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ORIGINAL RESEARCH COMMUNICATION

Copy Number Variation of the Gene *NCF1* Is Associated with Rheumatoid Arthritis

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Abstract

Aims: The aim of this study was to investigate genetic variants in the gene neutrophil cytosolic factor 1 (*NCF1*) for association with rheumatoid arthritis (RA). In rodent models, a single-nucleotide polymorphism (SNP) in *Ncf1* has been shown to be a major locus regulating severity of arthritis. *Ncf1* encodes one of five subunits of the NADPH oxidase complex. In humans the genomic structure of *NCF1* is complex, excluding it from genome-wide association screens and complicating genetic analysis. In addition to copy number variation of *NCF1*, there are also two nonfunctional pseudogenes, nearly identical in sequence to *NCF1.* We have characterized copy number variation and SNPs in *NCF1*, and investigated these variants for association with RA. ***Results:*** We find that RA patients are less likely to have an increased copy number of *NCF1*, 7.6%, compared with 11.6% in controls; $p = 0.037$. We also show that the T-allele of *NCF1*-339 (rs13447) is expressed in *NCF1* and significantly reduces reactive oxygen species production. ***Innovation:*** This is the first finding of genetic association of *NCF1* with RA. The detailed characterization of genetic variants in *NCF1* also helps elucidate the complexity of the *NCF1* gene. ***Conclusion:*** These data suggest that an increased copy number of *NCF1* can be protective against developing RA and add support to previous findings of a role of *NCF1* and the phagocyte NADPH oxidase complex in RA pathogenesis. *Antioxid. Redox Signal.* 16, 71–78.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder with a prevalence of 0.5%–1% and a heritability of 60% (25, 35). It is heterogeneous regarding symptoms as well as environmental and genetic contribution (29), making disease-associated genes difficult to identify (19, 37). To reduce genetic and environmental heterogeneity, animal models are used to study arthritis (16). In rats, a single-nucleotide polymorphism (SNP) in the neutrophil cytosolic factor 1 (*Ncf1)* gene was found to regulate severity of arthritis (18, 27). *Ncf1* encodes the p47phox subunit of the phagocyte NADPH oxidase (NOX2) complex, which produces reactive oxygen species (ROS) in phagocytes, including antigen presenting cells, in the immune system. The arthritis-regulating effect of the SNP in *Ncf1* stems from an altered capacity of the NOX2 complex to produce ROS (17, 27). We recently reported genetic association with RA of *NCF4*, encoding the p40phox subunit of the NOX2 complex (28). This was the first evidence of genetic association of the NOX2 complex with RA, but var-

Innovation

The gene neutrophil cytosolic factor 1 (*NCF1*) emerged as a possible genetic contributor to rheumatoid arthritis (RA), due to findings in rodent models of arthritis. The genetics of *NCF1* is complex in the human genome, complicating genetic analysis. In this study several new assays were developed to be able to investigate copy number variation of *NCF1* and the allelic distribution and functionality of three single-nucleotide polymorphisms in *NCF1*. This detailed characterization revealed that RA patients are less likely to have one extra copy of a functional *NCF1*-like gene compared with controls. This is the first finding of a genetic association of *NCF1* with RA. It was also found that the T-allele of *NCF1-339* is expressed in *NCF1* and encodes a greatly reduced capacity of the phagocyte NADPH oxidase (NOX2) complex to produce reactive oxygen species. These findings help elucidate part of the complexity of *NCF1* and add support to previous findings of a role of *NCF1* and the NOX2 complex in RA pathogenesis.

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iants in *NCF4* are also associated with Crohn disease (31). *NCF2*, encoding the p67phox subunit of the NOX2 complex, has been found to be associated with systemic lupus erythematosus (SLE) in a recent genome-wide association analysis (9), suggesting a role for the NOX2 complex in autoimmunity.

In the human genome, *NCF1* is located in the complex 7q11.23 region, characterized by deletions, duplications, and inversions (2, 26). Close to *NCF1* there are two pseudogenes, *NCF1B* and *NCF1C* (*NCF1B/C*), sharing 98% sequence similarity with *NCF1* (10, 15). The only genetic variant confirmed to be *NCF1B/C* specific is a GT deletion (ΔGT) in exon two, encoding a premature stop codon and a truncated protein (10). Patients with the *NCF1* (p47phox) form of the primary immunodeficiency syndrome chronic granulomatous disease (CGD) only have *NCF1B/C*-like genes, containing the ΔGT deletion, leading to a complete loss of NOX2 produced ROS (Fig. 1b) (32). In these patients, the functional GTGT sequence in *NCF1* has been exchanged with the ΔGT sequence from *NCF1B/C*, most likely due to genetic cross over events between *NCF1* and *NCF1B/C* (32). Due to the high sequence similarity between *NCF1* and *NCF1B/C*, it is difficult to specifically measure the number of functional *NCF1* copies. Instead, methods have been developed to estimate the ratio of *NCF1B/C* and *NCF1* genes, by segregating on the ΔGT/GTGT sequence (14). The most prevalent ΔGT/GTGT ratio, 2:1, reflects two copies each of *NCF1*, *NCF1B*, and *NCF1C*, whereas CGD carriers have five *NCF1B/C* like genes and only one *NCF1* gene, giving a 5:1 ratio (Fig. 1). A few studies have also reported 1:1 and 1:2 ratios, reflecting the presence of one or two copies of an additional *NCF1*-like gene (here denoted *NCF1-II)*, containing the functional GTGT sequence (Fig. 1a) (14). The *NCF1-II* gene reportedly expresses *NCF1B/C*-specific alleles (10), but is fully transcribed and believed to be functional (8, 14). Two of the *NCF1B/C*-specific alleles in *NCF1-II* encode amino acid replacements (14) and could therefore alter protein function and the capacity of the NOX2 complex to produce ROS.

The genetic variants in the human *NCF1* gene could have similar effect on NOX2 produced ROS, as the SNP in *Ncf1* found to regulate arthritis in the rats. To investigate if genomic or genetic variants of *NCF1* are associated with RA, we performed a detailed study of the different *NCF1* genes, by measuring the ΔGT/GTGT ratio in RA cases and controls. We also genotyped three nonsynonymous SNPs in 974 RA cases and controls to study association with RA.

Results

Copy number analysis reveals complex distribution of NCF1 *genes*

We used quantitative real-time polymerase chain reaction (qPCR) to measure the ratio between *NCF1B/C* and *NCF1* in 494 RA patients and 480 controls. The multiplex qPCR assays contain probes specific for either the GTGT sequence found in *NCF1* and *NCF1-II* or the ΔGT sequence found in *NCF1B/C* (39). The ratio between *NCF1B/C* and *NCF1* was estimated from the fold change (FC) values calculated using the $2^{-\Delta\Delta CT}$ method. As expected, the 2:1 ratio is the most common (85.3%), whereas 9.3% have a 1:1 ratio (Table 1). The increased ΔCT value of *NCF1/NCF1-II* and decreased of *NCF1B/C* confirm that the 1:1 ratio corresponds to three copies each of

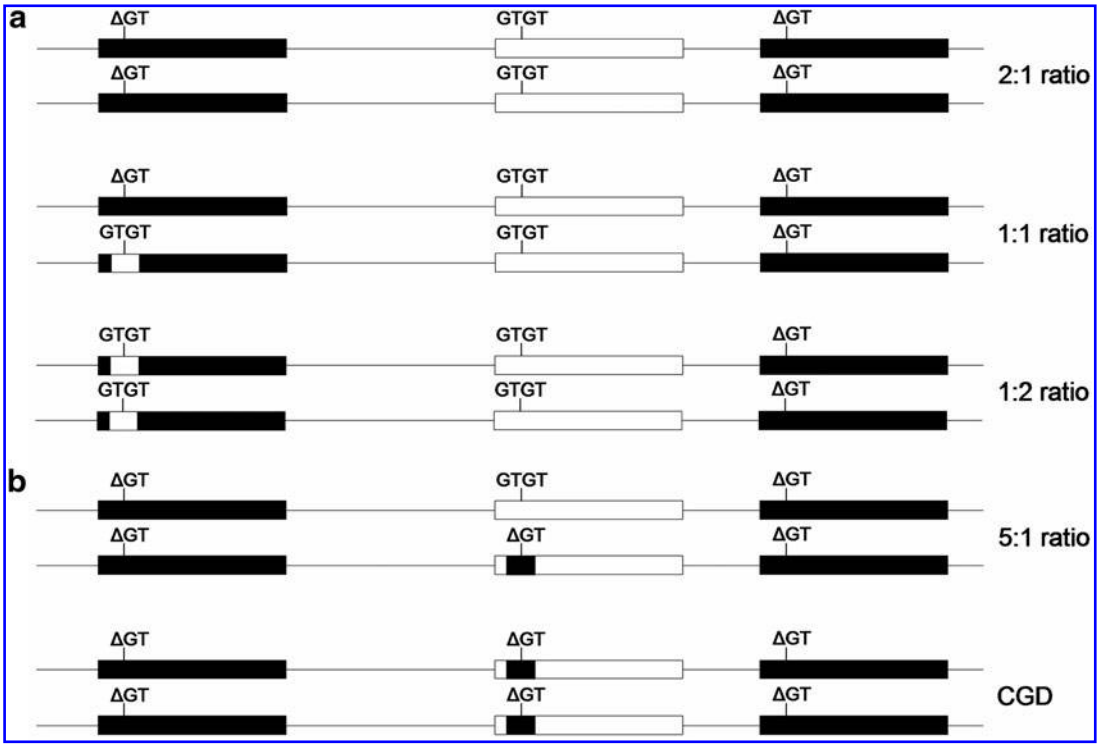

FIG. 1. A schematic drawing of the organization of the *NCF1* and *NCF1B*/C genes on the two chromosomes. The boxes represent *NCF1B/C* (*black*) and *NCF1* (*white*). **(a)** The genomic organization resulting in the 2:1, 1:1 and 1:2 ΔGT/GTGT ratios. **(b)** The genomic organization in CGD patients and of the 5:1 ΔGT/GTGT ratio found in CGD carriers. *NCF1*, neutrophil cytosolic factor 1; CGD, chronic granulomatous disease.

Table 1. Frequencies of Ratios

	All		Case		Control	
Ratio	#	%	#	%	#	%
2:1	822	85.1	422	86.5	398	83.6
1:1	92	9.5	37	7.6[a]	55	11.6[a]
1:2	12	1.2	7	1.4	5	1.1
3:2	12	1.2	5	1.0	7	1.5
4:3	3	0.3	2	0.4	1	0.2
5:2	22	2.3	14	2.9	8	1.7
5:1	3	0.3	1	0.2	2	0.4
Total	966	100	490	100	476	100

[a]*p*-value 0.037.
#, number of subjects.

Table 2. Genotyped Single-Nucleotide Polymorphisms

SNP ID	*rs number*	*Gene*	*mRNA*	*AA change*
NCF1-339	rs13447[a]	Exon 4	C339T	Arg90His
NCF1-365	rs17856077[a]	Exon 4	A365G	Gly99Ser
NCF1-566	rs4868	Exon 6	A566G	Asn166Asp

[a]Representative rs numbers.
NCF1, neutrophil cytosolic factor 1; SNP, single-nucleotide polymorphism.

NCF1B/C and *NCF1/NCF1-II*. Twelve samples had a 1:2 ratio, corresponding to two copies of *NCF1B/C* and four of *NCF1/NCF1-II*. Three CGD carriers, with a 5:1 ratio, were also detected. In addition, we found three ratios, 3:2, 4:3, and 5:2, not corresponding to a total of six *NCF1*-like genes, but instead seem to reflect five or seven genes (Fig. 2). The ΔCT values shows that the 3:2 and 5:2 ratios reflect changes in *NCF1B/C* copy number, with an increased ΔCT of *NCF1B/C* in the 5:2 ratio and a decreased in the 3:2 ratio. The 4:3 ratio instead reflects an increase in *NCF1/NCF1-II* copy number.

The NCF1-II *gene is less common in RA patients*

When comparing the ratio distribution between RA patients and controls, we found that RA patients have a significantly lower frequency of the 1:1 ratio, 7.6% compared with 11.6% in controls, $p = 0.037$, OR = 0.63, 95% CI: 0.40–0.97 (Table 1). No difference in frequency between cases and controls was seen for the other ratios (Table 1).

The T-allele of NCF1*-339 is expressed in* NCF1 *and not specific for* NCF1-II

A previous study has suggested that *NCF1-II* expresses *NCF1B/C*-specific alleles at two nucleotide positions, here denoted *NCF1*-339 and *NCF1*-566 (14). To confirm this and to investigate association with RA, these two SNPs and a third nonsynonymous SNP, *NCF1*-365, were genotyped in the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) cohort (Table 2). Genotyping of *NCF1* is complicated by the presence of *NCF1B/C*, requiring a nested PCR strategy, exclusively amplifying the GTGT sequence, to achieve *NCF1/NCF1-II* specificity (Supplementary Fig. S2; Supplementary Data are available online at www.liebertonline.com/ars). *NCF1-II* also complicates the interpretation of genotypes (Fig. 3) and the results from the ratio study were included to

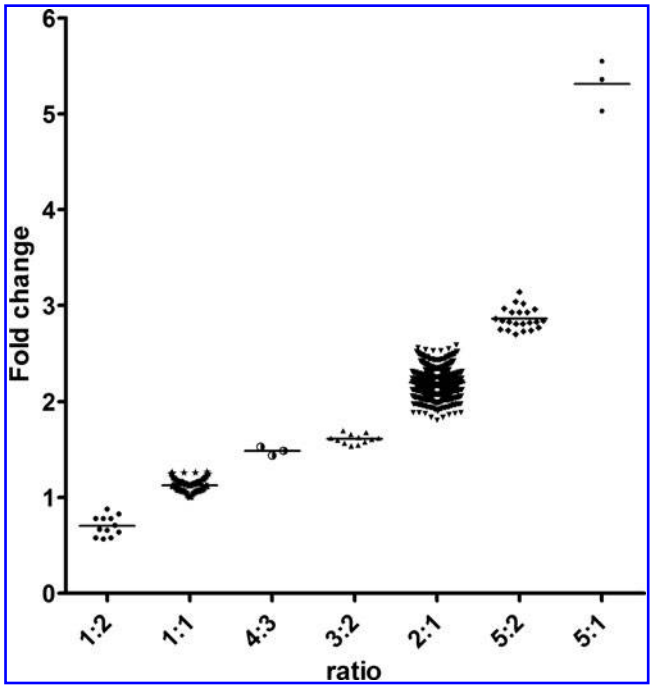

FIG. 2. Dotplot of the $2^{-\Delta\Delta CT}$ fold change of *NCF1B/C* and *NCF1*. The fold change ratio of *NCF1B/C* to *NCF1* is plotted and grouped according to assigned ratio. The values in the ratio groups are nonoverlapping except for the 4:3 ratio, which overlaps with the 3:2 ratio group.

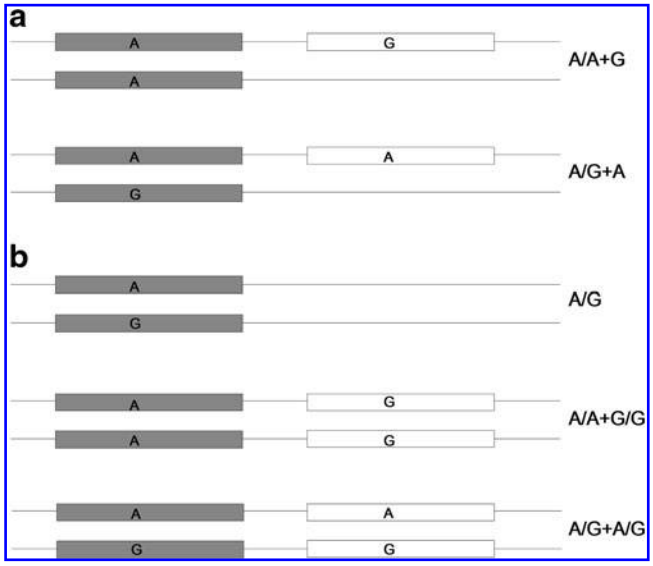

FIG. 3. Genotype analysis of the *NCF1*-566 SNP. The boxes represent *NCF1* (*gray*) and *NCF1-II* (*blank*). A and G represent the alleles of the *NCF1*-566 SNP. **(a)** The possible allelic distributions if the genotyping assay detects 2 A-alleles and 1 G-allele. **(b)** The possible allelic distributions if the genotyping assay detects an A/G ratio of 1. Because the genotyping assay for *NCF1*-566 cannot distinguish between *NCF1* and *NCF1-II*, the results from the ratio study were included in the analysis to determine the true genotypes as A/A+G in **a** and A/A+G/G in **b**. SNP, single-nucleotide polymorphism.

Table 3. Allele Distribution and Dosage of the Three Genotyped Single-Nucleotide Polymorphisms in Cases and Controls

	1 NCF1[a]		*2 NCF1*[b]				*2 NCF1+1 NCF1-II*[c]						*2 NCF1+2 NCF1-II*[d]						p-*value*[e]
NCF1-339	C		CC		C/T		TT		CCC		CC/T				CCCC		CCC/T		CC/TT		C *versus* T
	#	%	#	%	#	%	#	%	#	%	#	%			#	%	#	%	#	%	
Case	1	0.2	390	83.2	31	6.6	2	0.4	28	6	10	2.1			4	0.9	2	0.4	1	0.2	0.335
Control	2	0.4	366	81.3	23	5.1	2	0.4	38	8.4	14	3.1			4	0.9	0	-	1	0.2	
NCF1-365	A		AA		A/G		GG		AAA		AA/G		A/GG		AAAA		AAA/G		AA/GG		A *versus* G
	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	
Case	1	0.2	301	64.3	113	24.1	8	1.7	29	6.2	7	1.5	2	0.4	5	1.1	1	0.2	1	0.2	0.410
Control	2	0.4	290	63.7	96	21.1	10	2.2	39	8.6	13	2.9	0	-	3	0.7	2	0.4	0	-	
NCF1-566	A		AA		A/G		GG		AAA		AA/G						AAA/G		AA/GG		A *versus* G
	#	%	#	%	#	%	#	%	%	%	#	%					#	%	#	%	
Case	1	0.2	424	89.8	2	0.4	0	-	5	1.1	33	7					4	0.8	3	0.6	0.035
Control	2	0.4	396	86.7	1	0.2	0	-	2	0.4	51	11.2					1	0.2	4	0.9	

[a]5:1 ratio.
[b]2:1, 5:2, and 3:2 ratios.
[c]1:1 or 4:3 ratios.
[d]1:2 ratio.
[e]*p*-values from the combined *NCF1/NCF1-II* association analysis of allele frequencies.
#, number of subjects.

correctly call the genotypes. Only three individuals (0.4%), not having any copies of *NCF1-II*, were found to express the G-allele of *NCF1*-566 (Table 3), confirming that the G-allele is almost exclusively expressed in *NCF1-II*. The T-allele of *NCF1*-339, on the other hand, is not specific for *NCF1-II* as it is expressed in both *NCF1* and *NCF1-II*. *NCF1*-365 is also polymorphic in both *NCF1* and *NCF1-II* (Table 3). We analyzed allele and genotype frequencies for association with RA, in *NCF1* and *NCF1-II* separately as well as combined. No significant differences in genotype or allele frequencies between RA patients and controls were seen for *NCF1*-339 or *NCF1*-365 in any of the analyses. In the *NCF1/NCF1-II* combined association analysis, the G-allele of *NCF1*-566 had a significantly higher allele frequency in controls ($p=0.035$), reflecting the specificity of the G-allele for *NCF1-II* and the higher frequency of the 1:1 ratio in controls.

T-allele of NCF1-339 encodes loss of functionality of the NOX2 complex

NCF1-339, *NCF1*-365, and *NCF1*-566 encode amino acid shifts that could alter the functionality of the NCF1 protein and consequently also of the NOX2 complex (Table 2). We used three constructs of the human NOX2 complex in COS cells, in which the *NCF1* gene was mutated at either one of the three SNP positions, to estimate the effect of each allele on ROS production. The construct expressing the T-allele of *NCF1*-339 had significantly reduced ROS production, $p=0.0079$ (Fig. 4). The G-allele of *NCF1*-365 and the G-allele of *NCF1*-566 did not encode a significant difference in ROS production capacity.

Discussion

In this study we show that genomic variation of the *NCF1* gene is associated with RA. The 1:1 ΔGT/GTGT ratio, reflecting the presence of *NCF1-II*, is significantly less common in patients with RA compared with controls. We also show that the G-allele of *NCF1*-566 is specific for *NCF1-II*, but the T-allele of *NCF1*-339 is not, in contrast to previous reports (14). We also see that the T-allele of *NCF1*-339 greatly reduces ROS production by the NOX2 complex. These findings help elucidate part of the complexity of *NCF1* as well as adding support to previous findings of a role of *NCF1* and the NOX2 complex in RA pathogenesis.

Previous studies have investigated association of the 1:1 ratio with inflammatory bowel disease (12, 38), parasitic infection, and multiple sclerosis (11), with the presumption that the 1:1 ratio increases disease risk. Our results instead indicate a protective effect of the 1:1 ratio against RA and thus also of the presence of the additional *NCF1-II* gene. Having the 1:1 ratio increases expression of *NCF1* mRNA and protein (8, 13);

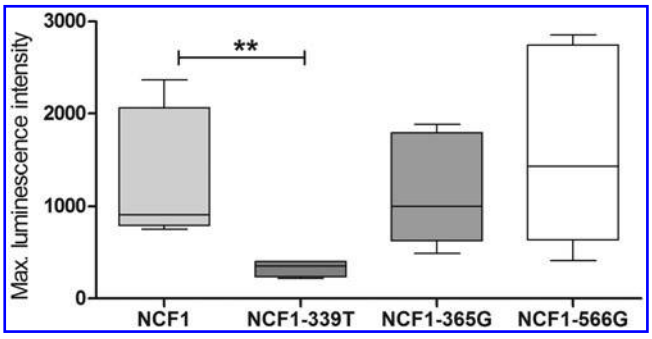

FIG. 4. ROS production in mutated NCF1 constructs. Three constructs of the *NCF1* gene, each mutated at one of the three detected polymorphic sites, *NCF1*-339 (Arg 90 His), *NCF1*-365 (Gly 99 Ser), and *NCF1*-566 (Asn 166 Asp) were created and transfected into COS-cells reconstituted with the other components of the NOX2 complex. The ROS production was stimulated and measured using an isoluminol detection method. The ROS production of the mutated Ncf1 constructs was compared with the nonmutated NCF1. Mann–Whitney: *NCF1 versus NCF1*-339 ($p=0.008$), *NCF1 versus NCF1*-365 ($p=0.84$), *NCF1*-566 ($p=0.69$). ROS, reactive oxygen species; NOX2, phagocyte NADPH oxidase. $^{**}p \le 0.01$.

however, it does not increase NOX2-produced ROS. Most likely this is because the efficiency of the NOX2 complex cannot be increased unless there is increased expression of the other subunits of the complex (22). Hence, there appears to be no difference in ROS production between the 1:1 or 2:1 ratio, explaining the RA-protective effect. However, a reduced number of functional *NCF1* copies negatively affect the functionality of the NOX2 complex (8). Due to a heterozygous deletion, some patients with Williams-Beuren syndrome have only one functional *NCF1* gene, and reduced levels of Ncf1 protein and ROS. We show that the T-allele of *NCF1*-339 is expressed in both *NCF1* and *NCF1-II* and could have a similar effect by encoding a complete loss of NOX2-produced ROS. In a 2:1 ratio context, heterozygous expression of the T-allele will likely reduce ROS levels, whereas in a 1:1 ratio context, the presence of the additional *NCF1-II* gene could restore ROS production to normal levels. In this study, the T-allele is most frequent in a 2:1 ratio context, and RA patients are slightly more likely to have a 2:1 ratio while expressing the T-allele, 72.3% compared with 64.3% of controls. Although not significant, this could indicate that the protective effect of the 1:1 ratio comes from the additional *NCF1-II* gene restoring reduced ROS levels, caused by loss-of-function alleles such as the T-allele of *NCF1*-339. Most likely, there are also other genetic variants in *NCF1* encoding reduced functionality of the NOX2 complex, from which *NCF1-II* could be protective.

The T-allele of *NCF1*-339 encodes an amino acid shift from arginine to histidine at position 90 in the protein sequence. Arg^{90} is evolutionary conserved and is a binding site to phosphatidylinositol (3,4)-biphosphate ($PI(3,4)P_2$) in the membrane (36). Mutation studies have shown that amino acid substitutions to Lys^{90}, Ala^{90}, and Leu^{90} greatly reduce the binding capability to $PI(3,4)P_2$, and decreases the production of ROS (20, 24). The substitution to His^{90} could be expected to have similar or more severe effects on binding to $PI(3,4)P_2$, than Lys^{90} based on the amino acid properties, which is supported by our results from the construct experiments.

There are reports of CGD patients showing symptoms resembling autoimmune syndromes—inflammatory bowel disease (1, 34), juvenile RA (23), and lupus (5)—indicating how important a functional NOX2 complex is to maintain tolerance toward self-antigens and preventing autoimmunity. The link to autoimmunity is strengthened by both *NCF4* and *NCF2* of the NOX2 complex being associated with other autoimmune diseases: *NCF4* with Crohn disease (31) and *NCF2* with SLE (9).

The region surrounding *NCF1* is highly dynamic and subject to segmental duplications, deletions, and inversions (2, 21). In combination with the presence of the *NCF1B* and *NCF1C* genes, this makes studying *NCF1* with PCR-based techniques difficult. The ΔGT sequence is the only genetic variant shown to be *NCF1B/C* specific, and therefore this was used to distinguish between *NCF1/NCF1-II* and *NCF1B/C*. The nested genotyping assay specifically amplifies the GTGT sequence, but analysis is further complicated by the presence of *NCF1-II*. To be able to determine the true genotypes we used an out-of-phase design of the genotyping assays, increasing the number of peaks defining the genotype. However, we are still unable to conclusively determine the exact genotypes of *NCF1*-566, when one or two copies of *NCF1-II* are present, as the genotyping assay used for *NCF1*-566 cannot separate *NCF1* from *NCF1-II*. However, as the G-allele is only seen in 3 individuals not having *NCF1-II*, we conclude that the G-allele is almost exclusively expressed in *NCF1-II* and thus the genotypes are interpreted as A/A+G and A/A+G/G. *NCF1*-339 and *NCF1*-365 could be genotyped more precisely by using an additional assay, specifically amplifying the region spanning exons three and four in *NCF1*, excluding *NCF1-II* (33). This assay enables correct calls of genotypes like C/C+T and C/T+C.

The qPCR assay used in the ratio study quantifies the number of *NCF1/NCF1-II* and *NCF1B/C* copies separately by two different probes, making it possible to determine changes in the number of *NCF1/NCF1-II* or *NCF1B/C* copies. However, due to a slight unspecificity of the *NCF1B/C* probe, the *NCF1B/C* copy number is overestimated, reflected by higher than expected ratios. Nonetheless, we have determined that the 1:1 ratio reflects an increase of *NCF1/NCF1-II* copies and a decrease of *NCF1B/C*, compared with the 2:1 ratio. The 1:2 ratio reflects a substantial increase in *NCF1/NCF1-II* and decrease in *NCF1B/C*. The 3:2 and 5:2 ratios reflect changes only in *NCF1B/C* copy number, whereas the 4:3 ratio indicate an increase of *NCF1/NCF1-II*, while *NCF1B/C* is unchanged. The finding of the 3:2, 5:2, and 4:3 ratios indicates that the distribution of *NCF1* genes is even more complex than previously believed and a recent study shows that this distribution also varies between populations (4).

In conclusion, our data show a complex inheritance of *NCF1*, *NCF1-II*, and *NCF1B/C*. We find that RA patients are less likely to have a copy of *NCF1-II*, possibly indicating that an additional copy of a functional *NCF1*-like gene is protective against RA. This finding is also supportive of the theory that increased levels of ROS are protecting against RA and autoimmunity.

Methods

Patient samples

The samples are from the EIRA, a population-based case–control study. The study base comprised the population, aged 18–70 years, in an area of the middle and southern parts of Sweden during May 1996–2005. A case was defined as a person who received a first time diagnosis of RA according to the American College of Rheumatology criteria of 1987 (3). About 494 RA patients and 480 controls, individually matched on sex and age were used. About 72% are women and the mean age is 53 ± 11 years for the patients and 56 ± 12 years for the controls.

Multiplex qPCR assay

To quantify the number of *NCF1* and *NCF1B/C* copies and to estimate the ΔGT/GTGT ratio, we used a multiplex qPCR assay (39). In each multiplex reaction the number of either *NCF1* or *NCF1B/C* copies is compared with the control gene albumin, which has no copy variation. The MBG probes (Applied Biosystems, Carlsbad, CA) for *NCF1/NCF1B/C* use the GTGT sequence as a distinguisher and are labeled with the FAM dye. The albumin probe is labeled with the VIC dye. Primer and probe sequences are listed in Supplementary Table S1. The reaction was run using the ABI PRISMA® 7000 Sequence Detection System and TaqMan Universal PCR Mastermix (Applied Biosystems). Each reaction was run in triplicate with 50 ng of DNA/reaction and a final volume of

25 μl. Optimized primer concentrations in the *NCF1*/Albumin reaction are 600/800 n*M* and the *NCF1B/C*/Albumin reaction are 300/800 n*M*. The probe concentrations are 100 n*M* in all reactions. ΔRn, the normalized emission intensity for a specific sample, was set at five different thresholds along the exponential phase of the curve, giving five CT values per sample. The mean and standard deviation were calculated and used in further calculations. ΔCT = (mean CT *NCF1*–mean CT albumin) or (mean CT *NCF1B/C*–mean CT albumin). About $2^{-\Delta\Delta CT}$ between *NCF1B/C* and *NCF1* was calculated using the formula: $^{\Delta\Delta}CT = (\Delta CT\ NCF1B/C - \Delta CT\ NCF1)$. To ensure specificity of the *NCF1* and *NCF1B/C* probes, plasmid DNA from the *NCF1*/*NCF1B/C*-specific bacterial artificial chromosome (BAC) clones (*NCF1*; RP11-186N20 and RP11-81J7, *NCF1B/C*; RP11-219M8 and RP11-396K3) was used.

Ratio analysis and validation

The $2^{-\Delta\Delta CT}$ FC values were divided into 0.1 windows and graphed (Supplementary Fig. S1). Based on the distribution of the FC values, ranges for the ratios were set (Supplementary Table S2). The 4:3 ratio FC values overlap with the 3:2 FC values and could only be assigned a ratio after analysis of the ΔCT values for the *NCF1/NCF1-II* probe. We were not able to assign ratios for eight samples, which were excluded from further analysis. The distribution of copy number of *NCF1-II* (0, 1, or 2) was in accordance with Hardy–Weinberg equilibrium in controls but not in patients ($p = 0.0002$), which is expected because of the detected association.

As a validation method we also ratio-typed all samples using the gene-scan method (7). We found that this method was not as sensitive as the qPCR method in distinguishing between the 5:2, 3:2, and 2:1 ratios, which contributed to a higher failure rate (~10%). Among the nonfailed samples 743/751 (98.9%) assigned a 2:1 ratio using the gene-scan method had also been assigned the 2:1 ratio using the qPCR assay. The genotyping assays were also used to confirm ratio assignment in samples where both alleles are expressed. A combined analysis of the gene-scan and genotyping results assigned the same ratio for 90/92 samples assigned 1:1 by the qPCR method.

Selection of SNPs for genotyping study

We selected two nonsynonymous SNPs, *NCF1*-339 and *NCF1*-566, reported to be of *NCF1B/C* origin and specifically expressed in *NCF1-II* (14). The nonsynonymous SNP *NCF1*-365 was also selected for genotyping. The SNPs have multiple rs-numbers and are therefore denoted according to their position in the mRNA sequence, *NCF1*-339 (rs13447 or rs17355366), *NCF1*-365 (rs17856077 or rs62475423), and *NCF1*-566 (rs4868).

Genotyping

Nested PCRs were used to ensure assay specificity (Supplementary Fig. S2). The first PCR specifically amplifies either *NCF1/NCF1-II* (primers; GTGT-specific) or *NCF1* (primers; *NCF1*-exon3-4; Supplementary Table S1). Reaction conditions for the GTGT-specific assay are 100 ng DNA, 300 n*M* primer, 500 n*M* dNTP, 1× Buffer 3, 3.75U Taq polymerase (Expand long template PCR system; Roche Diagnostic, Basel, Switzerland). Reaction cycling conditions were 2 min at 95°C, followed by 10 cycles of 10 s at 95°C, 6.5 min at 68°C, followed by 20 cycles of 15 s at 95°C, 6.5 min + 10 s extension each cycle at 68°C, followed by 68°C for 7 min. The specificity of the reactions was confirmed by using BAC clones specific for *NCF1* (RP11-186N20 and RP11-81J7) or *NCF1B/C* (RP11-219M8 and RP11-396K3). The second (nested) PCR amplifies the regions surrounding the SNPs following standard protocols for pyrosequencing (Qiagen, Hilden, Germany; Supplementary Fig. S2). About 0.5 μl of a 1:500 dilution of the first PCR was used in the pyrosequencing reaction.

Genotype analysis

The pyrosequencing method calculates genotypes by comparing peak heights, corresponding to the nucleotides in and around the SNP, in a pyrogram. Each peak is generated by the light emitting from incorporation of a nucleotide. At the SNP position nucleotides representing the two alleles are injected and incorporation of the correct nucleotide will generate a peak in the pyrogram. A peak height of 1 represents the insertion of one nucleotide and a height of two a double insertion. To interpret pyrograms created by incorporation of nucleotides in both *NCF1* and *NCF1-II*, we used an "out-of-phase" design for the three genotyping assays. In the out-of-phase assay the injection of nucleotides is out-of-phase for one of the two alleles, leading to incorporation only if the in-phase allele is present. This strategy creates a pyrogram with more peaks defining the genotype (Supplementary Fig. S3). To correctly call the genotypes, the results from the ratio analysis also had to be included in the analysis. The distribution of genotypes was in accordance with Hardy–Weinberg equilibrium.

ROS production assay

All reagents like isoluminol, HRP fraction II, and PMA were purchased from Sigma Aldrich (St. Louis, MI). COS cells reconstituted with *CYBB* (gp91phox), *CYBA* (p22phox), and *NCF2* (p67phox) (provided by Mary C. Dinauer, Indiana University, Indianapolis, Indiana) was cultured in Dulbecco's complete medium, 10% fetal calf serum, and penicillin–streptomycin (Gibco, Invitrogen, Paisley, United Kingdom). Human *NCF1* (BM457671) was isolated from the IMAGE clone 5498834 and inserted into the pcDNA3.1/Hygro (+) mammalian expression vector (Invitrogen). The three studied amino acid variants at position 339 (Arg to His), 365 (Ser to Gly), and 566 (Asn to Asp) were produced in the *NCF1* construct using *in vitro* mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's recommendations. Adherent COS-7 cells were harvested by incubating with trypsin/ethylenediaminetetraacetic acid for 5 min at 37°C. The cells were resuspended, washed in phosphate-buffered saline, and cultured in 96-well plates (NUNC, Roskilde, Denmark) at a density of 20,000 cells/well overnight. The cells were transiently transfected with *NCF1* with lipofectamin plus, optimum, and DNA PLUS system (Invitrogen), according to the manufacturer's instructions, using 100 ng DNA per well. Transfected cells were grown in complete media for another 48 h until assayed.

Functionality of the NOX2 complex and thus the function of the studied *NCF1* allele was assayed directly in the culture plate using an isoluminol detection method (6). Briefly, the cells were gently washed in Hank's balanced salt solution and

100 μl of isoluminol reagent buffer (final concentration isoluminol 50 μg/ml, horse radish peroxidase-type II 2.5 μ/ml, and PMA 400 ng/ml). Samples were gently mixed and the data collection was initiated immediately. The NOX2-produced ROS is initiated within minutes. Extra-cellular ROS production was followed at 37°C as produced luminescence signal (FluoStar Optima, BMG Labtechnologies, Offenburg, Germany) and presented as maximal relative signal during a measurement period of 30 min.

Statistical analyses

The Hardy–Weinberg analyses were done using the HWE test in the PLINK software (30). Association analyses of allele and genotype frequencies were done using the "Basic case/control association" test and the "Full model" tests in PLINK. Both these tests are based on contingency tables and Chi-square statistics. The combined *NCF1/NCF1-II* association analyses were done using the "CNP/SNP association" test in PLINK, which is based on regression analysis, and using contingency tables and Chi-square statistics in GraphPad InStat version 3.06. Ratio frequencies were also analyzed using contingency tables and Chi-square statistics in GraphPad InStat.

The statistical analysis of the ROS production differences in the mutated *NCF1* construct against nonmutated *NCF1* was performed using the Mann Whitney test with GraphPad Prism version 4.03.

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Author Disclosure Statement

Rikard Holmdahl and Peter Olofsson are founders of the company Redoxis AB, which is exploring possibilities for treatment by agents stimulating ROS production. No other authors declare conflict of interest.

References

1. Ahlin A, De Boer M, Roos D, Leusen J, Smith CI, Sundin U, Rabbani H, Palmblad J, and Elinder G. Prevalence, genetics and clinical presentation of chronic granulomatous disease in Sweden. *Acta Paediatr* 84: 1386–1394, 1995.
2. Antonell A, de Luis O, Domingo-Roura X, and Perez-Jurado LA. Evolutionary mechanisms shaping the genomic structure of the Williams-Beuren syndrome chromosomal region at human 7q11.23. *Genome Res* 15: 1179–1188, 2005.
3. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 31: 315–324, 1988.
4. Brunson T, Wang Q, Chambers I, and Song Q. A copy number variation in human NCF1 and its pseudogenes. *BMC Genet* 11: 13, 2010.
5. Cale CM, Morton L, and Goldblatt D. Cutaneous and other lupus-like symptoms in carriers of X-linked chronic granulomatous disease: incidence and autoimmune serology. *Clin Exp Immunol* 148: 79–84, 2007.
6. Dahlgren C and Karlsson A. Respiratory burst in human neutrophils. *J Immunol Methods* 232: 3–14, 1999.
7. Dekker J, de Boer M, and Roos D. Gene-scan method for the recognition of carriers and patients with p47(phox)-deficient autosomal recessive chronic granulomatous disease. *Exp Hematol* 29: 1319–1325, 2001.
8. Del Campo M, Antonell A, Magano LF, Munoz FJ, Flores R, Bayes M, and Perez Jurado LA. Hemizygosity at the NCF1 gene in patients with Williams-Beuren syndrome decreases their risk of hypertension. *Am J Hum Genet* 78: 533–542, 2006.
9. Gateva V, Sandling JK, Hom G, Taylor KE, Chung SA, Sun X, Ortmann W, Kosoy R, Ferreira RC, Nordmark G, Gunnarsson I, Svenungsson E, Padyukov L, Sturfelt G, Jonsen A, Bengtsson AA, Rantapaa-Dahlqvist S, Baechler EC, Brown EE, Alarcon GS, Edberg JC, Ramsey-Goldman R, McGwin G, Jr., Reveille JD, Vila LM, Kimberly RP, Manzi S, Petri MA, Lee A, Gregersen PK, Seldin MF, Ronnblom L, Criswell LA, Syvanen AC, Behrens TW, and Graham RR. A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. *Nat Genet* 41: 1228–1233, 2009.
10. Gorlach A, Lee PL, Roesler J, Hopkins PJ, Christensen B, Green ED, Chanock SJ, and Curnutte JT. A p47-phox pseudogene carries the most common mutation causing p47-phox- deficient chronic granulomatous disease. *J Clin Invest* 100: 1907–1918, 1997.
11. Greve B, Hoffmann P, Vonthein R, Kun J, Lell B, Mycko MP, Selmaj KW, Berger K, Weissert R, and Kremsner PG. NCF1 gene and pseudogene pattern: association with parasitic infection and autoimmunity. *Malar J* 7: 251, 2008.
12. Harbord M, Hankin A, Bloom S, and Mitchison H. Association between p47phox pseudogenes and inflammatory bowel disease. *Blood* 101: 3337, 2003.
13. Heyworth PG, Cross AR, and Curnutte JT. Chronic granulomatous disease. *Curr Opin Immunol* 15: 578–584, 2003.
14. Heyworth PG, Noack D, and Cross AR. Identification of a novel NCF-1 (p47-phox) pseudogene not containing the signature GT deletion: significance for A47 degrees chronic granulomatous disease carrier detection. *Blood* 100: 1845–1851, 2002.
15. Hockenhull EL, Carette MJ, Metcalfe K, Donnai D, Read AP, and Tassabehji M. A complete physical contig and partial transcript map of the Williams syndrome critical region. *Genomics* 58: 138–145, 1999.
16. Holmdahl R. Dissection of the genetic complexity of arthritis using animal models. *J Autoimmun* 21: 99–103, 2003.
17. Hultqvist M, Olofsson P, Gelderman KA, Holmberg J, and Holmdahl R. A new arthritis therapy with oxidative burst inducers. *PLoS Med* 3: e348, 2006.
18. Hultqvist M, Sareila O, Vilhardt F, Norin U, Olsson LM, Olofsson P, Hellman U, and Holmdahl R. Positioning of a polymorphic quantitative trait nucleotide in the Ncf1 gene controlling oxidative burst response and arthritis severity in rats. *Antioxid Redox Signal* 14: 2373–2383, 2011.
19. Kallberg H, Padyukov L, Plenge RM, Ronnelid J, Gregersen PK, van der Helm-van Mil AH, Toes RE, Huizinga TW, Klareskog L, and Alfredsson L. Gene-gene and gene-environment interactions involving HLA-DRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis. *Am J Hum Genet* 80: 867–875, 2007.

20. Karathanassis D, Stahelin RV, Bravo J, Perisic O, Pacold CM, Cho W, and Williams RL. Binding of the PX domain of p47(phox) to phosphatidylinositol 3, 4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction. *EMBO J* 21: 5057–5068, 2002.
21. Kriek M, White SJ, Szuhai K, Knijnenburg J, van Ommen GJ, den Dunnen JT, and Breuning MH. Copy number variation in regions flanked (or unflanked) by duplicons among patients with developmental delay and/or congenital malformations; detection of reciprocal and partial Williams-Beuren duplications. *Eur J Hum Genet* 14: 180–189, 2006.
22. Lapouge K, Smith SJ, Groemping Y, and Rittinger K. Architecture of the p40-p47-p67phox complex in the resting state of the NADPH oxidase. A central role for p67phox. *J Biol Chem* 277: 10121–10128, 2002.
23. Lee BW and Yap HK. Polyarthritis resembling juvenile rheumatoid arthritis in a girl with chronic granulomatous disease. *Arthritis Rheum* 37: 773–776, 1994.
24. Li XJ, Marchal CC, Stull ND, Stahelin RV, and Dinauer MC. p47phox Phox homology domain regulates plasma membrane but not phagosome neutrophil NADPH oxidase activation. *J Biol Chem* 285: 35169–35179, 2010.
25. MacGregor AJ, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, Aho K, and Silman AJ. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum* 43: 30–37, 2000.
26. Merla G, Howald C, Henrichsen CN, Lyle R, Wyss C, Zabot MT, Antonarakis SE, and Reymond A. Submicroscopic deletion in patients with Williams-Beuren syndrome influences expression levels of the nonhemizygous flanking genes. *Am J Hum Genet* 79: 332–341, 2006.
27. Olofsson P, Holmberg J, Tordsson J, Lu S, Akerstrom B, and Holmdahl R. Positional identification of Ncf1 as a gene that regulates arthritis severity in rats. *Nat Genet* 33: 25–32, 2003.
28. Olsson LM, Lindqvist AK, Kallberg H, Padyukov L, Burkhardt H, Alfredsson L, Klareskog L, and Holmdahl R. A case-control study of Rheumatoid arthritis identifies an associated SNP in the NCF4 gene supporting a role for the NOX-complex in autoimmunity. *Arthritis Res Ther* 9: R98, 2007.
29. Pedersen M, Jacobsen S, Klarlund M, Pedersen BV, Wiik A, Wohlfahrt J, and Frisch M. Environmental risk factors differ between rheumatoid arthritis with and without auto-antibodies against cyclic citrullinated peptides. *Arthritis Res Ther* 8: R133, 2006.
30. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, and Sham PC. PLINK: A tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81: 559–575, 2007.
31. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, Green T, Kuballa P, Barmada MM, Datta LW, Shugart YY, Griffiths AM, Targan SR, Ippoliti AF, Bernard EJ, Mei L, Nicolae DL, Regueiro M, Schumm LP, Steinhart AH, Rotter JI, Duerr RH, Cho JH, Daly MJ, and Brant SR. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 39: 596–604, 2007.
32. Roesler J, Curnutte JT, Rae J, Barrett D, Patino P, Chanock SJ, and Goerlach A. Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. *Blood* 95: 2150–2156, 2000.
33. Roos D, de Boer M, Koker MY, Dekker J, Singh-Gupta V, Ahlin A, Palmblad J, Sanal O, Kurenko-Deptuch M, Jolles S, and Wolach B. Chronic granulomatous disease caused by mutations other than the common GT deletion in NCF1, the gene encoding the p47phox component of the phagocyte NADPH oxidase. *Hum Mutat* 27: 1218–1229, 2006.
34. Schappi MG, Smith VV, Goldblatt D, Lindley KJ, and Milla PJ. Colitis in chronic granulomatous disease. *Arch Dis Child* 84: 147–151, 2001.
35. Seldin MF, Amos CI, Ward R, and Gregersen PK. The genetics revolution and the assault on rheumatoid arthritis. *Arthritis Rheum* 42: 1071–1079, 1999.
36. Stahelin RV, Burian A, Bruzik KS, Murray D, and Cho W. Membrane binding mechanisms of the PX domains of NADPH oxidase p40phox and p47phox. *J Biol Chem* 278: 14469–14479, 2003.
37. Steinsson K and Alarcon-Riquelme ME. Genetic aspects of rheumatic diseases. *Scand J Rheumatol* 34: 167–177, 2005.
38. Suraweera N, Zampeli E, Rogers P, Atkin W, Forbes A, Harbord M, and Silver A. NCF1 (p47phox) and ncf1 pseudogenes are not associated with inflammatory bowel disease. *Inflamm Bowel Dis* 10: 758–762, 2004.
39. Thiel CT, Kraus C, Rauch A, Ekici AB, Rautenstrauss B, and Reis A. A new quantitative PCR multiplex assay for rapid analysis of chromosome 17p11.2–12 duplications and deletions leading to HMSN/HNPP. *Eur J Hum Genet* 11: 170–178, 2003.

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Abbreviations Used

BAC = bacterial artificial chromosome
CGD = chronic granulomatous disease
EIRA = Epidemiological Investigation of Rheumatoid Arthritis
FC = fold change
NCF1 = neutrophil cytosolic factor 1
NOX2 = phagocyte NADPH oxidase
$PI(3,4)P_2$ = phosphatidylinositol (3,4)-biphosphate
qPCR = quantitative real-time polymerase chain reaction
RA = rheumatoid arthritis
ROS = reactive oxygen species
SLE = systemic lupus erythematosus
SNP = single-nucleotide polymorphism

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2. Lina M. Olsson, Rikard Holmdahl. 2012. Copy number variation in autoimmunity - importance hidden in complexity?. *European Journal of Immunology* **42**:8, 1969-1976. [CrossRef]
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4. Christine Deffert, Stephanie Carnesecchi, Huiping Yuan, Anne-Laure Rougemont, Tiina Kelkka, Rikard Holmdahl, Karl-Heinz Krause, Michela G Schäppi. 2012. Hyperinflammation of chronic granulomatous disease is abolished by NOX2 reconstitution in macrophages and dendritic cells. *The Journal of Pathology* n/a-n/a. [CrossRef]
5. Marianne K Kringen, Camilla Stormo, Runa M Grimholt, Jens P Berg, Armin P Piehler. 2012. Copy number variations of the ATP-binding cassette transporter ABCC6 gene and its pseudogenes. *BMC Research Notes* **5**:1, 425. [CrossRef]