Reactive Oxygen Species Produced by the NADPH Oxidase 2 Complex in Monocytes Protect Mice from Bacterial Infections

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Chronic granulomatous disease (CGD) is an inherited disorder characterized by recurrent life-threatening bacterial and fungal infections. CGD results from defective production of reactive oxygen species by phagocytes caused by mutations in genes encoding the NADPH oxidase 2 (NOX2) complex subunits. Mice with a spontaneous mutation in Ncf1, which encodes the NCF1 (p47^phox) subunit of NOX2, have defective phagocyte NOX2 activity. These mice occasionally develop local spontaneous infections by *Staphylococcus xylosus* or by the common CGD pathogen *Staphylococcus aureus*. Ncf1 mutant mice were more susceptible to systemic challenge with these bacteria than were wild-type mice. Transgenic MN+ mice gained the expression of NCF1 and functional NOX2 activity specifically in monocytes/macrophages, although minimal NOX2 activity was also detected in some CD11b+Ly6G+ cells defined as neutrophils. MN+ mice did not develop spontaneous infection and were more resistant to administered staphylococcal infections compared with MN− mice. Most strikingly, MN+ mice survived after being administered *Burkholderia cepacia*, an opportunistic pathogen in CGD patients, whereas MN− mice died. Thus, monocyte/macrophage expression of functional NCF1 protected against spontaneous and administered bacterial infections. The Journal of Immunology, 2012, 188: 000–000.
The NOX2 complex is functional in both neutrophils and macrophages. Although the critical role of neutrophil NOX2 in host defense is well established (13), the role of NOX2 in macrophages is less clear. Because the NOX2 complex is deficient in all myeloid cells in both CGD patients and mouse models, it was not possible to delineate the specific contribution of macrophage NOX2 to host defense in vivo. To our knowledge, we show for the first time that monocyte expression of functional NCF1 under the control of the human CD68 promoter protected mice from lethal infection by *S. xylosus* and *B. cepacia*.

In conclusion, a natural occurring mutation in *Ncf1*, which impairs the protein function, limits the antibacterial defense in mice similarly to the human CGD condition. Transgenic mice, in which functional NCF1 is targeted to monocytes, are protected from lethal bacterial infections. These findings are important in the dissection of the mechanisms of impaired host defense in CGD and for the development of targeted cures.

Materials and Methods

Ethics statement

All procedures performed on mice were approved by the respective institutes and complied with federal guidelines. The experiments conducted at Roswell Park Cancer Institute followed the federal Animal Welfare Act and the National Institute of Health’s Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (1135M). Experiments carried out in Finland were approved by the Regional state administrative agency in Southern Finland (ESAVI-0000497/041003/2011). The ones performed in Sweden followed the Animal Protection Law (DjurSkyddslag) and were approved by the Gothenburg committee (106-2009) and the Northern Stockholm committee (N169/10).

Animals

All mice used were genetically controlled and shared the C57Black background. The C57BL/10.Q/rdh background was confirmed to be homozygous, with the exception of the previously described (Ncf1<sup><s>M</s>Ncf1<sup>+</sup>) mutation in the Ncf1 gene, using a 10K SNP chip (12, 14). The MHC class II congenic C57BL/10.Q/rdh (B10.Q) mice express MHC class II H2-A<sup>Q</sup> that is encoded by a fragment from the DBA/1 strain. The Ncf1 mutation impaired the function of NCF1, as described earlier (12). B10.PMBQ mice were described previously (15); they are transgenic mice in which H-2<sup>A</sup>-<s>A</s> is expressed under the hCD68 promoter. B10.Q.B knockout (ko) mice were previously called B10.Q.<s>A</s>Mt mice; they were obtained from backcrossing the original Mt founder, kindly provided by Dr. Werner Müller (Institute of Genetics, Cologne, Germany) to B10.Q mice for >10 generations (16). TCR ko animals were bought from The Jackson Laboratory (Tcr<sup>−/−</sup>) animals and the TCR<sup>−/−</sup> mice for >10 generations to B10.Q mice. The B10.Q COMP ko mice were previously described and backcrossed to B10.Q mice for >10 generations (17). B10.Q.MN mice were described previously (18); the MN transgene encodes the functional NCF1 under the hCD68 promoter. Ncf1<sup>−/−</sup> (also denoted p47<sup>pseudox</sup>) mice, generated as previously described (19), were backcrossed to N14 in C57BL/6 mice and maintained at the Roswell Park Cancer Institute. Mice were housed under specific pathogen-free conditions.

Bacterial isolates

Bacterial growth of isolated samples from infected animals was assessed on various media under different incubation conditions. All identified isolates could be cultured on hematin agar at 37°C under aerobic conditions. Species identification was based on 16S rDNA sequence analysis and phenotypic tests. Cultured colonies were suspended in 100 μL H<sub>2</sub>O and heated for 10 min at 95°C. PCR<sup>−</sup> DNA<sup>−</sup> analysis was performed without further extraction of DNA, and amplification was carried out in a 50-μl reaction mixture containing 1× PCR buffer (QIAGEN), 3 nM MgCl<sub>2</sub>, 200 μM each deoxynucleoside triphosphate, 1.0 U HotStarTaq DNA polymerase (QIAGEN), 10 pmol each primer, and 5 μl DNA template. BF5 (5′-AGA GTT TGA TCM TGG TCG ATC AG-3′) and P911r (5′-CCC GTA TAC TTT GGT GAG T-3′) were used as PCR and sequencing primers. A PCR<sup>−</sup> PCR<sup>−</sup> step of 15 min at 95°C was followed by 40 cycles of 93°C for 30 s, 52°C for 50 s, and 72°C for 30 s. A final step of 5 min at 72°C terminated the amplification. Tubes with no target DNA and *Escherichia coli* DNA were included as negative and positive controls, respectively. Both strands of the ~800-bp PCR<sup>−</sup> product were sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) by BMlabet (Lund, Sweden). The sequences of the isolates were identical to the 16S rDNA genes of *S. aureus*, *Staphylococcus saprophyticus*, *xylosus*, or E. coli available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Strains determined to belong to the family of *S. saprophyticus* and *S. xylosus* with 16S were differentiated and identified to be *S. xylosus* by their ability to ferment xylose and mannose in oxidative-fermentative medium (21).

Strain typing

The *S. xylosus* isolates were typed using automated repetitive PCR (rep-PCR) (DiversiLab), as recommended by the manufacturer. DNA was extracted using the UltraClean Microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, CA), and a minimum of 50 ng DNA was amplified using the *Staphylococcus* fingerprinting kit (DiversiLab; bioMérieux) according to the manufacturer’s instructions. PCR<sup>−</sup> products were separated on DiversiLab LabChips (bioMérieux) using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) operating the DiversiLab v1.4 assay. Bioanalyzer data were exported to a DiversiLab Web site and analyzed with DiversiLab v3.4 software. Data analysis was performed with the Web-based software using the Pearson coefficients to determine distance matrices and the unweighted pair-group method with arithmetic mean to create dendrograms. The DiversiLab data generated for each organism included a dendrogram with virtual gel images, a graph of fluorescence intensity that corresponds to the organism’s banding pattern, and a similarity matrix. Cluster analysis was done by comparing the automated rep-PCR pattern for each isolate to a representative rep-PCR pattern obtained for each of the reference strains. Strain relatedness for the replicate studies was defined as a minimum of 90% similarity, with a difference of up to three bands.

Systemic administration of bacteria

*S. xylosus* strains 720 and 1056B and *S. aureus* strains LS-1 and 1056A were inoculated i.v. in the tail vein in a total volume of 200 μL PBS/mouse. Viable counts were performed to determine the number of bacteria injected. The mice received the following numbers of bacteria: *S. xylosus* 1056B, 0.8–1.2 × 10<sup>9</sup> bacteria/mouse; *S. xylosus* 720, 1.4 × 10<sup>9</sup> bacterial mouse; *S. aureus* LS-1, 5.7 × 10<sup>8</sup> bacterium/mouse; and *S. aureus* 1056A, 0.8 × 10<sup>8</sup> bacteria/mouse. We used a strain of *B. cepacia* isolated from a CGD patient, as previously described (22). Mice were administered 4.5 × 10<sup>9</sup> CFU i.p., an inoculum previously known to be lethal in p47<sup>pseudox</sup>-/- mice but not in wt mice (22).

Bacterial counts in organs

Organs were aseptically dissected, homogenized, serially diluted in PBS, and spread on agar plates. In *B. cepacia* experiments, peritoneal lavage was performed with 10 mL Dulbecco’s PBS. The number of CFU/site was determined after 24–48 h of incubation at 37°C.

Arthritis scoring

All mice were followed up individually and checked daily. Mice were scored blindly for arthritis severity and frequency. Finger/toe and ankle/ wrist joints were inspected, and arthritis was defined as visible erythema and/or swelling. To evaluate the intensity of arthritis, clinical scoring (arthritic index) was carried out using a system whereby macroscopic inspection yielded a score of 0–3 points for each limb (0, no swelling or erythema; 1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling and erythema). The total score was calculated by adding all of the scores within each individual tested.

The overall condition of each mouse was also examined daily by assessing signs of systemic inflammation (i.e., weight loss, reduced alertness, and ruffled coat). In cases of severe systemic infection when a mouse was judged too ill to survive another 24 h, it was culled and defined as death due to sepsis.

Enrichment of neutrophils from bone marrow with Percoll gradient

Bone marrow from femurs and tibiae were extracted by flushing the bones with PBS using a 26G needle. RBCs were lysed with ACK buffer, and the cells were washed with PBS before resuspending them in 3 ml 45% Percoll solution. A Percoll gradient was formed in a 15-ml tube, loading 3 ml 66% Percoll, 2 ml 55% Percoll, and, finally, the cells. After a 30-min centrifugation at 3000 rpm with brakes off, the 66–
60% interface layer of cells was recovered. All solutions and operations were performed at room temperature. This method leads to a myeloid preparation consisting of ~90% neutrophils based on cytology.

Abs
Pacific Blue-labeled anti-CD11b (Mac-1, M1/70) Ab and PE-Cy7– or PE-labeled anti-Ly6G (1A8, BD Biosciences) and anti-FcR (24G2; generated in the Medical Inflammation Research laboratory) were used to stain the blood samples. Intracellular staining for NCF1 was performed with Ab mouse anti-human Ncf1 D10 (Santa Cruz) and detected with anti-mouse IgGl-allophycocyanin (BD Biosciences).

Oxidative burst assay and flow cytometry
Twenty microliters of freshly drawn blood was stained for ROS production, as described previously (23). FcR interaction was blocked with anti-FcR blocker 24G2 before incubation with the surface markers Ab Pacific Blue anti-CD11b (Mac-1) Ab and PE-Cy7–labeled anti-Ly6G (BD Biosciences). Dihydrorhodamine 123 (DHR; Sigma) was added to the cells at a concentration of 3 nM, and the cells were stimulated with PMA at a concentration of 200 ng/ml. After washing, 2 × 10^5 cells were acquired with a BD LSR II flow cytometer, and the data were analyzed with FlowJo version 8.8.6.

For NCF1 intracellular staining, after incubation with surface marker Abs and washing in PBS, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and subsequently stained with anti-NCF1 Ab and anti-mouse IgGl-allophycocyanin, according to the manufacturer’s instructions. NCF1 expression was measured as the difference between the geometric mean of cells stained with anti-NCF1 Ab and anti-mouse IgGl-allophycocyanin and the geometric mean of cells stained with only anti-mouse IgGl-allophycocyanin: the geometrical mean is represented.

In vivo ROS detection
Naïve mice were anesthetized with isoflurane and injected i.p. with 20 mg/kg L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H) dione) probe (Wako Chemicals) dissolved in physiological saline (24). The luminescent signal was detected with an IVIS 50 bioluminescent system (Xenogen), consisting of an anesthesia unit in a light-tight chamber with a CCD camera (IVIS 132323, DW434). Image acquisition and analysis were performed with Living Image software (Xenogen).

Histology
Infected and healthy paws were collected and fixed in 4% paraformaldehyde-buffered solution. The skin was removed, and the paws were decalcified in a solution of 4% paraformaldehyde and formic acid (1:1) for 48 h. After dehydration, paws were embedded in paraffin and sectioned (10 or 4 μm) before staining with H&E or Gram staining.

Statistical methods
The Mann–Whitney U test was used to compare two groups. For comparison of three groups, the Kruskal–Wallis test with the Dunn comparison posttest was used. The Fisher exact test was used for incidence comparisons. Survival curves were displayed as Kaplan–Meier curves, and in-tergroup comparisons were assessed by the log-rank method. GraphPad Prism, version 5.0c, was used for statistical analysis. The p values < 0.05 were considered statistically significant.

Results
Spontaneous inflammation in soft tissue of Ncf1 mutant mice
We observed local spontaneous infections in Ncf1 mutant (Ncf1^+/−) mice, but not in Ncf1^−/− mice, in the our mouse colonies in two animal facilities: a conventional facility in Lund and a specific pathogen-free, Felasa II facility in Stockholm. All of the mice shared the C57BL/10.Q.rhd background and carried the Ncf1 mutation, but their genotype varied with respect to other congenic fragments and transgenes (Table I). The frequency of observed infections averaged 2% of 2600 B10.Q.Ncf1^+/− mice. Infections occurred mostly in the paws but were also found in the tail, limbs, head, and neck soft tissue. The inflamed tissue was swollen, red, and infiltrated with pus and was generally associated with scarring (Fig. 1A, 1B). Histology revealed massive cell infiltration in the skin and bone, sometimes associated with bone necrosis (Fig. 1C–E). In the abscesses, several bacterial colonies were present (Fig. 1F), where Gram-positive cocci were visible (Fig. 1H), together with a large number of neutrophils and macrophages (Fig. 1G). Splendore–Hoeppli material was observed around necrotic leukocytes and cell debris and inside necrotic macrophages (Fig. 1G). Infections were more common in males than in females.

Usually, several, if not all, mice in a cage developed infection, raising the possibility of transmitted infection. Interestingly, we also observed the same infection frequency in B10.Q.Ncf1^+/− mice (but not in NOX2-competent mice) with specific engineered cell or protein deficiencies, including deficiency in B cells or T cells, deletion of COMP (17), or deficient expression of specific MHC class II alleles on macrophages (15), all on the same B10.Q genetic background.

To characterize the bacterial strains responsible for the infections, samples from infected animals were assessed. All isolates could be grown on hematin agar at 37°C under aerobic conditions. Species identification was based on 16S rDNA sequence analysis and phenotypic tests. The sequences of the isolates were identical to the 16S rRNA genes either of S. aureus or S. saprophyticus/xylosus. The S. aureus strain was denominated 1056A. Strains determined to belong to the family of S. saprophyticus and S. xylosus with 16S were differentiated and identified to be S. xylosus by their ability to ferment xylose and mannose in oxidative-fermentative medium (21). Two S. xylosus strains were further

<table>
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<tr>
<th>Table I. Number and frequency of observation of local infections in Ncf1 mutant mice in the Stockholm mouse cohort</th>
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<td>Genotype</td>
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<tr>
<td>B10.Q.Ncf1^+/−</td>
</tr>
<tr>
<td>B10.Q.Pcf1^+/−</td>
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<tr>
<td>B10.Q.Ncf1^+/−,μMT</td>
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<tr>
<td>B10.Q.Ncf1^+/−,COMP ko</td>
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<tr>
<td>B10.Q.Ncf1^+/−,TIR ko</td>
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<td>B10.Q.Ncf1^+/−,background</td>
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Number of Ncf1^+/− mice from different lines with infections (most commonly in paws, nose, neck, and ear) during 2009 and 2010. No infections were seen in any other mice, including the B10.Q.Ncf1^+/− (wt) controls or heterozygous B10.Q.Ncf1^+/− mice. The frequency is calculated by dividing the number of observed cases by the total number of mice, males, and females are shown.

**Statistical significance for the gender difference was calculated with the χ² test: *p < 0.05, **p < 0.01, ***p < 0.001.

The number of B10.Q.Ncf1^+/−,μMT mice could only be estimated. For the other strains, the total number of males, females, and strains are shown.
characterized using the DiversiLab platform and were denominated \textit{S. xylosus} 720 and \textit{S. xylosus} 1056B. Interestingly, all investigated samples from the animal house in Lund carried the \textit{S. xylosus} 720 strain, whereas all samples from the Stockholm facility were either the \textit{S. xylosus} 1056B strain or the \textit{S. aureus} 1056A strain. Thus, B10.Q.\textit{Ncf1}^{	ext{+/+}} mice exhibited increased susceptibility to spontaneous staphylococcal infections, with the mouse facility influencing the acquisition of specific bacterial strains.

\textit{Administered \textit{S. xylosus} and \textit{S. aureus} infections induced higher mortality in \textit{Ncf1} mutant mice compared with \textit{Ncf1} wt mice}.

Based on our observations of spontaneous infections in \textit{Ncf1}^{	ext{+/+}} mice, we evaluated susceptibility of \textit{Ncf1}^{	ext{+/+}} mice versus \textit{Ncf1}^{	ext{+/+}} (\textit{Ncf1}+/+) mice to systemic infection. About $1 \times 10^8$ CFU of the isolated staphylococcal strains \textit{S. xylosus} 720 and 1056B or \textit{S. aureus} 1056A were injected i.v. in \textit{Ncf1}^{	ext{+/+}} and \textit{Ncf1}^{	ext{+/+}} mice. \textit{Ncf1}^{	ext{+/+}} mice were more sensitive to infection than were \textit{Ncf1}^{	ext{+/+}} mice based on mortality (Fig. 2B, 2D, 2F). The bacteria differed greatly in virulence: \textit{S. xylosus} 720 and \textit{S. aureus} 1056A caused lethal infection, whereas \textit{S. xylosus} 1056B did not result in lethality in \textit{Ncf1}^{	ext{+/+}} mice (Fig. 2B, 2D, 2F).

Weight loss observed during the infection was comparable in \textit{Ncf1}^{	ext{+/+}} and \textit{Ncf1}+/+ mice (Fig. 2A, 2C, 2E). However, the anal-

![Figure 1](image1.png)

**FIGURE 1.** Spontaneous infections in soft tissue of \textit{Ncf1} mutant mice. Photograph of inflamed ear (A) and paw (B) of \textit{Ncf1} mutant mice with spontaneous infection. (C-H) Histology of inflamed and healthy front paws. After fixation in paraformaldehyde and decalcification, the paws were sectioned and stained with H&E. Front paw from a healthy mouse (C) and from an infected one (D) (original magnification $\times$2.5). (E) Markedly enlarged, inflamed paw from an infected mouse shows profound, chronic inflammation with numerous abscesses (ABS) and cellulitis. Bones (B) and necrosis of bone (arrow) are visible. The abscess marked ABS* is shown in more detail in (F) (original magnification $\times$2.5). (F) Detail of the abscess marked ABS* in (E). The arrows point to some of the bacterial colonies. The structure at the periphery of the abscess (arrowhead) is shown in more detail in (G) (original magnification $\times$10). (G) Close view of the structure marked with an arrowhead in (F). It exhibits necrotic leukocytes, bacteria, and cell debris (*** surrounded by brightly eosinophilic, radial Sprodore–Hoeppli material (arrowhead). Part of this material also appears intracellularly in degenerate and necrotic macrophages (arrows) positioned at the periphery of the abscess. The vast majority of cells visible in this figure are neutrophils (original magnification $\times$60). (H) Detail of abscess in paw shows a colony of Gram-positive cocci immersed in exudate of neutrophils, as well as some macrophages (Gram stain, original magnification $\times$60).

![Figure 2](image2.png)

**FIGURE 2.** \textit{Ncf1} mutant mice have increased susceptibility to systemic \textit{S. xylosus} and \textit{S. aureus} infections. A total of 0.8–1.4 $\times$ 10^8 bacteria of the two strains of \textit{S. xylosus} (720 and 1056 B) and the \textit{S. aureus} 1056A isolated from infected paws of \textit{Ncf1} mutant mice were injected i.v. into \textit{Ncf1}^{	ext{+/+}} mice (\textit{Ncf1}^{	ext{+/+}}; \textit{n} = 10 for \textit{S. aureus} and \textit{S. xylosus} 720; \textit{n} = 5 for \textit{S. xylosus} 1056 A) and into wt (\textit{Ncf1}^{	ext{+/+}}; \textit{n} = 10). Mean and SEM are shown. Weight loss (A, C, E) and survival (B, D, F) were measured up to 7 d after bacterial challenge. Survival curves were displayed as Kaplan–Meier curves, and intergroup comparisons were assessed by the log-rank method. $^*$, $p < 0.05$, $^{**}$, $p < 0.01$
ysis of this parameter is affected by the high mortality in the 
Ncf1 \(+/+\) group, because only few surviving Ncf1 \(+/+\) mice could be 
evaluated for weight loss. These data indicate that Ncf1 \(+/+\) mice 
are more susceptible to systemic \(S.\) \(aureus\) (with variability in 
strain virulence) and \(S.\) \(aureus\) infection than are Ncf1 \(-/-\) mice.

S. aureus-induced arthritis was less severe in Ncf1 mutant mice 
compared with Ncf1 wt mice

We previously showed that experimental autoimmune arthritis is 
more severe in Ncf1 \(+/+) mice compared with Ncf1 \(+/+) mice (12). To 
investigate whether this is also the case with septic arthritis, we 
 injected \(5.7 \times 10^7\) \(S.\) \(aureus\) LS-1 bacteria i.v. into Ncf1 \(+/+) and 
Ncf1 \(+/+) mice. The LS-1 strain of \(S.\) \(aureus\) was selected because 
of its propensity to cause septic arthritis in mice (25). Both groups 
developed arthritis with a similar frequency, but Ncf1 \(+/+) mice had 
significantly greater arthritic severity (Fig. 3A, 3B). Ncf1 \(+/+) mice 
lost more weight compared with Ncf1 \(+/+) littermates (Fig. 3C) and 
had a higher bacterial count in the kidneys after bacterial infection 
(Fig. 3D). No significant difference between the genotypes was 
observed in bacterial counts from blood at days 1 and 3 postin-
fec tion (data not shown). Histology showed infiltration of cells 
into the joints and tissue and bone destruction (Fig. 3E, 3F). These 
data indicate that Ncf1 \(+/+) mice have impaired clearance of bacte-
ria but a milder septic arthritis than Ncf1 \(+/+) mice. Because 
Ncf1 \(+/+) mice are prone to more severe autoimmune chronic ar-
thritis (12), our results suggest that NOX2 has distinct roles in 
modulating the inflammatory response in autoimmune and septic 
arthritis.

Expression of Ncf1 in monocytes protects from infection after 
\(S.\) \(aureus\) and \(S.\) \(xylosus\) infection

Previously, we generated transgenic mice (transgene designated 
MN) on the B10.Q.Ncf1 \(+/+) background (Ncf1 \(+/+) MN\(^{-/-}\)) that 
express the functional form of NCF1 through the human CD68 
promoter, leading to restricted expression of NCF1 to monocytes 
and macrophages. Ncf1 \(+/+) MN\(^{-/-}\) mice were partially protected 
from autoimmune arthritis compared with Ncf1 \(+/+) mice (18). We 
observed that Ncf1 \(+/+) MN\(^{-/-}\) mice did not develop spontaneous 
infections; thus, it became relevant to investigate whether Ncf1 \(+/+) 
MN\(^{-/-}\) mice were protected from administered infections.

We previously reported that, in Ncf1 \(+/+) MN\(^{-/-}\) mice, NCF1 is 
specifically expressed on CD68\(^{+}\) macrophages and not on other 
cells, such as B cells and T cells (18); we have now confirmed that 
its expression is not detected in neutrophils, defined as CD11b\(^{+}\)Ly6G\(^{-}\)- 
expressing cells (26) (Fig. 4A, Supplemental Fig. 1A, 1D). In 
Ncf1 \(+/+) MN\(^{-/-}\) mice, there was full expression of NCF1 in 
monocytes/macrophages (CD11b\(^{+}\)Ly6G\(^{-}\) cells) comparable to 
the levels in wt monocytes/macrophages but no expression in neu-
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the presence of detectable NOX2-dependent ROS, even in naive animals, and the crucial role of the Ncf1 mutation in abolishing it.

We next evaluated susceptibility to administered infection in transgenic Ncf1\(+/+\) MN\(+/-\) mice compared with littermates negative for the transgene (Ncf1\(+/+\)) and wt Ncf1\(+/+\) mice. Mice were injected i.v. with S. xylosus (1 × 10^8 CFU) and monitored for 7-d survival. Ncf1\(+/+\) mice died within 24 h, whereas all Ncf1\(+/+\) mice survived. Mortality was observed in 30% of Ncf1\(+/+\) MN\(+/-\) mice on days 6 and 7 (Fig. 5A). Between the surviving groups, Ncf1\(+/+\) MN\(+/-\) mice lost more weight than did Ncf1\(+/+\) mice (Fig. 5B). Together, these data indicate that a functional NOX2 complex expressed in monocytes protects from S. xylosus challenge, although the transgene may not confer full protection compared with fully NOX2-competent Ncf1\(+/+\) mice.

Transgenic Ncf1\(+/+\) MN\(+/-\) mice and littermates negative for the transgene (Ncf1\(+/+\)), as well as Ncf1\(+/+\) mice, were next evaluated for their response to the arthriticogenic S. aureus LS-1 strain. Weight and arthritis were monitored for 7 d following bacterial administration. Male Ncf1\(+/+\) mice lost significantly more weight than did Ncf1\(+/+\) and Ncf1\(+/+\) MN\(+/-\) mice (Fig. 5C).

No difference in lethality was observed between the groups (data not shown). Bacterial load in the kidney at days 2, 4, and 7, in synovial fluid at day 7, and in blood at day 1 did not differ among the genotypes (data not shown). Arthritis developed in all groups but with very low scores (data not shown).

Ncf1 expression in monocytes rescued mice from lethal B. cepacia infection

B. cepacia is a Gram-negative rod that can cause severe infections in CGD patients (29, 30). Studies in isolated human neutrophils showed a key role for NOX2 in killing B. cepacia (31). Genetically engineered Ncf1 ko (Ncf1\(-/-\) or p47\(^{phox}\)\(-/-\)) mice had increased susceptibility to B. cepacia compared with wt mice (32).

To evaluate the specific contribution of monocyte NOX2 expression in the defense against B. cepacia, we evaluated survival and bacterial clearance in Ncf1\(+/+\) MN\(-/-\) mice and Ncf1\(+/+\) MN\(-/-\) mice. Mice were challenged i.p. with a B. cepacia strain isolated from a CGD patient. All Ncf1\(+/+\) mice died within 6 d postinfection, whereas Ncf1\(+/+\) MN\(-/-\) mice had long-term survival (Fig. 6A).

Engineered Ncf1\(+/+\) mice in the C57BL/6 lineage were tested in parallel to determine whether ko and spontaneous mutant mice differed in response to bacterial infection. Ncf1\(+/+\) mice had a similar time course as did Ncf1\(+/+\) mice, whereas wt C57BL/6 mice survived. Thus, spontaneous and engineered NOX2-deficient mice were highly susceptible to B. cepacia, whereas transgenic mice with monocyte NADPH expression were protected.

We next compared clearance of B. cepacia at a fixed time point after bacterial challenge in Ncf1\(+/+\) MN\(-/-\) and Ncf1\(+/+\) MN\(-/-\) mice. Mice administered the same inoculum were sacrificed at 24 h, according to their scatter and CD11b and Ly6G expression by flow cytometry. DHR fluorescence was measured at different time points after PMA stimulation. Different-colored graphs represent different time points. (D) Geometric mean of DHR fluorescence at different time points after PMA stimulation. (E) In vivo oxidative burst imaging: luminescent probe L-012 was injected i.p. in naive anesthetized mice, and the luminescent signal was detected over 35 min by a CCD camera. Images illustrate a representative example for each genotype. (F) To quantify the in vivo oxidative burst, the luminescent signal was quantified at 15 min as photons/cm^2/s/steradian in the circled areas surrounding the front paws, and the mean of the two front paw measurements was used. Mean and SEM of five mice/group. *p < 0.05, **p < 0.01, Kruskal–Wallis test with the Dunn comparison posttest.
Ncf1 curves were displayed as Kaplan–Meier curves, and intergroup comparisons were assessed by the log-rank method. ***p < 0.001. Mean and SEM are shown in (B). *p < 0.05, Mann–Whitney test. (C) Male Ncf1+/− mice (n = 9), as well as littermates negative for the transgene (Ncf1−/−, n = 10) and Ncf1 wt (Ncf1+/+) mice (n = 7), were injected i.v. with 5.7 × 10⁵ S. aureus LS-1. Weight was monitored for 7 d. Mean and SEM are shown. *p < 0.05, **p < 0.01, Ncf1−/− versus Ncf1+/+ mice, #p < 0.05, Ncf1−/+ MN−/− versus Ncf1+/+ mice, Kruskal–Wallis test with the Dunn comparison posttest.

FIGURE 5. NCF1 expression in monocytes protects from staphylococci. A total of 0.8 × 10⁸ S. xylosus 720 isolated from infected paws of Ncf1 mutant mice were injected i.v. into Ncf1−/− MN−/− mice and nontransgenic littermate Ncf1−/− mice, as well as Ncf1 wt (Ncf1+/+) mice (n = 6–9). Survival (A) and weight change (B) were monitored over 7 d. Survival curves were displayed as Kaplan–Meier curves, and intergroup comparisons were assessed by the log-rank method. ***p < 0.001. Mean and SEM are shown in (B). *p < 0.05, Mann–Whitney test. (C) Male Ncf1+/− MN−/− mice (n = 7), were injected i.v. with 5.7 × 10⁵ S. aureus LS-1. Weight was monitored for 7 d. Mean and SEM are shown. *p < 0.05, **p < 0.01, Ncf1−/− versus Ncf1+/+ mice, #p < 0.05, Ncf1−/+ MN−/− versus Ncf1+/+ mice, Kruskal–Wallis test with the Dunn comparison posttest.

FIGURE 6. NCF1 expression in monocytes protects Ncf1−/− mice from lethal B. cepacia infection. B10.Q.Ncf1−/− MN−/− mice and B10.Q.Ncf1+/− littermates negative for the transgene, as well as Ncf1−/− and C57BL/6 mice (n = 10), were challenged i.p. with 4.5 × 10⁸ B. cepacia. (A) Survival curves were displayed as Kaplan–Meier curves, and intergroup comparisons were performed between groups using the log-rank method. ***p < 0.001. (B) In separate studies, mice were administrated 4 × 10⁸ B. cepacia i.p., and bacteria load in peritoneal lavage, spleen, kidneys, and lung cell suspensions were quantified at 24 h. Data are the combined results of two independent experiments (n = 8 mice/genotype). Mean and SEM are shown. *p < 0.004, Mann–Whitney U test, **p < 0.04, Wilcoxon signed-rank test, which was used when bacterial cultures from a group were negative.

Discussion

Our results show that monocyte-specific expression of the NOX2 regulatory component, NCF1, protected mice from lethal infections with staphylococci and B. cepacia, pathogens that commonly affect CGD patients. Interestingly, a similar phenomenon was recently described in humans with regard to susceptibility to tuberculosis. A macrophage-specific impairment of the core subunit of the NOX2 complex, gp91phox, resulted in susceptibility to tuberculous mycobacterial disease (33). These observations suggest that a functional NOX2 complex in monocytes is important for clearance of specific intra- and extracellular bacteria.

Transgenic mice, in which a gene of interest is expressed under the control of the human CD68 promoter, have been widely used to achieve monocyte/macrophage-restricted expression (34, 35) based on the monocyte predominance of CD68 expression. Using the same promoter, we confirmed a monocyte/macrophage-specific NCF1 expression in Ncf1−/− MN−/− mice versus Ncf1+/+ mice. A previous publication from our group showed that, in Ncf1−/− MN−/− mice, the expression of Ncf1 was undetectable in dendritic cells (DCs) (18). A more sensitive analysis revealed expression of functional NCF1 protein in DCs from Ncf1−/− MN−/− transgenic mice (A. Pizzolla and R. Holmdahl, unpublished observations). Because myeloid DCs and monocytes have a shared lineage, it is not unexpected that CD68 promoter activity would be present in both cells, as reported recently (36). NOX2 is expressed in DCs and could have a role in Ag display and priming T cell responses (37). However, the role of DCs in controlling acute bacterial infection, if any, is unclear. Ncf1−/− MN−/− mice showed a low, but significant, DHR staining, indicating the presence of ROS in neutrophils after activation in vitro. In this article, neutrophils are defined as CD11b+Ly6G+ cells. Although it is difficult to formally exclude any NCF1 expression in neutrophils, we could not detect the protein by flow cytometry in blood CD11b+Ly6G+ cells. It is possible that a fraction of those cells transcribes the human CD68 promoter or that NCF1 is expressed as a result of promoter-independent expression. Kuhns et al. (38) recently showed that, among CGD patients, even very low levels of neutrophil NOX2 was associated with better outcomes compared with patients with complete NOX2 deficiency. Studies of isolated neutrophils showed that H₂O₂ generated by NOX2-competent neutrophils necessary to protect the host is difficult to determine. Studies on CGD carriers and genetically corrected CGD mouse models showed that a small fraction of ROS-producing neutrophils, ~10% of the total, could efficiently protect from bacterial and fungal infection (41, 42). Nevertheless, in these studies, all phagocytes, both neutrophils and monocytes, expressed the functional NOX2 complex and, most commonly, only the neutrophils’ ROS production was determined. Therefore,
the immunological relevance of neutrophils and monocytes separately could not be assessed.

The NOX2 complex accounts for most of the ROS production that is essential for antimicrobial defense, because mutations in any of the genes coding for the subunits of the complex lead to a CGD phenotype in humans (4, 7). The NOX2 complex also generates the majority of the ROS observed in vivo, because the mutation in Ncf1 abolishes the visible ROS production in both naïve (Fig. 4E, 4F) and arthritic mice (24, 43). In addition, ROS produced by other sources, such as the mitochondrial respiratory chain, were described to have bactericidal activity in macrophage cell lines (44).

Similar to the situation in CGD patients, a naturally occurring mutation in Ncf1 mice increases the risk for uncontrolled bacterial infections. The infections observed in Ncf1 mutant mice were caused by S. aureus and S. xylosus. S. aureus is a common pathogen in CGD patients (3) and is commonly found on the skin of healthy individuals, but it has not been reported in mouse models of CGD. S. xylosus was found to cause abscesses in soft tissue of Ncf1-/- mice similar to those observed in Ncf1+/+ mice (8). Environmental factors could be relevant in determining the type and frequency of infections. The conditions under which the mice were maintained and the bacterial flora carried by the handlers could explain the difference in infectious strains observed in various colonies of Ncf1+/+ and Ncf1-/- mice. Staphylococci are normally nonpathogenic bacteria for mice, because wt mice have not developed any infection. The infection spreads rapidly in the cage between homozygous Ncf1+/+ offspring of Ncf1+/+ parents. Males are affected more often than females, which could be due to the fact that males fight more than females, resulting in wounds. A wound was always observed in the infected area, and it is likely to be the access for bacteria into the soft tissue. Recently, it was reported that NOX2-deficient mice develop spontaneous inflammation in their paws (45), which phenotypically resembled the infections observed in Ncf1-/- (11) and Ncf1+/+ animals (Fig. 1A).

The involvement of cells other than phagocytes in the NOX2-dependent clearance of pathogens does not seem to play a role in spontaneous infections. The absence of B cells or T cells did not dramatically alter the frequency of spontaneous infection by staphylococci. In both mice and humans, it is known that an effective immunological memory is not built after S. aureus infections [mechanisms reviewed by Foster (46)]. In addition to antimicrobial host defense, NOX2 is a critical modulator of inflammation. Indeed, one of the characteristics of CGD is excessive inflammation, including inflammatory bowel disease, granulomatous cystitis, and pneumonitis. Macrophage NOX2 may be important in controlling inflammation. We previously showed that monocyte/macrophage expression of NCF1 protects against autoimmune chronic inflammatory disease (18). There are a number of mechanisms by which macrophage NOX2 may limit inflammation, including internalization of apoptotic neutrophils (47–49); modulation of transcriptional factors, such as NF-kB and NFκB (50); modification of Ag processing (43, 51); or regulation of T cells during Ag presentation (52, reviewed in Ref. 43). A long-standing question has been whether this is secondary to the deficient protection against infections or whether the lack of oxidative burst promotes inflammatory disorders. In mice, arthritis directly caused by S. aureus infection is decreased in ROS-deficient mice, in contrast to autoimmune arthritis, for which we previously found increased inflammation (12).

In conclusion, monocyte NOX2 expression is associated with protection from spontaneous and induced bacterial infections. Further characterization of cell-specific molecular mechanisms of bacterial clearance is important for elucidating the pathways involved in microbial defense and for the development of targeted cures for CGD.

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Disclosures

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