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ORIGINAL RESEARCH COMMUNICATION

Positioning of a Polymorphic Quantitative Trait Nucleotide in the *Ncf1* Gene Controlling Oxidative Burst Response and Arthritis Severity in Rats

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Abstract

The *Ncf1* gene, encoding the $P47^{PHOX}$ protein that regulates production of reactive oxygen species (ROS) by the phagocyte NADPH oxidase (NOX2) complex, is associated with autoimmunity and arthritis severity in rats. We have now identified that the single-nucleotide polymorphism (SNP) resulting in an M153T amino acid substitution mediates arthritis resistance and thus explains the molecular polymorphism underlying the earlier identified *Ncf1* gene effect. We identified the SNP in position 153 to regulate ROS production using COS^{PHOX} cells transfected with mutated *Ncf1*. To determine the role of this SNP for control of arthritis, we used the Wistar strain, identified to carry only the postulated arthritis resistant SNP in position 153. When this *Ncf1* allele was backcrossed to the arthritis susceptible DA strain, both granulocyte ROS production and arthritis resistance were restored. Position 153 is located in the hinge region between the PX and SH3 domains of $P47^{PHOX}$. Mutational analysis of this position revealed a need for an −OH group in the side chain but we found no evidence for phosphorylation. The polymorphism did not affect assembly of the $P47^{PHOX}/P67^{PHOX}$ complex in the cytosol or membrane localization, but is likely to operate downstream of assembly, affecting activity of the membrane NOX2 complex. *Antioxid. Redox Signal.* 14, 2373–2383.

Introduction

A major challenge in medical science is to reveal the genetic cause of common complex disorders, such as rheumatoid arthritis (RA). Recent large-scale genome-wide association studies start to give some insight into the genes involved, achieving statistical evidence on association between disease and single-nucleotide polymorphism (SNP) markers [for review see (27, 62)]. Most of the indicated loci have, however, minor influence as indicated by low odds ratios. This implies that they operate in concert, and analysis in a properly controlled genetic and environmental context is necessary for the understanding of the function of these genes. To accomplish this, animal models are essential. In addition to being a highly valuable tool for genetic studies, animal models provide the possibility to experimentally test pathogenic mechanisms and therapeutic possibilities (3).

Genetic segregation experiments, using strains with variable susceptibility to complex diseases, have shown high efficiency in linkage analysis where the major loci have been indicated (4, 26). Due to the lack of dense recombinations in inbred strain crosses, it has, however, been difficult to position the genes underlying these loci. By using a congenic strategy, in which the linked fragment has been minimized through recombinations and isolated through backcrossing, a limited number of genes have been identified, including the major histocompatibility complex haplotype and the complement factor C5 (8, 13, 34, 44, 49, 66). Another example is the *Ncf1* gene, identified in a cross between the pristane-induced arthritis (PIA) susceptible DA and the resistant E3 rat strains (49, 63). The recombinant fragment was reduced to 300 kb containing only two genes, *Ncf1* and *Gtf2i*. Neither of the genes showed differential expression and only *Ncf1* contained nonsynonymous SNPs (49). The congenic rat, with a resistant E3-derived fragment on susceptible DA background, did not

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only show reduced arthritis severity but also increased oxidative burst response. This is compatible with known functions of the $P47^{PHOX}$ protein (also called NCF1), a subunit of the phagocyte NADPH oxidase (NOX2) complex (21), which is responsible for production of reactive oxygen species (ROS) in response to invading pathogens (6, 16). The complex consists of five subunits of which the membrane-integrated catalytic core protein $GP91^{PHOX}$ (CYBB) forms a stable heterodimer with $P22^{PHOX}$ (CYBA), constituting the enzymatic flavocytochrome b_{588} (CYT b_{558}) (7). The other subunits [$P40^{PHOX}$ (NCF4), $P47^{PHOX}$, and $P67^{PHOX}$ (NCF2)] are in the resting state residing in the cytosol where an auto-inhibitory conformation of the $P47^{PHOX}$ protein prevents the cytosolic complex to bind to the membrane subunits (18, 28). This auto-inhibitory conformation is released upon activation and consequent phosphorylation of $P47^{PHOX}$, which transport the cytosolic subunits to the membrane to form the functional complex (1, 57).

The finding that low ROS production-mediated arthritis (49) was surprising, as it required a mechanism in which ROS, produced by the NOX2 complex, protected against, rather than exaggerated inflammation, as the general dogma proposes [for an overview see (37, 41)]. These findings were strengthened by the discovery that a spontaneous mutation in the mouse *Ncf1* gene had similar effects on arthritis (36).

The three SNPs identified in *Ncf1* were all highly polymorphic both in the natural rat population and between the DA and E3 strains (49, 50). To understand the molecular mechanisms underlying the effect on arthritis, we needed to identify the arthritis causative SNP. To do so, we first investigated which of the three SNPs controlled ROS production *in vitro* by mutating recombinant *Ncf1*. We found that the amino acid shift from methionine to threonine at position 153 led to a restored ROS production capacity. Next, we selected recombinant haplotypes from inbred strains and made a new congenic strain that had an *Ncf1* variant differing only at position 153. Lastly, we investigated the functional difference between threonine and methionine at this position and found that threonine regulated the capacity of the already membrane bound NOX2 complex to produce ROS.

Materials and Methods

Cells and reagents

Calyculin A was from Cell Signaling Technology (Danvers, MA). All other reagents were purchased from Sigma-Aldrich if nothing else is stated. COS-7 cells reconstituted with $GP91^{PHOX}$, $P22^{PHOX}$, and $P67^{PHOX}$ (hereafter referred to as $COS^{phox\text{-}Ncf1}$), and COS-7 cells stably expressing $P47^{PHOX}$ in addition to $GP91^{PHOX}$, $P22^{PHOX}$, and $P67^{PHOX}$ (referred to as COS^{phox}) (52) kindly provided by Prof. Mary C. Dinauer (Indiana University) were cultured in Dulbecco's complete medium, 10% fetal calf serum, and penicillin–streptomycin (Gibco, Invitrogen). The murine Ra2 microglia cell line (licensed by the Japan Science and Technology Agency, Patent ID US6.673,6,5; JP3410738; EP10/602,234) was kindly provided by Dr. Makoto Sawada (Department of Brain Function, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan).

Vector constructs

DA (AF547392) and E3 (AF547393) *Ncf1* alleles were PCR amplified and inserted in the pcDNA3.1/Hygro (+) mammalian expression vector (Invitrogen). Mutations in the *Ncf1* cDNA were generated using QuickChange II Site-Directed M mutagenesis Kit (Stratagene) according to the manufacturer's recommendations. In all transfection experiments pcDNA3.1/Hygro (+) mammalian expression vector (Invitrogen) was used as control vector. Primer sequences are provided in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertonline.com/ars).

Cell transfection and ROS assay

Adherent COS-7 cells were harvested by incubation with trypsin/EDTA for 5 min at 37°C. The cells were resuspended, washed in PBS, and cultured in 96-well plates (NUNC) at a density of 20,000 cells/well overnight. The cells were transiently transfected with *Ncf1* with lipofectamine plus, optimum, and DNA PLUS system according to procedures recommended by the manufacturer (Invitrogen) using 100 ng DNA per well. Transfected cells were grown in a complete medium for another 48 h until assayed. Empty pcDNA3 vector was used as a negative control.

Functionality of the NOX2 complex and thus the function of the studied *Ncf1* allele was assayed directly in the culture plate using an isoluminol-enhanced chemiluminescence assay (17). Briefly, the cells were gently washed in Hanks balanced salt solution (HBSS) and 100 μl of isoluminol reagent buffer [isoluminol 10–50 μg/ml, HRP type II 2, 5–4 μ/ml, and phorbol 12-myristate 13-acetate (PMA) 200–400 ng/ml, final concentration] was added. Samples were gently mixed and data collection was initiated immediately. The ROS production from the NOX2 complex is initiated within minutes. Extracellular ROS production was followed at 37°C as produced luminescence signal (FluoStar Optima; BMG Labtechnologies) and presented as maximal relative signal during a measurement period of 30 min.

Western blot

Western blot was performed using standard protocols. $P47^{PHOX}$ was detected using a mouse monoclonal anti-$P47^{PHOX}$ (D-10) (sc-17845) from Santa Cruz Biotechnology followed by secondary antibody Rabbit anti-mouse IgG HRP-conjugated (P0260) from DAKO A/S (Glostrup). The sc-17845 antibody is generated against amino acids 196–390 of $P47^{PHOX}$ of human origin, therefore not interfering with the polymorphic area under the study. $P67^{PHOX}$ was detected using goat polyclonal anti-$P67^{PHOX}$ (N-19, sc-7663; Santa Cruz) followed by secondary antibody ImmunoPure® mouse anti-goat IgG HRP-conjugated (31400; Thermo Fisher Scientific).

Animals

Rats, DA and Wistar (originating from Harlan Europe), were kept in a climate-controlled environment with 12 h light/dark cycles and fed standard rodent chow and water *ad libitum* in the animal facility of Medical Inflammation Research, Lund University, Lund, Sweden, or Karolinska Institute, Solna, Sweden. The Wistar *Ncf1* ($Ncf1^{W}$) allele was backcrossed to the DA background for 10 generations. The rats were found to be free from common pathogens, including Sendai virus, Hantaan virus, coronavirus, reovirus, cytomegalovirus, and *Mycoplasma pulmonalis*. The experiments

were approved by the local ethical committee license M70/04 and M107/07 (Malmö/Lund, Sweden) and N67-10 (Stockholm, Sweden).

Genotyping

DNA samples were prepared from toe biopsies and assayed on a MegaBACE 1000 (GE Healthcare). Identification of SNPs in the *Ncf1* region has earlier been described (49). The SNPs were analyzed using a Pyrosequenser PSQ 96 (Quiagen) according to manufacturer's protocol.

Induction and evaluation of disease

Arthritis was induced at the age of 6–12 weeks. Rats were sex- and age-matched within all experiments. PIA was induced by an s.c. injection at the base of the tail with 200 μl of pristane (Acros Organics). Arthritis development was monitored by inspection evaluating the number of joints affected by arthritis according to a macroscopic scoring system of the four limbs ranging from 0 to 15 (1 point for each swollen or red toe, 1 point for a swollen or red midfoot digit or knuckle and 5 points for a swollen ankle), resulting in a maximum total score of 60 for each rat (33). All scoring was performed in a blinded manner to avoid biased results.

Determination of ROS production ex vivo

The level of intra-cellular oxidative burst *ex vivo* was measured by preparing single-cell suspensions, hemolyzed with ammonium chloride (0.84% pH 7.4), from blood. Oxidative burst in granulocytes was determined by incubation of cells for 30 min (4°C) with biotin-labeled antibody HIS-48 (anti-granulocytes) (BD Biosciences Pharmingen). According to manufacturer's information the HIS-48 antibody reacts with an antigen expressed on all granulocytes. After washing with PBS, cells were incubated with allophycocyanin-conjugated streptavidin (BD Pharmingen) for 20 min at 4°C. To determine the level of NOX2 activity we used a modified version of the oxidative burst activity flow cytometry assay previously described (64). Briefly, cells were resuspended in Dulbecco's complete medium without FCS after staining, and incubated for 10 min at 37°C with 3 μM dihydrorhodamine-123 (Molecular Probes), which after oxidization by hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hydroxyl radicals ($OH^\bullet$) to rhodamine-123 emits a bright fluorescent signal upon excitation by blue light. The cells were then stimulated for 20 min at 37°C with PMA (200 ng/ml). Cells were washed with PBS and then acquired on a FACSort (BD Biosciences) and gated on HIS-48-positive cells (granulocytes), R-123 fluorescence intensity measured on FL-1, and results expressed in relative fluorescence units.

Extracellular ROS production was detected in thioglycolate-recruited peritoneal neutrophils using an isoluminol-enhanced chemiluminescence (17). Neutrophils were attracted to the peritoneum with an injection of autoclaved thioglycolate medium (2.4%). About 24 h after injection, peritoneum was washed with ice-cold HBSS and cell concentration was determined. Samples were also analyzed for frequency of granulocytes (HIS-48-positive cells) among white blood cells [Leukocyte common antigen, CD45-positive cells (Mouse anti-rat CD45 clone OX-1; BD Biosciences)] using antibody staining and flow cytometry detection as described above. Cells were washed in HBSS and diluted to a concentration of 2×10^6 cells/ml and 50 μl of the cell suspension was added to the wells of a white 96-well plate (LumiNUNC; NUNC) and mixed with 50 μl of isoluminol reagent buffer (isoluminol 350 μg/ml, horse radish peroxidase-type II 3.5 μ/ml, and PMA 60 ng/ml or formyl-Met-Leu-Phe (fMLP) 400 n*M*. Samples were gently mixed and data collection was initiated immediately. Extracellular ROS production was followed at 37°C as produced luminescence signal (Synergy 2 ELISA reader (BioTek Instruments).

Immunoprecipitation

$COS^{phox\text{-}Ncf1}$ cells were seeded on 10-cm dishes (3×10^6 cells/dish), and grown on and transfected when 90%–95% confluence was reached. The cells were transiently transfected with plasmids coding for rat $P47^{PHOX}$ protein (DA or E3) using Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations. After 48 h incubation, cells were washed twice with ice-cold PBS, and lysed with 1 ml of ice-cold RIPA Buffer (Sigma) containing phenylmethylsulfonylfluoride, protease inhibitor cocktail (Roche), and sodium orthovanadate for 5 min. These whole cell lysates (kept at +4°C throughout the protocol) were collected by centrifugation (8000 *g* 10 min) and the supernatants were precleared with 5 μg of rabbit IgG and 15 μl of Protein A/G PLUS-Agarose (Santa Cruz) for 1 h. Beads were pelleted and an aliquot (0.5 ml) of the precleared supernatant was incubated with 4 μl of the primary antibody ($P67^{PHOX}$ (N-19), sc-7663 or $P47^{PHOX}$ (D-10), sc-17845; Santa Cruz) that had already been complexed with 20 μl of the Protein A/G PLUS-Agarose by incubating them at +4°C for 1 h with gentle agitation overnight. After 4 h, immunoprecipitates were collected, and the pellets washed 2 times with 1 ml of PBS. Proteins were released into 40 μl of Laemmli sample buffer by boiling the samples for 5 min. Equal aliquots of immunoprecipitates were analyzed by Western blot.

Lentiviral transduction

Ra2 microglia were transduced with separate pLOX TW lentiviral vectors (65) expressing either the tetracycline-inducible transactivator protein or the cDNAs of $P47^{PHOX/DA}$ and/or $P47^{PHOX/E3}$, the latter two vectors in different ratios as indicated. Virus production and transduction was performed essentially as described (54). Expression levels of $P47^{PHOX}$ after induction with 0.1 μg/ml doxycycline overnight were determined by Western blotting and flow cytometry with rabbit polyclonal anti-$P47^{PHOX}$ antibody poly-B. Superoxide production of the generated $P47^{PHOX/DA}$ and/or $P47^{PHOX/E3}$ Ra2 cell lines was measured at 37°C by luminol-enhanced chemiluminescence recorded before and after stimulation with 100 ng/ml PMA or 4–8 μM fMLP delivered through the injector module of the Synergy HT microplate reader (54). Luminol has a similar reactivity as isoluminol but is in contrast to isoluminol membrane permeable and thus reacts with both with ROS released from the cell as well as with ROS produced into phagosomes (17). Two series of separately transduced Ra2 cell populations were used for all figures with essentially the same results.

P47^{PHOX} localization and translocation

Ra2 microglia expressing *Ncf1^{E3}* or *Ncf1^{DA}* were seeded in LabTech tissue culture 8-chamber slides with glass bottom and incubated with nonopsonized zymosan particles in full growth medium at 4°C for 30 min to allow zymosan attachment to the cell surface. Subsequently, cells were washed thoroughly with cold medium and incubated for 10 minutes in medium at 37°C to allow internalization of bound zymosan particles. Cells were then fixed and immunofluorescence performed with anti-GP91^{PHOX} mAb 54.1 (15), rabbit pAb anti-P47^{PHOX} poly-B (generated against the peptide (NH_2) CRRNSVRFLQQRRRP (−COOH)), and Alexa633-conjugated phalloidin.

Statistics

Quantitative data are expressed as mean ± SEM; significance analysis was performed using Mann-Whitney test. All results were compared to those from the control group if nothing else is stated. Statistical significance is represented by $*p \le 0.05$, $**p < 0.01$, and $***p < 0.001$ throughout the article.

Results

The M153T mutation restores ROS production capacity in vitro

Three SNPs were previously found to be polymorphic between the arthritis susceptible DA strain and the resistant E3 strain. Only two of these polymorphisms were nonsynonymous: M106V and M153T (49). The M106V alteration is located within the P47^{PHOX} membrane phospholipid binding Phox homology (PX) domain (2, 39), whereas the M153T alteration is located between the PX domain and the P22^{PHOX} binding Src homology 3 (SH3) domains (42, 43, 46, 58, 59) (Fig. 1a). To identify which of the SNPs caused the effect on ROS production, we used COS cells expressing the subunits of the functional human NOX2 complex, except for P47^{PHOX} (COS$^{phox\text{-}Ncf1}$ cells) (52). These cells were subsequently reconstituted with the *Ncf1* wild-type DA or E3 allele, or with the DA allele mutated at the different polymorphic positions. ROS producing capacity of the cells was assayed using an isoluminol-enhanced chemiluminescence assay reacting with the superoxide anion ($O_2^{\cdot -}$), produced by the NOX2 complex, exclusively in the extracellular compartment (45). Cells expressing P47$^{PHOX/DA}$ presented with an impaired ROS production compared to cells expressing P47$^{PHOX/E3}$ (Fig. 1b). When the effect of the two polymorphisms was investigated separately, we found that a substitution of methionine to threonine at position 153 (M153T) restored the ROS producing efficiency of the *Ncf1^{DA}* allele. The methionine to valine amino acid substitution in position 106 of the DA *Ncf1* allele did not significantly alter ROS production (Fig. 1b). After concluding that the SNP resulting in amino acid replacement M153T controlled the induced ROS production, we asked whether the same SNP would also control arthritis.

M153T alteration restores ROS production in vivo *and protection from severe arthritis*

To conclusively determine that the M153T alteration is responsible for the genetic effect of the *Pia4* locus (63), we screened a large number of inbred rat strains and found that a substrain of the Wistar strain had *Ncf1* DA alleles at two of the three SNPs found to differ between DA and E3. Position 153 was found to be occupied by threonine, which is found in E3 P47^{PHOX} and not DA (Fig. 2a). The Wistar *Ncf1* allele (*Ncf1^{W}*) was backcrossed to the DA genetic background for 10 generations and the resulting congenic strain (DA.*Ncf1^{W}*) (Supplementary Fig. S2) was investigated for ROS production and susceptibility to PIA. Introduction of the *Ncf1^{W}* allele on the DA genetic background resulted in enhanced ROS production capacity in blood granulocytes, analyzed by a flow cytometry-based assay where intracellular oxidation of rhodamin-123 is measured (55, 64) (Fig. 2b). We could also see an enhanced extracellular ROS production in peritoneal thioglycolate recruited granulocytes in response to PMA and fMLP when using the isoluminol-enhanced chemiluminescence assay in cells that originated from DA.*Ncf1^{W}* congenic rats (Fig. 2c). This effect was not due to a larger capacity of granulocytes to infiltrate the peritoneum since neither cell concentration in the cell samples (not shown) nor granulocyte frequency differed between the two strains (Fig. 2d). In addition to increasing the capacity of granulocytes to produce ROS, the *Ncf1^{W}* allele also mediated resistance to severe PIA (Fig. 2e), verifying that the M153T alteration is crucial for the function of the P47^{PHOX} protein, activation of the NOX2 complex, and protection against autoimmunity and arthritis severity.

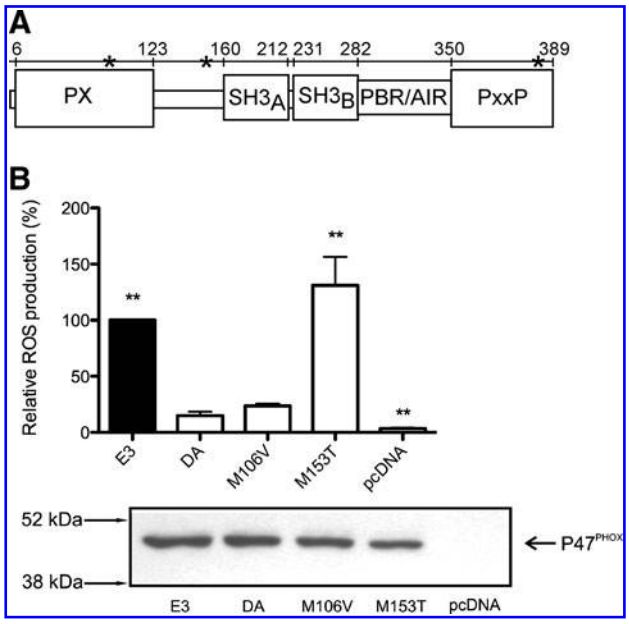

FIG. 1. The M153T mutation restores the ROS production capacity of the NOX2 complex *in vitro*. (A) Schematic figure of the P47^{PHOX} protein with the approximate location of position 106, 153, and 383 marked by *. Sequence structure was taken from SWISS-prot entry AAH61810. **(B)** COS cells, transfected with the NOX2 complex but lacking P47^{PHOX}, were reconstituted with the cDNAs of the different genetic variants of *Ncf1* (*Ncf1^{E3}*, *Ncf1^{DA}*, *Ncf1^{DA_M106V}*, or *Ncf1^{DA_M153T}*). Values are presented as percentage of ROS production after the *Ncf1^{E3}* reconstitution (100%) and represent a mean ± SEM of five separate transfection experiments. Transfection efficiency was monitored by detecting the expression levels of P47^{PHOX} by Western blot. Statistical significance is compared to *Ncf1^{DA}* and $**p < 0.01$. ROS, reactive oxygen species.

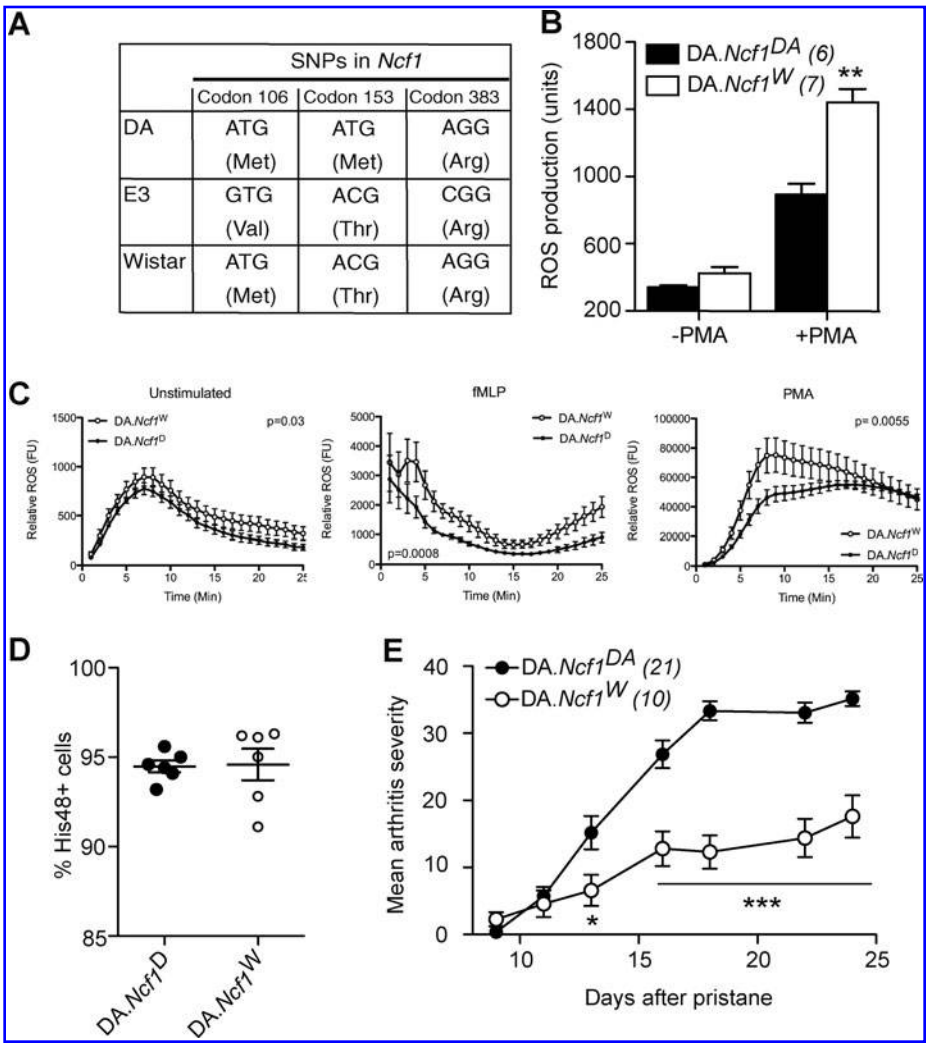

FIG. 2. The M153T alteration protects from severe arthritis in rats. (A) The Wistar rat strain was found to differ from DA rats only in the single-nucleotide polymorphism at position 153 of the tree identified single-nucleotide polymorphisms in *Ncf1* polymorphic between DA and E3 rats. The Wistar allele was backcrossed to the DA background for 10 generations and littermates were investigated for **(B)** *ex vivo* intracellular ROS production (relative units) with and without PMA stimulation using the DHR-123 flow cytometry-based assay and **(C)** *ex vivo* extracellular ROS production using isoluminol-enhanced chemiluminescence in unstimulated cells as well as after PMA and formyl-Met-Leu-Phe stimulation. Statistics are calculated for all values during the assay ($n=6$). **(D)** Frequency of granulocytes in the cell suspensions used for analysis of extracellular ROS production did not differ between the strains. **(E)** Susceptibility to pristane-induced arthritis (PIA). Results are expressed as mean ± SEM. The numbers within parentheses represent number of animals in each group. Significance is calculated for each day of scoring separately. *represent a p-value <0.05, **represent a p-value <0.01 and ***represent a p-value <0.001. PMA, phorbol 12-myristate 13-acetate. FU, fluorescence units.

Only threonine and serine at position 153 can activate the NOX2 complex

To analyze the structural consequences controlled by amino acid 153 of the $P47^{PHOX}$ protein, we created $P47^{PHOX/DA}$ constructs and altered the amino acids at this position. The constructs were used to reconstitute $COS^{phox\text{-}Ncf1}$ cells and ROS production in PMA-stimulated cells was measured using the isoluminol-based chemiluminescence assay (Fig. 3). Our results confirmed earlier published *in vitro* and *in vivo* results (24, 35, 49, 60) that cells expressing $P47^{PHOX/DA}$ are not completely deficient of ROS production since cells reconstituted with the negative control, pcDNA, had significantly lower ROS production (Fig. 3a).

We reconstituted the cells with $P47^{PHOX/DA}$ having serine, tyrosine, or cysteine at position 153 to introduce polar, uncharged amino acids (similar to threonine). Serine and tyrosine have a hydroxyl group and could theoretically be phosphorylated. Cysteine is similar in size with serine, whereas tyrosine has a bulky side chain. However, cysteine also introduces a reactive thiol into the protein that may alter functional outcome. These were also used to assess the importance of the size of the side chain on ROS production. We found that serine, but not tyrosine or cysteine, restored the ROS production, suggesting that activation by a threonine/serine-specific protein kinase(s), such as MAP kinases and protein kinase C, is of importance for a fully functional NOX2 complex. Since both serine and threonine can be phosphorylated, we inserted aspartate and glutamate that are also negative in charge. These alterations did not affect ROS production arguing that phosphorylation is not mediating the effect (Fig. 3a). However, to directly test whether the threonine residue could be phosphorylated, we utilized matrix-assisted laser desorption/ionization-TOF/TOF mass spectrometry to analyze purified $P47^{PHOX/E3}$ protein from PMA stimulated cells. Phosphopeptides were enriched using TiO_2 micro columns in an effort to enrich potential phosphopeptides followed by analysis using dihydroxy benzoic acid, spiked with 1% phosphoric acid as matrix: no peptide with a phosphoryl group was detected. To further enrich the possible phosphorylation, the PMA-stimulated cells were treated with Calyculin A, a potent serine/threonine phosphatase inhibitor (38), to preserve phosphorylated status. Phosphopeptides were this time enriched with TiO_2/ZrO_2-coated tips and analyzed by mass spectrometry using the dihydroxy benzoic acid matrix as above (see Supplementary Data for further details). Despite these extensive efforts to enrich the phosphorylated peptides, we could not identify phosphorylation at position 153 (data not shown). In addition, analysis of the amino acid sequence of $P47^{PHOX/E3}$ protein

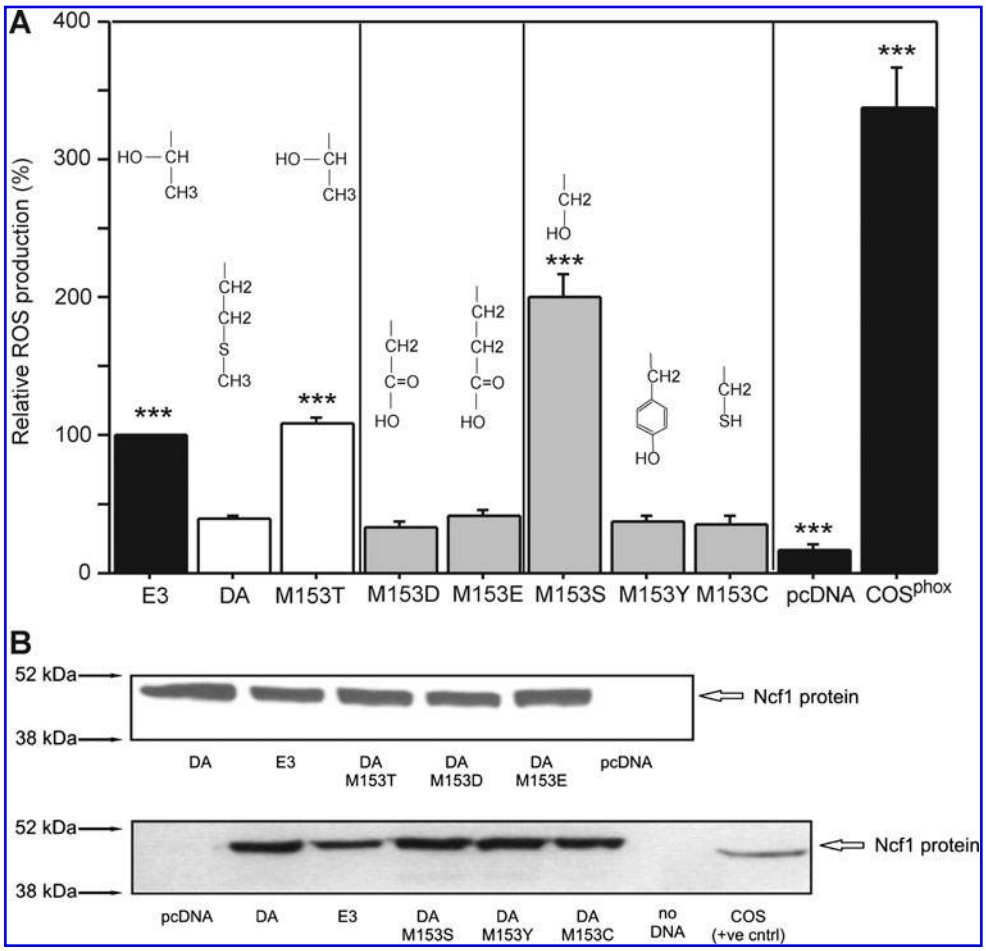

FIG. 3. Position 153 in P47^{PHOX} is of importance for ROS production: threonine and serine enable oxidative burst. COS^{phox} cells lacking P47^{PHOX} were transfected with the different *Ncf1* cDNA constructs. **(A)** The functionality of the NOX2 complex was assayed using the isoluminol detection method. **(B)** Cells were lysed and P47^{PHOX} expression was analyzed by Western blot (47 kDa). Control COS cells are stably expressing P47^{PHOX}. Data are expressed as percentage compared to E3 and are pooled from 4 separate experiments ($n = 7$–13). All groups were compared to DA. Results are expressed as mean ± SEM. *** represent a p-value < 0.001.

(NetPhos, Center for Biological Sequence Analysis, Denmark) predicted that Threonine 153 is not likely to be phosphorylated (Supplementary Fig. S3) by any kinases, including PKC, p38MAPK, GSK3, PKA, and casein kinase II (NetPhosK).

The M153T mutation does not alter localization or recruitment to phagosomes

The exact effect of mutations in the hinge region between the PX and the SH3 domains is still unknown, but altered localization of the protein has been suggested (56). In resting cells, three of the subunits of the NOX2 complex are residing in the cytosol (P47^{PHOX}, P67^{PHOX}, and P40^{PHOX}), where an auto-inhibited state of P47^{PHOX} prevents binding to the integral membrane subunits (P91^{PHOX} and P22^{PHOX}) (7, 18, 28). Upon activation, phosphorylation of the P47^{PHOX} protein releases the auto-inhibition and permits translocation of the cytosolic subunits to the membrane where they together with CYT b_{558} and the small GTPase RAC (1/2) (29) form the fully assembled and activated complex (1, 57). Phosphorylation of the auto-inhibitory domain, the tandem SH3 domain, is exposed and allows P47^{PHOX} to translocate to the plasma membrane and bind P22^{PHOX} (11). We wanted to investigate if altered localization of the protein was the reason for the effect of the M153T alteration. First, we investigated if the binding of P47^{PHOX} to P67^{PHOX} was affected by the mutation. However, anti- P67^{PHOX} co-immunoprecipitates analyzed by Western blotting with anti-P47^{PHOX} antibodies showed no major differences in binding capacity between the two genotypes and the same was observed when P47^{PHOX} was pulled down and the co-immunoprecipitated P67^{PHOX} detected (Fig. 4a, b).

To investigate if the M153T alteration affected the capacity of P47^{PHOX} to translocate to the membrane after activation, Ra2 microglia expressing either P47$^{PHOX/E3}$ or P47$^{PHOX/DA}$ were allowed to phagocytose surface-bound zymosan particles (heat killed yeast particles) for up to 10 min at 37°C. After cell fixation, immunofluorescence was performed with anti-GP91^{PHOX} (15) and anti-P47^{PHOX} antibodies in combination with Alexa633-conjugated phalloidin (Fig. 4c). Phalloidin specifically binds F-actin and is used here to indicate recently formed phagosomes, as associated F-actin is shed rapidly (within minutes) postinternalization. However, P47$^{PHOX/DA}$ and P47$^{PHOX/E3}$ were recruited to newly formed phagosomes (co-localizing with CYT b_{588}), with similar kinetics and efficiency, suggesting that the negative effect of the M153T mutation on superoxide production is not due to altered capacity of P47^{PHOX} for stimulus-induced redistribution and binding to the membrane. We found no differences in uptake of particles per cell between P47$^{PHOX/DA}$ and P47$^{PHOX/E3}$-expressing cells (data not shown).

It is believed that continuous cycling of cytosolic NADPH oxidase subunits is necessary to sustain the oxidative burst (20, 61). It could therefore be speculated that P47$^{PHOX/DA}$ inhibits ROS production in the assembled NOX2 complex due

to greatly reduced off-kinetics from the CYT b_{558} binding sites, effectively preventing new cytosolic subunits to exchange. A similar scenario was also proposed by Shen *et al.* where $P47^{PHOX}$ protein with glycines in positions 151–158 showed increased binding to liposomes (56). If so, expression of the $P47^{PHOX/DA}$ variant should be dominant negative as the CYT b_{558} sites available becomes saturated with this $P47^{PHOX}$ species. To test this hypothesis, $P47^{PHOX/DA}$ and $P47^{PHOX/E3}$ were co-expressed in Ra2 microglia cells at different ratios and the effect on fMLP and PMA-induced superoxide release analyzed. The results, however, do not support a dominant negative role of $P47^{PHOX/DA}$ as $P47^{PHOX/E3}$ over-expression

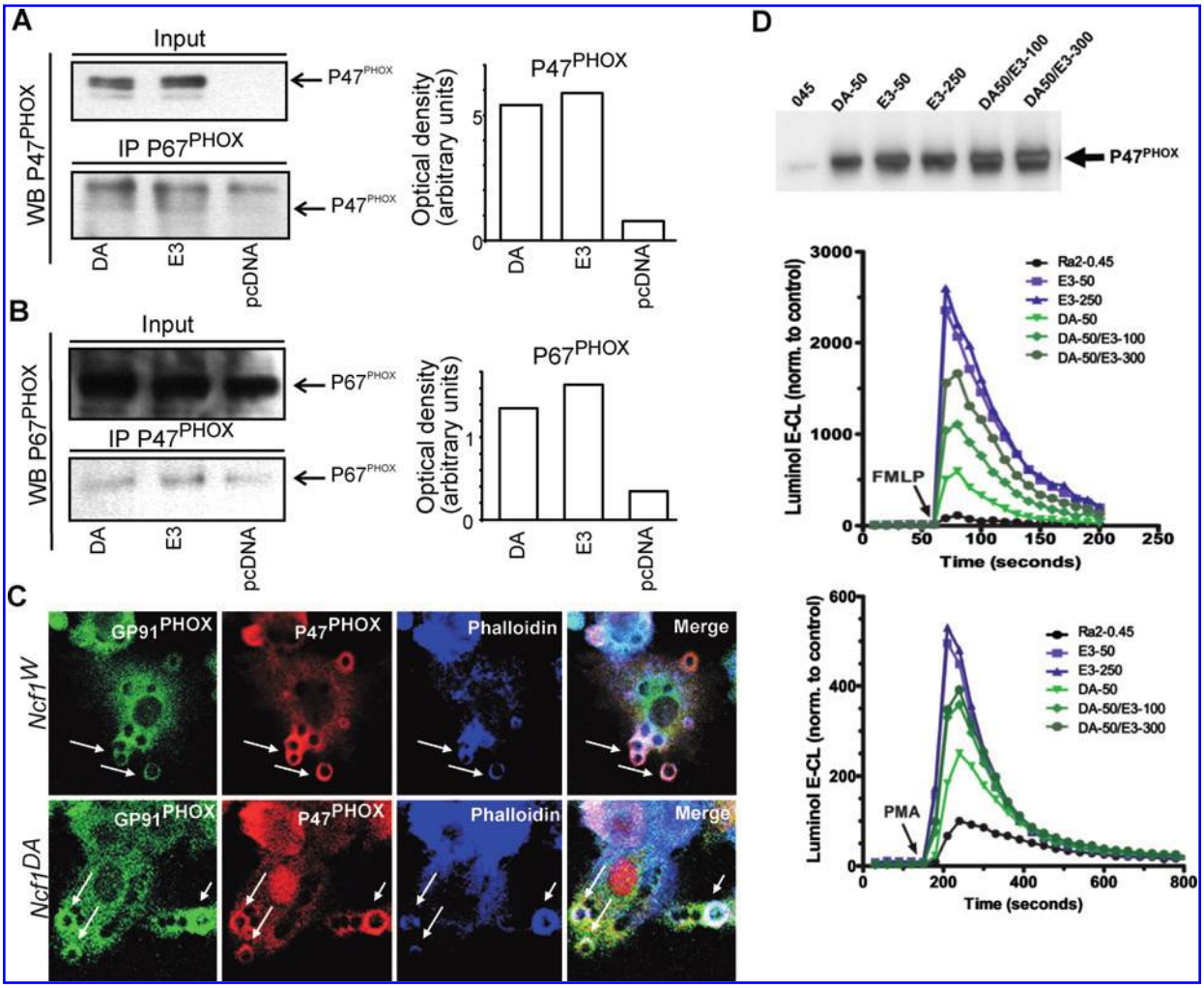

FIG. 4. The effect of Ncf1 mutation is not mediated by altered localization or binding to the membrane. (A) Both variants of $P47^{PHOX}$ bind to $P67^{PHOX}$. Immunoprecipitation of $P67^{PHOX}$ followed by a Western blot stained with anti-$P47^{PHOX}$ antibody resulted in detection of the $P47^{PHOX}$ protein in similar levels of both variants. The bands corresponding to the heavy chain (about 50 kDa) of the immunoprecipitating antibody were detected by the secondary antibody and are seen just above $P47^{PHOX}$. **(B)** Similar co-immunoprecipitation was done by pulling down $P47^{PHOX}$ and detecting the associated $P67^{PHOX}$. Signals from the co-immunoprecipitated proteins were densitized (panels on the right in **A** and **B**). $P47^{PHOX}$ and $P67^{PHOX}$ levels in lysates before immunoprecipitation were detected to verify similar transfection efficiency and to control the input levels for the immunoprecipitation procedure (*upper blots*). The experiment was repeated with similar results. **(C)** $P47^{PHOX/DA}$ is recruited efficiently to phagosomes. Ra2 microglia expressing $Ncf1^{E3}$ or $Ncf1^{DA}$ were incubated with nonopsonized zymosan particles before washing and phagocytosis for 10 min. Cells were fixed and immunofluorescence performed with anti-$GP91^{PHOX}$ mAb, rabbit pAb anti-$P47^{PHOX}$ poly-B, and Alexa633-conjugated phalloidin. *Arrows* point to phagosomes with surrounding actin-ring, indicative of their recent formation. **(D)** $P47^{PHOX/DA}$ is not dominant-inhibitory over $P47^{PHOX/E3}$ for NADPH oxidase activity. Ra2 microglia were transduced with lentiviral vectors for conditional overexpression of $P47^{PHOX/DA}$, $P47^{PHOX/E3}$, or both in the indicated ratios (numbers denote viral supernatant volume used for transduction). Western blotting with rabbit polyclonal anti- $P47^{PHOX/DA}$ antibody poly-B shows that transgene expression in all cell lines is considerably higher than endogenous mouse $P47^{PHOX}$ in control Ra2 045 cells. Production of superoxide of the different cell lines after stimulation with formyl-Met-Leu-Phe (*upper graph*) or PMA (*lower graph*) as measured by luminol-enhanced chemiluminescence. Agonists were added at the time point indicated by *arrow*, and results are expressed as chemiluminescent counts normalized to Ra2 045 control cells. Result is from one of two experiments with similar results. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

returned the $P47^{PHOX/DA}$ response to near $P47^{PHOX/E3}$ levels alone (Fig. 4d).

These observations support the idea that the decreased ROS production associated with $P47^{PHOX/DA}$ does not relate to compromised translocation to the membrane, but to an inherently diminished ability to support the catalytic mechanism of superoxide production in the assembled NOX2 complex.

Discussion

For the first time, we have identified a natural polymorphic quantitative trait nucleotide (QTN) in an earlier identified quantitative trait locus in a disease model for a complex disorder. The identified QTN replaces a threonine at position 153 in the $P47^{PHOX}$ protein with methionine, which leads to a reduced ROS response and increased arthritis severity.

The effect of a polymorphism at position 153 has been unclear as the hinge region between the PX and the SH3 domains for a long time was considered not to be of importance for the function of $P47^{PHOX}$. Upon activation, $P47^{PHOX}$ is phosphorylated on several serine residues resulting in release of the auto-inhibitory conformation (1, 57) that prevents the cytosolic NOX2 complex subunits to bind CYT b_{558} and phosphoinositides in the membrane. As a consequence of phosphorylation, the subunits translocate to the membrane where the active NOX2 complex is formed together with the small GTPase RAC (1 or 2) (29). $P47^{PHOX}$ can move by itself to the membrane to interact with the cytochrome and/or phosphoinositides, but $P67^{PHOX}$ is recruited only in association with $P47^{PHOX}$ (31). $P47^{PHOX}$ is, thus, normally regarded to be an organizer of the complex and has been shown to be necessary for ROS production in cellular systems, even though ROS can be produced without involvement of $P47^{PHOX}$ in cell-free systems if $P67^{PHOX}$ and RAC are in excess (22, 40).

There are only a few studies investigating the linker region of $P47^{PHOX}$ that contains the Thr^{153}. A snap-lock function of this region has been proposed, based on the fact that in a coupling model between SH2 and SH3 domains in the Src kinase, the linker region forms a snap-lock that locks the orientation of these two domains together (67). Lately, amino acids 151–158 were shown to be of importance for binding of the PX domain to membrane phosphoinositides and functional ROS production in a cell-free system, whereas region 143–150 did not affect either ROS production or binding to the membrane (56). The fact that neither association with $P67^{PHOX}$ nor redistribution of the $P47^{PHOX}$ protein is altered by the M153T amino acid substitution suggests that this region is not of importance for translocation to the enzymatic core at the membrane sites. The QTN might also alter binding of $P47^{PHOX}$ to the $P22^{PHOX}$ subunit in the membrane. However, the amino acid in position 153 has not been suggested to bind directly to $P22^{PHOX}$ (47).

The importance of this region is also strengthened by the fact that substitution of alanine to Ile^{152} is not affecting binding to $P67^{PHOX}$ or $P22^{PHOX}$ but inhibits ROS production from the NOX2 complex (60). This is interesting since $P47^{PHOX}$ is often proposed to function just as an adaptor protein to tether $P67^{PHOX}$ to $P22^{PHOX}$. Our work, together with the work of Taura *et al.* (60), proposes a direct effect on the activation of the complex. As both serine and threonine, without detectable phosphorylation on threonine, enabled ROS production, a hydroxyl group in this position might be needed for full activity of NOX2 complex. Even though we could not detect phosphorylation on threonine, we cannot completely exclude the possibility that the moiety is phosphorylated upon activation and thus controls the activity of the NOX2 complex. Another possible post-translational modification is glycosylation, but Thr153 in $P47^{PHOX/E3}$ is not predicted to be glycolysated (NetOGlyc 3.1, Center for Biological Sequence Analysis, Denmark). Our finding that also serine 153 is functional is supported by the fact that serine is found in this position in a $P47^{PHOX}$ ortholog (Supplementary Fig. S4).

Taken together, we have now conclusively identified a naturally occurring polymorphism in rats, resulting in low ROS production and lack of resistance to arthritis development. This polymorphism results in a T153M alteration in the hinge region, located between the PX and SH3 domains that has lately been suggested to be crucial for activation of the NOX2 complex (56, 60). This effect is not mediated by an altered translocation of the $P47^{PHOX}$ protein, but rather depend on the nature of the amino acid and possible conformational effect. Importantly, the identified QTN is highly polymorphic in the wild rat population and is located within the strongest locus controlling development of both models for RA (PIA and collagen-induced arthritis) (48) as well as for multiple sclerosis (experimental autoimmune encephalomyelitis) (10, 12). The increased risk of severe arthritis due to a reduced ROS production is certainly against previous postulations concerning the role of ROS on inflammation, but is now well validated (37). The most likely explanation involves a regulatory effect on autoreactive T cell activation. We hypothesize that ROS production during antigen presentation regulates the reactivity of the responding T cell toward that antigen by altering the T cells redox status (24, 25). A regulatory role of ROS is most presumably an evolutionary conserved mechanism, likely to operate also in other species, such as mice and humans. A corresponding polymorphism is not present in the mouse *Ncf1* gene although the pathway has been shown to be functionally similar to rats. In humans, the *NCF1* gene is in a region of high instability, with numerous deletions and duplications (5, 9). The human *NCF1* gene has several functional and nonfunctional copies, which exists in varying frequencies in different populations (14, 32). Due to this complexity any large-scale genetic analysis, such as a genome-wide association scan, of *NCF1* has been impossible. However, this large degree of genetic variability in the *NCF1* gene and its duplicates could in numerous ways reduce the functionality of $P47^{PHOX}$ in much the same way as is seen in the rat (14, 19, 32). Two other genes of the NADPH oxidase complex have been found to be genetically associated with RA or other autoimmune conditions. We and others have reported associations of the *NCF4* gene (encoding $P40^{PHOX}$) with RA (51) and Crohn disease (53), and in a large-scale replication analysis, *NCF2* (encoding $P67^{PHOX}$) was found to be strongly associated with Systemic Lupus Erythematosus (23). This study, identifying the underlying QTN causing the *Ncf1* effect, now opens a detailed analysis of the role of this important new pathway regulating autoimmune-mediated chronic inflammation.

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Author Disclosure Statement

This publication reflects only the authors' views. The European Community is not liable for any use that may be made of the information herein. The founders had no role in study design, data collection, and analysis; decision to publish; or preparation of the article. M.H., P.O., and R.H. declare competing interests. No competing financial interests exist for the other authors.

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Abbreviations Used

PIA = pristane-induced arthritis
PMA = phorbol 12-myristate 13-acetate
QTN = quantitative trait nucleotide
RA = rheumatoid arthritis
ROS = reactive oxygen species
SNP = single nucleotide polymorphism

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