL-1) gene cluster has been consistently reported by independent research groups. Others, such as the genes of ARTS1 (also known as ERAP1) and IL-23 receptor (IL-23R) have been described by the Wellcome Trust Case-Control Consortium (WTCCC) study group that had formerly performed the genome-wide association study of 14000 cases of seven common diseases. Yet, less information is available regarding the genetics of AS in comparison to, for example, rheumatoid arthritis (RA).

Despite of the increasing amount of data about genetic contributors, AS is a multifactorial disease, where the „conspiracy" of genes and environmental factors lead to the development of the well known clinical symptoms. These include both skeletal (axial and oligoarticular) and extraskeletal manifestations (uveitis, inflammatory bowel disease, cardiac and pulmonary symptoms). Without proper treatment AS can lead to disability and even life threatening complications.

In this study, first we summarize data on the genetic basis of AS based on both human and rodents studies. Than, we present our experimental results based on the investigation of a murine model of spondylitis. We hope that our findings can contribute to the better understanding of development of spondyloarthropathies and promote further steps in order to overcome this disease.

2. Review of recent literature

2.1. Role of HLA-B27 and other MHC genes

2.1.1. The significance of HLA-B27

The association between HLA-B27 and AS was first reported in the early 1970s. The prevalence of HLA-B27 is about 6-8% in the general population and >90% among AS patients. Numerous studies indicate that AS occurs in 1.2-1.3% of HLA-B27$^+$ individuals in the general population compared to 15-21% of HLA-B27$^+$ first degree relatives of AS patients. As estimated by linkage analysis, as well as HLA-B27-dependent multiplicative model, the genetic contribution of HLA-B27 is about 5-6%. The concordance rates for HLA-B27$^+$ mono- and dizygotic twins are 63% and 23%, respectively. HLA-B27 contributes to 20-30% of the total genetic risk for AS.

Although there is no doubt that HLA-B27 is the major susceptibility gene for AS, its mechanism of action is still not known. All manifestations of SpA spontaneously develop in HLA-B27 transgenic rats indicating a direct role of this gene in disease susceptibility. Among the 25 known HLA-B27 alleles, HLA-B*2705, the predominant allele in the Caucasian population, may be the original allele and all other alleles may be derived from HLA-B*2705 by mutation. Most allelic mutations affect the variable region and thus result in altered interactions between T cell receptors and antigenic peptides. While most other HLA-B27 alleles have been associated with SpA, HLA-B*2706 and HLA-B*2709 occurring in South-East Asia and Sardinia, respectively, show no association with SpA.

2.1.2. Other HLA-B alleles

Among other HLA-B alleles, HLA-B*1403 has been associated with AS in Togo, while HLA-B60, -B35 and -B39 may play a minor role in susceptibility to SpA.

2.1.3. The role of HLA-B molecules in antigen presentation

In HLA-B alleles that confer susceptibility to SpA a presence of glutamic acid at position 45 and that of cysteine at position 67 of the HLA-B molecule is the specific pattern present in all alleles associated with SpA but absent in SpA-independent alleles. Based on these structural alterations, functional theories have emerged. The arthritogenic peptide theory suggests that this molecular structure enables the presentation of specific peptides that induce an autoimmune response. Regarding the impaired folding theory, disulfide bridges are formed between two cysteines at position 67 resulting in altered intracellular trafficking of the molecules.

2.1.4. Possible role of other MHC genes

MHC genes other than HLA-B may also be involved in the development of SpA. These genes may include class II MHC alleles (HLA-DR genes), tumor necrosis factor-α (TNF-α) and complement genes, as well as some genes involved in antigen presentation by class I MHC molecules including TAP, LMP2 and LMP7. Unfortunately, the predominant role of HLA-B27 highly influences the interpretation of these results as the reported associations may rather be attributable to linkage disequilibrium between the mentioned loci and HLA-B27. Only the direct additional effect of HLA-DR4 has been confirmed in HLA-B27$^+$ relatives of SpA patients.

2.2. Non-MHC alleles in ankylosing spondylitis

As discussed above, MHC accounts for less than 50% of the genetic risk for AS. Various techniques have been used to study the contribution of non-MHC genes to susceptibility to and severity of human AS.

2.2.1. Linkage studies

Linkage exists when a candidate gene and another known locus are very close to each other, therefore, the two loci are transmitted together. Such linkage studies can be carried out in large families with many family members affected by a given disease. In these studies, results are presented as a non-parametric linkage score (NPL), which is then converted to a log odds ratio (LOD) score. High LOD values (LOD≥3.6) indicate significant associations, while LOD≥2.2 values are suggestive.

There have been four large linkage studies with respect to susceptibility to AS. In the North-American Spondylitis Consortium (NASC) study, 185 families with 255 affected sibling pairs were analyzed. The most significant associations were attributed to the MHC locus located on chromosome 6 (LOD=15.6) and a single non-MHC locus on chromosome 16 (LOD=4.7). Other loci with suggestive LOD values were located on chromosomes 1, 3, 4, 5, 10, 11, 17 and 19. In the French AS genetics cohort (GFEGS) 180 families with 244 affected sibling pairs were assessed. Again, the MHC locus had the strongest linkage. Also in this cohort, a region on the short arm of chromosome 9 was significantly associated with acute anterior uveitis but not with AS. Two studies from Oxford studies confirmed the strongest linkage with the MHC region and suggested linkage with loci on chromosomes 2, 3, 9, 10, 11, 16 and 19.

A pooled metaanalysis indicated the most clear evidence for linkage to MHC on chromosome 6. Additional strong linkage was observed with regions on chromosomes 16 and 10, while moderate linkage was seen with loci on chromosomes 3, 4, 5, 6, 11 and 17.

Some loci were also associated with disease activity and functional severity. While MHC showed no linkage, regions on chromosome 18 were significantly associated with the BASDAI score. In addition, regions on the long arm of chromosome 2 exerted suggestive linkage with the BASFI functional impairment score.

2.2.2. Candidate gene associations

There have been conflicting results regarding the interleukin-1 (IL-1) gene cluster. This gene complex is located on chromosome 2 and includes genes encoding IL-1α (*IL-1A*), IL-1β (*IL-1B)*, IL-1 receptor antagonist (*IL-1RN)* and other genes (*IL1F5.IL1F10*. This gene cluster corresponds to the region on chromosome 2 identified in linkage studies described above. IL-1α and IL-1β are pro-inflammatory cytokines primarily produced by monocyte/macrophages, which stimulate the release of other inflammatory mediators including prostaglandins, matrix metalloproteinases and other cytokines, as well as the expression of various adhesion receptors. IL-1Ra competitively block the binding of IL-1α and IL-1β to their receptor and thus antagonize the effects of these cytokines (44). While early small studies suggested association between AS and the *IL1RN* gene encoding IL-1Ra, further larger studies could not confirm this association. However, some small studies and a recent metaanalysis showed higher carriage of a variable nucleotide tandem repeat (VNTR) in intron 2 of the *IL1RN* gene in AS patients compared to controls. Moreover, two SNPs in exon 6 of the *IL1RN* gene were also associated with AS (51,52). Regarding other genes in the IL-1 cluster, altogether 14 SNPs in the *IL-1A* and *IL-1B* genes exerted significant associations with AS. Among these SNPs, SNP rs3783526 in the *IL-1A* and

rs1143627 in the *IL-1B* gene showed the most significant associations. In addition, SNPs rs2856836, rs17561 and rs1894399 in the *IL-1A* gene also showed very strong associations.

2.2.3. Genome-wide association studies

As described above, the WTCCC initiative identified two new loci strongly associated with AS, IL-23R and ARTS1. IL-23R has been implicated in the pathogenesis of RA, psoriasis and inflammatory bowel diseases (IBD). IL-23 is a potent pro-inflammatory cytokine that stimulates the generation of Th17 cells, as well as the production of other cytokines including TNF-α, IL-6, IL-17 and IL-22. The gene for the IL-23R protein is located on chromosome 1. Susceptibility to Crohn's disease and psoriasis has been associated with the SNP rs11209026. In addition, SNP rs7530511 is also associated with psoriasis. Apart from the SNP mentioned above, several other SNPs including rs10889677 and rs2201841 also had significantly increased prevalence in Crohn's disease in comparison to controls. We have recently confirmed that SNPs rs10889677 and rs2201841 are not only associated with IBD, but also with RA. In the WTCCC cohort, eight IL-23R SNPs were genotyped in 1000 AS patients and 1500 controls. Seven out of these 8 SNPs showed association with AS. Highly significant associations were found with SNPs rs11209032, rs11209026 and rs10489629. Associations between IL-23R gene polymorphisms and AS have recently been confirmed in a Spanish cohort. The IL-23R gene is responsible for 9% of the population-attributable risk of AS.

As far as ARTS1 (Aminopeptidase Regulator of TNF receptor Shedding) is concerned, this protein is an aminopeptidase in the endoplasmic reticulum. ARTS1, also known as ERAP1 (Endoplasmic Reticulum-associated Aminopeptidase 1) cleaves receptors for cytokines including TNF-α (TNF-R1), IL-1 (IL-1R2) and IL-6 (IL-6Rα) from the cell surface. ARTS1 is also involved in the processing of antigenic peptides to optimal lenghth for antigen presentation. The

three genes encoding ARTS1 are located on chromosome 5. In the WTCCC cohort and follow-up studies, five SNPs including rs27044, rs30187, rs17482078, rs10050860 and rs2287987 were associated with AS. In addition, there is no association between any ARTS1 SNPs and either Crohn's disease or ulcerative colitis. Thus, ARTS1 may not be involved in the pathogenesis of various SpAs but its effects may be specific for AS within the SpA family. The ARTS1 gene is responsible for 26% of the overall risk of AS.

2.2.4 Other genes with unconfirmed associations

As discussed above, the associations of IL-1 cluster genes, IL-23R and ARTS1 genes have been confirmed in large cohorts. There have been small studies suggesting the associations of other genes with AS.

Some alleles of the cytochrome P450 CYP2D6 gene located on chromosome 22 have been weakly associated with AS. There have been controversies regarding possible associations of AS with the transforming growth factor-β (TGF-β), ANKH and Toll-like receptor 4 (TLR4) genes. While some studies suggested marginal associations of these genes with AS, other studies could not confirm this. Previous studies suggested that some IL-10 allele polymorphisms may be protective against the development of reactive arthritis. However, no such association was confirmed in AS.

Finally, NOD2/CARD15 mutations have been associated with Crohn's disease, however, several studies confirmed that there were no such associations with AS.

2.3. Animal models of spondylarthropathies and their use in genetic studies

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, but the *ank* gene, either in humans or mice, is not involved in autoimmune processes. Other models of SpA have been developed in HLA-B27 transgenic rodents, or in transgenic mice expressing a mutant type IX collagen or a truncated form of TNF-α.

In our experiments we used proteoglycan (PG)-induced spondylitis (PGIS), an autoimmune murine model of SpA. According to our results PGIS model can also provide useful data about the genetic background and pathogenesis of SpA.

2.4. Lessons from the proteoglycan-induced spondylitis (PGIS) model

Polyarthritis and spondylitis can be induced in susceptible mouse strains by immunization with human cartilage proteoglycan (PG). PGIS shows similarities to AS in terms of clinical and radiological features. Segregation of susceptibility to PG-induced arthritis (PGIA) from that to PGIS in different genetic crosses suggests that PGIA and PGIS are two separate diseases. Therefore, this model allows for the elucidation of genetic components involved in the etiology of SpA, independent of those controlling the susceptibility to PGIA. PGIS was induced in susceptible BALB/c and C3H/HeJCr (C3H) strains of mice, and in their F1 and F2 generations derived from intercrosses with arthritis- and/or spondylitis-resistant DBA/2 and DBA/1 parent strains, by systemic immunization with cartilage PG. Almost all (97-100%) PG-immunized BALB/c and C3H mice developed peripheral arthritis by 2 weeks after the third antigen injection. Massive inflammatory cell infiltration, pannus formation, and cartilage and bone erosion characterized the histopathologic picture of the affected joints. None of the DBA/1 or DBA/2

parents nor the (BALB/c x DBA/2)F1 hybrids developed arthritis until the end of the 14–18-week experimental period.

The incidence of PGIS was 62–70% in inbred BALB/c and C3H mice, whereas only weak and sporadic peridiscitis involving only 1 or 2 intervertebral discs occurred in a few PG and DDA–immunized DBA/2 mice. No spondylitis was found in PG- or human CII–immunized animals of the DBA/1 strain. The incidence and severity of spondylitis were highly comparable in both PGIS-susceptible inbred strains (BALB/c and C3H).

Recently, a new extensive longitudinal study, using *in vivo* ProSense and OsteoSense probes confirmed by X-rays images, and then ex vivo using histopathology, indicates that DBA/2 mice are susceptible to SpA, but not to arthritis (authors unpublished observation).

Although F1 hybrids of the BALB/c x DBA/2 intercross were fully resistant to peripheral PGIA, unexpectedly, more than 30% of them developed PGIS, whereas none of the F1 hybrids of BALB/c x DBA/1 developed PGIS. Even more surprising was that F2 hybrids of BALB/c x DBA/2 (PGIS-susceptible versus PGIS-resistant parent strains, respectively) and (BALB/c x C3H)F2 mice (both of which have PGIS-susceptible parent strains) showed a similarly high incidence (63–70%) and severity of spondylitis, but none of the F2 hybrids of the BALB/c x DBA/1 intercross developed spine involvement. These obervations were unexpected as the F2 hybrids of both genetic intercrosses (BALB/c x DBA/2 and BALB/c x DBA/1) exhibited a similar incidence and severity of PGIA when immunized using the same protocol (28). These observations suggest that the DBA/1 strain carries very strong protective genes against SpA, while the DBA/2 genome may contain both spondylitis susceptibility and protective genes that might be silent in the original background. BALB/c and DBA/2 strains carry the same H-2d allele. This indicates that the MHC alone (e.g., in DBA/2 mice) is insufficient to control susceptibility to PGIA, but supportive to PGIS. This notion was also supported by the results in

the (BALB/c x DBA/1)F2 generation, in which 25% of the immunized mice were homozygous for the H-2d allele (79), but none of the F2 hybrids developed spondylitis.

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3. Materials and Methods

3.1. Animals, antigens (Ag), and immunization

Inbred female BALB/c and male DBA/2 mice were purchased from the National Cancer Institute and mated to generate F1 and then F2 offspring. All animal experiments were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center. All mice were maintained in a pathogen-free environment. At 12 week of age, mice were immunized intra-peritoneal (i.p.) with 100 μg of PG (measured as protein) emulsified with 2 mg of dimethyldioctadecylammonium bromide (DDA) adjuvant in 100 μl of 1x PBS (0.15 M NaCl, 0.01 M potassium phosphate, pH 7.4) as described formerly. Mice received total of four injections every 3 week (days 0, 21, 42, and 63) and were sacrificed 3–4 week after the fourth PG injection, i.e., on days 84–91 of the experiment.

3.2. Clinical and immunological phenotypes

The axial skeleton spanning the spine from the midcervical to distal lumbar region was dissected, fixed in neutral buffered 10% formalin, decalcified, and embedded in paraffin. Spine sections were stained with H&E and individual IVDs scored as described earlier. In brief, enthesitis, inflammatory cell accumulation around the IVD (discitis), and/or infiltration of the annulus fibrosus with or without evident tissue damage was recorded as severity score 1; massive but <50% resorption/erosion of the IVD received a score of 2; nearly complete resorption (>50%) of the IVD was recorded as score 3; and cartilaginous/bony ankylosis was given a score of 4. An average of 18 IVDs per mouse was scored. Finally, a spondylitis index (SPI) for each animal was calculated by dividing the sum of scores (all IVD scores) with the number of IVDs examined histologically. In addition, a specific index for mice exhibiting late onset of spondylitis (SPI_{LS}) was also calculated. A mouse was assigned SPI_{LS}"1" if at least one of the IVDs had a mild

severity score of 1. Otherwise a mouse with progressive spondylitis was assigned SPI_{LS}"0", even if IVDs had a more advanced form of the disease indicated by severity scores 2, 3, or 4.

Ag (PG)-specific T cell proliferation was measured by [3H]thymidine incorporation in response to in vitro PG treatment as described. Ag induced cytokine production (IL-2, IFN-γ, IL-4, and TNF-α), and serum levels of Abs, amyloid A, and cytokines (IL-1, IL-4, IL-6, and TNF-α) were determined using commercially available ELISA-s as described previously.

3.3. Genomic markers

Markers were selected for detectable simple sequence length polymorphism between the parent BALB/c and DBA/2 strains from the mouse genome database (www.informatics.jax.org) or alternatively were designed using primers flanking regions of short, usually <100 bp, tandem repeats in the mouse genome. Differences in length between PCR fragments of *BALB/c* and *DBA/2* alleles were >3%. Polymorphism between the strains was detected in 3.5% high resolution Aquapore agarose gel (National Diagnostics) upon staining with ethidium bromide and UV illumination. All 20 mouse chromosomes, except chromosome Y, were covered with a total of 224 polymorphic markers at an average spacing of 6.2 cM (10.8 Mbp).

3.4. Genome screening and statistical analysis

Genomic DNA was isolated from mouse kidney using proteinase K and sodium lauryl sulfate. DNA was genotyped with simple sequence length polymorphic markers (MWG Biotech) using conventional PCR and gel-electrophoresis as described previously. Initial linkage map was generated with Map Manager QTX (25) using the Kosambi mapping function. The order of markers was further adjusted using the "ripple" command and then confirmed according to

physical positions of oligonucleotide primers in the National Center for Biotechnology Information mouse genome assembly (www.ensembl.org/Mus_musculus/) and the Celera Discovery System genome database (www.celeradiscoverysystem.com/). Single marker effect was estimated using marker regression in Map Manager QTX. For traits that demonstrated association with genomic markers stronger than $\chi^2 > 10$ ($p < 0.01$), both simple and complex interval mappings were performed on the entire genome using Windows QTL Cartographer. Experiment-specific empirical thresholds for likelihood ratio statistic (LRS) were established for each trait with a permutation test ($n = 2000$, 1 cM walk speed) according to an algorithm proposed by Churchill and Doerge and implemented in Map Manager QTX and Windows QTL Cartographer. Levels for genome-wide highly significant ($\alpha < 0.001$) and significant ($\alpha < 0.05$) linkage were used. For a suggestive linkage, we used a $p < 0.05$ chromosome-wise significance level ($\alpha < 0.63$), which corresponds to one false-positive QTL for the entire genome.

Statistical analysis was performed using the SPSS statistical software package. As spondylitis indices demonstrated nonparametric distribution in the F2 hybrid population, we used the Mann-Whitney *U* test to examine differences between groups and the Spearman's correlation coefficient (r_s) to evaluate biases between traits. χ^2 statistics and Kruskal-Wallis H tests were used to determine the difference between distributions of traits in the genotypes. The two-sample Student's *t* test was used for comparison of means of two groups, when data showed normal distribution. The significance level was set at $p < 0.05$.

4. Results

4.1. Clinical and immunological parameters of spondylitis in parental BALB/c and DBA/2 strains

Upon systemic immunization with cartilage PG, BALB/c mice develop spondylitis with an incidence of 61.5%. Using the same immunization protocol, DBA/2 mice demonstrated much lower susceptibility (4%) with notably less severe IVD damage than BALB/c mice, and spine ankylosis was never detected in the DBA/2 strain.

The major immunological characteristics were measured in progenitor mice and correlated with disease susceptibility. The DBA/2 strain had almost three times higher serum levels of Igs as compared with BALB/c mice (17.8 vs 6.5 mg total Ab per milliliter of serum with IgG1 being the major Ig isotype in both strains). The serum level of IgG2a isotype showed an opposite distribution between the strains: it was three times more abundant in BALB/c. Based on the IgG1/IgG2a Ig isotype ratio, both BALB/c and DBA/2 strains demonstrated a Th2-type immune response; however, in Pg-immunized DBA/2 mice, the Th2-type response was more prominent. Interstrain differences found in histological scores were highly correlated with the elevated concentration of proinflammatory cytokines IL-1β and IL-6 in sera of spondylitis-susceptible BALB/c mice, although serum levels of IL-4 and TNF-α were comparable.

Ag (PG)-stimulated lymphocytes of BALB/c mice demonstrated significantly higher production of IFN-γ and IL-4 when compared with lymphocytes from DBA/2, and the IFN-γ/ IL-4 ratio was 2.2-times higher in BALB/c (5.1) than in DBA/2 mice (2.3). This observation also indicates that BALB/c mice with PG-induced spondylitis (PGIS) exhibit a more significant shift to Th1 dominance, whereas the virtually resistant DBA/2 strain does not.

4.2 Correlations between clinical and immunological traits in F1 and F2 hybrid populations

The incidence of spondylitis in F1 hybrids of BALB/c and DBA/2 mice was 35.5%, approximately the median value between the progenitor strains, although the severity of the disease was relatively low (SPI 0.16). Unexpectedly, the incidence of spondylitis in F2 hybrid mice was the same as in the susceptible parent BALB/c strain with a severity score nearly twice higher than in BALB/c mice.

Because mice of the F1 generation are genetically homogeneous, only nongenetic environmental factors might be responsible for trait variance. In contrast, F2 hybrid mice are genetically heterogeneous and both genetic and environmental factors might be in action. Thus, the correlation between spondylitis and immunological parameters in F1 and F2 populations must come from different sources. One of the best examples is the SPI, which very tightly correlated with SPI_{LS} in the F1 population (r_s 0.97), but this correlation was notably weaker in F2 hybrid mice (r_s 0.54) because the heterozygous combination of genes differently affected the SPI and SPI_{LS} in (BALB/c x DBA/2) F2 hybrids.

To determine the most important immunological parameters that might be associated with spondylitis in F1 and F2 hybrid populations, we calculated correlation coefficient r_s. The strongest positive correlation of PGIS was found with serum concentrations of amyloid A (SAA; an acute phase protein in mice) and IL-6, r_s 0.28 and 0.43, respectively, which is consistent with an even stronger correlation between SAA and IL-6 (r_s 0.74). Spondylitis correlated positively with Ab production (both for IgG1 and IgG2a isotypes) in the F2 hybrid population. Surprisingly, spondylitis correlated negatively with all measured T cell responses (T cell proliferation, IFN-γ, IL-4, and TNF-α production) but only in the F2 mice. There was no correlation between spondylitis and Ag-specific T cell responses (or the serum levels of most Abs) in homozygous F1 hybrid mice. In contrast, these correlations are strong in the segregating F2 population,

suggesting that spondylitis is controlled by immune response-related genes and/or allele combinations in this animal model. In summary, enhanced serum levels of IL-6, SAA, and Ab, together with decreased production of IFN-γ, IL-4, and TNF-α by PG-stimulated T cells, could be considered the strongest predictors of spondylitis in this MHC-matched cross of PGIS-susceptible and resistant mice.

4.3. Linkage analysis for spondylitis in (BALB/c x DBA/2)F2 hybrids

To map non-MHC spondylitis-susceptibility genes, we used a BALB/c x DBA/2 cross, where both progenitor strains carry the same H-2d haplotype, thus deliberately excluding the effects of the *MHC* genes from linkage analysis. To perform an effective scan for murine genes regulating SPI and SPILS clinical traits, F2 hybrid male and female mice were immunized with PG, scored for spondylitis, and a set of immune-related parameters was measured. Accordingly, all mice were genotyped for the entire mouse genome (20 chromosomes) with 224 markers. Interval mapping and single marker effect analysis indicated the presence of a very limited number of chromosome loci controlling spondylitis (SPI, SPI_{LS}), serum level of Ab (IgG2a isotype to mouse PG), and IL-6 in the sera of F2 mice, whereas other traits did not show significant linkage.

The major genetic locus controlling spondylitis was identified on the telomeric part of chromosome 18 (*Pgis1*). The LRS reached a value of 31, which exceeds the highly significant cut-off level introduced by Lander and Kruglyak and empirically established a highly significant threshold at $\alpha < 0.001$. Composite interval mapping (Windows QTL Cartographer, standard model 6 with control of five markers inside of a 10 cM window) confirmed the QTL peak position for markers D18Mit51 and D18Mit142.

The second major spondylitis QTL was identified on chromosome 2 (*Pgis2*). This locus, however, was fairly vague, when simple interval mapping was used, and occupied up to one-third of the chromosome. Using composite interval mapping, we found the peak for this QTL (LRS 16.9, $\alpha < 0.05$) located near marker D2Mit241. When the integrated effects of each chromosome upon spondylitis susceptibility was calculated, chromosomes 2 and 18 appeared to jointly control 40.5% of the entire SPI trait variance in the F2 population.

The additional spondylitis phenotype index, SPI_{LS}, was introduced in this study. This is a binary index with score "1" for mice with late onset of spondylitis, and it was tightly correlated with low PGIS severity. Despite the high correlation between SPI and SPI_{LS}, the genetic basis of these two clinical traits seems to be different. For example, there is no QTL for SPI_{LS} on chromosome 18, whereas the major QTL controlling late/weak spine inflammation was found at the same position as the *Pgis2* locus. However, the *Pgis2* locus was more prominent and narrower for SPI_{LS} than for SPI. *Pgis2* alone contributed to 28.7% of the entire SPI_{LS} trait variance in the F2 population. The *Pgis2* peak was near the D2Mit296 marker (LRS 21.1, $\alpha<0.05$, simple interval mapping), and the position was confirmed by composite interval mapping (LRS 25.9, $\alpha < 0.001$).

Because spondylitis in F2 hybrid mice correlated significantly with certain immunological phenotypes (T and B cell responses, serum cytokines), we performed linkage analysis for intermediate immunological traits as well. Serum concentration of IgG2a isotype autoantibodies seemed to be under control of several QTLs. Two major loci were mapped close to each other on mouse chromosome 11 (LRS 17.7 and 19.4, $\alpha< 0.001$), and two weaker QTLs were found on chromosomes 1 and 5 (LRS 11.8 and 10.8, $\alpha < 0.63$). Serum concentration of the proinflammatory cytokine IL-6 was under the control of a single QTL located in the central part of chromosome 14 (LRS 14.9, $\alpha < 0.05$), and composite interval mapping confirmed the peak position.

4.4. Interaction between the two major clinical QTLs on chromosomes 2 and 18

Although both BALB/c and DBA/2 strains carry genes for susceptibility to spondylitis, these strains responded very differently to PG immunization. To determine the source of disease-controlling alleles in the F2 population, which might come either from the BALB/c or the DBA/2 strain, we calculated the average SPI separately for BALB/c homozygous ($Pgis^{B}$), DBA/2 homozygous ($Pgis^{D}$), and BALB/c-DBA/2 heterozygous mice ($Pgis^{H}$), and for each QTL. Unexpectedly, we have found that F2 mice, which were homozygous for the DBA/2 allele of *Pgis1* ($Pgis1^{D}$), developed spondylitis at a significantly higher incidence and severity rate than BALB/c homozygous animals ($Pgis1^{B}$ vs $Pgis1^{D}$, $p < 0.000003$), which suggested that the major disease susceptibility allele was derived from a resistant DBA/2 strain. Another QTL, *Pgis2* on chromosome 2, showed "normal" phenotype-genotype relationship, and mice with BALB/c homozygosity for this region were significantly more spondylitis-susceptible than F2 hybrid mice with DBA/2 homozygosity ($Pgis2^{B}$ vs $Pgis2^{D}$, $p < 0.0005$), thus indicating that the disease allele was derived from the spondylitis-susceptible BALB/c strain. For both *Pgis1* and *Pgis2* QTLs, the BALB/c alleles were dominant over the *DBA/2* allele, since BALB/c-DBA/2 heterozygous mice had as high SPI as BALB/c homozygous animals.

Thus, in F2 hybrid mice the highest susceptibility to spondylitis was observed in DBA/2 homozygous animals ($Pgis1^{D}$); that seems to contradict the virtual resistance of the parental DBA/2 mice having the same genotype of the *Pgis1* locus. As we found only two major loci controlling spondylitis in the BALB/c x DBA/2 cross, to explain the silence of the $Pgis1^{D}$ allele in the parental DBA/2 strain, we examined the influence of one locus to the other. The SPI and incidence of the disease were calculated based on the hypothesis of cooperation between *Pgis1* and *Pgis2* loci; average values for phenotypes were calculated separately for each of the nine

allele combinations. When the effects of *Pgis1* genotypes upon clinical traits were analyzed independently of the *Pgis2* locus, $Pgis1^{D}$ homozygous mice were most spondylitis-susceptible, $Pgis1^{B}$ homozygous mice demonstrated the lowest susceptibility to the disease, and $Pgis1^{H}$ heterozygous animals were in-between. Surprisingly, the relationship between *Pgis1* alleles was found critically dependent upon the genetic composition of the *Pgis2* locus. It seemed that at least one copy of the *Pgis2* of BALB/c origin ($Pgis2^{B}$) was necessary for the high penetrance of the $Pgis1^{D}$ allele and its function as a spondylitis-permissive gene. Thus, mice bearing the $Pgis1^{D}$ allele were the most spondylitic (average SPI = 0.96) and mice with the $Pgis1^{B}$ allele were the least affected (SPI = 0.13). However, the phenotypic difference between $Pgis1^{D}$ and $Pgis1^{B}$ allele-carrying mice vanished on a pure $Pgis2^{D}$ genetic background. Thus, the spondylitis-promoting $Pgis1^{D}$ allele needs the $Pgis2^{B}$ allele for the complete trait penetrance and full action. F2 hybrid mice that carry the most malicious $Pgis1^{D}$–$Pgis2^{B}$ allele combination demonstrated the highest spondylitis severity and 100% incidence of the disease, further confirming the importance of both loci for disease development.

5. Discussion, new results

In this study, we present for the first time a complete genome scan for genetic loci controlling spondylitis susceptibility in mice. We determined the genetic basis of trait variance in a complex phenotype that developed from natural genetic variation instead of artificial modification, i.e., gene deficiency. Despite a number of murine models for spondyloarthropathy established and studied earlier, the genomic basis of disease susceptibility is not known, except for the contribution of *HLA-B27* and a few other genes to disease pathology.

Earlier we demonstrated that spondylitis susceptibility of mice of different parental strains and genetic crosses was associated with certain H-2-permissive haplotypes, which indicates the leading role of the MHC in murine arthritis and spondylitis. The major goal of this study was finding non-MHC genes. We have found only two major genetic loci regulating spondylitis in the BALB/c x DBA/2 cross: the *Pgis1* disease-controlling allele originating from the DBA/2 strain (although the gene remained silent in this strain), and the *Pgis2* allele which is derived from the BALB/c strain. Obviously, the combined effects of these two loci/genes resulted in high disease incidence in BALB/c x DBA/2 F1 hybrids (35.5%), and even higher susceptibility in F2 hybrid mice (61.7%) when two spondylitis-promoting alleles of two genes supplemented each other. Similarly, disease severity, which was defined only in SPI-positive mice, was even higher in F2 hybrids than in BALB/c progenitor mice due to a cumulative effect of two permissive genes in the F2 population.

Despite the differences between spondylitis-susceptible and resistant mice in immune function-related traits (T and B cell responses, and serum cytokines), and the known pattern of interaction among SPI-linked genes inside the *Pgis1* and *Pgis2* loci, at this stage of investigation it is not possible to identify spondylitis contributing primary causative genes within these loci.

Comparison of genomic maps of disease-controlling loci, in human patients and in mice and syntenic mapping, might aid in identification of gene candidates in future studies.

The major SPI locus in the BALB/c x DBA/2 cross was found on the telomeric region of mouse chromosome 18. The *Pgis1* locus is flanked by D18Mit55 and D18Mit80 markers and occupies the region from 54 Mbp to the chromosome telomere. The *Pgis1* locus overlaps with murine QTLs for a number of autoimmune diseases such as murine lupus (*Lbw6*), PG- and collagen induced arthritis (*Pgia11* and *Cia18*), experimental allergic encephalomyelitis (*Eae25*), wound healing (*Heal9*), and chronic multifocal osteomyelitis (*Cmo*).

The murine *Pgis1* locus (54–81 Mbp) is homologous with human chromosomes 5q (segments 110–129 Mbp and 137–150 Mbp) and 18q (segments 17–52 Mbp and 64–76 Mbp) (www.ncbi.nlm.nih.gov/mapview/). These human chromosome segments contain two loci for AS; one on chromosome 18q was found in Oxford pedigree *AS*(*Ox*), and the *AS*(*Eu*) locus was found in the kindred of European origin on chromosome 5q. The list of gene candidates in the locus includes metalloproteinase *Adamts19*, IL *IL-17b*, MHC class II-associated invariant chain *Cd74*, macrophage CSF I receptor *Csf1r*, and others. The region also contains the *Nfatc1* gene encoding calcineurin-dependent NF 1 of activated T cells. This gene regulates IL-2 and IL-4 gene transcription, differentiation, proliferation, and activation-induced cell death in T lymphocytes (http://harvester.embl.de/harvester/). Because we have found a significant correlation between SPI and T cell responses, this gene inside the *Pgis1* locus is a plausible candidate as a primary causative factor.

The size of the second major locus, *Pgis2*, is larger than *Pgis1*. The *Pgis2* locus is flanked by the D2Mit293 and D2Mit156 markers located at 26.4 and 57.0 Mbp, respectively. Simple interval mapping suggested slightly different positions for SPI and SPI^{LS} on mouse chromosome 2. However, we maintain the hypothesis that a single gene/locus in this region controls disease

susceptibility based on the following considerations. First, the mode of inheritance for SPI and SPI^{LS} peak position markers is identical, and demonstrated a dominant action of the *BALB/c* allele. Second, we did not succeed in splitting *Pgis2* into two loci by varying the number of markers in composite interval mapping. Third, both SPI and SPI^{LS} clinical traits were closely correlated both in the F1 and F2 hybrids of the BALB/c x DBA/2 cross.

The murine *Pgis2* locus (24–57 Mbp) contains numerous autoimmune loci linked to Ag-induced bronchial hyperresponsiveness (*Abhr1* and *Abhr2)*, *e*xperimental autoimmune gastritis (*Aig*), collagen-induced arthritis (*Cia2* and *Cia4*), serum transfer-induced arthritis (*Stia2*), and experimental allergic encephalomyelitis (*Eae21*). Interestingly, *Pgis2* also overlaps with QTLs controlling the anatomical development of the skeleton such as femoral cross-sectional area (*Fcsa5)* and periosteal circumference and femur length (*Pcfm1*).

Combining data from publicly available online genome resources and analysis of published literature (www.ncbi.nlm.nih.gov/mapview/), we found that the *Pgis2* locus is syntenic with two segments in the human genome: one located on chromosome 2q between 143 and 161 Mbp, and the second on chromosome 9 between 120 and 137 Mbp. Both intervals on chromosomes 2 and 9 contain QTLs for AS identified in British pedigree. Besides this, linkage with C5 deficiency monogenic syndrome resulting in recurrent local and systemic infections and systemic lupus erythematosus (Mendelian inheritance in man) was also found in the syntenic human loci.

Gene candidates in the locus include complement component 8 γ (*C8* γ) and an *IL-1* gene cluster, namely IL-1R antagonist and five members of the IL-1 family. The latter gene cluster is considered the major gene candidate for AS on human chromosome 2 in the Oxford study. After initial genome scan and identification of positive linkage on chromosome 2, the Oxford group found significant association between single polymorphisms and IL-1-related haplotypes. In our

study, IL-1 itself did not show any significant linkage, probably because the difference in serum concentrations of IL-1 was not significantly different in BALB/c and DBA/2 mice. Any of the gene candidates located between the telomeric end of *Pgis2* locus and the IL-1 cluster might also be functionally associated with the spondylitis phenotype.

The best-known gene proposed to have a crucial role in ankylosis in mice is the *Ank* gene. Spontaneous mutation(s) on chromosome 15, resulting in progressive ankylosis, was described in 1981 and 1988. Later, the mutation was mapped inside the *Ank* gene. At present it is believed that mutation of this gene is responsible for craniometaphyseal dysplasia and crystal deposition arthropathy (Mendelian inheritance in man). In our genome scan we found only a suggestive QTL on mouse chromosome 15, on which the *Ank* gene is located. However, this locus was linked with PgIA in our earlier studies, suggesting that chromosome 15 loci might be partially involved in spondylitis in this murine model.

In addition to the two major SPI loci on chromosomes 2 and 18, and a suggestive locus on chromosome 15, four more loci demonstrated suggestive linkage. We found QTLs on chromosomes 11, 12, and 19 (LRS 11.0, 12.5 and 12.1, respectively). Suggestive spondylitis QTL on the telomeric part of chromosome 12 contains a cluster of Ig H chain genes (www.ensembl.org/Mus_musculus/). This locus has been found in several crosses produced in our laboratory to control PgIA or serum concentration of IgG2a, or both traits, although this QTL reached a significant level of linkage with arthritis only in the C3H x C57BL/6 cross.

Major QTLs for IgG2a and IL-6 did not fall into either *Pgis1* or *Pgis2* regions on chromosomes 2 and 18. However, there is an overlap between spondylitis and immunological QTLs when a suggestive threshold for linkage is considered. The telomere part of chromosome 11 carries suggestive a spondylitis-susceptibility locus, which coincides with the major peak for IgG2a. Additionally, our earlier studies showed two arthritis-controlling QTLs on chromosome

11 (*Pgia7* and *Pgia28*), which occupy the same regions as newly discovered QTLs for IgG2a in this murine cross. Chromosome 14 carried the arthritis *Pgia29* locus which shares a position with IL-6 QTL.

The pivotal role of both T and B cell populations in the induction of murine arthritis and spondylitis was shown in the experiments with adoptive disease transfer. Depletion of donor lymphocytes with Abs specific to Th and T suppressor populations, similar to depletion of B cells, prevented the successful disease transfer. Neither anti-PG Abs nor PG-specific B cells alone were able to transfer disease, but the cooperation between T and B cell subpopulations is necessary. Progenitor BALB/c mice upon immunization with PG produced twice more IL-6 than DBA/2 mice (57.8 pg/ml vs 30.1 pg/ml, $p < 0.013$); and the IgG2a Ig isotype concentration was three times higher whereas IgG1 concentration was three times lower in BALB/c than in DBA/2 mice. The correlations between disease severity and Th2-supported Ag-specific Ig levels were significant in (BALB/c x DBA/2)F2 hybrid mice as well. The strongest correlations with spondylitis were found for the IL-6 and IgG1 Ab isotype: the SPI vs IL-6 coefficient of correlation r_s was 0.43, and for SPI vs IgG1 was 0.23. Th1-type associated IFN-γ and TNF-α production by PG stimulated lymphocytes in vitro were found to be negatively correlated with spondylitis in the F2 hybrid mice further supporting the leading role of Th2-type cells in disease pathogenesis. Therefore, we conclude that a strong genetic predisposition toward a Th2 response and susceptibility to arthritis and spondylitis is similar in progenitor strains and in genetically mixed F2 hybrid mice, and the F2 population is modeling similar pathology as seen in the parental BALB/c strain.

Colocalization of QTLs for clinical and immunological traits, as was demonstrated for chromosomes 11 and 12, is a powerful approach to the effective mapping of spondylitis-

susceptibility genes and better understanding of the involvement of these loci, thus shedding light upon the mechanisms of spondylitis.

6. Summary

The HLA-B27 antigen has been accounted for 20-50% of the total genetic risk for ankylosing spondylitis (AS). However, susceptibility to AS cannot be fully explained by associations with the major histocompatibility complex (MHC). Recent studies including linkage analyses as well as candidate gene and, most recently, genome-wide association studies indicate significant associations of the interleukin-1 gene cluster, interleukin-23 receptor and ARTS1 genes, as well as other possible loci with AS.

In our experimental model autoimmune spondylitis was induced in BALB/c mice and their MHC-matched (BALB/c x DBA/2)F1 and F2 hybrids by systemic immunization with cartilage/intervertebral disk proteoglycan (PG). As in human ankylosing spondylitis, the MHC was the major permissive genetic locus in murine PG-induced spondylitis (PGIS). Two major non-MHC chromosome loci with highly significant linkage were found on chromosomes 2 (*Pgis2*) and 18 (*Pgis1*) accounting for 40% of the entire F2 trait variance. The dominant spondylitis-susceptibility allele for *Pgis2* locus is derived from the BALB/c strain, whereas the *Pgis1* recessive allele was present in the disease-resistant DBA/2 strain. The *Pgis1* locus significantly affected the disease-controlling *Pgis2* locus, inducing as high incidence of spondylitis in F2 hybrids as was found in the spondylitis-susceptible parent BALB/c strain. Additional disease-controlling loci with suggestive linkage were mapped to the chromosomes 12, 15, and 19. Severity of spondylitis in F2 mice positively correlated with serum levels of amyloid A, IL-6, and Pg-specific Abs, and showed negative correlation with Ag-induced T cell proliferation, IFN-_, IL-4, and TNF-_ production. A major locus controlling serum IL-6 was

found on chromosome 14 near osteoclast differentiation factor *Tnfsf11*. Locus on chromosome 11 near the *Stat3* and *Stat5* genes controlled serum level of the Ig IgG2a isotype. The two major genetic loci *Pgis1* and *Pgis2* of murine spondylitis were homologous to chromosome regions in human genome, which control ankylosing spondylitis in human patients. Thus, this animal model of experimentally induced spondylitis might facilitate the identification of spondylitis-susceptibility genes in humans.

7. Publications

7.1. Papers on the topic of the thesis:

1. **Vegvari, A.,** Szabo, Z., Szanto, S., Nesterovitch, A.B., Mikecz, K., Glant, T.T., Adarichev, V.A.: Two major interacting chromosome loci control disease susceptibility in murine model of spondylarthropathy. J Immunol, 2005, 175(4):2475-2483. (**IF: 6.48)**

2. **Végvári A**, Szabó Z, Szántó S, Glant TT, Mikecz K, Szekanecz Z: The genetic background of ankylosing spondylitis. Joint Bone Spine, in press. **(IF 1.66)**

Impact factor: 8.14

7.2. List of other publications:

1. Szabo, Z., Szanto, S., **Vegvari, A.,** Szekanecz, Z., Mikecz, K., Glant, T.T.: Genetic control of experimental spondylarthropathy. Arthritis and Rheumatism, 2005, 52(8): 2452-2460. (**IF: 7.41)**

2. Glant TT, Szabó Z, **Végvári A,** Szántó S, Mikecz K. A TSG-6/Tnfip6 gyulladásgátló hatása arthritisben. Magyar Reumatológia, 2005, 46: 5-13. (IF 0)

3. Glant TT, Szántó S, **Vegvari A,** Szabo Z, Kis-Toth K, Mikecz K, Adarichev VA. Two loci on chromosome 15 control experimentally induced arthritis through the differential regulation of IL-6 and lymphocyte proliferation. J Immunol. 2008 Jul 15;181(2):1307-14. **(IF 6.29)**

4. Adarichev VA, **Vegvari A,** Szabo Z, Kis-Toth K, Mikecz K, Glant TT. Congenic strains displaying similar clinical phenotype of arthritis represent different immunologic models of inflammation. Genes Immun. 2008 Oct;9(7):591-601. **(IF 4.53)**

5. Kapitány A., Szabó Z., Lakos G., Aleksza M., **Végvári A.,** Soós L., Karányi Z., Sipka S., Szegedi G., Szekanecz **Z.:** Associations between serum anti-CCP antibody, rheumatoid factor levels and HLA-DR4 expression in Hungarian patients with rheumatoid arthritis. Isr Med Assoc J (IMAJ), 10: 32-36, 2008. **(IF 0,58)**

6. Szekanecz **Z.,** Soós L., Szabó Z., Fekete A., Kapitány A., **Végvári, A.,** Sipka, S., Szűcs G., Szántó S., Lakos, G.: Anti-citrullinated protein antibodies in rheumatoid arthritis: as good as it gets? Clin Rev Allergy Immunol, 34: 26-31, 2008. **(IF 2.07)**

7. Besenyei T, **Végvári A,** Szabó Z, Szekanecz **Z.:** Az endothelsejtek, leukocita migráció, chemokinek és angiogenezis jelentősége gyulladásos reumatológiai kórképekben. Magy Immunol, 2008/1-2: 4-21, 2008. **(IF 0)**

8. Szántó S., Aleksza M., Mihály E., Lakos G., Szabó Z., **Végvári A.,** Sipka S., Szekanecz **Z.:** Intracytoplasmic cytokine expression and T cell subset distribution in the peripheral blood of patients with ankylosing spondylitis. J Rheumatol, 2008 Nov 1 [Epub ahead of print] **(IF 3.15)**

9. Szekanecz**, Z.,** Aleksza, M., Antal-Szalmás, P., Soltész, P., Veres, K., Szántó, S., Szabó, Z., **Végvári, A.,** Szamosi, S., Lakos, G., Sipka, S., Szegedi, G., Varga, J., Szűcs G. Combined plasmapheresis and high-dose intravenous immunoglobulin treatment in systemic sclerosis for 12 months: follow-up of immunopathological and clinical effects. Clin Rheumatol, 2008 Dec 6 [Epub ahead of print] **(IF 1.64)**

10. Kerekes G, Soltész P, Dér H, Veres K, Szabó Z, **Végvári A,** Shoenfeld Y, Szekanecz **Z.:** Effects of biologics on vascular function and atherosclerosis associated with rheumatoid arthritis. Ann NY Acad Sci, in press. **(IF 1.73)**

11. Kerekes G, Soltész P, Dér H, Veres K, Szabó Z, **Végvári A,** Szegedi G, Shoenfeld Y, Szekanecz Z. Effects of rituximab treatment on endothelial dysfunction, carotid atherosclerosis and lipid porofile in rheumatoid arthritis. Clin Rheumatol 2009 March 25 [Epub] **(IF 1.64)**

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