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Positional Cloning of *Ncf1* – a Piece in the Puzzle of Arthritis Genetics

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Abstract

Positional cloning of susceptibility genes in complex diseases like rheumatoid arthritis in humans is hampered by aspects like genetic heterogeneity and environmental variations, while genetic studies in animal models contain several advantages. With animal models, the environment can be controlled, the genetic complexity of the disease is minimized and the disease onset can be predicted, which simplify diagnosis and characterization. We use pristane-induced arthritis in rats to investigate the inheritance of arthritis. Until now, we have identified 15 loci that significantly predispose rats to the development of arthritis. One of these arthritis loci has been isolated and confirmed to be caused by a polymorphism in the *Ncf1* gene. In this review, we outline the methods used to identify *Ncf1* as one single susceptibility gene in a complex puzzle of inherited factors that render susceptibility to a complex autoimmune disorder like arthritis.

Introduction

Rheumatoid arthritis (RA) is a complex disease that despite decades of research still remains to be of unknown aetiology [1]. RA is known to depend both on environmental factors [2] and on familial inheritance [3–5]. With the genetic revolution that has culminated during the last few years, through the release of the full genome sequence of humans [6, 7] and mice [8], great expectations are set upon researchers to resolve complex inherited diseases like RA [9]. However, linkage analysis in RA patients has so far only been able to detect the human leucocyte antigen (HLA) region to be one important genetic factor in the predisposition to RA [10]. Besides HLA, which has been estimated to account for one-third of the inherited susceptibility to RA [11], no other loci of significant contribution to RA have been detected despite several linkage analysis being performed in human patient studies [12–15]. Multifactorial environmental influence and genetic and phenotypic heterogeneity together with the impracticality of obtaining individual samples in high enough number make the majority of human linkage analyses of complex diseases predestined to end short of significant disease-associated chromosomal loci [16, 17]. Another major cumbersome problem that human geneticists have to face when dissecting complex traits is the impracticality to proceed further once a significant locus has been identi-

fied. The need for additional informative individuals might be an overwhelming obstacle, as it is necessary to obtain additional samples within the same ethnic group as the original study [18]. Despite these difficulties, there have been some occasions of successful identifications of autoimmunity-regulating genes in humans [19–21], but overall it is difficult to identify genes in complex human diseases [22]. Furthermore, if a significant genetic association is identified, it is needed to identify candidate genes for positional cloning. Positional cloning approaches will most often demand additional large number of patient family members to identify single predisposing genes. In human genetic research, this is only a science fiction utopia, while in animal models it is the reality [23]. For studies of arthritis, there exist several animal models. The most common model of arthritis is induced both in mice [24] and in rats [25] with collagen type II emulsified in incomplete Freund's adjuvant (i.e. mineral oil) or complete Freund's adjuvant (i.e. mycobacteria cell walls in mineral oil). Besides the arthritis induced with cartilage-specific proteins like the type II collagen-induced arthritis ($C^{II}IA$), type XI collagen-induced arthritis ($C^{IX}IA$) [26] or the cartilage oligomeric matrix protein-induced arthritis (COMPIA) [27], it is also possible to induce arthritis with mycobacterium emulsified in adjuvant (MIA), or for that matter even with mineral oil only (oil-induced

arthritis, OIA) or with synthetic adjuvants like pristane (pristane-induced arthritis, PIA) [28–32]. All models of arthritis in rats have different characteristics and upon comparison with criteria used for diagnosing RA [33], it is clear that the consensus knowledge of all these models can be used to study most aspects of RA (Table 1). However, when analysing genetic regulation of arthritis in animal models, one must keep in mind the evolutionary difference between humans and rodents, as well as the fact that an animal model of RA is not the same as the human disease. Hence, the most important finding, obtained from positional cloning of arthritis-regulating genes in animal models for future studies of RA, is not the identified genes themselves, but rather the knowledge of previously unknown pathological mechanisms and pathways of disease regulation. Since the first publication, in 1996, of genetic linkage analysis of CIA in rats [34], studies of linkage analysis of arthritis in rats have been ongoing, using various models of arthritis and different inbred rat strains ([29, 35]). Genetic projects based on linkage analysis of complex disorders are time and lab consuming, especially as isolation of associated loci in congenic strains demands years of breeding. Hence, there have been doubts concerning the potential of reverse genetics of polygenic diseases both in humans and in animal models [22, 36]. However, we recently reported the successful identification of a functional polymorphism of *Ncf1*, explaining the effect on arthritis severity by the quantitative trait locus (QTL) *Pia4*, through positional cloning [37]. We appreciate this to be the first in a long range of genes that will be identified in animal models of arthritis, that all will be important pieces in a puzzle of the complex inheritance of autoimmune diseases. Eventual assembly of this puzzle will give deeper understanding of the pathogenesis of many common diseases. We here review the positional cloning of *Ncf1* and discuss the methods used to go from the characterization of an animal model to the identification and verification of *Ncf1* as one major gene in the regulation of adjuvant-induced arthritis.

Linkage analysis and positional cloning of Ncf1 in pristane–induced arthritis

Establishment of pristane-induced arthritis

PIA was established as a model that mimics several aspects of diagnosed RA [33]. The main pathological features of PIA include oedema, infiltration into the joint of mononuclear and polymorphonuclear cells, pannus formation and erosion of cartilage and bone [32]. PIA is a disease that is largely T-cell dependent, while for example CIA has a more complex pathology also involving antibodies directed against type II collagen [38].

In the original characterization of PIA, several inbred strains as well as major histocompatibility complex (MHC) congenic strains were tested for PIA susceptibility [32]. In this work, a high degree of variation in susceptibility and severity was observed between the different strains. The first genetic association for PIA regulation was also observed to the MHC region on chromosome 20 (*Pia1*). To identify non-MHC loci that control arthritis susceptibility or severity by linkage analysis, it is most suitable to choose animals with large difference in susceptibility to ensure a strong genetic effect on disease regulation and also to choose animals that are the most distantly related [39]. In the present work, we used the 100% susceptible DA rat in combination with the totally resistant E3 rat to unravel the underlying genetic mechanism of DA susceptibility to arthritis.

Linkage analysis and quantitative trait locus detection

In the first linkage analysis based on E3 and DA rats, it was clear how different QTLs regulated different aspects of the disease [40]. One of the strongest linkages was obtained to the *Pia4* locus on chromosome 12. The main effect of the *Pia4* locus was observed on arthritis severity, but it had also an influence on the onset of disease and the following chronic disease. Later, we followed up the first F2 intercross with a DA(E3 × DA) backcross experiment where we increased the number of animals to 650 rats [41]. With the backcross approach, we were only able to detect loci with E3-dominant effect, but with a statistically higher significance, as there is lower degree of freedom [42]. In both the intercross and the backcross, *Pia4* was highly significantly linked to severity of arthritis (Fig. 1). However, the penetrance of the effect of *Pia4* is more pronounced in the backcross. This suggests an interacting effect by *E3* genes that is observed in the F2

Table 1 Comparison of arthritis models in rats with American Rheumatism Association criteria for rheumatoid arthritis (RA)

	RA	MIA	OIA	PIA	CIA
Early morning stiffness	+	ND	ND	ND	ND
Arthritis of at least three areas (PIP-MC, wrist, elbow, knee, ankle, PIP-MT) >6 weeks	+	–	–	+	+
Arthritis of hand joints >6 weeks	+	–	–	+	+
Rheumatoid nodules	+	–	–	–	–
Symmetric arthritis	+	–	+	+	–
Serum rheumatoid factors	+	–	–	+	+
Radiographic changes	+	+	–	+	+
Classical RA (>4 criteria)	+	–	–	+	+
Additional observations					
MHC association		+	+	+	+
Anti-CII antibodies	+	–	–	–	+
Enthesopathy	–	+	–	+	+

OIA, oil-induced arthritis; PIA, pristane-induced arthritis; CIA, collagen-induced arthritis; MHC, major histocompatibility complex; ND, not determined; MIA, mycobacteria cell walls in mineral oil-induced arthritis; PIP-MC, proximal interphalangeal metacarpophalangeal joint; PIP-MT, proximal interphalangeal metatarsophalangeal joint.

Figure 1 Limit of detection score plots of chromosome 12 detecting *Pia4* using the trait maximal arthritis severity in (A) (E3 × DA)F2 and (B) DA(E3 × DA). The thin vertical line shows the threshold for highly significant linkage, as determined by permutation analysis (1000 permutations). The circle on the chromosome indicates the location of *Ncf1*. The lower panel shows rats in the respective crosses segregating for *Pia4* (a = E3 allele and b = DA allele). In panel A, a simple scoring method was used to evaluate the arthritis severity in the rats. With the simple scoring method, a maximum disease severity is 12, while in the extended scoring system otherwise used the maximum severity is 60 for each rat [71].

intercross, where also E3 homozygosity at interacting loci is involved.

From QTL detection to congenic strain

Once a significant linkage for a QTL has been identified, the real challenging work to isolate the susceptibility gene begins [43]. In animal models, the progress from QTL detection to candidate genes will run in two separate, but connected, pathways (Fig. 2). The first important task is to reproduce the identified QTL in congenic strains. A congenic strain is produced by insertion of the genetic fragment of interest from the resistant parental strain onto the genome of the susceptible parental strain, or vice versa (reciprocal congenics), through conventional backcross breeding or marker-assisted breeding [44, 45]. Once a pure background genome of the congenic strain has been created, the disease trait can be analysed in the congenic strain and compared with wild-type animals.

In the case of *Pia4*, the chromosome 12 region of *Pia4* from E3 was introgressed into the DA background genome through conventional breeding. After five generations of backcrossing, the interfering background is approximately 3% [45], the reason why the DA.*Pia4* congenic strain also was verified for DA homozygosity at other previously

identified *Pia* QTLs, i.e. *Pia1–3* and *Pia5–6*, before the rats were subjected to PIA (Fig. 3A). As there was significantly less arthritis severity in the DA.*Pia4* congenic strain compared with that in the littermate DA, further backcross breeding with the aim of positional cloning of *Pia4* was justified. As previously observed in the linkage analysis, there was no gender effect of *Pia4* (data not shown), the reason why all ensuing experiments on congenic strains were performed on gender-mixed populations. The penetrance of *Pia4* is not 100%, the effect is overlapping between the groups, but it needs to be emphasized that the use of a limited number of animals in the testing is crucial for a successful narrowing of the fragment and subsequent positional cloning. At this stage, we realized that the *Pia4* gene influences arthritis severity in a DA genetic environment, i.e. there is no major suppressive trans-interaction. However, it is still possible that there are several linked interacting genes that could dilute, but hopefully also enhance, the effect of the gene as has been apparent in some other QTL projects [46, 47].

The confidence interval of a linked QTL could be quite broad, the reason why almost half of chromosome 12 was included in the initial verification experiment. After another five generations of breeding (to N10), the congenic interval was reduced to about 20 cM (Fig. 3B), still

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                          Quantitative trait
                                 │
                        Verify genetic inheritance
                                 │
                        Genomes to investigate
                                 │
                      Linkage analysis to identify QTLs
                          ╱                    ╲
   Homologous comparison with other models        Establish congenic strains
   and other strains or species                            │
                    │                          Reproduce and fine map locus in congenic strain
           Test congenic strain with other traits          │
                    │                              Produce physical map
                     ╲                           ╱
                          Identify candidate genes
                                 │
                    Sequence candidate genes for polymorphisms
                          ╱                    ╲
   Analyse function of candidate genes      Sequence other strains and species for polymorphism
                     ╲                           ╱
                          Validate identified gene
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Figure 2 Schematic pathway of how to identify susceptibility genes in complex genetic diseases using animal models.

encompassing, maybe, 30 million basepairs [48]. To narrow down this *Pia4* region, an approach of collecting and testing small cohorts of animals having recombinations within this fragment was initiated [49]. With this method, a consensus regarding the location of the *Pia4* gene and a congenic line (13*n*) with an E3 fragment of approximately 2 cM was reached (Fig. 4).

Subphenotypes, where and when

During the process of fine mapping the congenic interval fragment and production of physical maps, there are means of obtaining a deeper understanding of the trait in question, thereby building up a stronger rationale for candidate evaluation. For example, it is possible to search for identified disease linked QTLs to syntenic regions of other species [3, 50]. The *Pia4* region had been identified in models for multiple sclerosis and uveitis [51–54] and to be homologous to a locus on mouse chromosome 5 (*Bb2*), which was identified using a model of Borrelia-induced Lyme disease [55], indicating *Pia4* to be involved in general autoimmune regulation. Through the analysis of disease-relevant subphenotypes, an understanding of the mechanism of the QTL as well as an indication concerning where and when the gene might be of importance is

achieved. During the characterization of PIA, we had developed biochemical analyses in plasma that reflect systemic inflammation (α_1-acid glycoprotein (AGP) [56]) and cartilage erosion (COMP [57]). Through the use of adoptive transfer of mitogen-activated T cells [58, 59], we could also divide the phases of the disease into activation and effector stage of the disease. Using these three methods, we were able to show that the E3 allele of the *Pia4* locus, besides having an ameliorating effect on clinically observable arthritis like swollen red and deformed peripheral joints, also caused decreased level of AGP and COMP, when introduced into the DA genome [37]. This showed that *Pia4* had a profound effect on arthritis that included a systemic inflammatory response as well as severe erosive arthritis. The question about when the action of *Pia4* is of importance was clarified through adoptive transfer where transfer of mitogen-activated spleen cells from the immunized DA strain to a susceptible as well as a resistant strain was possible. The reversal transfer, when an immunized resistant strain was used as spleen cell donor, did not transfer disease [37]. In contrast to our expectations, we had to conclude that the defect in *Pia4* must involve the activation of autoreactive T cells in the donor rats, i.e. before they reach the joints.
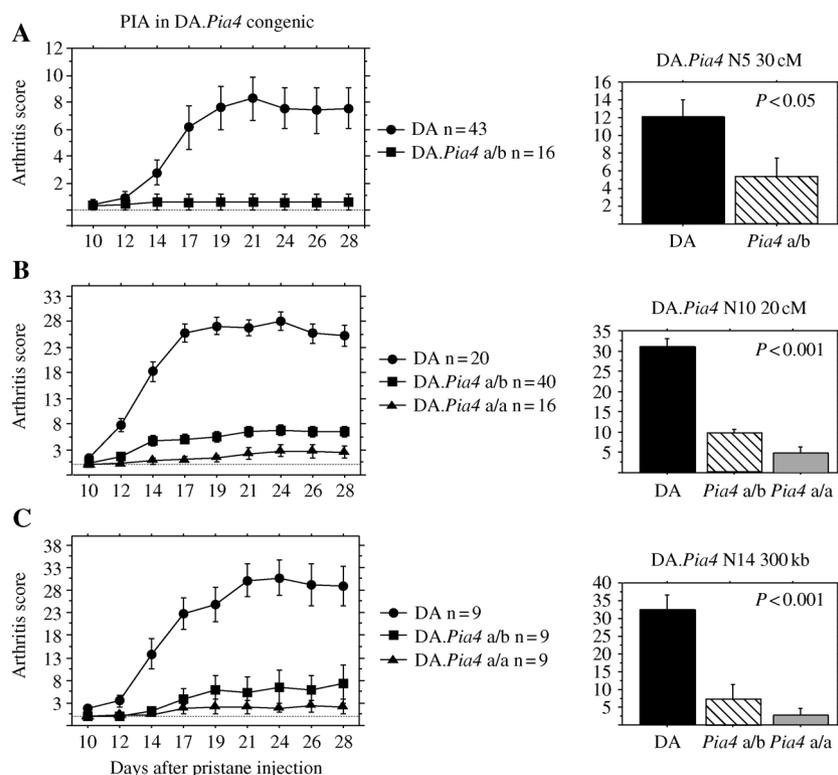
Figure 3 Isolation of *Pia4* in a congenic strain. The introgressed alleles in the DA.*Pia4* congenics (a = E3 allele and b = DA allele) represent copies of E3-derived *Pia4* alleles. Left panel shows the arthritis development after pristane injection and the right panel shows maximal arthritis severity during the experimental period. All data are presented as mean values ± standard error of the mean for the indicated genotype. In panel A, a simple scoring method was used to evaluate the arthritis severity in the rats. With the simple scoring method, a maximum disease severity is 12, while in the extended scoring system otherwise used the maximum severity is 60 for each rat [71]. (A) First analysis of *Pia4* in DA background after five generations of backcrossing. At this stage, the congenic strain still contains a few per cent interfering background genome from the E3 rat, besides the linked region in chromosome 12. Still, there is a significant ($P < 0.05$) amelioration of pristane-induced arthritis. (B) After 10 generations, the DA.*Pia4* is considered as a pure congenic strain. Now, the effect of *Pia4* is more obvious ($P < 0.0001$), however, the *Pia4*-inserted genomic region is still quite large, encompassing about 20 cM. (C) The final DA.*Pia4* congenic that after 14 generations of backcrossing only has an insert of 300 kb E3 genomic segment, containing only the *Ncf1* and the *Gtf2i* genes.

Physical mapping

The decision concerning the extension of breeding to obtain further recombinants with the aim of narrowing down the genomic region that will be searchable for possible candidate genes is a cutoff between benefit of getting smaller genomic fragment and animal breeding/screening costs.

The problem with linkage maps for rodents consisting of simple sequence length polymorphism (SSLP) markers was that there is usually no knowledge about physical distances or exactly which genes are within a certain locus. Therefore, once a small (<2 cM) QTL has been isolated in a congenic strain, there is a need to convert the genomic map into physical distances and to include actual genes into the SSLP map. Reliable sequence information is highly valuable at this stage, but the *Pia4* project was performed before publication of the rat genome sequence, and therefore a physical map and partial sequences had to be performed without this help. We produced the physical map through standard technology,

by arranging overlapping genomic BAC and PAC clones, into an overlapping contig. The specific genomic clones for the locus were obtained by hybridization screening of a clone library using available SSLP markers and also EST clones from the region. Subsequently, the identified artificial chromosomal clones were end-sequenced and arranged into a physical contig covering the *Pia4* locus. With the recent releases of full mammalian genome sequences [6–8] including those of the rat, conversion from genetic map based on SSLP markers into a gene informative physical map is, in the future, merely a matter of bioinformatics (http://www.ncbi.nlm.nih.gov/mapview/). Nevertheless, not until the physical map and the total number of annotated genes in the isolated QTL are known is it possible to start the evaluation of candidate genes.

Candidate gene analysis

In the *Pia4* locus, the density of genes was fairly low (GenBank accession number NW_042785 for rat
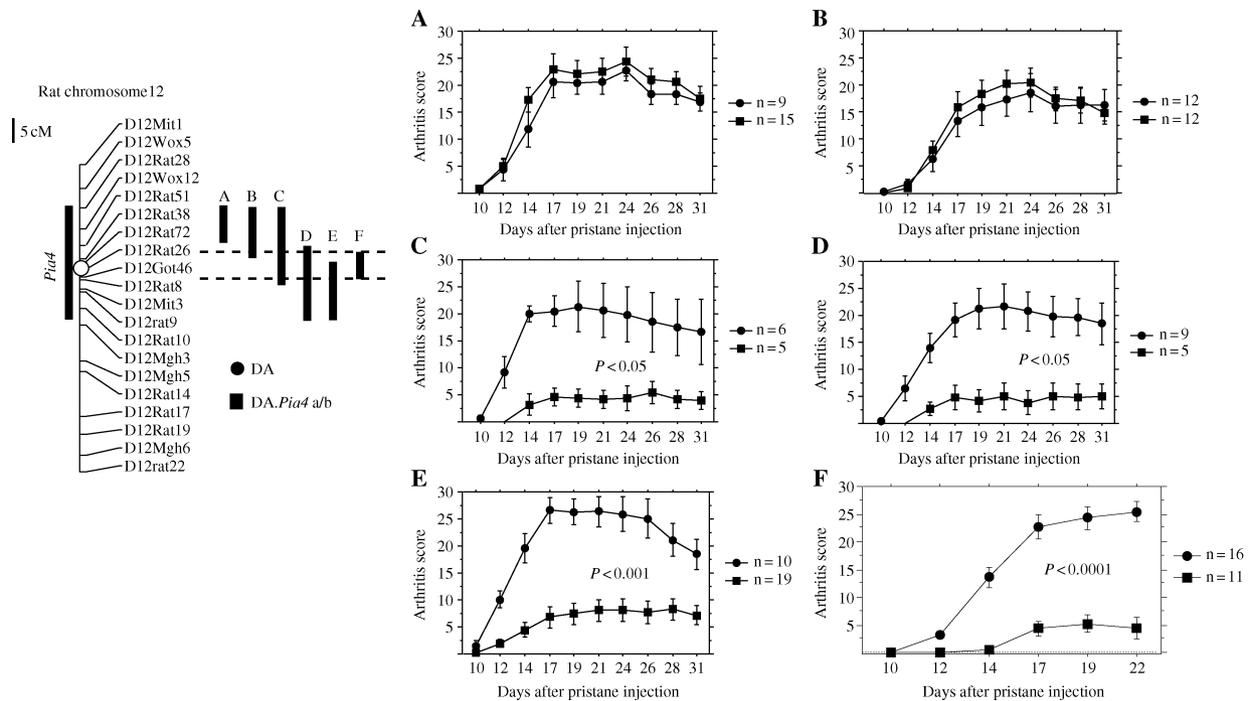
Figure 4 The genomic interval of the protective *Pia4* region in the DA background was gradually shortened through stepwise recombinations. All new congenic strains were tested for arthritis susceptibility to obtain the smallest region of protective fragment. With this method, although time consuming, the protective fragment was taken from approximately 20 to 2 cM, a genomic region more suitable for physical mapping. The circle on the chromosome indicates the location of *Ncf1*. All data are presented as mean values ± standard error of the mean for the indicated genotype.

chromosome 12 contig), and with the use of several congenic strains with recombinations within the region, one of them containing the disease-protecting E3 allele of the gene together with only 300 kb of E3 genome inserted into DA (Fig. 3C). With these congenics, we could conclusively reduce the *Pia4* locus to only two genes.

The two candidate genes in the *Pia4* region that had to be carefully investigated were *Ncf1* (alias *p47phox*) and *Gtf2i*. The most straightforward approach for the validation of candidate genes is to test for the expression on transcriptional level and on translational level using quantitative polymerase chain reaction (PCR) and Western blots, respectively. The other possible basis for variation in gene function is through structural alterations because of altered amino acid sequence. This has to be analysed for every candidate gene by cDNA sequencing of the parental strains. No differential expression of *Ncf1* or *Gtf2i* could be detected, and only *Ncf1* had polymorphism alterations in the coding sequence, leading to changes in the amino acid sequence of the translated protein. Two of the polymorphisms in *Ncf1* resulted in amino acid substitutions: amino acid residue 106 ATG/GTG (single-nucleotide polymorphism (SNP) 330 bp) (DA/E3) that resulted in a Met/Val alteration and amino acid residue 153 ATG/ACG (SNP 472 bp) that resulted in a Met/Thr alteration. The third identified polymorphism did not lead to an amino acid difference. SNP analyses of these three polymorph-

isms in the *Ncf1* gene in various inbred strains, especially BN, suggested that the alteration in amino residue 153 from methionine to threonine was the essential one in *Ncf1* for arthritis susceptibility (Table 2).

Validation of the proposed QTL gene identification

To prove the accuracy of an identified gene as the true causative gene in an isolated QTL will always be the most difficult. It has been suggested that the burden of proof for identified genes of complex diseases must be heavier [60]. Evidence of correct annotation of a disease-regulating gene of a complex trait locus should consist of *in vitro* functional assay, similar phenotypes associated with naturally occurring or engineered mutations in other species, complementation or inhibition [60]. We regard the established DA.*Pia4* congenic strain to be more appropriate than any transgenic or knock in mice, as these manipulations include more genome insertions either through the construct itself or through the use of inappropriate embryonic stem cells (ES) cells [61]. Thus, only the *Ncf1* gene could explain the arthritis effect, although we have not formally excluded an additional influence of the linked *Gtf2i* gene through expression effects at time points or in tissues not investigated. In the case of functional confirmation of *Ncf1*, which is part of the NADPH oxidase complex and involved in the production of reactive oxygen species

Table 2 Single-nucleotide polymorphism (SNP) genotyping of *Ncf1* polymorphisms in inbred and wild rats

	SNP 330 bp	SNP 472 bp	SNP 1161 bp
Inbred rats			
DA	**DA**	**DA**	**DA**
E3	**E3**	**E3**	**E3**
ACI	E3	E3	E3
BDE	E3	E3	DA
BDII	E3	E3	DA
BDIX	E3	E3	DA
BDV	DA	DA	DA
BH	DA	E3	DA
BN	**DA**	**E3**	**DA**
BS	DA	E3	DA
BUF	DA	DA	DA
COP	E3	E3	E3
F344	DA	DA	DA
GK	DA	DA	DA
LEW.1F	DA	DA	DA
LOU	E3	E3	E3
MNS	DA	E3	DA
MWF	E3	E3	DA
NAR	E3	E3	DA
NEDH	DA	DA	DA
NZNU	E3	E3	E3
OM	DA	DA	DA
PVG	E3	E3	E3
SHR	E3	E3	E3
WC	E3	E3	DA
WKY	DA	DA	DA
Wild rats			
KL-1	E3	E3	DA/E3
KL-2	E3	DA/E3	DA/E3
KL-3	DA	DA	DA
KL-4	DA	DA	DA
KL-6	DA/E3	DA/E3	DA
KL-7	DA/E3	DA	DA
KL-8	DA/E3	DA	DA
KL-9	E3	DA	DA
KL-10	DA	DA	DA
KL-11	E3	DA	E3
KL-12	DA	E3	–
KL-13	DA/E3	DA/E3	DA
KL-14	E3	DA	DA
KL-15	E3	DA	DA
KL-17	E3	DA	DA/E3
KL-18	E3	DA	DA
JH-1	DA/E3	DA	DA/E3
JH-2	DA	DA/E3	DA
JH-3	DA	E3	DA
JH-4	DA	E3	DA
Ax-1	E3	DA	E3

BN rats share all genotypes but one (SNP 472 bp) with DA but nevertheless are arthritis resistant and show normal oxygen burst levels. This strongly suggests SNP 472 bp (aa 153 Met/Thr) to be the important site of polymorphism concerning *Ncf1* activation and arthritis regulation. The presence of both alleles of *Ncf1* in wild rats, collected at many different locations in both Sweden and Germany, argues against a bottleneck of inbreeding laboratory animals but indicate that the arthritis-susceptible allele of *Ncf1* is compatible with wildlife.
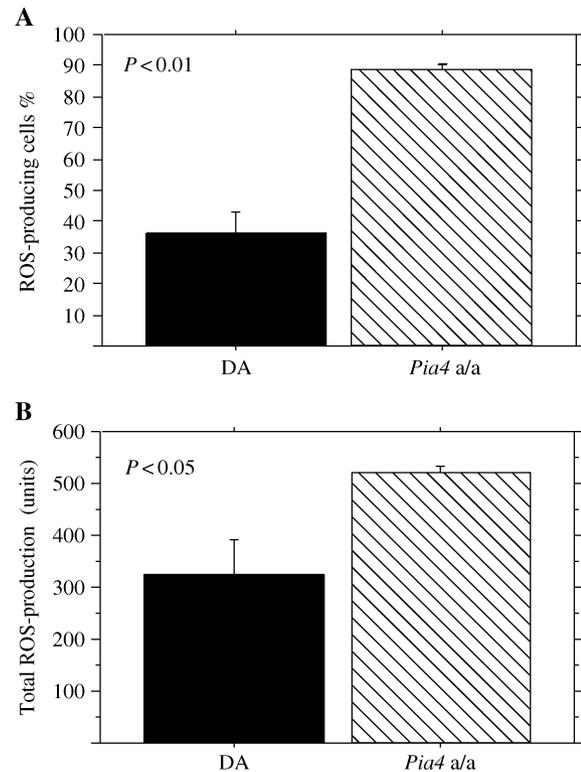
Figure 5 Analysis of reactive oxygen species (ROS) production after phorbol 12-myristate 7-acetate activation in peripheral blood leucocytes obtained from DA and DA.*Pia4* rats. The frequency of ROS-producing cell (A) as well as the actual ROS production in the cells (B) was significantly reduced in DA compared with DA.*Pia4* rats.

(ROS), the most appropriate was to investigate its effect on the oxidative burst. Indeed, it was found that the arthritis-susceptible DA rat had 50% lower ROS production as compared with the DA.*Pia4* congenic strain and the E3 strain (Fig. 5). The effect on oxidative burst followed the same inheritance pattern as the effects on arthritis severity. Furthermore, as discussed above, the polymorphism of *Ncf1* is pronounced in both inbred rat strains and in the wild rat population. The finding that the effect of the *Ncf1* gene operates before T-cell transfer of arthritis and not after are, however, not consistent with the current dogma of *Ncf1* function. The NADPH oxidase-determined oxidative burst is believed to mainly operate through phagocytes, enhancing the inflammatory attack in tissues. Nevertheless, it is clearly possible that oxidative burst reactions play an important regulatory role in antigen-presenting cell (APC)–T-cell interactions. To further dissect the mechanisms of the *Ncf1* gene on the pathway leading to arthritis, it is important to get access to the mouse system in which gene manipulations are more efficiently performed and in which molecular pathways are better defined. There are *Ncf1* knockout mice available, but surprisingly these are protected from inflammatory diseases, as tested using a myelin oligodendrocyte
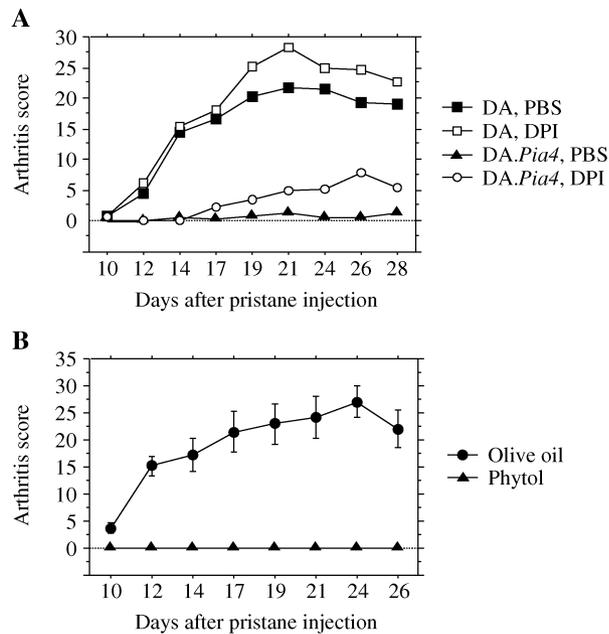
Figure 6 Treatment of pristane-induced arthritis (PIA) in DA rats was attempted through inhibition of NADPH oxidase complex activity (A) or activation of the NADPH oxidase complex activity (B). (A) Administration intraperitoneal of 5 μmol DPI/kg rat diluted in minimal volume of dimethyl sulphoxide with phosphate-buffered saline (PBS) into 1 ml/injection at day 0, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 17, 19, 21 after pristane injection had no ameliorating effect on PIA. No statistical significant differences were observed. (B) Administration intradermal of 200 μl phytol at day 5 or at the day of pristane injection completely prevents the development of PIA.

glycoprotein peptide-induced allergic encephalomyelitis (EAE) model [62]. There are, however, several differences with this experiment and the results using the DA.*Pia4* rats. Firstly, the *Ncf1* knockout mouse is a mix of *129* and *B6* genes, thus having a large linked fragment around the deleted *Ncf1* gene and of course the *Ncf1* gene is also totally depleted, whereas in the DA rat it is still partially functional. Secondly, it is not known whether the *Ncf1* gene will operate the same way in the mouse or even in other genomic backgrounds than the DA rat, as it might specifically interact with other polymorphic genes. Thirdly, the used inflammatory model is different than PIA as it is an EAE model induced with an immunogenic peptide together with both mycobacteria cell wall components and pertussis toxin. Hence, further investigations are necessary to evaluate the role of *Ncf1* in models of autoimmune disease in mice.

Approaching pathway mechanisms

The strategy to dissect the mechanism of inheritance through the analysis of predetermined candidate genes in arthritis has so far been fruitless [63], whereas the identification of *Ncf1* shows how important it is to address complex inheritance with an unbiased mind. We, however,

do not know how *Ncf1* regulates arthritis severity. *Ncf1* is known to be a part of the NADPH oxidase complex responsible for the one-electron reduction of oxygen-yielding ROS [64]. The primary generated O_2^- from the NADPH oxidase complex serve as starting material for the production of reactive oxidants including free radicals and singlet oxygen [65]. The identified polymorphism in the *Ncf1* gene of DA rats makes the NADPH oxidase complex less functional to produce ROS than other rat strains, suggesting a link between low oxygen burst and arthritis severity. This result goes against the general dogma concerning ROS and arthritis [66, 67]. Accordingly, it has been shown that experimental arthritis is suppressed by diphenyleneiodonium chloride inhibitors (DPI) of the NADPH oxidase complex [68, 69]. Nevertheless, when attempting to inhibit the NADPH oxidase complex with equal concentrations of DPI, in our PIA model in rats, the arthritis was not suppressed, on the contrary it escalated. However, the increased arthritis severity because of DPI treatment did not reach statistical significance (Fig. 6A).

Based on these findings, we analysed whether NADPH-activating substances would then have a disease-ameliorating effect. In our initial study of the *in vivo* effect of the NADPH-activating oil phytol, we showed a clear preventing or ameliorating effect of PIA on rats treated with phytol (Fig. 6B). However, in line with the fact that *Pia4/Ncf1* is of major importance in the activation phase of PIA, most ameliorating effect of PIA is achieved when phytol is administered before the onset of disease. Hence, we propose that polymorphisms in genes of the NADPH oxidase complex that alter the function to produce ROS will have effects not only in the effector phase in the joints, where produced radicals may cause damage to the tissue and increase an ongoing inflammation. An altered production of ROS in secondary lymphoid organs might either directly or, as previously shown by altering the internal environment through influx of ROS secrete numerous proteases [70], cause different processing of circulating antigens and thus elicit an immunological response to self antigens that might precipitate in autoimmunity. The potential of NADPH oxidase activators in future development of anti-inflammatory/rheumatic drugs is thus a challenging task as the outcome of such treatments might be highly dependent upon delivery and tissue distribution. As treatment of PIA with phytol was most effective in the early stage of the disease, the potential use of such treatments in RA would be to prevent and ameliorate phases of more severe inflammation, when new formation of autoreactive T cells might occur. However, validation of *Ncf1* as a drug target requires a deeper understanding of the pathogenic pathway, associated with *Ncf1* polymorphism, leading to severe arthritis. Obviously, decreased oxygen burst is associated with effects on T-cell priming, whereas the possibility remains that an increased oxygen burst may increase inflammation in the target tissue. An oxygen burst response may serve very

different functions if operating in an APC in a lymphoid organ than operating through a phagocyte cell in an arthritic joint. If so, the connected pathways are likely to be different and the drugs must be developed in a more intelligent way based on a deeper understanding of the genetically controlled pathogenesis of the disease.

In conclusion, the use of animal models of arthritis facilitated positional cloning of a polymorphism in *Ncf1*, which in the studied models proved to be one crucial factor for arthritis susceptibility. The identified involvement of ROS in arthritis induction is an overlooked mechanism in this aspect, and future analyses of animal models as well as investigation of RA patients will detail the importance of *Ncf1* in autoimmune disorders.

Acknowledgments

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