Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the *Ncf1* gene

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The *Ncf1* gene was recently identified as a strong regulator of severe arthritis in rat. This finding was surprising, because the disease-promoting allele mediated a lower level of reactive oxygen species in NADPH oxidase-expressing cells. We have now investigated a splice mutation of the *Ncf1* gene in B10.Q mice, causing a truncated and nonfunctional Ncf1 protein. We found that the mutated Ncf1 led to a more severe and chronic relapsing collagen-induced arthritis. Enhanced IgG and delayed-type hypersensitivity responses against type II collagen were seen, indicating increased activity of autoreactive T cells. Interestingly, female *Ncf1*-mutated mice spontaneously developed severe arthritis during the postpartum period. The arthritis was accompanied by an increased antibody response to type II collagen, with the same fine specificity as in collagen-induced arthritis. The enhancing effect of the mutated Ncf1 could also be shown to be more general in that it enhanced myelin oligodendrocyte glycoprotein protein-induced experimental autoimmune encephalomyelitis, a model for multiple sclerosis. These results show that *Ncf1*, a gene important for oxidative burst, regulates the susceptibility and severity of both arthritis and encephalomyelitis and modulates, directly or indirectly, the level of T cell-dependent autoimmune responses.

rodent | T lymphocytes | NADPH

Rheumatoid arthritis (RA) is a common autoimmune disease that is partially genetically regulated. Like many other autoimmune diseases, a significant part of the genetic contribution is the association with the MHC region (1, 2). However, despite decades of research of linkage and association studies, including genetic dissection of the MHC region, successful molecular understanding of these complex diseases, including single mutations and polymorphisms, is rare (3–5). Recently, we identified a polymorphism of *Ncf1* (alias *p47phox*) that regulates the severity of arthritis in rats (6). A surprising fact with the *Ncf1* polymorphism in the rat is that the susceptibility allele of *Ncf1* is accompanied by lower level of reactive oxygen species (ROS) than the resistant strain, contradictory to general dogma where high levels of radicals have been considered to promote inflammation (7). This association between ROS and autoimmunity is interesting, because it opens up the possibilities for new insight into the pathogenesis of complex autoimmune disorders. The Ncf1 protein is an essential component of the NADPH oxidase complex that catalyzes the transfer of a single electron from NADPH to oxygen, generating ROS. The release of ROS and its downstream products from phagocyting cells is known as the respiratory burst and is regarded as part of the protection against invading pathogens (7). The importance for the innate immune defense of having a functional phagocyte NADPH oxidase is clearly exemplified in chronic granulomatous disease (CGD), a rare genetic disorder characterized by severe recurrent infections due to the inability of neutrophils and macrophages to mount a respiratory burst and thereby kill the invading pathogens (8). The second most common genetic defect, responsible for ≈30% of CGD cases, is an autosomal recessive form caused by mutations in *Ncf1* (9). Knockouts of *Ncf1* (10) have been created through gene-targeting technologies, creating mouse models with a CGD phenotype. Targeted gene disruption in mice is the most common method to study the effects of gene defects. The selection of which genes to analyze by genetic manipulations, however, must be chosen through highly qualified predictions based on knowledge of the molecular pathology of the immune system. This approach is most feasible in monogenic diseases like CGD. However, in polygenic diseases, which cause most common autoimmune disorders like RA, the underlying molecular mechanism is more complex due to the contributions and interactions of several genes. Hence, other ways to unravel the genetic regulation must first be considered. Through linkage analysis followed by positional cloning, naturally occurring polymorphisms that predispose susceptibility to diseases can be identified without prior determinations about regulating genes (3).

Given the identified linkage between arthritis and *Ncf1* polymorphism in the rat, careful analysis of arthritis severity in NADPH oxidase-deficient animals is of interest. To this intent, we have used an earlier identified natural mutation in the *Ncf1* gene in C57BL/6J mice (11). This mutation, located at a splicing site at position −2 before exon 8 of the *Ncf1* gene, results in various forms of transcripts. One of these gave rise to low levels of protein but was truncated, lacking eight amino acids in the second SH3 domain of critical importance for interaction with the Ncf2 protein. Consequently, the mice had no detectable NADPH oxidase function, measured by ROS response of neutrophils. We have backcrossed this mutation to B10.Q strains, allowing study of its role in experimental models for RA and multiple sclerosis. These *Ncf1*-mutated mice showed augmented severity of both chronic experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA). An enhancing effect on T cell-dependent autoimmunity was observed. Interestingly, these mice spontaneously developed severe arthritis in the postpartum period, i.e., in a phase of life when both mice and humans are most susceptible to developing certain autoimmune diseases, such as arthritis (12–15).

Materials and Methods

Animals. Mice [B6.Cg-m+/+ Lepr (db), formerly known as C57BL/6J-m+/+ Lepr^{db}] with a relative deficiency of Ncf1 caused by a point mutation in the splice site for exon 8 (11) were

Abbreviations: RA, rheumatoid arthritis; ROS, reactive oxygen species; EAE, experimental autoimmune encephalomyelitis; CIA, collagen-induced arthritis; CFA, complete Freund's adjuvant; CII, collagen II; COMP, cartilage oligomeric matrix protein; OVA, ovalbumin; DTH, delayed-type hypersensitivity; APC, antigen-presenting cells; MOG, myelin oligodendrocyte glycoprotein.

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purchased from The Jackson Laboratory. The mice were backcrossed to B10.Q (originally from Jan Klein, Tübingen University, Tübingen, Germany) for at least six generations to introduce the $H2^q$ haplotype in the MHC locus and to breed out the leptin receptor ($lepr^{db}$) defect present in these mice. Offspring from a B10.Q.*Ncf1*+/* (* denotes the mutation, and + denotes the wild type) intercross were used. All mice were kept and bred in a climate-controlled environment with a 12-h light/dark cycle, housed in polystyrene cages containing wood shavings, and fed standard rodent chow and water ad libitum in the animal house of Medical Inflammation Research. The experiments were approved by a local (Malmö/Lund, Sweden) ethical committee (license M7-01).

Genotyping. DNA was prepared from toe biopsies by heating the sample in 1 ml of 50 mM NaOH for 1 h (16). The DNA solution was neutralized with 100 μl of 1 M Tris buffer and used directly in PCR reactions. Single-nucleotide polymorphism markers that distinguish between the wild-type allele and the mutation in the splice site of exon 8, causing exclusion of this exon, were performed by using *Ncf1*-ex8-specific markers. The reaction was amplified by PCR using forward 5′-bio-TAG AAA GGG AAA GCC AGA AAG AAT-3′ and reverse 5′ACG CTT TGA TGG TTA CAT ACG GT-3′ primers yielding a DNA template of 365 bp that, after separation with streptavidin, can be detected in a sequencing reaction by the sequencing primer 5′-ACG CTT TGA TGG TTA CAT ACG GT-3′ using Pyrosequencing equipment according to protocols supplied by the manufacturer (Biotage, Uppsala).

Induction and Evaluation of Arthritis. The mice were age-matched (9–15 weeks) and given an intradermal injection at the base of the tail with 100 μg of rat collagen II (CII) purified from the Swarm rat chondrosarcoma, as described (17), emulsified in complete Freund's adjuvant (CFA; Difco). At day 35 after primary CII immunization, mice were boosted at the same location with 50 μg of rat CII in incomplete Freund's adjuvant. Arthritis development was monitored in all four limbs by using a macroscopic scoring system. Briefly, one point was given for each swollen or red toe, one point for each swollen joint (metatarsal phalangeal joints, metacarpal phalangeal joints, proximal interphalangeal joints, and distal interphalangeal joints), and five points for a swollen ankle (maximum score per limb 15, 60 per mouse). The mice were examined one to four times per week for 2 months after immunization. At days 40 and 102, serum was obtained through tail bleeding and kept at −20°C until assayed. For histopathologic analyses, ankle joints were taken on day 31 after CII immunization. Paws were fixed in 4% formaldehyde, decalcified in EDTA, embedded in paraffin, sectioned, and stained with hematoxylin and erythrosine (18).

Induction and Evaluation of EAE. The mice were age-matched (12–15 weeks) and immunized at the base of the tail with 25 μg of recombinant rat MOG_{1-125} protein or 25 μg of mouse myelin oligodendrocyte glycoprotein $(MOG)_{79-96}$ peptide emulsified in CFA (Difco), containing 50 μg of *Mycobacterium tuberculosis*. Clinical signs of EAE were assessed by using a standard scoring protocol (19). The recombinant MOG_{1-125} protein was a generous gift from Robert Harris (Karolinska Institute, Stockholm). The MOG_{79-96} peptide corresponds to amino acids of the mouse sequence (GKVTLRIQNVRFSDEGGY) and was synthesized by Shafer-N, Copenhagen, with a purity of >97%.

Determination of Serum Levels of Cartilage Oligomeric Matrix Protein (COMP). Serum concentration of COMP was determined by a competitive enzyme-linked immunosorbent assay (ELISA) (20). Rat COMP was used for coating of the microtiter plates and for preparing the standard curve included in each plate. Plasma COMP was detected by using a polyclonal antiserum against rat COMP (generously provided by Dick Heinegård, Lund University, Lund, Sweden).

Antibody Response Against CII and Ovalbumin (OVA). Antibodies against CII or OVA in plasma were analyzed with ELISA in 96-well plates (Costar), coated overnight at 4°C with 50 μl of PBS per well containing 10 μg/ml rat CII, epitopes of CII [prepared as described (21)] or OVA (Sigma). All washings were performed by using Tris-buffered saline (NaCl 1.3 M/Tris 0.1 M, pH 7.4) containing 0.1% Tween 20 (Tris/Tween). The plasma was diluted in PBS/0.1% Tween and analyzed in duplicate. The amounts of bound IgM and IgG isotype antibodies (Southern Biotechnology Associates) were estimated after incubation with biotin-conjugated isotype-specific antibodies and 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt as substrate followed by detection in a Spectra Max (Molecular Devices). The relative amount of antibodies was determined in comparison with a positive control of pooled serum. Because different pools of serum were used for the different experiments, no comparisons should be made among the experiments.

Delayed-Type Hypersensitivity (DTH) Response. Mice, at age 9–15 weeks, received an intradermal injection at the base of the tail with either 100 μg of CII emulsified in CFA (Difco) or 100 μg of OVA (Sigma) emulsified in 50 μl of PBS and 50 μl of CFA. At day 13 after primary injection, the CII-immunized mice received an injection containing 20 μg of CII in 0.05 M acetic acid in the right ear. Acetic acid (0.05 M) was injected in the left ear as a control. The OVA-immunized mice were injected with 20 μg of OVA in PBS in the right ear. The control ear was injected with PBS. After 48 h, the DTH response was measured as the difference in percent in swelling of the ears between the right and left ears.

Statistics. Quantitative data are expressed as mean ± SEM, and significance analysis was performed by using Student's *t* test or the Mann–Whitney test. All results were compared to those from B10.Q.*Ncf1**/* mice.

Results

An *Ncf1* Splicing Mutation Backcrossed on B10.Q Mice Prohibits Ncf1 Expression and the ROS Response. A mutation at position −2 before exon 8 generating a splice defect of the *Ncf1* gene in a C57BL/6J colony also carrying a leptin receptor mutation has been reported to have a dramatically reduced expression of Ncf1 protein and an undetectable ROS response (11). To determine its effect on CIA and EAE, we backcrossed the mouse strain carrying the *Ncf1* mutation to the B10.Q strain. The B10.Q mouse carries the C57BL/10 background and a DBA/1-derived congenic fragment containing the MHC class II gene A^q allowing an immune response to CII and MOG (19, 22). The experiments were performed on an intercrossed cohort obtained after four to six generations of backcrossing. Remaining differences in the genome background were excluded by littermate-controlled experiments. Differences in the linked fragment on chromosome 5 were unlikely to play a role, because none of the microsatellites defined on this chromosome differ in length between C57BL/6J and B10.Q (Mouse Genome Informatics, www.informatics.jax.org, data not shown). Consequently, no autoimmune quantitative trait loci have been found on chromosome 5 that differs between C57BL/6J and C57BL/10 (23). Analysis of spleen neutrophils from the backcrossed *Ncf1*-mutated B10.Q mice confirmed that the homozygous mutation led to a reduced Ncf1 expression and undetectable ROS response (see *Supporting Text* and Fig. 5, which are published as supporting information on the PNAS web site), as investigated in detail by Huang *et al.* (11).

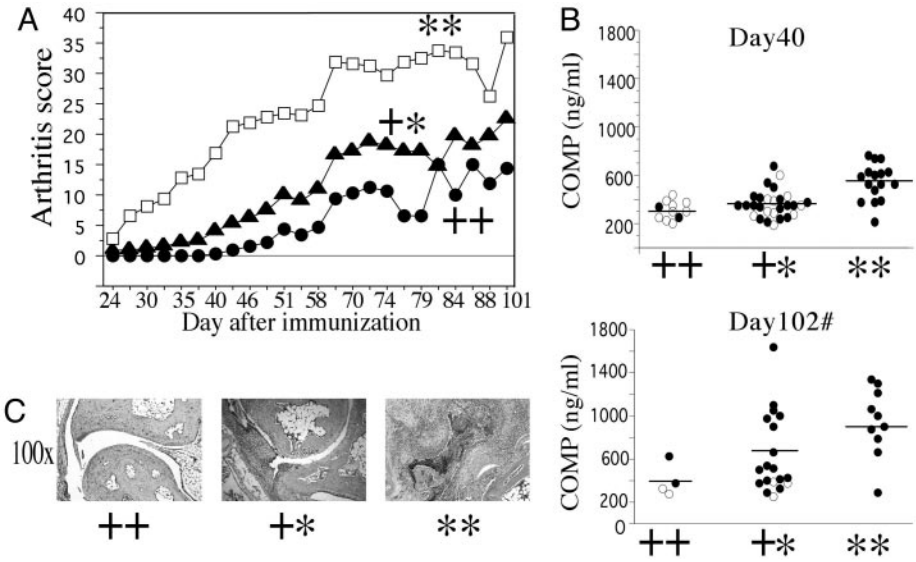

Fig. 1. Mice with mutated *Ncf1* develop enhanced CII autoimmunity and chronic CIA. Development of CIA in B10.Q.*Ncf1**/* ($n = 19$) mice compared with B10.Q.*Ncf1*+/* ($n = 28$) and B10.Q.*Ncf1*+/+ ($n = 11$) littermates. Mice were immunized with 100 μg of rat CII in CFA injected intradermally at the base of the tail. At day 35, a boosting with an intradermal injection containing 50 μg of rat CII in incomplete Freund's adjuvant was performed. After day 58, fewer animals are scored due to scarification for histology; B10.Q.*Ncf1**/* ($n = 10$), B10.Q.*Ncf1*+/* ($n = 19$), B10.Q.*Ncf1*+/+ ($n = 4$). (*A*) Arthritis scores plotted against the different days after CII immunization. (*B*) Serum concentration of COMP used as a measurement of ongoing cartilage destruction days 40 and 102. Filled circles represent mice with arthritis, and lines represent the mean values [B10.Q.*Ncf1**/* ($n = 19$), B10.Q.*Ncf1*+/* ($n = 28$), B10.Q.*Ncf1*+/+ ($n = 11$)]. #, fewer animals analyzed on day 102 [B10.Q.*Ncf1**/* ($n = 10$), B10.Q.*Ncf1*+/* ($n = 19$), B10.Q.*Ncf1*+/+ ($n = 4$)]. $P > 0.001$ for both wild-type and heterozygous mice in comparison with mutated mice on day 40. Day 102 $P < 0.01$ for wild-type and $P < 0.5$ for heterozygous mice. (*C*) Representative morphological sections of ankle joints taken at day 102 after CIA induction. Sections were stained with hematoxylin and erythrosine (×100). Severe arthritis with cartilage and bone destruction is seen in the B10.Q.*Ncf1**/* mice, whereas there was milder cartilage destruction in the B10Q.*Ncf1*+/* and B10.Q.*Ncf1*+/+ control mice.

Severe Chronic CIA in *Ncf1* Mutated Mice. To investigate the effect of aberrant Ncf1 function and NADPH oxidase complex activity on arthritis, we investigated the development of CIA in *Ncf1*-mutated mice. Wild-type mice normally develop mild/medium severe arthritis with onset after booster (24). We observed more severe arthritis with earlier onset ($P < 0.001$) in B10.Q.*Ncf1**/* compared to wild-type mice (Fig. 1). *Ncf1**/+ mice showed significantly milder arthritis than the homozygously mutated *Ncf1**/* mice, indicating that one fully functional allele of the *Ncf1* gene is enough to protect from the severe form of the disease. Interestingly, the *Ncf1*-mutated mice showed an increasingly active chronic development of arthritis characterized by severe inflammation in a relapsing pattern.

The serum levels of COMP, which is regarded as a measurement of ongoing cartilage erosion of peripheral joints (20), were highly elevated ($P < 0.0001$) in the B10.Q.*Ncf1**/* mice compared to B10.Q.*Ncf1**/+ and B10.Q.*Ncf1*+/+ littermate controls. Elevated levels of COMP in the *Ncf1*-mutated mice were detected both in the early severe phase (day 40) as well as in the chronic relapsing phase of the disease (day 102), confirming a role for *Ncf1* as protecting against both acute and chronic inflammation (Fig. 1*B*). Morphology examination of hind-paw ankle joints taken on day 102 after primary CIA induction, i.e., at the chronic relapsing period, also revealed active inflammation in the *Ncf1*-mutated mice, whereas the wild-type control and heterozygous mice were only mildly affected (Fig. 1*C*).

In contrast to the investigations of *Ncf1* polymorphisms that were performed in rats by using the pristane-induced arthritis (PIA) (6), in the CIA mouse model, it is possible to evaluate the levels of antibodies directed against the injected CII antigen. Levels of antibodies to CII were consistently higher in the B10.Q.*Ncf1**/* mice as compared with both heterozygous and normal littermate mice at both days 40 and 102. Analysis of the antibody isotypes on day 40 showed that the effect was most pronounced for the more T cell-dependent production of IgG and not IgM. The increased levels of anti-CII IgG involved all isotypes (IgG1, IgG2b, IgG2c, and IgG3) ($P < 0.01$) (Fig. 2*A*). Elevated responses against the major C1 epitope as well as against the J1 and U1 epitopes ($P < 0.05$) were found in B10.Q.*Ncf1**/* (Fig. 2*B*). To evaluate the influence of an inflammatory reaction depending on T but not B cells, we analyzed the mice for DTH response to CII and OVA. As in the case of antibody production, also the DTH response against CII was elevated in the B10.Q.*Ncf1**/* mice as compared with both heterozygous and normal littermate mice ($P < 0.05$) (Fig. 2*C*). However, when DTH and antibody responses to OVA were tested, no enhancing effect of homozygous mutated *Ncf1* was seen (Fig. 2 *D* and *E*). These results indicate an overall increased activity of CII-reactive T cells to be of importance for the enhancement of inflammation and the chronic arthritis in the *Ncf1* mutated mice.

Enhancement of MOG Protein-Induced EAE and Suppression of MOG Peptide-Induced EAE. Our results of an enhancement of CIA were surprising in light of earlier reported findings that EAE, induced with the MOG_{35-55} peptide, was completely protected in mice deleted for the *Ncf1* gene (25). To clarify this, we immunized our *Ncf1*-mutated mice with MOG protein or MOG_{79-96} peptide, both known to induce EAE in $H2^q$ mice (19). MOG_{79-96} peptide-induced EAE was milder in the *Ncf1*-mutated mice than in the wild-type control (Fig. 3*A*), confirming the results reported by van der Veen *et al.* (25). However, induction of EAE with MOG protein induced a more severe chronic EAE in the *Ncf1*-mutated mice, corroborating our results in the CIA model (Fig. 3*B*). This finding shows that *Ncf1* has a more general role in regulating autoimmune inflammation and also suggests that the effect of *Ncf1* is related to the function of antigen-presenting cells (APC) in processing and presenting autoantigens to T cells.

Spontaneous Arthritis in B10.Q.*Ncf1/* Mice.** The *Ncf1*-mutated mice showed no abnormalities apart from one consistent obser-

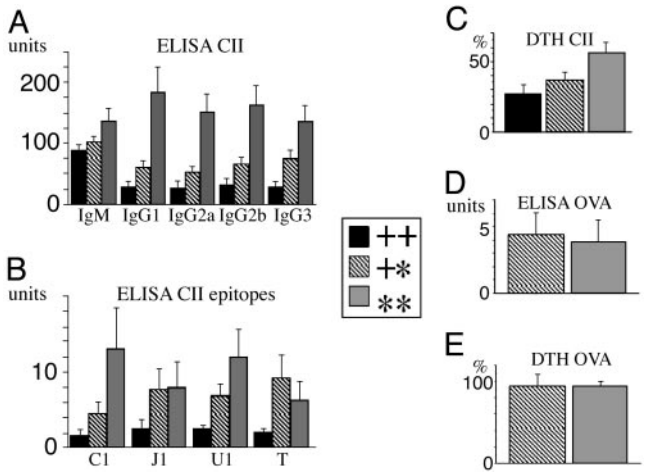

Fig. 2. T cell-dependent autoimmune response to CII, but not to OVA, is enhanced in *Ncf1*-mutated mice. Antibody response directed against (*A*) CII and (*B*) epitopes of CII was measured in serum at day 40 after CIA induction in B10.Q.*Ncf1**/* ($n = 19$), B10.Q.*Ncf1*+/* ($n = 28$) and B10.Q.*Ncf1*+/+ ($n = 11$) mice. A significant difference ($P < 0.05$) in levels of antibodies against C1, J1, and U1 can be seen between wild-type and homozygously mutated mice. (*C*) DTH response against CII in B10.Q.*Ncf1**/* ($n = 17$), B10.Q.*Ncf1*+/* ($n = 20$) and B10.Q.*Ncf1*+/+ ($n = 7$) was evaluated for reactivity 48 h after stimulation with CII in the ear. DTH result is presented as percent volume increase of the stimulated ear in comparison to control injected left ear. A significantly lower response can be seen in B10.Q.*Ncf1*+/* and B10.Q.*Ncf1*+/+ compared with B10.Q.*Ncf1**/* ($P < 0.05$). (*D*) DTH response presented as percent volume increase of the ear stimulated with OVA in comparison with control-injected left ear after 48 h and (*E*) antibody response measured in sera on day 18 after immunization with OVA in B10.Q.*Ncf1*+/* ($n = 7$) and B10.Q.*Ncf1**/* ($n = 11$).

vation. Many female mice, during the first days after partus (the so-called postpartum period), were observed to develop severe arthritis. After these occasional observations, we followed 12 *Ncf1**/* mice during their postpartum period, of which three developed clinical arthritis. The arthritis started a few days after delivery and was as severe and chronic as CIA (Fig. 4 *A* and *B*). Histology showed a massive destruction of bone and cartilage in the ankle joint compared to healthy B10.Q.*Ncf1**/* and B10.Q.*Ncf1**/+ mice (Fig. 4*C*). An elevated amount of antibodies against CII was detected in sera from the spontaneous arthritic mice compared to the healthy B10.Q.*Ncf1**/* mice, although not significant ($n = 3$). However, when the sera reactivity to different epitopes of CII was tested, an increased level of antibodies against the major CII epitope C1 as well as against epitopes J1 and U1 in the mice with spontaneous arthritis was found ($P < 0.05$), resembling the response in CIA (Fig. 4*D*).

Discussion

The identification of a single-nucleotide polymorphism in the rat *Ncf1* gene leading to a reduced oxidative burst and severe arthritis was indeed a surprising but also challenging finding. We have now found similar effects in a mouse strain carrying another mutation in *Ncf1*, also leading to a reduced ROS response. We are now able to show that the *Ncf1* polymorphism also enhanced autoreactive T cell functions and the development of chronic CIA and EAE. Interestingly, the mutated *Ncf1* mice developed chronic severe arthritis spontaneously postpartum.

The oxidative burst, induced in phagocytic cells after exposure of bacterial components, is generally thought to be a local powerful component in the inflammatory response. It has also been widely anticipated that the ROS response promotes an autoimmune inflammation. Recently, however, further complexity of the redox status and the induced ROS response in particular has been revealed. Thus, the ROS response may occur not only in phagocytes but also in other cell types, including APC such as dendritic cells and class II-expressing macrophages (26). Ncf1 expression and functional activities in APC but not in T cells have earlier been reported (27, 28). Oxidation effects have also been shown to modulate T and B cell function, and interestingly on T cells a major effect is oxidation of cell membranes that are exposed for ROS from interacting cells (29). It is clear that oxidation effects on T cell receptor signaling pathways, such as in the linker for activation of T cells molecule (30), modulate T cell functions, and it is theoretically possible that ROS, in particular hydroxide radicals, could be transferred over the synapse from APC to T cells (31, 32). It is also possible that an oxidative burst response in an APC may change the antigen-presentation capacity of that cell. An interaction between APC and T cells occurs at several stages during T cell development *in vivo*; in the thymus, during migration into peripheral lymphoid organs, during priming, and maybe also during subsequent activation in the target tissue. The enhanced T cell autoimmune activities could be explained by an influence at any of these occasions *in vivo*. It also remains to be investigated whether the modulated T cell function by ROS leads to an enhanced regulatory function or a decreased activity of autoreactive T cells. Sakaguchi *et al.* (33) have recently reported a similar situation; the occurrence of spontaneous arthritis in mice was found to be caused by a structural polymorphism in the *Zap70* gene. This mutation caused a decreased T cell receptor signaling, resulting in an enhanced T cell autoreactivity to joint antigens such as CII, and with the development of arthritis depending on an interactive effect with MHC class II. Thus, polymorphism in both the *Zap70* and *Ncf1* genes in rat and mouse argues for an important effect during T cell priming and selection as a cause for the enhancement of arthritis. The *Ncf1*

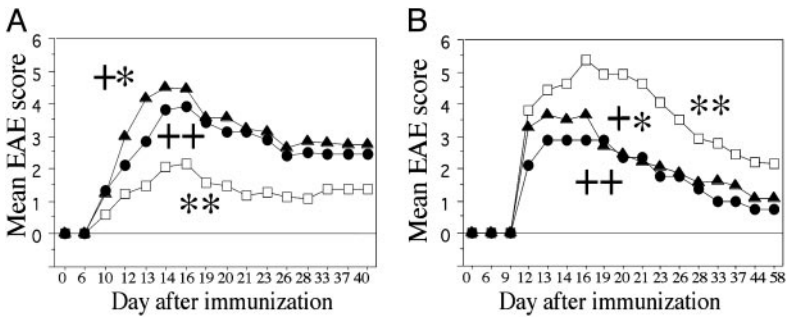

Fig. 3. Enhancement of EAE induced with native MOG but reduction of EAE induced with a MOG peptide in the *Ncf1*-mutated mice. Influence of the *Ncf1* mutation on experimental EAE. (*A*) Development of EAE in mice immunized with the MOG peptide 79–96 in CFA B10.Q.*Ncf1**/* ($n = 13$), B10.Q.*Ncf1*+/* ($n = 9$) and B10.Q.*Ncf1*+/+ ($n = 12$). (*B*) Development of EAE in mice immunized with native MOG recombinant protein in CFA B10.Q.*Ncf1**/* ($n = 13$), B10.Q.*Ncf1*+/* ($n = 7$) and B10.Q.*Ncf1*+/+ ($n = 4$).

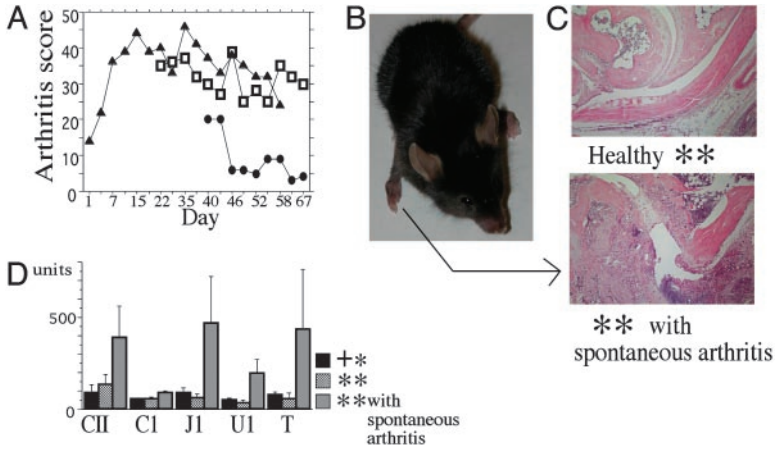

Fig. 4. Spontaneous arthritis and CII autoimmunity develops in B10.Q.*Ncf1*∗/∗ mice. (*A*) Development of arthritis in three individual mice followed by clinical scoring. (*B*) A naïve *Ncf1*-mutated mouse with spontaneous arthritis. (*C*) Representative morphological sections of ankle joints taken from the mouse shown in *B* and a control mouse. The sections were stained with hematoxylin and erythrosine (×100). (*D*) The antibody response directed against CII and epitopes of CII in B10.Q.*Ncf1*∗/∗ mice with spontaneous arthritis compared to healthy B10.Q.*Ncf1*+/∗ and B10.Q.*Ncf1*∗/∗ mice ($n = 3$). The mutated mice with spontaneous arthritis have significantly higher levels of C1, J1, and U1 epitopes ($P < 0.05$).

gene, however, is likely to have divergent influence in various tissues and in different cell types. Thus, the local oxidative environment in the inflammatory focus as a drive for vascularization and cell toxicity and, on the other hand, its role in the APC–T cell interaction shifting the function of inflammatory cells and T cells may lead to contrasting outcomes of a ROS response. Recent results using mice unable to mount a ROS response, due to deficiency in a critical component in the NADPH oxidase complex, such as Ncf1, show a different effect on an inflammatory response. The observation that zymosan-induced arthritis is enhanced in *Ncf1*-knockout B6/129 mice (34) is not likely to be an effect on the selection of autoreactive T cells, because this is a local T cell-independent type of arthritis. Possibly a complete deficiency of the ROS response may also lead to a feedback regulation, increasing the number of circulating neutrophils. A contrasting effect is the observation that *Ncf1*-knockout B6/129 mice are completely protected from MOG-peptide-induced EAE (25). This finding is difficult to explain in light of our recent identification of a polymorphic *Ncf1* allele in the rat, which promotes a reduced ROS response and increased arthritis severity. Our results using a mutation in the mouse *Ncf1* gene may partly clarify these issues.

First, combining our results in rats and mice strengthens the hypothesis that increased arthritis severity is directly related to reduced oxidative burst. The structural polymorphism in the rat *Ncf1*, giving amino acid replacements at position 106 and 153, is different from the mutation in the mouse *Ncf1*, giving a splice defect. Nevertheless, both the rat and the mouse mutations reduce oxidative burst and promote arthritis severity.

Second, the results from the *Ncf1*-mutated mouse show that the reduced Ncf1-mediated ROS response enhances T cell-dependent autoimmunity. This is clearly compatible with the observations in the pristane-induced arthritis model in the rat in which the effect could be transferred with $\alpha\beta$ T cells. After immunization with CII in the mouse, both a CII-specific DTH reaction and T cell-dependent IgG responses are enhanced. Interestingly, the immune response to the foreign antigen OVA is not affected. Taken together, our results suggest that Ncf1 plays a role in the selection of T cell autoreactivity.

Third, the finding that MOG protein-induced EAE is also enhanced in a similar way as CIA shows that the *Ncf1* gene has a more general effect on T cell-dependent autoimmune disease. The finding is compatible with earlier results in the rat in which a strong linkage between EAE severity and the *Pia4* locus (containing *Ncf1*) was identified (35). Interestingly, the enhancement occurred only if the disease was induced by immunization with the native MOG protein and not with the immunodominant MOG peptide. This indicates that immune recognition of native epitopes or uptake and processing of the protein is of importance for the Ncf1 function, because administration of the peptide will bypass such steps before binding to the MHC class II molecule on the APCs. This hypothesis may partly explain why *Ncf1* knockout B6/129 mice are completely resistant to MOG peptide-induced EAE. Still, there are differences, and it is possible that embryonic stem cell-derived 129 genes linked to the deleted *Ncf1* gene influence the stronger effect in the *Ncf1* knockout mice as compared with the *Ncf1*-mutated mouse. There are clearly several linked genes in this particular gene region that could influence autoimmunity, as discussed concerning the diverse results obtained using various osteopontin-deficient mouse strains, possessing a 129 fragment in the same region (36). Another difference between the *Ncf1* knockout and the mutated mouse is that the Ncf1 protein is still expressed in the mutated mouse, although at low levels and as truncated protein.

The last point to be made is the observation that spontaneous arthritis in fact develops in the *Ncf1*-mutated B10.Q mouse strain. Interestingly, this occurs only in the postpartum period. We have earlier observed a similar effect postpartum on the susceptibility to classical CIA in the DBA/1 mice in a study of the influence of various pregnancy factors (15). These mice developed relapses of arthritis postpartum, relapses that could be prevented by a continued estrogen replacement. Similarly to RA in humans (12, 13), mice are protected from arthritis during pregnancy (14) but are more vulnerable immediately after partus (15), and these effects seem to be mainly hormonally regulated through the estrogen receptor (37). In the case of the *Ncf1*-mutated mice, they develop severe arthritis spontaneously, and this arthritis continues along a chronic disease course with an erosive inflammatory arthritis. That this occurs only in a minority of the mice does not necessarily reflect remaining genetic heterogeneity of B6 and B10 genes, because the same reduced frequency of severe arthritis also occurs in inbred mice with an autologous CIA, i.e., mice induced with mouse CII (38) or mice expressing the immunodominant CII peptide in cartilage (39). The observation of a low frequency of spontaneous severe arthritis postpartum in fact confirms previous observations of DA rats, which carry the arthritis-promoting *Ncf1* allele, also

occasionally developing arthritis postpartum (unpublished observations). Interestingly, the mice also develop a joint-specific autoimmune response, as seen with increased levels to CII, in fact to epitopes also recognized in human RA (21). Antibodies to these epitopes (C1, J1, and U1) do in fact induce arthritis upon passive transfer (40).

The *Ncf1* polymorphism in the rat was found to be associated with an arthritis model, pristane-induced arthritis, with many features in common with RA, in particular during the chronic relapsing disease course (18). The CIA model in the mouse, despite being the most commonly used animal model for RA, usually develops only an acute disease without a subsequent chronic active disease course. In the present study, we show that *Ncf1*-mutated mice develop a chronic relapsing disease, thereby better resembling RA in humans. This, in fact, was achievable in a mouse strain that normally develops only mild arthritis with low frequency, but that tends to show a chronic disease course, as has been earlier shown in IL-4-deficient mice of the same genetic background (41).

Taken together, we show that the *Ncf1* gene is associated with a previously undescribed pathway involving autoreactive T cells and modulating inflammatory diseases like arthritis and encephalomyelitis. It provides possibilities of generating immunotherapeutic drugs to target RA and multiple sclerosis, and the finding that it operates in both mice and rats increases the likelihood that similar genetics also operate in humans.

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