

Variation in IL-1 β gene expression is a major determinant of genetic differences in arthritis aggressivity in mice

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Contributed by Diane Mathis, July 8, 2005

In humans and in animal models, susceptibility to arthritis is under complex genetic control, reflecting influences on the immunological processes that initiate autoimmunity and on subsequent inflammatory mechanisms in the joints. The effector phases are conveniently modeled by the K/BxN serum transfer system, a robust model well suited for genetic analysis where arthritis is initiated by pathogenic Ig. Here, we mapped the genetic loci distinguishing the high-responder BALB/c vs. low-responder SJL strains. After computational modeling of potential breeding schemes, we adapted a stepwise selective breeding strategy, with a whole-genome scan performed on a limited number of animals. Several genomic regions proved significantly associated with high sensitivity to arthritis. One of these regions, on distal *chr2*, was centered on the interleukin 1 gene family. Quantitation of transcripts of the *Il1a* and *Il1b* candidate genes revealed a 10-fold greater induction of *Il1b* mRNA in BALB/c than in SJL splenocytes after injection of LPS, whereas *Il1a* showed much less difference. The differential activity of the *Il1b* gene was associated with a particular sequence haplotype of noncoding polymorphisms. The BALB/c haplotype was found in 75% of wild-derived strains but was rare among conventional inbred strains (4/33 tested, one of which is DBA/1, the prototype arthritis-susceptible strain) and was associated with vigorous *Il1b* responses in a panel of inbred strains. Inbred strains carrying this allele were far more responsive to serum-transferred arthritis, confirming its broad importance in controlling arthritis severity.

genetics | autoimmunity | inflammation | Mouse Phenome Project

As might be expected for a complex disease that involves a number of cellular and molecular players, the genetic basis of arthritis has proven very complex and, despite significant efforts, remains even more uncharted than for many other multigenic diseases (reviewed in refs. 1–3). The importance of the genetic component of human rheumatoid arthritis (RA) has been well established by studies on familial aggregation, twins, and population segregation. The MHC, in particular the HLA-DR locus, is a major contributor to disease predisposition, accounting for one-third of the genetic effect (2). Although many non-MHC chromosomal regions exhibited suggestive linkage in several large-scale genetic studies worldwide, none has proven convincingly reproducible between cohorts (2).

In animal models, progress has also been hampered by the complexity of pathogenesis and of the underlying genetics. A strong influence of the MHC region was established for rats and mice (4, 5), but contributions from non-MHC genes have proven complex and more difficult to map (6). For rats, a major locus influencing the severity of pristane-induced arthritis localized to the *Ncf1* gene, which controls oxidative burst (7). For mice, the *C5* locus that encodes Complement Factor 5 was identified in both the collagen-induced arthritis (CIA) and K/BxN models as a key element for susceptibility and severity (8, 9), and the *Ncf1* gene was recently

found to affect CIA severity in mice as well (10). Yet, beyond these rare identified loci, it seems the surface has only been scratched, and there are many more arthritis modifier genes to identify (6, 11).

K/BxN T cell receptor transgenic mice spontaneously develop an autoimmune disease with many of the clinical, histological, and immunological features of RA in humans (12, 13). K/BxN arthritis is critically dependent on both T lymphocytes, at the apex of the autoimmune process, and B lymphocytes, which constitute an essential relay that produces arthritogenic Abs. K/BxN mice might be thought of as a generic model of inflammatory arthritis mediated by Ab and immune complex deposition in the joint. This pathophysiological mechanism is a classic one for RA, but one which had fallen in disfavor until interest was recently rekindled by a number of observations, in particular the impact of B cell-based therapy (reviewed in ref. 14).

Transfer of serum from arthritic K/BxN mice into healthy animals provokes arthritis within days (13), providing a powerful experimental tool for analyzing effector-phase pathways, without the confounding influence of the immunological phase. The rapidity of the assay allows relatively facile genetic analysis, particularly because the disease does not completely depend on a single genetic background, in contrast to the situation with most other murine models of autoimmunity. Analysis of a panel of inbred mouse strains revealed a rich variability in the patterns of responses to K/BxN serum, ranging from complete resistance to explosive arthritis (8). An initial F₂ analysis on a responder/nonresponder combination (C57BL/6 vs. NOD) highlighted a complex genetic determinism and revealed the critical role of the *C5* locus. Indeed, the presence of the inactive variant of the *C5* allele explains the complete nonresponsiveness of several inbred strains (8).

Here, we wished to explore the genetic basis for the very explosive arthritis induced in BALB/c mice by K/BxN serum, a rapidly progressing disease initiating within 1 day after serum injection. The SJL/J strain was chosen as a comparator, because preliminary data indicated that arthritis appears slowly after introduction of K/BxN serum, and the inflammation always remains mild (8). We mapped the intervals and genes responsible for aggressive arthritis with a selective breeding strategy, resulting in the identification of IL-1 β as a key determinant of susceptibility.

Methods

Mice and Arthritis Induction. BALB/cJ, SJL/J, and NOD/LtJ mice were obtained from The Jackson Laboratory; all breedings and experimentation were performed in our facility (protocol 3024). *In silico* breedings were performed on S-PLUS (INSIGHTFUL; see *Supporting Text*, which is published as sup-

Abbreviations: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; QTL, quantitative trait loci; maxAT, maximum ankle thickening.

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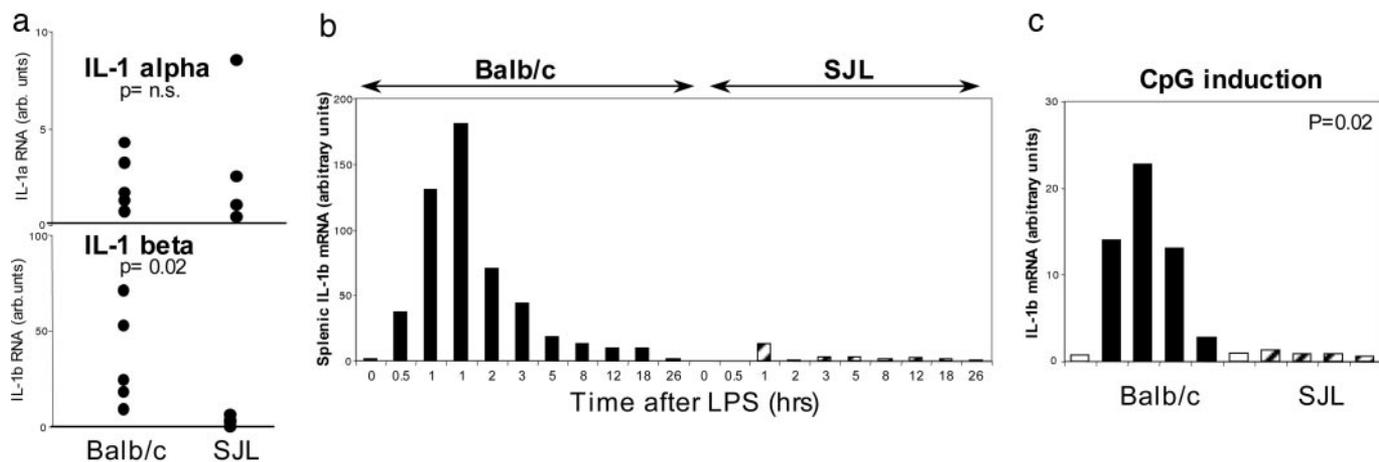


Fig. 2. The *Il1b* gene is differentially induced in BALB/c and SJL mice. (a) mRNA levels of the *Il1a* and *Il1b* candidate genes measured by quantitative real-time PCR in spleen RNA, from BALB/c, and SJL mice 6 h after i.p. injection of LPS; levels are standardized relative to hypoxanthine guanine phosphoribosyl transferase (HPRT) transcripts [units defined as $2^{\exp(\Delta-\Delta CT)}$]. Each dot represents an individual mouse. (b) Time course of IL-1 β transcripts after induction by i.p. LPS; each bar represents an individual mouse (data are representative of two similar experiments). (c) IL-1 β mRNA levels (quantitative PCR) 6 h after injection of CpG oligonucleotide; white bars, untreated mice. *P* value from Student's *t* test.

founder F₂ cohort, the mice used for breeding at each generation up to H₆, and a larger subset of mice of the H₅ cohort, encompassing individuals that display more extreme phenotypes and were therefore likely to be more informative. The markers used were a combination of microsatellites and SNPs (105 markers altogether, with an average spacing of 18.3 Mb; Table 1, which is published as supporting information on the PNAS web site). In most cases, we used a composite “severity index” to represent disease aggressivity, which incorporated rate of onset, maximum clinical score, and maxAT, as a phenotypic metric.

Statistical methods are detailed in *Supporting Text*. The first test was designed to match the particular aspects of the pedigree, deriving combinatorial information from different generations in the cohort. Significance was estimated by comparison with a large number of randomly generated virtual pedigrees of identical structure, asking how frequent the phenotypic correlations and segregation patterns of markers in the true dataset would prove to be when a large number of neutral loci were simulated to segregate down an identical pedigree. Significant association ($P < 0.001$) was discerned from the combined information (Fig. 1*b*), primarily on *Chr2* (50–160 Mb) and *Chr6* (50–90 Mb). The analysis also highlighted loci where the BALB/c allele was negatively correlated with arthritis severity (not shown). This was the case on *Chr5* and *Chr1*, quite close to a region that is positively associated with severity.

The second analysis used more conventional family-based tests, treating the entire pedigree as a group of families, and searching for linkage using QTDT software (19). Fig. 1*c* presents the regions with logarithm of odds scores >2 found on *Chrs1*, -2, -6, and -7, with a particularly robust association on *Chr2*. Similar association signals were observed for both time of onset and degree of inflammation.

Thus, the two analytical methods produced fairly concordant results, and the whole-genome screen identified prominent regions of significant linkage on *Chr2* and *Chr6*, with the possibility of secondary regions on *Chr1* and *Chr7*.

***Il1b* as a Candidate Gene for the Chr2 Region?** Animal models of arthritis depend, to varying degrees, on one of the major inflammatory cytokines, TNF α and IL-1 (20). We previously showed that the K/BxN model requires IL-1, because mice deficient in the IL-1 receptor are refractory to disease (21). The two principal members of the IL-1 family, *Il1a* and *Il1b*, are encoded within 30 kb of each other, within the peak of the interval on *Chr2* (129 Mb), and thus made immediate suspects for the major QTL on this chromosome. Hence, we measured the expression of both cytokines in BALB/c

and SJL splenocytes (resting or after *in vivo* LPS challenge) by quantitative real-time PCR. There was a striking difference in levels of IL-1 β transcripts that were induced up to 20-fold more in BALB/c than in SJL spleen cells, but the difference in IL-1 α transcripts was less marked (Fig. 2*a*). Time-course experiments (Fig. 2*b*) indicated that the peak of IL-1 β mRNA appeared at about the same time in both strains but was more pronounced in BALB/c than in SJL splenocytes (IL-1 α mRNA followed the same kinetics). Because the induction of the IL-1 α mRNA message did not show such divergence between the strains, the difference in IL-1 β inducibility was unlikely to result from a variable sensitivity to LPS, as exists in the mutant C3H/HeJ mouse (22). This notion was confirmed in Fig. 2*c* by showing that IL-1 β mRNA was also induced more strongly in BALB/c than in SJL mice after challenge with an independent inducer, a CpG dinucleotide-containing oligonucleotide that activates IL-1 transcription via the TLR9 receptor (23). This different response was specific to IL-1, because no such differential was seen in spleen IFN γ and TNF α mRNA levels in BALB/c vs. SJL after LPS challenge (data not shown).

These results suggested that the explosive responsiveness of BALB/c mice to K/BxN serum might be due, at least in part, to a much greater inducibility of the *Il1b* gene. To search for underlying sequence variation, we performed DNA sequence analysis on the *Il1b* locus of the BALB/c and SJL genomes. Recent findings indicate that genetic variation among inbred strains is dichotomous: some regions show very high divergence between any two strains, on the order of five SNPs per kb, whereas other areas have a far lower rate of sequence divergence (<0.05 SNP per kb) (24–26). This dichotomy is thought to reflect the mixed input from two main ancestral mouse genomes before the fixation of inbred mouse strains over the last century (27), such that any inbred strain is a mosaic of genetic material of *domesticus* and *non-domesticus* origin. We sequenced all *Il1b* exons and substantial stretches of promoter/intron sequences (Fig. 3*a*), focusing on the intronic regions conserved between human and mouse genomes. A number of differences were found, involving 39 of 4,011 bp sequenced, clearly in the range of high diversity. None of the polymorphisms alter the protein sequence, most belonging to flanking or intronic regions. The SJL sequence was always identical to, and BALB/c always different from, the database B6 sequence (in contrast, sequencing of a few segments of *Il1a* showed a far more intricate pattern of variation, with no simple dichotomy).

The mosaic genomes of inbred mice, because of the patchwork nature of their genetic variation, offer great potential for QTL

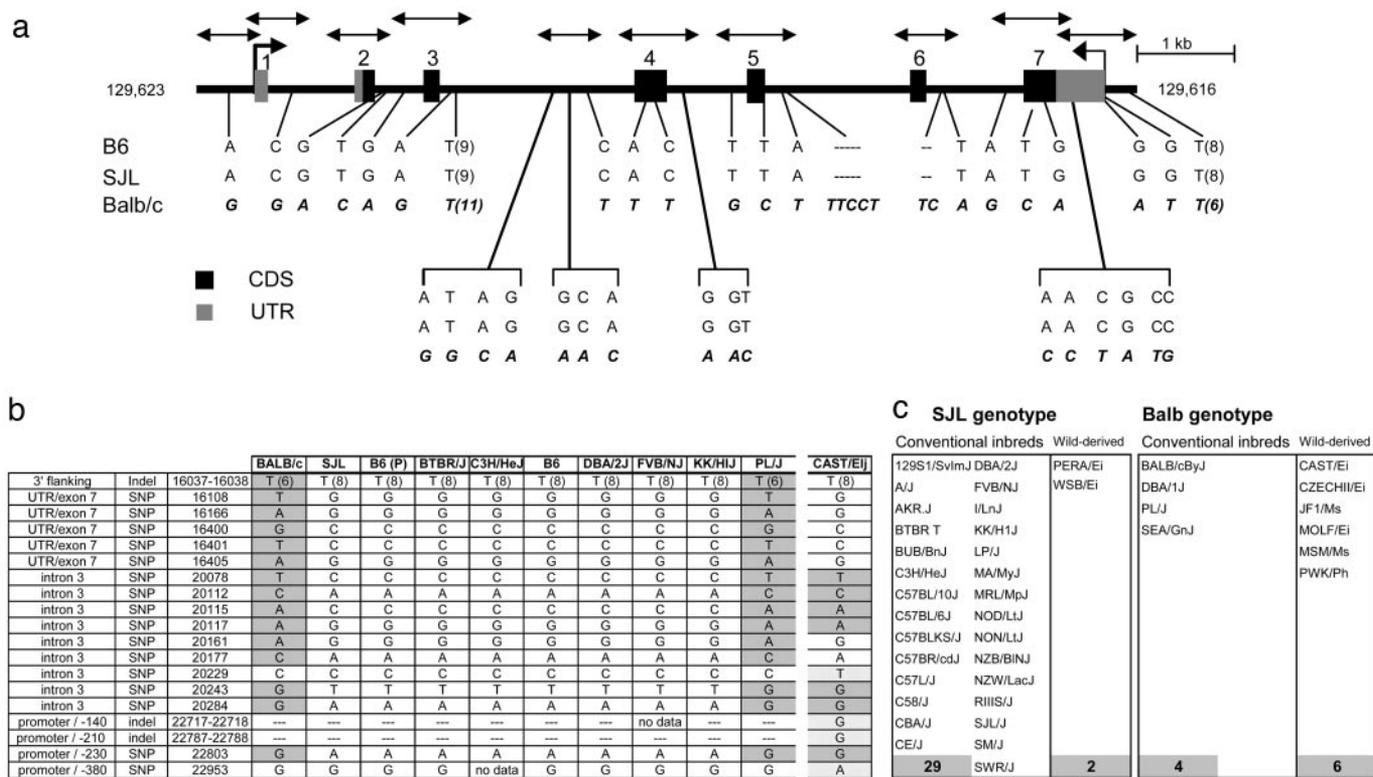


Fig. 3. Sequence analysis of the *IL-1β* gene in inbred mouse strains. (a) Sequencing of the *Il1b* gene in three inbred strains. The intron–exon structure of the gene is schematized, with its position on mouse Chr2; double-headed arrows indicating the PCR amplicons sequenced after amplification from tail DNA of Balb/cJ, SJL/J, and C57BL/6J mice. The bases at variable positions for each of the three strains are shown. (b) Additional sequencing in a broader panel of strains. Three of the *Il1b* amplicons shown in a were sequenced in DNA from a broader panel of inbred strains. White boxes, SJL haplotype; shaded, BALB/c haplotype; dotted, “private” variability of the non-*domesticus* strain CAST/EiJ; B6(P), reference sequence from public databases. (c) Genotyping for the -230 SNP of the *IL-1β* promoter, in the full panel of strains from the Mouse Phenome Project (plus DBA/1J and MRL/MpJ). The strains are separated into conventional and wild-derived inbreds.

mapping (24–26). We thus asked whether the two haplotypes observed in the BALB/c vs. the SJL and B6 strains were conserved as a whole in other inbred strains, or whether strains carrying intragenic recombinant sequences might pinpoint the difference in inducibility. PCR amplicons located at each extremity and in the middle of the *Il1b* gene were sequenced in a set of eight inbred strains chosen for their distinct genealogies (27). The sequences partitioned cleanly into either BALB/c or SJL haplotypes; all had exactly the same sequence as either BALB/c or SJL at all positions sequenced (Fig. 3b). Only the wild-derived non-*domesticus* CAST/EiJ mouse (*Mus musculus castaneus*) had a more complex sequence. The SJL haplotype seemed more frequent, because only the PL/J strain carried the BALB/c haplotype. To better estimate the frequency of these haplotypes among strains, we determined the genotype of one diagnostic SNP (at -230 in the promoter region) in a broader panel of 42 strains constituting the reference panel of the Mouse Phenome Project (28). This analysis confirmed the skewed distribution of the *Il1b* haplotype (Fig. 3c). The BALB/c genotype was found in the majority of (6/8) “wild-derived” non-*domesticus* strains in the panel. In contrast, most conventional inbred strains carry the SJL haplotype; only four of them had the BALB/c haplotype: BALB/c, PL/J, SEA/J, and DBA/1. The identity of these exceptions is suggestive. DBA/1 is the prototypical high susceptibility strain in the CIA model, indicating that the present observations may apply to CIA too; SEA/GnJ derives from a BALB/cJ (27); PL/J is known to share similarities to wild-derived inbred strains in haplotype and disease susceptibility, notably West Nile Virus (29). Thus, the BALB/c haplotype at *Il1b* and the explosive transcriptional induction it seems to confer are quite

frequent among wild-derived mice but appear to have been counterselected during fixation of the conventional inbred strains.

At this point, it was important to establish whether there was a direct relationship between the unusual *Il1b* haplotype of BALB/c mice, its high inducibility, and susceptibility to arthritis. The difference in *Il1b* gene transcription in BALB/c and SJL mice could theoretically be due to polymorphism at the locus itself or to differences in factors that regulate its expression, affecting upstream events in the cascade of IL-1 induction. We tested whether IL-1β mRNA levels segregated with the *Il1b* locus in a set of F₂ intercross mice. The mice were genotyped and challenged with LPS, and IL-1β responsiveness in spleen mRNA was measured by real-time PCR. A clear relationship was found (Fig. 4a), because mice with the BALB/c allele at *Il1b* showed higher levels of splenic IL-1β mRNA and serum cytokine than those with the SJL-derived allele. We extended this observation by testing the panel of eight inbred mouse strains whose *Il1b* gene had been partially sequenced (Fig. 4b). Here again, mice that shared the BALB/c allele (PL and CAST) showed higher IL-1β mRNA expression levels than did strains with the SJL allele (with the marked exception of BTBR). Thus, the strain difference in IL-1β expression is associated, at least in part, with the *Il1b* locus itself, in both BALB/c vs. SJL segregants and in a diverse panel of inbred strains.

Finally, we asked whether the *Il1b* haplotype correlates with responsiveness to K/BxN arthritis in mouse strains in general. We had previously analyzed the susceptibility of several inbred strains, and Fig. 5a collates these data together with the newly determined *Il1b* genotype; quite clearly, the BALB/c allele at *Il1b* correlates with high disease susceptibility. This analysis was extended by comparing the course of arthritis in additional strains of mice after

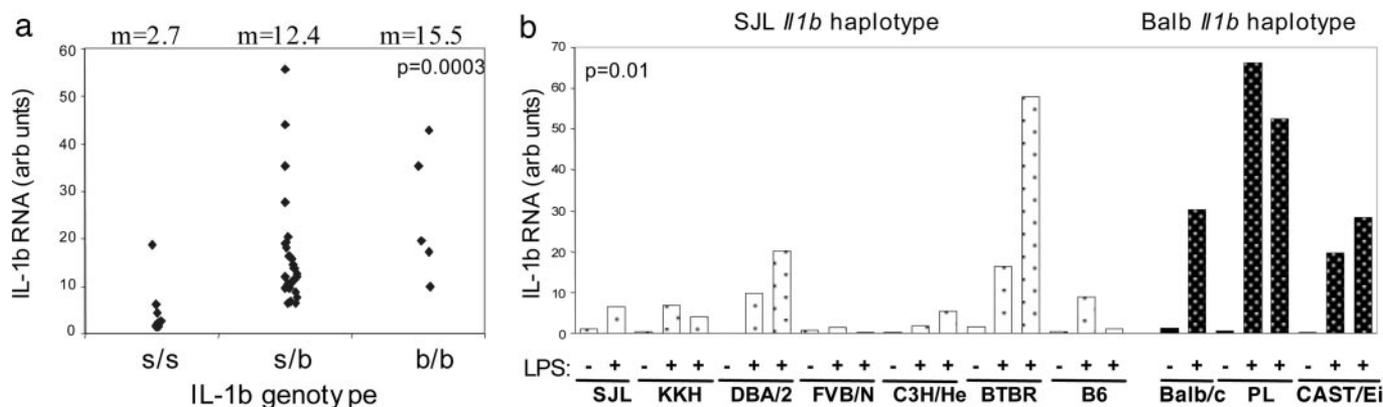


Fig. 4. Higher inducibility of the *IL-1 β* gene in the BALB/c haplotype. (a) *Il1b* mRNA was measured by real-time PCR in spleen RNA 6 h after LPS in a cohort of (BALB/c \times SJL) F₂ mice genotyped for the -230 promoter region SNP. *P* values from a Kruskal-Wallis rank sum test. (b) A panel of inbred strains, carrying either the SJL or BALB/c allele at *Il1b*, were tested by LPS injection. *Il1b* transcripts were measured (spleen RNA, 4 h postLPS) in control (-) and LPS-injected (+) mice. *P* values are from Student's *t* test.

transfer of limiting doses of K/BxN serum (Fig. 5*b*). Strains with the BALB/c haplotype all showed high responsiveness, whereas strains with the SJL haplotype responded more weakly as a group. Thus, the analysis relying on inbred strain variability confirmed and extended the results of the intercross between BALB/c and SJL mice: the *Il1b* locus is associated with aggressive arthritis.

Discussion

The experiments described in this paper, initiated by *in silico* modeling of mouse genealogies, have resulted in the identification of *Il1b* as a gene whose variation strongly conditions mouse susceptibility to Ab-mediated arthritis. How worthwhile was the selective breeding approach in this endeavor? In one sense, it was successful; the major regions of significant linkage were detected, and <100 mice were genotyped, substantially less than would be required in conventional F₂ crosses. But, quite clearly, the selection

did not work as smoothly in breathing as in virtual mice (see *Supporting Text*). The rate of fixation of neutral markers largely matched the expected, but the association scores were significantly lower than had been hoped for, and it is only on the strongest QTL that significance could be established. Part of the discrepancy between the expected and the obtained can be attributed to uneven breeding and part to a higher phenotypic variability than had been modeled. Furthermore, the distribution of phenotypes at successive generations also indicated that more loci were at play than had been simulated, with a likely occurrence of dominant susceptibility loci from SJL. Quite clearly, the study did not exhaust all of the relevant polymorphism in the BALB/c vs. SJL combination but identified the major regions on *Chr2* and *Chr6*.

As in previous studies on the K/BxN and other arthritis models (reviewed in refs. 6 and 11), the picture that emerged is one where a complex array of genetic polymorphisms determines the differential responsiveness of BALB/c and SJL mice, one that differs from previous results. We detected no sign of association with the region of the *Ncf1* gene (10) or with the *C5* locus (8). The region of association on *Chr6* may be related to the *Cia6* and *Pgia19* loci identified in previous studies (30, 31); it may be relevant that a broad peak of association with CIA was detected on *Chr6*, suggesting the possibility of two independent QTL, compatible with the distribution observed here. Previous studies did not uncover a major effect of the *Il1b* locus, as observed herein. This discrepancy is readily explained by the strains used; the previously published experiments used a BALB/c vs. DBA/1 comparison, precisely the strains that shared the rare overinducible *Il1b* allele identified here (as discussed below, probably not by coincidence). The present results also differ from those of our previous analysis in a (B6xNOD)F₂ intercross, which brought forth loci on *Chr1* and proximal *Chr2* (at *C5*) (8). The *Chr1* QTL found in the two studies might be the same (our recent results point to a complex organization with two independent subregions, both of which are compatible with the QTL found in the Balb/SJL combination), but the *C5* and *Il1b* loci are highlighted in only one of the two strain pairs. These different results illustrate the importance of testing multiple strain combinations (or out-bred crosses) to fully capture relevant genetic variation.

Definition of a susceptibility interval on *Chr2* was followed by candidate gene analysis that resulted in the identification of a polymorphism in *Il1b* and consequently a difference in IL-1 β mRNA induction as a major determinant of genetic susceptibility to K/BxN arthritis. We cannot exclude that other closely linked loci on *Chr2* are involved as well, but *Il1b* had a major influence on the severity of arthritis in the backcross/intercross cohort and in inbred

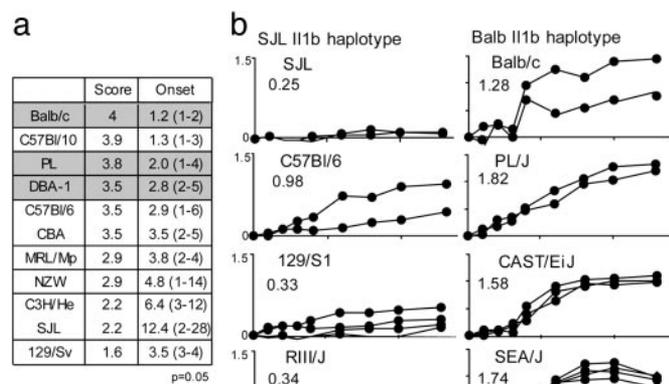


Fig. 5. The BALB/c allele at *IL-1b* confers high responsiveness to arthritis induction. (a) Several strains that had been previously phenotyped for sensitivity to arthritis induction by K/BxN serum were genotyped for the -230 *Il1b* SNP. Strains with the BALB/c allele at *Il1b* are shaded. The values shown are the average maxAT (on a 0-4 scale) and the average (range) day of onset (phenotypic values reproduced from ref. 8); *P* value from Wilcoxon's rank sum test comparing scores in strains with either *Il1b* haplotype. (b) Additional mice carrying either the SJL or BALB/c alleles at *Il1b* (left and right columns, respectively) were challenged with K/BxN serum. Disease progression was measured by ankle thickening; the values in the boxes represent integrated ankle thickening (days 1-7); *P* value is from Student's *t* test comparing both groups.

lines. This assignment fits well with the known role of IL-1 in this arthritis model; mice carrying knockout mutations in the *IL-1 receptor* locus or in the combined *Il1a/Il1b* loci were fully resistant to K/BxN arthritis (21, 32). An impact of quantitative variations in IL-1 expression on the unfolding of arthritis is also very consistent with the disease that developed when IL-1RA, the natural antagonist of IL-1 activity, was eliminated (ref. 33; interestingly, the phenotype was detected on the BALB/c but not on the C57BL/6 background) and with the protection against CIA afforded by overexpression of IL-1RA as a transgene (34). In fact, many interventions that modify IL-1 availability have had an impact on most mouse models of arthritis (reviewed in ref. 20).

Sequence analysis revealed variation at many positions of the BALB/c and SJL *Il1b* genes. These variations did not affect the protein sequence, occurring rather in promoter and intron sequences, and may thus influence mRNA levels, consistent with the very different inducibility of the *Il1b* haplotypes by ligands of Toll-like receptors. The time-course analysis suggested a difference not in mRNA stability but instead in transcriptional or splicing efficiency, because the timing of induction was essentially identical in the two strains. We had hoped that the analysis of inbred strains would allow us to determine the relevant variation by providing recombinant loci for SNP/phenotype correlation. This proved impossible, because the whole gene was transmitted *en bloc* in all conventional inbred strains sequenced, i.e., they all carried either the full BALB/c or the full SJL haplotype. The assignment of causal differences will require old-fashioned promoter dissection.

The BALB/c haplotype at the *Il1b* locus showed a strikingly imbalanced distribution among mouse strains; it was common in non-*domesticus* wild-derived mice but rare among the conventional inbreds, most of which were derived from mouse-fancier colonies of the late 1800s (27). Such a shift in haplotype distribution suggests that the BALB/c haplotype was for some reason subject to negative selective pressure during this domestication period. It is possible that the higher activity of the BALB/c *Il1b* locus is advantageous in wild mice, permitting them to respond to a particular set of pathogens encountered in the varied environments in which they reside, but that the activity of the *Il1b* locus also needs to be balanced at the population level by an allele that confers lower IL-1 responses. In the sheltered environment of pet or laboratory colonies, the lower IL-1 response might have been favored. Alternatively, this cytokine may somehow participate in the expression

of behavioral traits, one set of phenotypes that most obviously distinguishes domesticated from wild mice and that must have been strongly selected; IL-1 β has been implicated in murine behavioral traits in many studies (e.g., refs. 35 and 36).

Although variation at the *Il1b* locus has a strong effect on the IL-1 β response, it is not the only determinant; even when homozygous for the BALB/c *Il1b* haplotype, mice of the (BALB/c \times SJL), the H₈ cohort did not recapitulate the typical BALB/c expression levels, suggesting that other elements from the SJL genome introduced in the pedigree may dampen the full expressivity of the *Il1b* haplotype. Similarly in inbred lines, whereas there was a correlation between the *Il1b* haplotype and mRNA inducibility, it was not absolute, and there were some clear exceptions (the high responsiveness of the SJL-like BTBR/J strain, for instance). Thus, other genes must influence the transcriptional activity conditioned by local variation at the *Il1b* locus. Genes encoding transcription or signaling factors that activate or silence the *Il1b* promoter/enhancer could modify the expression phenotype and may correspond to some of the other influences revealed in the genome scan.

The convergence of results from the genetic mapping and the inbred strain analyses demonstrate that genetic polymorphism at *Il1b* is an important determinant of arthritis severity in a broad sense, not merely when comparing the BALB/c and SJL inbred strains. What might be the relevance to arthritis in outbred humans? There is already evidence of suggestive association between RA severity and SNPs in genes encoding members of the *IL-1* gene family (e.g., refs. 37–39). Although the human IL-1 family region obviously requires more profound analysis, these data do suggest that the role of *IL1b* in controlling arthritis development in mice may well have a parallel in humans. It will be of great interest to ascertain whether genetic variation in the human species harbors the same range of IL-1 expression levels found in mice, and how this diversity distributes among human populations and diseases.

We thank Q. M. Pham, T. Rogers, V. Brucklich, and J. Gile for help with the mice; G. Losyev for flow cytometry; the Arthritis Group for inspiring discussions; and S. Deveau (The Jackson Laboratory) for DNA. This work was supported by grants from the National Institutes of Health and by William T. Young Chair funds (to D.M. and C.B.), Joslin Diabetes Center's National Institute of Diabetes and Digestive and Kidney Diseases-funded Diabetes and Endocrinology Research Core, and the Mouse Phenome Project. K.O. received postdoctoral support from the Uehara Memorial Foundation and the National Arthritis Research Foundation.

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