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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 (p47phox) 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 x under the human CD68 promoter (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.

C hronic granulomatous disease (CGD) is an inherited disorder characterized by recurrent life-threatening bacterial and fungal infections and persistent inflammation (1). The pathogens vary according to the geographical area. In North America and Europe, the majority of the infections are caused by the following pathogens: Staphylococcus aureus, Burkholderia cepacia, Serratia marcescens, Nocardia spp., and Aspergillus spp. and other molds reviewed by Grimm et al. (2) and Holland (3) and van den Berg et al. (4). The impaired host defense is due to defective production of reactive oxygen species (ROS) by phagocytes caused by mutations in genes encoding the phagocyte NADPH oxidase 2 complex (NOX2) protein subunits (5, 6). CGD can result from recessive mutations in any of the five genes encoding subunits of the NOX2 complex (4, 7). X-linked CGD results from mutations in the cytochrome b-245 H chain catalytic subunit (also known as gp91phox or NOX2 and coded by CYBB) and accounts for ~70% of CGD cases. The most common form of autosomal recessive CGD results from mutations in the regulatory protein NCF1 (coted by NCF1 and also known as p47phox) and accounts for 20% of CGD cases (4). Knocking out Cybb or Ncf1 (the latter referred to as Ncf1−/−) generated mouse models of CGD that showed increased susceptibility to bacterial and fungal infections compared with wild-type (wt) mice (8–11). Spontaneous bacterial and fungal infections occurred in Ncf1−/− mice, with soft tissue infections by Staphylococcus xylosus being the most common (11).

We previously reported that a spontaneous single nucleotide mutation in the Ncf1 gene (referred to as Ncf18/8) results in impaired phagocyte NADPH oxidase activity and enhanced inflammatory responses in autoimmune chronic inflammatory diseases, such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (12). Because human CGD is most commonly caused by single mutations, we investigated whether Ncf18/8 mice exhibit the CGD phenotype regarding increased susceptibility to infections.

We observed that Ncf18/8 mice in separate mouse facilities occasionally developed spontaneous soft tissue infections with similar features as the ones reported in Ncf1−/− mice. The most common cultured isolates were S. xylosus, the same strain previously isolated in Ncf1−/− mice (11), as well as S. aureus, a frequent pathogen in CGD patients. Taken together, these results show that Ncf18/8 mice manifest both increased susceptibility to spontaneous infections and a more severe inflammatory phenotype in experimental models of autoimmune chronic inflammation.
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 antibiotic 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 at 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. 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 C57Black6 background. The C57BL/10.Qrhd background was confirmed to be homozygous, with the exception of the previously described (Ncf1<sup>m1J/Ncf1<sup>+</sup>) mutation in the Ncf1 gene, using a 10K SNP chip (12, 14). The MHC class II congenic C57BL/10.Qrhd (B10.Q) mice express MHC class II H2-A<sup>Z</sup> that is encoded by a fragment from the DBA/1 strain. The Ncf1<sup>m1J</sup> mutation impairs the function of NCF1, as described earlier (12). B10.PMBQ mice were described previously (15); they are transgenic mice in which H-2A<sup>d</sup> is expressed under the hCD68 promoter. B10.Q.B knockout (ko) mice were previously called B10.Q,μMT mice; they were obtained from backcrossing the original μMT 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>) and Btk<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>phox</sup>−/− mice), generated as previously described (11), 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 5 min at 95°C. PCR amplification was performed without further extraction of DNA, and amplification was carried out in a 50-μL reaction mixture containing 1× PCR buffer (QIAAgen), 3 mM 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. PCR was performed on a Biometra Thermo Cycler 9600, using a thermal cycler and a temperature program of 5 min at 95°C followed by 30 sec at 95°C, 1 min at 60°C, and 1 min at 72°C, with a final extension step of 10 min. One PCR product was 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 BMBlabett (Lund, Sweden). The sequences of the isolates were identical to the 16S rRNA 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 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* strain 720, 0.8–1.2 × 10<sup>7</sup> bacteria/mouse; *S. xylosus* strain 720, 1.4 × 10<sup>7</sup> bacteria/mouse; *S. aureus* LS-1, 5.7 × 10<sup>5</sup> bacteria/mouse; and *S. aureus* strain 1056A, 0.8 × 10<sup>7</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>7</sup> CFU i.p., an inoculum previously known to be lethal in p47<sup>phox</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 graded 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 (arthritis 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; and 3, marked swelling and erythema). The total score was calculated by adding up the scores for each of the forepaws 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, followed by 2 ml 60% 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 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 IgG1-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 Abs 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 LSRII 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 IgG1-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 IgG1-allophycocyanin and the geometric mean of cells stained with only anti-mouse IgG1-allophycocyanin: the geometric mean is represented.

**In vivo ROS detection**

Naive 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<sup>−/−</sup>) mice, but not in Ncf1<sup>−/−</sup> wt 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<sup>−/−</sup>/mice. Infections occurred most commonly 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<sup>−/−</sup>/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 RNA genes either of <i>S. aureus</i> or <i>S. saprophyticus</i> or <i>S. xylosus</i>. The <i>S. aureus</i> strain was denominated 1056A. Strains determined to belong to the family of <i>S. saprophyticus</i> and <i>S. xylosus</i> with 16S were differentiated and identified to be <i>S. xylosus</i> by their ability to ferment xylose and mannose in oxidative-fermentative medium (21).

Two <i>S. xylosus</i> strains were further

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total No. of Mice</th>
<th>Frequency (%)</th>
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<tr>
<td>B10.Q.Ncf1&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>~1.3</td>
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<tr>
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<td>3</td>
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<tr>
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<tr>
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<td>3</td>
</tr>
<tr>
<td>B10.Q.Ncf1&lt;sup&gt;−/−&lt;/sup&gt;/TCR ko</td>
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<td>2</td>
</tr>
<tr>
<td>Total B10.Q.Ncf1&lt;sup&gt;−/−&lt;/sup&gt; background</td>
<td>~2767</td>
<td>~2</td>
</tr>
</tbody>
</table>

Number of Ncf1<sup>−/−</sup> 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<sup>+/−</sup> (wt) controls or heterozygous B10.Q.Ncf1<sup>−/−</sup>/ mice. The frequency is calculated by dividing the number of observed cases by the total number of Ncf1<sup>−/−</sup> mice. B10.Q.Ncf1<sup>−/−</sup>/ TCR ko mice are lacking T cells (deficient in TCRβ), B10.Q.Ncf1<sup>−/−</sup>/μMT mice are lacking B cells (deficient in the membrane part of IgM), B10.Q.Ncf1<sup>−/−</sup>/COMP ko mice are deficient in COMP, and B10.P.Ncf1<sup>−/−</sup>/MBQ mice express H2-A<sup>Q</sup> specifically on macrophages. They are all on the B10.Q.Ncf1<sup>−/−</sup> genetic background and carried the C57BL/10.Q.rhd background and shared the C57BL/10.Q.rhd background.

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

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

Based on our observations of spontaneous infections in Ncf1+/− mice, we evaluated susceptibility of Ncf1+/− mice versus Ncf1 wt (Ncf1+/+) mice to systemic infection. About 1 × 10⁸ CFU of the isolated staphylococcal strains S. xylosus 720 and 1056B or S. aureus 1056A were injected i.v. in Ncf1+/− and Ncf1+/+ mice. Ncf1+/− mice were more sensitive to infection than were Ncf1+/+ mice based on mortality (Fig. 2B, 2D, 2F). The bacteria differed greatly in virulence: S. xylosus 720 and S. aureus 1056A caused lethal infection, whereas S. xylosus 1056B did not result in lethality in Ncf1+/− mice (Fig. 2B, 2D, 2F).

Weight loss observed during the infection was comparable in Ncf1+/− and Ncf1+/+ mice (Fig. 2A, 2C, 2E). However, the anal-

![Figure 1](http://www.jimmunol.org/Downloaded_from_http://www.jimmunol.org)  
**FIGURE 1.** Spontaneous infections in soft tissue of Ncf1 mutant mice. Photograph of inflamed ear (A) and paw (B) of 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 ×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 ×2.5). (G) 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 ×10). (H) Close view of the structure marked with an arrowhead in (F). It exhibits necrotic leukocytes, bacteria, and cell debris (*** surrounded by brightly eosinophilic, radial Splendore–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 ×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 ×60).

![Figure 2](http://www.jimmunol.org/Downloaded_from_http://www.jimmunol.org)  
**FIGURE 2.** Ncf1 mutant mice have increased susceptibility to systemic S. xylosus and S. aureus infections. A total of 0.8–1.4 × 10⁸ bacteria of the two strains of S. xylosus (720 and 1056B) and the S. aureus 1056A isolated from infected paws of Ncf1 mutant mice were injected i.v. into Ncf1 mutant (Ncf1+/−) mice (n = 10 for S. aureus and S. xylosus 720; n = 5 for S. xylosus 1056 A) and into wt (Ncf1+/+) mice (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 injection (Fig. 3D). No significant difference between the genotypes was observed in bacterial counts from blood at days 1 and 3 postinfection (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 bacteria but a milder septic arthritis than Ncf1+/+ mice. Because Ncf1−/− mice are prone to more severe autoimmune chronic arthritis (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 it is not expressed 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 neutrophils (CD11b−Ly6G− cells), as observed in neutrophils from bone marrow, blood, and spleen (Fig. 4B, Supplemental Fig. 1B, 1E). Intracellular ROS production was measured by flow cytometry using the probe DHR (27), which, upon oxidation, emits a fluorescent signal. PMA was used as activator of the NOX2 complex. Blood monocytes (CD11b+Ly6G+) from Ncf1+/+ mice showed almost the half of the DHR staining of monocytes from Ncf1+/− mice (Supplemental Fig. 1C). Blood neutrophils (CD11b+Ly6G+) from Ncf1−/− MN+/− mice had ~15% of the DHR staining of neutrophils from Ncf1−/− mice (Supplemental Fig. 1C). This observation was confirmed in splenic and thiglycollate-elicited peritoneal neutrophils (data not shown, Supplemental Fig. 1F). The discrepancy between the lack of NCF1 expression and the detectable DHR staining of neutrophils could be explained by leakage of ROS or other DHR-stained products from macrophages to neutrophils after PMA stimulation in vitro. To examine this hypothesis, neutrophils from bone marrow were purified with a Percoll gradient, stained for CD11b and Ly6G, and analyzed via flow cytometry for their capacity to oxidize DHR. PMA-stimulated ROS production was measured from CD11b+Ly6G+ cells from Ncf1+/+ MN+ mice was minimal (<1% of Ncf1+/+ neutrophils) but distinguishable from ROS production in CD11b+Ly6G+ cells from Ncf1−/− mice (Fig. 4C, 4D). To confirm that the ROS production in monocytes in Ncf1−/− MN+/− mice was also detectable in vivo, whole-body imaging of ROS production was performed using the probe L-012 (24), which is a sensitive luminal derivative that detects ROS in biological samples, including NOX2-derived superoxide (28). L-012 was injected i.p. in sedated naïve mice, and the chemiluminescent signal was recorded over time with a CCD camera. With the exception of the peritoneal area where the probe was injected, the luminescent signal was most easily detected in the paws, where fur is absent. Therefore, the signal was quantified around the front paws, as represented in Fig. 4E. In vivo oxidative burst was higher in paws from naïve Ncf1+/− and Ncf1−/− MN+/− mice compared with Ncf1+/+ mice. The signal from Ncf1−/− MN+/− mice was reduced compared with wt mice, as expected, because ROS-producing cells other than macrophages are present in healthy joints (Fig. 4F). This observation illustrates
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 × 10^7 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 arthritogenic 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 died with 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.

**FIGURE 4.** NCF1 expression and ROS quantification in bone marrow neutrophils from Ncf1+/+ MN−/− mice. (A) Bone marrow cells were stained for surface markers CD11b (Mac-1, M1/70) and Ly6G (1A8) and gated accordingly. CD11b+Ly6G− cells are considered monocytes, and CD11b+Ly6G+ cells are considered neutrophils. (B) Intracellular NCF1 expression was measured in both cell populations. Mean and SEM of five animals/group is shown. (C) Intracellular ROS production was measured in purified neutrophils from pooled bone marrows. After enrichment for neutrophils with Percoll gradient, cells were identified as neutrophils by 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/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.

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NCF1 expression in monocytes protects from staphylococci.

A total of $0.8 \times 10^8$ S. xylosus 720 isolated from infected paws of Ncf1 mutant mice were injected i.v. into Ncf1$^{+/+}$ MN-/- mice and transgenic 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 ($n = 9$), as well as littermates negative for the transgene (Ncf1$^{+/+}$, $n = 10$) and Ncf1 wt (Ncf1$^{+/+}$) mice ($n = 7$), were injected i.v. with $5.7 \times 10^7$ 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, and **$p < 0.05$, Ncf1$^{+/+}$ MN-/- versus Ncf1$^{-/-}$ mice. Kruskal–Wallis test with the Dunn comparison posttest.

a time that precedes the onset of clinical morbidity. Higher numbers of live bacteria were recovered from the peritoneum and spleens of Ncf1$^{+/+}$ MN-/- mice compared with Ncf1$^{+/+}$ MN-/- mice (Fig. 6B). No difference in bacterial counts in kidneys and lungs was observed between the genotypes (data not shown). The majority of blood cultures showed no growth, and no significant difference was observed between genotypes (data not shown). Taken together, the survival and bacterial clearance data demonstrate protection associated with monocyte NOX2 expression against B. cepacia. We conclude that NCF1 expression in monocytes plays an essential role in the systemic response to both spontaneous and induced bacterial infections.

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 neutrophil NOX2 was associated with better outcomes compared with Ncf1$^{-/-}$ MN-/- mice, ROS produced by monocytes could penetrate into low-bursting neutrophils, increasing their antimicrobial activity.

The importance of neutrophil ROS production in antimicrobial defense is well established (13). However, the critical amount of ROS-producing 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,
Further characterization of cell-specific molecular mechanisms of protection from spontaneous and induced bacterial infections. Increased inflammation (12). In contrast to autoimmune arthritis, for which we previously found the access for bacteria into the soft tissue. Recently, it was shown that males fight more than females, resulting in wounds. 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 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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