Neutrophils from p40<sup>phox</sup>−/− mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing

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The generation of reactive oxygen species (ROS) by the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex plays a critical role in the antimicrobial functions of the phagocytic cells of the immune system. The catalytic core of this oxidase consists of a complex between gp91<sup>phox</sup>, p22<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, and rac–2. Mutations in each of the phox components, except p40<sup>phox</sup>, have been described in cases of chronic granulomatous disease (CGD), defining their essential role in oxidase function. We sought to establish the role of p40<sup>phox</sup> by investigating the NADPH oxidase responses of neutrophils isolated from p40<sup>phox</sup>−/− mice. In the absence of p40<sup>phox</sup>, the expression of p67<sup>phox</sup> is reduced by ~55% and oxidase responses to tumor necrosis factor α/fibrinogen, immunoglobulin G latex beads, Staphylococcus aureus, formyl-methionyl-leucyl-phenylalanine, and zymosan were reduced by ~97, 85, 84, 75, and 30%, respectively. The defect in ROS production by p40<sup>phox</sup>−/− neutrophils in response to S. aureus translated into a severe, CGD-like defect in the killing of this organism both in vitro and in vivo, defining p40<sup>phox</sup> as an essential component in bacterial killing.

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of neutrophils and macrophages plays an essential role in the mechanisms by which these cells destroy engulped pathogens (1, 2). The delivery of superoxide anion (O<sub>2</sub>−) by this enzyme complex into the phagosome is thought to indirectly activate several classes of protease (3), and O<sub>2</sub>− itself can be directly converted into a variety of destructive molecular species (reactive oxygen species [ROS] and halide derivatives) (4, 5). Together with the delivery of other nonoxidant-dependent microbicidal agents into the phagosome, e.g., defensins (6), the NADPH oxidase plays a central role in our defense mechanisms against invading microbes.

Although the role of the phagocyte NADPH oxidase in microbe killing is clear, it has become apparent that the same, or closely homologous, enzyme complexes also exist in many other cell types, e.g., in lymphocytes and endothelia (1, 5, 7). The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of neutrophils and macrophages plays an essential role in the mechanisms by which these cells destroy engulfed pathogens (1, 2). The delivery of superoxide anion (O<sub>2</sub>−) by this enzyme complex into the phagosome is thought to indirectly activate several classes of protease (3), and O<sub>2</sub>− itself can be directly converted into a variety of destructive molecular species (reactive oxygen species [ROS] and halide derivatives) (4, 5). Together with the delivery of other nonoxidant-dependent microbicidal agents into the phagosome, e.g., defensins (6), the NADPH oxidase plays a central role in our defense mechanisms against invading microbes.

Although the role of the phagocyte NADPH oxidase in microbe killing is clear, it has become apparent that the same, or closely homologous, enzyme complexes also exist in many other cell types, e.g., in lymphocytes and endothelia (1, 5, 7). The role of ROS produced by the NADPH oxidases in these other cell types is still largely undefined but is likely to include intra- and peri-cellular signaling via H<sub>2</sub>O<sub>2</sub>-mediated oxidation of critical cysteine residues in target proteins. Thus far, these target proteins are largely thought to be members of the protein tyrosine phosphate phosphatase superfamily that are intermediaries in cell surface receptor-regulated signaling pathways (8).

The core molecular components of the phagocyte NADPH oxidase are a b-type membrane-bound cytochrome consisting of gp91<sup>phox</sup> and p22<sup>phox</sup> subunits (cytochrome b<sub>558</sub>) and four soluble components: rac–2, p67<sup>phox</sup>, p47<sup>phox</sup>, and p40<sup>phox</sup> (1, 2). On activation, the soluble components form an active complex with the b-type cytochrome and electrons are transferred from NADPH across the membrane and are delivered to molecular oxygen to generate the superoxide anion, O<sub>2</sub>−. The importance of the NADPH oxidase is clearly witnessed by molecular defects in components of the NADPH oxidase, which lead to chronic granulomatous disease (CGD), a genetic disorder in which patients suffer recurrent fungal and bacterial infections.
infections (9, 10). Additionally, a phagocyte immunodeficiency has also been described in a patient with dysfunctional rac-2 (11). The molecular events that underlie NADPH oxidase activation are still incompletely understood, but there is now overwhelming evidence that key aspects are GTP-rac-2 binding to p67phox and multiple phosphorylation of p47phox (1, 2, 12), which promote complex formation with each other and the cytochrome to form a catalytically competent enzyme.

There is a very large range of stimuli that activate the oxidase in cells such as neutrophils and macrophages, and hence there is a large variety of receptor subtypes and associated signaling pathways that underlie this capability. Microbes are phagocytosed via a series of overlapping and redundant receptors, such as members of the integrin and antibody receptor families, depending on the organisms themselves and the type and degree of opsonization (13, 14), resulting in subsequent activation of NADPH oxidase on both the plasma and phagosomal membranes. Cells such as neutrophils also undergo rapid activation of their NADPH oxidase in response to soluble stimuli, such as formylated peptides (e.g., FMLP), leukotrienes (e.g., LTB4), and components of complement (C5a) (15). Furthermore, a variety of other inflammatory agents, which include cytokines such as TNF-α, GM-CSF, IL-8, and low doses of the primary stimuli themselves, collectively “prime” the oxidase, resulting in substantial up-regulation of NADPH oxidase activity in response to primary stimuli at the site of inflammation (15–17). As a consequence, a very large number of intracellular signaling pathways have been shown to influence NADPH oxidase activation. Progress has been made defining some of these pathways, e.g., the coupling of receptor activation to guanine nucleotide exchange on rac (18–21) and protein kinase C-mediated lipid binding driven by mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (25–31).

Among the NADPH oxidase subunits, p40phox is perhaps the least well understood. It is known to bind p67phox via reciprocal PB1 domain dimerization (32, 33), forming a tight complex in the cytosol of resting neutrophils (34, 35); however, there is some debate as to whether it is also found in a trimeric complex with p67phox and p47phox or whether this complex only occurs on activation or priming (22, 36, 37). p40phox is not required to reconstitute anionic detergent-stimulated oxidase activity in recombinant assays (34), and mutations in the p40phox gene have not been described in cases of CGD, leading to the view that it is not essential for NADPH oxidase activity. There are several reports, however, that suggest that, under some assay conditions, p40phox can influence oxidase activity in vitro (37–40) and several conflicting reports using cell-based assays that have suggested that p40phox is either a positive or negative regulator of the NADPH oxidase (41–43). p40phox has a clearly defined domain structure (PX, PB1, and SH3 domains) that is evolutionarily conserved and undergoes stimulated phosphorylation paralleling NADPH oxidase activation (44, 45). The PX domain has high specificity and affinity for binding the class III PI3K product PtdIns3P (37, 46, 47), but the physiological relevance of this interaction has yet to be shown in vivo. Similarly, the SH3 domain has been shown to bind a proline motif in p47phox with low affinity, but the true in vivo ligand for this domain has yet to be conclusively identified (36, 48, 49). Furthermore, although several binding partners for p40phox have been described, including moesin (50), corotinin (51), thioredoxin (52), and Ku70 (53), these have not immediately provided a molecular explanation for the role of p40phox. To define the requirement for p40phox in NADPH oxidase regulation, we have created mice with a lesion in the p40phox gene and present the first characterization of NADPH oxidase activation and bacterial killing in neutrophils from p40phox−/− animals.

RESULTS

p40phox−/− mice are viable and produce differentiated neutrophils

p40phox−/− mice were produced by standard gene-targeting strategies (see Materials and methods and Fig. S1 A, which is available at http://www.jem.org/cgi/content/full/jem.20052069/DC1). Two independent embryonic stem cell lines were used to derive two independent strains of p40phox−/− mice. These two strains could not be distinguished on the basis of any of the experiments presented, and, where appropriate, data is compiled from several animals of each strain. p40phox−/−, p40phox+/−, and p40phox+/+ animals were produced from the breeding of p40phox−/− heterozygotes in the expected Mendelian ratios and appeared healthy and fertile when kept under barrier conditions. p40phox−/− mice had normal organ weights (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20052069/DC1) and normal blood cell counts (Fig. 1 A).

We used a mouse monoclonal antibody to p40phox to investigate the tissue distribution of p40phox protein. Amongst the tissues examined, we could only find strong evidence for immuno-detectable protein that was absent in equivalent p40phox−/− samples (Fig. 1 B, arrowheads) in both bone marrow–derived neutrophils (BMNs) and in the spleen, suggesting that p40phox expression is highly restricted. We could not detect expression of p40phox in BMNs from p40phox−/− mice using three different monoclonal and polyclonal antibodies raised against p40phox (Fig. 1 C and unpublished data), indicating successful targeting of the p40phox gene. Expression of p47phox was unaffected by the absence of p40phox but expression of p67phox was significantly reduced (Fig. 1 C). Careful quantitation indicated that p67phox levels in BMNs from p40phox−/− mice were reduced to 44.8 ± 4.3% of wild-type levels (Fig. 1 E). p40phox−/− BMNs demonstrated 71.9 ± 3.9% and 15.3 ± 4.0% reductions in p40phox and p67phox, respectively (Fig. 1, D and E).
BMNs from p40phox−/− mice were able to support normal phagocytic uptake of Staphylococcus aureus (Fig. 2 A) and zymosan (unpublished data), and normal activation of PKB, p38 MAPK, and p42/44 Erk. Graphs represent quantification of phospho-protein levels ± the range from a single experiment representative of three experiments. Black bars, +/+; white bars, −/−. Units are arbitrary.

**Figure 1.** Blood cell counts and phox protein expression in p40phox−/− mice. (A) Peripheral blood from p40phox+/+ and p40phox−/− animals was analyzed in a Vetabc animal blood cell counter. WBC, white blood cells; RBC, red blood cells; Plt, platelets; Lymph, lymphocytes; Mono, monocytes; Gran, granulocytes. Data represented are mean percentage of wild-type counts ± SE (n = 12). (B) 30 μg of clarified, homogenized tissues from p40phox−/− and p40phox+/+ animals was subjected to SDS-PAGE and immunoblotted for p40phox. Arrowheads indicate position of p40phox. We routinely observed immunoreactive bands in the spleen and thymus from p40phox−/− animals. We do not know whether they are products of alternative splicing of p40phox gene transcripts. (C) BMNs from p40phox−/−, p40phox+/−, and p40phox−/− animals were sonicated into SDS sample buffer, subjected to SDS-PAGE, and immunoblotted for p40phox, p47phox, and p67phox. Graphs represent quantitation of p40phox (D) and p47phox (E) levels in BMNs as mean percentage of wild-type samples ± SE (n = 10–13) from 6–10 independent experiments using three to six mice per preparation.

**Figure 2.** p40phox−/− neutrophils exhibit normal S. aureus phagocytosis and FMLP-induced signal transduction. (A) Primed p40phox+/+ and p40phox−/− BMNs were adhered to glass and allowed to phagocytose serum-opsonized, FITC-labeled S. aureus for 40 min. Coverslips were washed and fixed, and the mean number of internalized bacteria per neutrophil was determined. 300 neutrophils were examined (n = 2). (B) p40phox+/+ and p40phox−/− BMNs were incubated for 1min in the presence (+) or absence (−) of 10 μM FMLP. Resultant lysates were split between two blots and probed with phospho- (P) and total (T) antibodies against PKB, p38 MAPK, and p42/44 Erk. Graphs represent quantification of phospho-protein levels ± the range from a single experiment representative of three experiments. Black bars, +/+; white bars, −/−. Units are arbitrary.

**p40phox−/− neutrophils show substantially reduced oxidase activation in response to several soluble stimuli**

We characterized production of ROS in response to FMLP in either unprimed, mTNF-α−, or mGM-CSF–primed BMNs isolated from p40phox−/−, p40phox+/−, and p40phox+/+ mice. ROS production was measured by a horseradish peroxidase (HRP)-dependent chemiluminescence assay that provides a rate measure of O₂⁻ production into the extracellular space. ROS production under each of these circumstances was severely reduced in the p40phox−/− mice (~69–84% defective; Fig. 3, A–C). The kinetics of the remaining ROS production were similar in p40phox−/− neutrophils compared with the wild-type, and the dose-response curves to FMLP showed only a small shift to higher concentrations (Fig. 3, A–C). PMA is often used to bypass cell surface receptors and induce a more direct activation of the NADPH oxidase via, amongst less well-defined pathways, a direct protein kinase C-mediated phosphorylation of p47phox. PMA-induced production of ROS in p40phox−/− neutrophils was also substantially reduced compared with the wild-type (Fig. 3 D) and showed negligible shifts in the dose-response curves. **p40phox−/− neutrophils show variable reductions in oxidase activation in response to different particulate stimuli**

We characterized ROS responses in both primed and unprimed BMNs from p40phox−/− and p40phox+/+ mice in response to various particulate stimuli. We initially measured ROS production using luminol-dependent luminescence in the absence of HRP, which previous work has indicated is a rate measure of ROS production in the vicinity of endogenous peroxidases, largely thought to arise from the coincident deposition of ROS and myeloperoxidase into the phagosome (54). This is supported by our own data indicating little effect of adding extracellular HRP in these assays (unpublished data). Under these circumstances, there were significant differences in the relative reductions in ROS production seen with p40phox−/− neutrophils compared with the wild-type, depending on the nature of the stimulus (Fig. 4).

Priming the cells with mTNF-α and mGM-CSF caused a significant but relatively minor increase in the rate of ROS
generation in response to both unopsonized and opsonized zymosan and *S. aureus*, and a major increase in both the rate and magnitude of ROS generation in response to soluble stimuli. BMNs from p40<sup>−/−</sup>, p40<sup>/+</sup>, and p40<sup>+/+</sup> animals were analyzed for soluble stimulus-induced ROS production using HRP-dependent, luminol-dependent chemiluminescence. Duplicate wells of BMNs (3.75 × 10<sup>5</sup>/well) were stimulated in a luminometer and ROS production (measured in relative light units per second, rlu/sec) was followed over time. 10 μM of unprimed FMLP (A), 10 μM mTNF-α–primed FMLP (B), 10 μM mGM-CSF–primed FMLP (C), and 300 nM PMA (D). Response kinetics (top line graph), total integrated responses as percentage of wild-type (bar graph), and agonist dose-response curves (bottom graph) are shown. Dose-response curves are standardized to 100% with 300 nM PMA and 30 μM FMLP, respectively. Black, +/++; gray, +/−; white, −/−. All data are means ± SE (*n* = 6) from three independent experiments using three to six mice per preparation.

**Figure 3.** p40<sup>−/−</sup> neutrophils have severe defects in ROS production in response to soluble stimuli. BMNs from p40<sup>−/−</sup>, p40<sup>/+</sup>, and p40<sup>+/+</sup> animals were analyzed for soluble stimulus-induced ROS production using HRP-dependent, luminol-dependent chemiluminescence. Duplicate wells of BMNs (3.75 × 10<sup>5</sup>/well) were stimulated in a luminometer and ROS production (measured in relative light units per second, rlu/sec) was followed over time. 10 μM of unprimed FMLP (A), 10 μM mTNF-α–primed FMLP (B), 10 μM mGM-CSF–primed FMLP (C), and 300 nM PMA (D). Response kinetics (top line graph), total integrated responses as percentage of wild-type (bar graph), and agonist dose-response curves (bottom graph) are shown. Dose-response curves are standardized to 100% with 300 nM PMA and 30 μM FMLP, respectively. Black, +/++; gray, +/−; white, −/−. All data are means ± SE (*n* = 6) from three independent experiments using three to six mice per preparation.

To confirm that ROS accumulation by p40<sup>−/−</sup> BMNs in response to IgG–Zym was directed into the phagosome, we took advantage of the fact that O<sub>2</sub>−–catalyzed reduction of soluble formazan salts to form insoluble, colored products can be used as a semiquantitative assay for the location of NADPH oxidase activity. Substantial formation of dark purple nitroblue tetrazolium (NBT) deposits could be seen in the IgG coating (Fig. 4); thus, it is reasonable to assume that an Fc receptor of some type plays a central role in the ROS response to this stimulus. p40<sup>−/−</sup> neutrophils exhibited a very severe defect in ROS production to IgG latex beads (~85%; Fig. 4), indicating that the signaling pathways from Fc receptors to the oxidase are highly dependent on p40<sup>−/−</sup>. Given the requirement for both ROS and peroxidase activity in the luminol-dependent chemiluminescence assay, and the potential ambiguity in interpretation of a deficiency that this measurement provides, we also sought to measure NADPH oxidase activity in p40<sup>−/−</sup> and p40<sup>/+</sup> neutrophils by measuring the rate of O<sub>2</sub> consumption. It has been shown previously that NADPH oxidase–catalyzed consumption of O<sub>2</sub> during a stimulated “respiratory burst” dominates O<sub>2</sub> consumption by other routes, e.g., mitochondrial respiration (55), and hence provides a measure of substrate use that should be independent of the location of the oxidase or the pathways of ROS use. PMA−, IgG−Zym−, and S. aureus–stimulated O<sub>2</sub> consumption showed similar relative differences between p40<sup>−/−</sup> and p40<sup>+/+</sup> BMNs as those previously described by the chemiluminescence measurements (Fig. 5, A and B). Thus, the p40<sup>−/−</sup> neutrophils showed large deficiencies in O<sub>2</sub> consumption when challenged with PMA and *S. aureus* yet a relatively minor deficiency when challenged with zymosan, despite similar absolute rates of O<sub>2</sub> consumption for each stimulus (Fig. 5 A).
Neutrophils attached to a variety of surfaces via their cell surface integrins can generate substantial quantities of extracellular ROS in response to particulate stimuli (e.g., TNF-α), chemoattractants (e.g., FMLP), or Fc receptors (FcyRIII in murine neutrophils) (56–58). We investigated the ability of BMNs from p40phox−/− mice attached to a fibrinogen surface to produce ROS in response to mTNF-α, a response previously established to be dependent on β2 integrin engagement (59, 60). p40phox−/− neutrophils exhibited a remarkable deficiency in this response compared with their wild-type controls (96.7 ± 0.7% reduced; Fig. 6, A and C). Similar defects were seen upon costimulation with FMLP and mGM-CSF (unpublished data). p40phox−/− neutrophils also exhibited a severe deficiency in activation of their oxidase in response to direct cross-linking of their β2 integrins by an anti-β2 antibody (95.1 ± 2.7% reduced; Fig. 6, B and C), a response that recent work has suggested is probably mediated via cooperation between β2 integrin and FcyRIII activation (58). p40phox−/− neutrophils expressed normal levels of cell surface β2 integrins and demonstrated no defect in TNF-α–induced adhesion and spreading on fibrinogen (Fig. 6, D and E).

Figure 4. p40phox−/− neutrophils have differing defects in ROS production in response to particulate stimuli. BMNs from p40phox−/+ and p40phox−/− animals were analyzed for particulate stimulus-induced ROS production using luminol-dependent chemiluminescence (filled symbols, +/+; open symbols, −/−). Triplicate wells of mock-primed or mTNF-α−, mGM-CSF–primed BMNs (3.75 × 10⁶ BMN/well for latex beads stimulations, 1.25 × 10⁵ BMN/well for zymosan stimulations, and 1.25 × 10⁵ BMN/well for latex beads stimulations) were stimulated in a luminometer and ROS production (measured in relative light units per second, rlu/sec) was followed over time (A). Each horizontal panel of graphs is shown on the same scale. Data are means ± SE (n = 3–5) from three to five independent experiments using three to six mice per preparation.
Thus, p40\textsuperscript{phox} plays an essential role in the pathways between integrin engagement and oxidase activation in these contexts of neutrophil activation.

**p40\textsuperscript{phox−/−} neutrophils exhibit a severe deficiency in bacterial killing in vitro**

Killing of *S. aureus* by neutrophils in vitro is highly dependent on NADPH oxidase activity, consistent with the high frequency with which *S. aureus* infections present in cases of CGD (9, 10). In contrast, killing of *Escherichia coli* is relatively independent of neutrophil NADPH oxidase activity (61). In line with these conclusions, we found that the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) had a much greater effect on the killing of *S. aureus* by BMNs than the killing of *E. coli* (Fig. 7, A and B). BMNs from p40\textsuperscript{phox−/−} mice had a clear defect in the killing of *S. aureus* (~75% relative to wild-type) but an insignificant defect in the killing of *E. coli* (Fig. 7, A and B). This defect was mirrored in the killing of *S. aureus* by whole blood (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20052069/DC1). Using a range of DPI concentrations to construct a relationship between BMN NADPH oxidase activity and *S. aureus* killing (61), the NADPH oxidase deficiency of p40\textsuperscript{phox−/−} neutrophils correlates well with their deficiency in *S. aureus* killing (Fig. 7 C), suggesting that the two are causally linked.

**p40\textsuperscript{phox−/−} mice are deficient in clearing an *S. aureus* infection in vivo**

We sought to establish whether the substantial defect in the in vivo killing of *S. aureus* by p40\textsuperscript{phox−/−} neutrophils translated into a defect in the killing of this organism in the whole animal. We injected live *S. aureus* into the peritoneum of p40\textsuperscript{phox−/−} and p40\textsuperscript{phox+/-} mice and measured the number of surviving bacteria over the subsequent 24 h. We reproducibly recovered significantly more live *S. aureus* from the p40\textsuperscript{phox−/−} mice than their wild-type controls (Fig. 7 D).

**DISCUSSION**

We observed very substantial defects in NADPH oxidase activity in neutrophils isolated from p40\textsuperscript{phox−/−} mice, defining an essential role for p40\textsuperscript{phox} in the physiological regulation of this important enzyme complex. The degree of defect in NADPH oxidase activity varied greatly depending on the nature of the stimulus. Large defects were observed in intracellular ROS production in response to *S. aureus* or IgG latex beads, and smaller defects were observed in response to the uptake of zymosan. Extracellular ROS generation was absent in response to TNF-α/fibrinogen and greatly reduced in response to FMLP or PMA. ROS stimulation by TNF-α/fibrinogen is thought to occur via costimulation of β2 integrins and TNF-α receptors (59, 62). FMLP is thought to activate the oxidase via formyl-peptide receptors (63) and IgG to act via Fcγ receptors (64). *S. aureus* and zymosan are complex stimuli that are engulfed and activate the oxidase through a combination of inaccurately defined receptor types, but these are likely to include β2 integrin receptors (64) and β glucan receptors (65). Furthermore, these receptor types are likely to be extended in vivo through opsonization with complement or antibodies. Thus, it is clear that loss of p40\textsuperscript{phox} has a differential impact in the signaling pathways from different receptor subtypes. The extent of p40\textsuperscript{phox} involvement in the various physiological mechanisms of NADPH oxidase activation in neutrophils and other cell types will only be resolved with much further work, but our in vitro and in vivo...
models of oxidant-dependent bacterial killing already indicate that it is a critical component of our innate defense mechanism (Fig. 7). Importantly, the extent of the S. aureus killing defect is as severe as that observed with neutrophils from p47phox−/− CGD model mice (3, 66).

A key question is clearly the molecular mechanism by which p40phox expression is reduced (34, 67, 68), suggesting that p40phox and p67phox mutually regulate each other’s steady-state expression, and this is supported by the reduced p67phox levels in p40phox−/− neutrophils. Whether this regulation occurs at the level of protein stability, mRNA stability, or transcriptional/translational control is unknown. It is established that p67phox is an absolute requirement for reconstituted NADPH oxidase activity in vitro and, through characterized cases of CGD, also in vivo. Thus, the drop in p67phox levels in the p40phox−/− neutrophils is a confounding issue in trying to ascribe molecular consequences to the loss of p40phox. There is the possibility that this reduction in p67phox has an impact directly on the oxidase, and there is also the possibility that this has an impact indirectly, via altering the dependence on other subunits. Unfortunately, the absence of a p67phox−/− mouse model prevents a simple assessment of this impact in p67phox−/− mice; however, we note that human carriers of p67phox−/− deficient CGD do not present with a CGD phenotype (69). Further, the very large reductions in oxidase responses to TNF-α/fibrinogen and S. aureus in p40phox−/− neutrophils suggest that p40phox has a substantial role in these responses under normal circumstances.

Earlier work has suggested that PtdIns3P binding to the PX domain of p40phox may play a role linking PI3K signaling pathways to NADPH oxidase activation (22, 37, 46, 47). Furthermore, the discovery that PtdIns3P synthesis can occur on phagosomal membranes provided a context for this potential mechanism (70, 71). Evidence that this pathway does indeed operate in FcγRII receptor stimulation of the NADPH oxidase in the COS phox system is provided in an accompanying article by Suh et al. (on p. 1915 of this issue; reference 72). In this elegantly engineered cell model, an intact Ptd-Ins3P-binding PX domain of p40phox−/− neutrophils provides strong in vivo corroboration of this model. However, further work must clearly be done measuring elements of the known pathways to NADPH oxidase activation (e.g., p47phox/p67phox/p40phox phosphorylation, p47phox/p67phox/ββ-2 translocation to cytochrome b558) before the molecular consequences of the absence of p40phox can be properly ascribed, and it is likely that these will need to be done in the context of replenishment of p67phox and/or knock-in mutations of p40phox before clear answers emerge. However, the development of the p40phox−/− mouse does appear to offer a potential route to answering these questions.

The absence of examples of CGD patients characterized by mutations in p40phox together with enigmatic data arising from attempts to reconstitute the NADPH oxidase in cellular and a-cellular systems lead to the general view that p40phox does not have a critical role in NADPH oxidase activation. A counter argument arising from data describing widespread tissue expression of p40phox suggested that this protein may have a wider role outside of phagocytes (73), precluding the presentation of p40phox dysfunction as CGD. This argument is
not supported by our initial characterization of p40<sub>phox</sub> expression in the p40<sub>phox</sub>-/- mice, which did not provide evidence for high expression of p40<sub>phox</sub> outside the haemopoetic system, nor by the fact that p40<sub>phox</sub>-/- mice appear healthy when raised under pathogen-free conditions. It seems plausible that either a loss of p40<sub>phox</sub> presents a mild phenotype in humans or, indeed, that several uncharacterized cases of CGD-like immunodeficiencies may arise from as yet undescribed mutations in the p40<sub>phox</sub> gene.

**MATERIALS AND METHODS**

Murine GM-CSF (mGM-CSF), FMLP, luminol, DPI, and HRP were from Sigma-Aldrich. Murine TNF-α (mTNF-α) was from R&D Systems. All buffer components were from Sigma-Aldrich and were endotoxin free or low endotoxin, as available. The following antibodies were used: anti-phospho-Ser473 PKB (Ab4802; Abcam), anti-PKB (9272; Cell Signaling), anti-phospho-T180/Y182 p38 (9211S; Cell Signaling), anti-p38 (9212; Cell Signaling), anti-phospho–PKB (sc-7383; Santa Cruz Biotechnology, Inc.), p47<sub>phox</sub> (07-500 polyclonal; Upstate Biotechnology), and p67<sub>phox</sub> (07-502 polyclonal; Upstate Biotechnology). Signal was detected (Image Reader LAS-1000; Fujifilm) and quantified using Aida Image Analyser 2.2. For tissue Westerns, tissues were collected and immediately macininated into ice-cold 20 mM Heps, pH 7.1, at 4°C. 0.1% SDS, 0.4% cholate, 0.1% NP-40, 0.1M NaCl, 0.2 mM PMSF, and 10 μg/ml each of pepstatin A, leupeptin, antipain, and aprotinin were added for 12,000 g (for 30 min at 4°C). Protein concentrations were determined (BCA; Pierce Chemical Co.), and 30 μg of each protein was subjected to SDS-PAGE, transferred, and immunoblotted for p40<sub>phox</sub>.

**Preparation of BMNs.** BMNs were prepared as described previously with minor modifications (75). Bone marrow, from at least three mice per preparation, was dispersed in HBSS (without Ca<sup>2+</sup> and Mg<sup>2+</sup>), 0.25% fatty acid-free BSA, 15 mM Heps, pH7.4, at room temperature and purified over discontinuous Percoll (GE Healthcare) gradients. After washes, mature neutrophils were resuspended in Dulbecco’s PBS with Ca<sup>2+</sup> and Mg<sup>2+</sup>, 1 g/liter glucose, 4 mM sodium bicarbonate (DPBS<sup>+</sup>). Purity was typically 70–80% as assessed by cytospin and REASTAIN Quick-Diff (Reagena) staining (non-neutrophils were ~50% immature white cells, 25% monocytes, and 25% lymphocytes). All assays were performed in DPBS<sup>+</sup>. BMNs were primed at 37°C with 500 U/ml mTNF-α, 100 ng/ml mGM-CSF, and 100 μg/mouse serum for 60 min. In some experiments, as indicated, BMNs were primed individually with 500 U/ml mTNF-α for 30 min, 100 ng/ml mGM-CSF for 60 min, or 500 U/ml mTNF-α and 100 ng/ml mGM-CSF for 60 min.

**Neutrophil and multiple tissue Western blots.** 5 × 10<sup>5</sup> BMNs were sonicated into 1× SDS loading buffer, and 5 × 10<sup>5</sup> cell equivalents were subjected to SDS-PAGE, transferred, and blotted for p40<sub>phox</sub> (05-539 monocular; Upstate Biotechnology), i-cadherin (sc-18252 and sc-18253 polyclonal; Santa Cruz Biotechnology, Inc.), p47<sub>phox</sub> (07-500 polyclonal; Upstate Biotechnology), and p67<sub>phox</sub> (07-502 polyclonal; Upstate Biotechnology). Signal was detected (Image Reader LAS-1000; Fujifilm) and quantified using Aida Image Analyser 2.2. For tissue Westerns, tissues were collected and immediately macininated into ice-cold 20 mM Heps, pH 7.1, at 4°C. 0.1% SDS, 0.4% cholate, 0.1% NP-40, 0.1M NaCl, 0.2 mM PMSF, and 10 μg/ml each of pepstatin A, leupeptin, antipain, and aprotinin were added for centrifugation (12,000 g for 30 min at 4°C). Protein concentrations were determined (BCA; Pierce Chemical Co.), and 30 μg of each protein was subjected to SDS-PAGE, transferred, and immunoblotted for p40<sub>phox</sub>.

**PKB, p38 MAPK, and p42/44 Erk activation assays.** 5 × 10<sup>5</sup> unprimed BMNs from p40<sub>phox</sub>-/- and p40<sub>phox</sub>-/-/- mouse were primed for 3 min at 37°C at 5 × 10<sup>5</sup> in ml in DPBS<sup>+</sup> in duplicate. After 1 min of stimulation with prewarmed FMLP (10 μM final) or salts, reactions were stopped by the addition of excess ice-cold PBS, followed by immediate centrifugation (12,000 g for 10 s). Cell pellets were lysed in 20 mM Tris, pH 7.5 at 4°C, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.2 mM PMSF, and 10 μg/ml each of pepstatin A, leupeptin, antipain, and aprotinin and incubated by centrifugation (12,000 g for 20 min at 4°C). Lysates were split between two SDS-PAGE gels and blotted for phospho- and total protein, respectively. Blots were imaged and quantified as described above.

**Preparation of mouse serum.** Mouse blood was collected and left to clot at room temperature for 45 min in a glass container before transfer to a 15-ml tube followed by centrifugation (1,500 g for 10 min at room temperature). Serum was removed to a fresh tube, recentrifuged, recovered, placed on ice, aliquoted, and stored at −80°C.

**Chemiluminescent detection of ROS.** ROS production was measured by luminol-dependent chemiluminescence in polystyrene 96-well plates (no. 23300; Berthold Technologies Ltd.) as described previously (75) in DPBS<sup>+</sup>, except final concentrations of luminol and HRP were 150 μM and 18.75 U/ml, respectively. Prewarmed stimuli were added manually and measurement started immediately. Assays using soluble stimuli (PMA, FMLP, and mTNF-α) were conducted in the presence of exogenous added HRP; the signal was >95% HRP dependent, indicating predominantly extracellular ROS production (unpublished data). Assays using particulate stimuli (zymosan, IgG latex beads, and live S. aureus) were conducted without HRP and thus represent intracellular ROS production. The addition of HRP revealed little extracellular ROS production (unpublished data). Final particle/BNM ratios were as follows: zymosan, 5:1; S. aureus, 20:1; and IgG latex beads, 50:1. In some S. aureus experiments, BMNs were preincubated
with varying concentrations of DPI or vehicle (DMSO) alone before stimulation. Data output is in relative light units per second (rlu/s).

Adhesion-dependent responses. Surfaces (96-well plates or 12-well tissue culture plates) were coated overnight at 4°C with sheep fibrinogen (F9754; Sigma-Aldrich) at 150 μg/ml in PBS (100% FCS was used as a non-specific control). Before use, surfaces were washed three times with PBS. For adhesion-dependent ROS assays, neutrophils at 5 × 10⁶/ml were incubated at 37°C for 1 h before the addition of prewarmed 2× HRP/luminol followed by mTNF-α (final concentration of 20 ng/ml), anti-CD18 (anti-β2 integrin, M18/2; Chemicon), or IgG2a isotype control antibodies (final concentration of 18 μg/ml). Cells were immediately aliquoted into the plate (100 μl/well) and counted. For spreading experiments, preincubated neutrophils were incubated at 37°C in coated 12-well tissue culture plates (6.25 × 10⁵/well) in the presence of 20 ng/ml mTNF-α. After 40 min, wells were aspirated and cells were fixed in 3.8% formaldehyde. Non-adhered cells were washed away and remaining cells were visualized by light microscopy. In both ROS and spreading assays, FCS-coated wells produced minimal responses (unpublished data). For analysis of β2 integrin expression, neutrophils were stimulated with mTNF-α for 30 min at 37°C, placed on ice, and processed for FACS analysis using the anti-CD18 antibody, its isotype control, and an FITC anti-rat secondary.

Preparation of particulate stimuli. IgG-opsinized zymosan particles (IgG-Zym) were prepared as per the manufacturer’s instructions (unlabeled zymosan A, Z-2849, and rabbit anti-zymosan A, Z-2850; Invitrogen). Zymosan and S. aureus were serum opsonized or mock opsonized by incubation in DPBS* with or without 50% mouse serum at 37°C with end-over-end mixing for 1 h (zymosan) or 15 min (S. aureus) followed by washing. Carboxylate-modified latex beads (0.9-μm diameter; Sigma-Aldrich) were cross-linked to sulfhydryl-modified BSA and coated with an anti-BSA monoclonal antibody (Sigma-Aldrich) or not, as described previously (IgG latex beads) (76). Where appropriate, S. aureus was washed and resuspended in DPBS* (4 × 10⁵/ml), heat killed at 60°C for 30 min, and opsonized in mouse serum as described above.

S. aureus phagocytosis assay. 10⁵ primed BMNs were allowed to adhere to glass coverslips for 20 min at 37°C. They were then aspirated, 10⁵ FITC-labeled, serum-opsonized S. aureus was added, and they were returned to 37°C (FITC labeling of bacteria as described previously [77] and opsonization as detailed above). After 40 min, coverslips were washed, fixed in 4% paraformaldehyde, and mounted. Postfixation probing with a rabbit anti-S. aureus antibody (S-2860; Invitrogen) and goat anti-rabbit Alexa Fluor 568 secondary antibody (Invitrogen) revealed that >95% of bacteria present were internalized. Phagocytosed bacteria were visualized by fluorescence microscopy and enumerated.

Oxygen consumption. Oxygen consumption was measured in a Clark-type oxygen electrode (Rank Brothers Ltd.) at 37°C with rapid stirring. Primed BMNs were added to the rapidly stirred chamber at 5 × 10⁶/ml and equilibrated for 5 min before the addition of prewarmed stimuli. Final concentrations were 1 μM PMA; IgG-Zym, 20:1; and heat-killed S. aureus, 20:1 (particles/BMNs).

NBT microscopy. Primed BMNs were adhered to a coverslip in 0.5 mg/ml NBT at 37°C. IgG-Zym particles were added, and dark purple formazan deposition was followed during phagocytosis by bright field microscopy.

In vitro bacterial killing assays. Bacteria (S. aureus Wood 46 and E. coli E2348169) were subcultured at 37°C to logarithmic growth from an overnight culture. 4 × 10¹⁰ bacteria were washed in DPBS* and opsonized as described above. Opsonized bacteria (1.5 × 10⁶ S. aureus and 6 × 10⁶ E. coli) were added to 6.2 × 10⁵ primed BMNs (2.5 × 10⁵/ml) at 37°C with rapid orbital mixing. After the indicated times, 50-μl aliquots were removed to 950-μl ice-cold Luria broth (LB) containing 0.05% saponin. Samples were sonicated (output 1.5 for 10 s; Sonicator 3000; Misonix) to liberate intracellular bacteria and returned to ice. Suspensions were serially diluted and plated on LB-agar to enumerate surviving bacteria. A parallel bacterial incubation was also run in the absence of neutrophils. In some experiments, neutrophils were incubated for 5 min with DPI at varying concentrations or vehicle (DMSO) alone before the addition of bacteria (oxidant-dependent killing control).

For S. aureus killing assays in whole blood, 2.5 × 10⁸ bacteria in 1 ml DPBS* were added to 1 ml of fresh whole blood (mixed from at least three animals) and incubated for 20 min at 37°C with end-over-end mixing. 0.8 ml of blood/bacteria mix was added to tubes containing lysostaphin (a final concentration of 2.5 U/ml to kill extracellular bacteria) in duplicate and returned to mixing. Samples were taken after 1 h, added to ice-cold PBS, and pelleted by centrifugation, and the pellets were resuspended in 0.5 ml nutrient broth, 0.05% saponin. Samples were then processed and quantified as described above.

In vivo S. aureus survival assays. S. aureus (LS-1) was subcultured at 37°C to logarithmic growth from an overnight culture. Bacteria were washed and resuspended in injection-grade PBS at 2.5 × 10⁸/ml. Three animals of each genotype per time point were injected intraperitoneally with 0.2 ml of bacterial suspension (5 × 10⁹ bacteria). After 4 or 24 h, mice were killed and the peritoneal cavity was thoroughly flushed with 10 ml ice-cold PBS, 5 mM EDTA, and 5 U/ml heparin. Aliquots were diluted, sonicated, and plated, and bacteria were enumerated as for the in vitro killing assays.

Online supplemental material. Fig. S1 illustrates the targeting strategy used to generate p40⁰/−/− mice and the subsequent screening strategies used. Fig. S2 denotes normal organ weights of p40⁰/−/− animals. Fig. S3 describes the S. aureus killing deficiency of whole blood from p40⁰/−/− animals.

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