PtdIns3P binding to the PX domain of p40<sup>phox</sup> is a physiological signal in NADPH oxidase activation

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The production of reactive oxygen species by the NADPH oxidase complex of phagocytes plays a critical role in our defence against bacterial and fungal infections. The PX domains of two oxidase components, p47<sup>phox</sup> and p40<sup>phox</sup>, are known to bind phosphoinositide products of PI3Ks but the physiological roles of these interactions are unclear. We have created mice which carry an R58A mutation in the PX domain of their p40<sup>phox</sup> gene, which selectively prevents binding to PtdIns3P. p40<sup>phoxR58A</sup>/p47<sup>phox</sup> embryos do not develop normally but p40<sup>phoxR58A</sup>/−/− mice are viable and neutrophils from these animals exhibit significantly reduced oxidase responses compared to those from their p40<sup>phox</sup>+/−/− sibings (e.g. 60% reduced in response to phagocytosis of <i>Staphylococcus aureus</i>). Wortmannin inhibition of the S. aureus oxidase response correlates with inhibition of phagosomal PtdIns3P accumulation and overlaps with the reduction in this response caused by the R58A mutation, suggesting PI3K regulation of this response is substantially dependent on PtdIns3P-binding to p40<sup>phox</sup>. p40<sup>phoxR58A</sup>/−/− mice are significantly compromised in their ability to kill S. aureus in vivo, defining the physiological importance of this interaction.

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Introduction

The NADPH oxidase in phagocytic cells, such as neutrophils and macrophages, plays a key role in our innate immune system (Nathan, 2006). This oxidase is a multisubunit enzyme complex that transfers electrons from NADPH, across the membrane on which it is assembled, to molecular oxygen (Cross and Segal, 2004; Quinn and Gauss, 2004; Sheppard et al., 2005). The superoxide anions (O<sub>2</sub>−) thus formed can then be converted, depending on their location, by both enzymatic and non-enzymatic routes to a variety of other reactive oxygen species (ROS) and halide derivatives (e.g. HOCI). This transfer of electrons is electrogenic and there is much debate over the nature of the compensating currents, which must occur to allow significant quantities of ROS to be generated, with evidence to suggest both H<sup>+</sup> and K<sup>+</sup> channels are involved (Cross and Segal, 2004; Murphy and DeCoursey, 2006). It is thought that a compensating K<sup>+</sup> current generated by NADPH oxidase activity on phagosomal membranes leads to substantial accumulation of K<sup>+</sup> inside the phagosome, leading to subsequent activation of several proteases (Reeves et al., 2002), which, together with the toxicity of the ROS and halide derivatives themselves, plays a key role in the killing of pathogens in this organelle (Hampton et al., 1998). The importance of the NADPH oxidase in the killing of several species of bacteria and fungi, in particular, is clear from the recurrent, life-threatening infections, which occur in chronic granulomatous disease (CGD), a condition caused by germ line mutations in several of the oxidase subunits (Meischl and Roos, 1998; Heyworth et al., 2003).

Analysis of the nature of the mutations in CGD, together with classical biochemical purification and in vitro reconstitution, has defined a core set of components sufficient for NADPH oxidase activity, these include a two subunit, membrane bound cytochrome b<sub>558</sub> (p91<sup>phox</sup> and p22<sup>phox</sup>) and three soluble components, p67<sup>phox</sup>, p47<sup>phox</sup> and GTP-rac (Cross and Segal, 2004; Quinn and Gauss, 2004). Additionally, p67<sup>phox</sup> is known to bind tightly to another oxidase subunit, p40<sup>phox</sup> (Matute et al., 2005). The function of p40<sup>phox</sup> has been unclear; no mutations in p40<sup>phox</sup> have been found to cause CGD, heterologous expression of this protein in model cell systems has been shown to both promote and inhibit oxidase activity in response to different agonists and in vitro reconstitution experiments have suggested p40<sup>phox</sup> has a positive or neutral role in NADPH oxidase activation (Matute et al., 2005). We have recently created p40<sup>phox</sup>−/− mice and reported large reductions in oxidase responses to a variety of agonists in their bone marrow-derived neutrophils (Ellson et al., 2006). However, these mice also show substantial reductions in the expression of p67<sup>phox</sup> (approx. 55% reduced) which, given the established importance of p67<sup>phox</sup> in the catalytic mechanism of the oxidase, makes it difficult to accurately ascribe a role for p40<sup>phox</sup> alone in these responses. The scale of the reductions in oxidase activation in p40<sup>phox</sup>−/− neutrophils to some phagocytic stimuli (e.g. 85% reduced in response to <i>Staphylococcus aureus</i> and IgG-latex beads) (Ellson et al., 2006), together with recent data describing a clear dependency of an IgG-SRBC-induced oxidase burst on heterologous expression of p40<sup>phox</sup> in Cos<sup>phox</sup> cells (Suh et al., 2006), indicates p40<sup>phox</sup> is likely to have an important role, at least, in the assembly of an active NADPH oxidase on phagosomes.

Many different cell surface receptors are able to regulate the neutrophil’s NADPH oxidase activity at sites of infection.
and inflammation. These include receptors for opsonin coats (e.g. Fcγ receptors and integrins for antibodies or complement, respectively), the so-called pattern receptors for complex molecules exposed on the surface of microbes themselves and also a range of soluble inflammatory stimuli, such as combinations of chemokines and cytokines found at sites of inflammation (e.g. IMLP and TNFα) (Sheppard et al., 2005). Hence, the signalling pathways that couple this array of cell surface receptors to activation of the NADPH oxidase complex are necessarily complex, with evidence to suggest many different, interacting, signalling pathways are involved, for example PLC, PI3K, PLD, PLA2, MAPKs (Quinn and Gauss, 2004; Sheppard et al., 2005). At the level of the oxidase, key events are thought to be multiple phosphorylation of the C-terminus of p47phox, which relieves an intramolecular SH3 domain/C-terminus constraint, allowing interaction with p22phox (Faust et al., 1995; Perisic et al., 2004; Groemping and Rittinger, 2005). Further, GTP/GDP exchange on Rac promotes complex formation with p67phox and gp91phox (Lapouge et al., 2000; Diebold and Bokoch, 2001; Perisic et al., 2004). Thus, through a series of mutual contacts, complex formation between soluble and membrane bound oxidase components is promoted at a particular membrane location, allowing efficient transfer of electrons from NADPH to O2. There is still much to be done, however, in identifying the relative balance of their contributions in what must be a robust, and hence probably redundant, signalling web to this important component of our innate immune response.

There has been much interest in the role of PI3K signalling pathways in NADPH oxidase regulation. Wortmannin, a relatively specific and broad range catalytic inhibitor of class I PI3Ks, is of Gi-coupled receptors on neutrophils (Perisic et al., 2004, 2005). There is good evidence that receptor-stimulated class I PI3Ks activation (Dinauer, 2003; Welch et al., 2001; Ellson et al., 2001; Kanai et al., 2001; Karathanassis et al., 2002; Perisic et al., 2004). The binding of PtdIns3P to the PX domain of p47phox is, in particular, of sufficient affinity and specificity to suggest it must play a physiological role in the regulation of p47phox function. Further, a crystal structure for short-chain PtdIns3P bound to the isolated PX domain of this molecule is available (Bravo et al., 2001), which provides an opportunity to test this role through specific mutagenesis. PtdIns3P is an established product of class III PI3K activity in the endosomal system of eukaryotic cells but has more recently been shown also to accumulate on phagosomal membranes, shortly after fission from the plasma membrane (Vieira et al., 2001; Ellson et al., 2001b). Hence, it appeared plausible that PtdIns3P might act as a signalling molecule, which regulates NADPH oxidase activity at this location via binding to p47phox. We generated mice expressing a version of p47phox carrying a single point mutation in its PX domain, which prevents binding to PtdIns3P, to test this hypothesis.

Results

Generation of p40phoxR58A/−/− mice

We generated two independent ES cell lines each possessing one p40phoxR58A allele. The targeting strategy introduced a single codon change in exon 3, resulting in conversion of arginine at amino-acid position 58 to alanine (Figure 1). Mutation of R58 to (either Q or A) has been shown to prevent PtdIns3P binding to the PX domain of p40phox (Bravo et al., 2001; Zhan et al., 2002), with minimal predicted changes to the overall fold of the protein (Bravo et al., 2001).

These two ES cell lines were used to generate male chimeras via blastocyst injection and p40phoxR58A/+ heterozygotes were created by breeding with C57BL/6 females. The ‘floxed’ neomycin resistance cassette introduced into the intron between exons 3 and 4 (Figure 1) was removed at this stage via Cre-mediated excision in the testes (see Materials and methods). Interbreeding of the p40phoxR58A/+ heterozygotes generated further p40phoxR58A/+ and p40phoxR58A/−/− animals, but only approximately 1 in 50 predicted p40phoxR58A/−/− mice live births (Table I). Further analysis of 70 embryos derived from timed matings indicated p40phoxR58A/−/− embryos failed in development before day 10 (data not shown).

Breeding p40phoxR58A/−/− mice with p40phoxR58A/+ heterozygotes generated p40phoxR58A/+ and p40phoxR58A/−/− mice in the expected 1:1 ratio (Table I), indicating that embryonic lethality requires two copies of the p40phoxR58A allele. Importantly, comparison of the p40phoxR58A/−/− siblings allows a direct comparison of the protein products of p40phoxR58A and p40phoxR58A/−/− allelles.

p40phoxR58A/−/− mice were apparently normal, healthy and fertile when kept under pathogen-controlled conditions. Blood cell counts from p40phoxR58A/−/− mice were normal (Figure 2A) and bone marrow derived neutrophils (BMNs) from p40phoxR58A/−/− mice exhibited normal differentiated phenotypes with respect to their morphology and phagocytosis of bacteria, zymosan and IgG-latex beads (data not shown and see below). p40phoxR58A/+ neutrophils showed comparable expression of p42phox and, importantly, p67phox (Figure 2B and D). R58A/p40phox protein itself exhibited a slightly retarded mobility on SDS–PAGE and was expressed at a slightly greater level in p40phoxR58A/−/− neutrophils than wild-type p40phox in p40phoxR58A/−/+ neutrophils (Figure 2B and C).

p40phoxR58A/−/− neutrophils exhibit significant reductions in ROS production in response to TNFα/IMLP and IgG-latex beads

Several soluble inflammatory stimuli (e.g. fMLP, PAF, LTβ4) are known to induce the assembly of the NADPH oxidase at the plasma membrane and the generation of extracellular ROS. The responses to these stimuli are characteristically augmented by prior exposure to the so-called ‘priming’ cytokines (e.g. GM-CSF, TNFα), a phenomenon thought to localise the production of potentially self-damaging ROS to sites of inflammation (Hallett and Lloyds, 1995; Sheppard et al., 2005). We measured the generation of ROS in response to fMLP in TNFα primed, BMNs from p40phoxR58A/−/− and p40phoxR58A/−/− mice using a luminol-dependent, chemiluminescence assay

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in the presence of horse radish peroxidise (HRP), an assay that provides a sensitive, rate-measure of extracellular ROS production (Dahlgren and Karlsson, 1999). The kinetics of these responses in the p40\textsuperscript{phoxR58A/C0} and p40\textsuperscript{phox+/C0} neutrophils were very similar but the total ROS accumulated in the p40\textsuperscript{phoxR58A/—} neutrophils was reduced by about 19% (Figure 3A).

It is well established that neutrophils undergoing Fc\textsubscript{yR}-mediated phagocytosis of IgG-coated particles assemble an active NADPH oxidase at the phagosome (Zhou and Brown, 1994; Jakus \textit{et al.}, 2004; Ueyama \textit{et al.}, 2004). We measured the generation of intracellular ROS in primed, BMNs from p40\textsuperscript{phoxR58A/—} and p40\textsuperscript{phox+/—} mice in response to uptake of IgG-latex beads using luminol-dependent chemiluminescence in the absence of added HRP. This assay provides a rate measure of intracellular ROS production in the vicinity of endogenous peroxidases, which previous work has indicated is dictated under these conditions by co-incidental release of ROS and myeloperoxidase into the phagosome (Dahlgren and Karlsson, 1999). Intracellular ROS production in response to phagocytosis of IgG-latex beads was substantially reduced in p40\textsuperscript{phoxR58A/—} neutrophils compared to p40\textsuperscript{phox+/—} neutrophils (approximately 42% reduction; Figure 3B).
Phagocytosis itself was not influenced by the R58A mutation (data not shown), indicating a specific signalling defect to NADPH oxidase activation under these circumstances.

**p40phoxR58A/− neutrophils exhibit a large reduction in intracellular ROS production in response to S. aureus that is equivalent to a large, wortmannin-sensitive element in this response**

We measured the production of intracellular ROS in response to phagocytosis of *S. aureus* in primed, BMNs from p40phoxR58A/− and p40phox+/− mice. We observed a large reduction, of about 60%, in this response in p40phoxR58A/− neutrophils (Figure 4A). Phagocytic uptake of *S. aureus* was normal in these neutrophils (Figure 4D) suggesting that, as with the oxidase response to IgG-latex beads, there is a specific defect in the phagosomal oxidase response in neutrophils from p40phoxR58A/− animals.

We also measured the sensitivity of intracellular ROS production in response to *S. aureus*, in both p40phoxR58A/− and p40phox+/− neutrophils, to inhibition by the general PI3K inhibitor wortmannin (Figure 4A). Phagocytosis of *S. aureus* was not significantly inhibited at the concentrations of wortmannin used (Figure 4D), consistent with previous reports that phagocytic uptake of small particles, requiring minimal membrane extension, is relatively insensitive to PI3K inhibition (Cox et al., 1999). This allows a window in which the potential for more direct involvement of PI3Ks in this oxidase response can be evaluated.

The inhibition of *S. aureus* oxidase responses by wortmannin was complex. The initial phase of ROS production was substantially inhibited by wortmannin, but an increasingly significant proportion of the total response at later times was insensitive to doses of wortmannin up to 300 nM (Figure 4A), suggesting differential requirement for PI3K signalling in these responses with time. Importantly, the initial phases of ROS production in p40phoxR58A/− and p40phox+/− neutrophils were differentially sensitive to wortmannin inhibition (Figure 4B and C). The R58A mutation did not reduce further the residual ROS response in the presence of 100 nM wortmannin, suggesting the wortmannin-sensitive components in this response are on the same pathway to oxidase activation as the R58A lesion. The initial ROS response in p40phoxR58A/− neutrophils was partially inhibited by low concentrations of wortmannin, but an increasingly significant proportion of the total response at later times was insensitive to doses of wortmannin up to 300 nM (Figure 4A), suggesting differential requirement for PI3K signalling in these responses with time. Importantly, the initial phases of ROS production in p40phoxR58A/− and p40phox+/− neutrophils were differentially sensitive to wortmannin inhibition (Figure 4B and C). The R58A mutation did not reduce further the residual ROS response in the presence of 100 nM wortmannin, suggesting the wortmannin-sensitive components in this response are on the same pathway to oxidase activation as the R58A lesion. The initial ROS response in p40phoxR58A/− neutrophils was partially inhibited by low concentrations of wortmannin, but an increasingly significant proportion of the total response at later times was insensitive to doses of wortmannin up to 300 nM (Figure 4A), suggesting differential requirement for PI3K signalling in these responses with time. Importantly, the initial phases of ROS production in p40phoxR58A/− and p40phox+/− neutrophils were differentially sensitive to wortmannin inhibition (Figure 4B and C). The R58A mutation did not reduce further the residual ROS response in the presence of 100 nM wortmannin, suggesting the wortmannin-sensitive components in this response are on the same pathway to oxidase activation as the R58A lesion. The initial ROS response in p40phoxR58A/− neutrophils was partially inhibited by low...
Figure 4 4p40phoxR58A/– neutrophils exhibit reduced intracellular ROS production in response to phagocytosis of S. aureus, which equates with a wortmannin-sensitive component of the response. Primed BMNs from p40phoxR58A/– and +/+ animals were analysed for S. aureus-induced intracellular ROS production. Where appropriate, cells were pretreated with indicated wortmannin concentrations for 10 min prior to addition of S. aureus. (A) Time course of ROS production in the presence of increasing concentrations of wortmannin. Data are means ± s.d. from a single experiment, performed in triplicate, representative of three independent experiments. (B, C) Total integrated ROS responses for the first 16.5 min of each condition (as indicated by dashed lines in A) were calculated and expressed as a percentage of p40phox+/+ response (B), or as a percentage of relevant control response for p40phox+/+ and p40phoxR58A/– (C). Data are mean ± s.e. (n ≥ 8). (D) Phagocytosis of S. aureus by p40phox+/+ and p40phoxR58A/– BMN and the effects of wortmannin. Data shown are mean ± s.e. from at least 100 cells at each condition, expressed as a percentage of p40phox+/+ phagocytosis.

**p40phoxR58A/– neutrophils exhibit differential reductions in intracellular versus extracellular ROS production in response to PMA**

PMA is often used as a receptor-independent stimulus of oxidase assembly and activation. The mechanism of action of PMA in this respect is incompletely understood but must, at least in part, involve PKC-mediated phosphorylation of p47phox (Dekker et al, 2000; Dang et al, 2001; Fontayne et al, 2002; Bey et al, 2004). Using chemiluminescent detection methods for ROS, PMA has previously been shown to stimulate a large extracellular production of ROS, which is insensitive to inhibition of PI3Ks and a smaller, intracellular production, which is potently inhibited by wortmannin (Karlsson et al, 2000; Brown et al, 2003). We used luminol-dependent chemiluminescence in the presence and absence of added HRP to compare the oxidase responses to PMA in neutrophils from p40phoxR58A/– and p40phox+/+ mice.

The kinetics of both intracellular and extracellular ROS production in response to PMA are clearly complex with at least two clear phases (Figure 5 and data not shown). We found that both phases of extracellular ROS production in response to PMA were extremely similar in p40phoxR58A/– and p40phox+/+ neutrophils (Figure 5A and data not shown). However, the initial phase of intracellular ROS formation in response to PMA was reduced by almost half in neutrophils expressing a GFP-tagged, isolated PX domain of PtdIns3P-regulates the NADPH oxidase (Ellson et al, personal communication, 2006). The presence of the GFP-tagged, PtdIns3P-probe allows a semiquantitative determination of intracellular ROS (Figure 5). The initial phase of the intracellular ROS response in p40phox+/+ neutrophils was inhibited by wortmannin, to a level very similar to that seen in the p40phoxR58A/– neutrophils. Importantly, wortmannin was unable to reduce significantly this phase of the response any further in p40phox+/+ neutrophils (Figure 5B and D). These results suggest there is a large component of the initial phase of PMA-stimulated intracellular ROS accumulation that is dependent on PtdIns3P-binding to p40phox.
measure of PtdIns3P accumulation around S. aureus-containing phagosomes (Figure 6). PLB-985 cells expressing GFP-iPX show equivalent oxidase responses to a variety of soluble and particulate stimuli, compared to cells with no exogenous protein expression, suggesting that, at the expression levels achieved, the concentration of the iPX-domain does not influence oxidase activation (data not shown). At the concentrations tested, we observed little effect of wortmannin on the rate of S. aureus phagocytosis, consistent with our observations above with mouse neutrophils. In contrast, both the accumulation of PtdIns3P on S. aureus-containing phagosomes and the rate of intracellular ROS production were similarly and substantially sensitive to inhibition by wortmannin, suggesting they may both be equivalently dependent on PI3K activity (Figure 6).

**p40phoxR58A/C0 mice are defective in clearance of peritoneal S. aureus in vivo**

Previous studies have defined an important role for the NADPH oxidase in the killing of S. aureus in vitro and in vivo, consistent with the prevalence of S. aureus infections in cases of CGD (Meischl and Roos, 1998; Heyworth et al, 2003; Rada et al, 2004). We investigated the ability of p40phoxR58A/– and p40phox+/– neutrophils to kill S. aureus in suspension cultures in vitro and to clear intraperitoneal injection of S. aureus in vivo. We have previously observed a severe defect in the ability of p40phox+/– neutrophils and p40phox−/− mice to kill S. aureus in these assays (Ellson et al, 2006). We observed a small but significant defect in the ability of p40phoxR58A/– neutrophils to kill S. aureus in vitro (Figure 7A). This defect was only a relatively small proportion (approx. 20%) of the total oxidase-dependency of S. aureus killing in these assays (revealed by comparison with the NADPH oxidase inhibitor DPI; Figure 7A). However, this is consistent, given the nonlinear relationship between ROS production and S. aureus killing (Rada et al, 2004), with the relative sizes of the reductions in ROS production observed in p40phoxR58A/– and p40phox−/− neutrophils (Ellson et al, 2006). We also observed a highly significant defect in the ability of p40phoxR58A/– mice to clear a S. aureus infection in vivo (Figure 7B), demonstrating the physiological importance of an intact PX domain for NADPH function.

**Discussion**

Our observation that most p40phoxR58A/R58A embryos die early in development is very surprising. Mice lacking gp91phox, p47phox or, indeed, p40phox are born in the expected...
Mendelian ratios (Jackson et al., 1995; Pollock et al., 1995; Ellson et al., 2006). This suggests that the p40phoxR58A allele has a lethal ‘gain of function’. Further, our observation that p40phoxR58A/C0 animals are viable suggests that expression from both alleles is required to achieve a lethal threshold of R58A-p40phox protein. There are no previous reports of NADPH oxidase function during early embryogenesis, and there are no previous indications as to why high expression of R58A-p40phox would be lethal. The crystal structure of the PX domain of p40phox gives no clues as to how the R58A mutation might cause a significant change to the fold of p40phox (Bravo et al., 2001). Indeed, comparison of NADPH oxidase activity and the expression levels of oxidase subunits in p40phoxR58A/−, p40phox+/- and p40phox−/− mice indicates R58A-p40phox is expressed at similar levels to wild-type protein, is correctly folded and is able to bind functionally to p67phox. Heterologous expression of oxidase subunits in PLB-985 cells also confirms that R58A-p40phox and wild type protein can bind equivalently to p67phox (data not shown). Further work is clearly needed to clarify this effect of R58A-p40phox during embryo development, including its dependency on NADPH oxidase activity.

The production of fully differentiated neutrophils in p40phoxR58A/− mice and the normal expression of oxidase subunits in these cells compared to p40phox+/- neutrophils allow a direct comparison of p40phoxR58A and p40phox alleles and the potential to ascribe differences in neutrophil NADPH oxidase activity to the function of the PX domain in p40phox. We observed large reductions in intracellular ROS production in response to S. aureus and IgG-latex beads but much smaller reductions in extracellular ROS produced in response to TNFα/fMLP. A comparison of the scale of these reductions with those we observed in equivalent oxidase responses in p40phox−/− neutrophils suggests that the larger defects in ROS production seen in p40phox−/− neutrophils, particularly extracellular ROS in response to TNFα/fMLP and PMA (75 versus 25%, 80 versus 5%, for p40phox−/− versus p40phoxR58A/−, respectively) may be caused by the loss of a non-PtdIns3P-binding function of p40phox or the significant decrease in p67phox expression in these cells (Ellson et al., 2006).

Given the clarity, both with which PtdIns3P is defined as a ligand for the PX domain of p40phox and with which the R58A mutation abolishes this binding, our observations start to define the contribution that PtdIns3P binding could, in principle, make to the role of p40phox in NADPH oxidase regulation. We measured a large reduction in intracellular ROS production in response to phagocytosis of S. aureus in the p40phoxR58A/− neutrophils. Importantly, this reduction overlapped substantially with the inhibition of a large element of this response by the general PI3K inhibitor wortmannin.

**Figure 6** PtdIns3P accumulation and NADPH oxidase activation in response to S. aureus are inhibited in parallel by wortmannin. (A) $5 \times 10^4$ differentiated GFP-IPX PLB-985 cells were pretreated with DMSO (vehicle control) or 100 nM wortmannin prior to incubation with RITC-labelled, serum-opsonised S. aureus for 5 min. Samples were cytopun on glass slides, fixed and mounted, and GFP-positive phagosomes and phagocytosed bacteria visualised by fluorescence microscopy; the scale bar represents 1 μm. (B) Intracellular ROS production in response to S. aureus, enumeration of GFP-positive phagosomes encapsulating phagocytosed S. aureus, and number of phagocytosed S. aureus/cell, in the presence of the indicated wortmannin concentrations, are expressed as a percentage of the relevant control (DMSO) responses. The wortmannin insensitive component of ROS production (approximately 22%) was subtracted from the ROS responses shown; data are means ± s.e.; at least 60 cells were analysed for each condition.
Previous work has described an accumulation of the PI3K product, PtdIns3P, in phagosomes containing bacterial and IgG-opsonised targets in macrophage-like cell lines (Ellson et al, 2001b; Vieira et al, 2001). In agreement with the concept that PtdIns3P synthesis may be a general phenomenon in phagosomes, under the conditions studied. This raises the question of whether oxidase-relevant rises in PtdIns3P are so small in response to these agonists that they have evaded detection or that the oxidase assembles on existing PtdIns3P-rich structures (Kobayashi et al, 1998; Karlsson et al, 2000), such as components or derivatives of the endosomal/lysosomal compartments.

The large defect in the S. aureus-induced oxidase response in neutrophils from p40phoxR58A/− mice translated into a smaller defect in the in vitro killing of this organism. This is not surprising given previously established relationships between oxidase activity and in vitro killing of S. aureus by neutrophils (Rada et al, 2004). We did, however, observe a significant defect in the clearance of S. aureus in vivo in p40phoxR58A/− animals. These experiments are limited to single modes of bacterial delivery, single doses of bacteria and limited times of sampling, and thus give only a small ‘snapshot’ of how these animals may deal with bacterial infections. They do however provide very strong evidence that the PtdIns3P:PX domain interaction in p40phox has a significant physiological role in the regulation of NADPH oxidase activity and provide a basis for future work aimed at a more comprehensive evaluation of this signalling element in NADPH oxidase function.

Materials and methods

Murine GM-CSF (mGM-CSF), fMLP, luminol, DPI, PMA, HRP were from Sigma. Murine TNFα (mTNFα) was from R&D. Cell culture
Generation of p40phoxR58A/- mice
Several clones encoding p40phox genomic NT-cDNA sequence were isolated from the RPC1 mouse PAC Library 21 (Pieter de Jong; UK HGMP Resource Centre) by Southern screens using an NT-cDNA probe. An 11.9 kb Spel-Spel fragment encompassing exons 3-4 was isolated from clone RP21-641C7 and inserted into the low copy number module and a NeoR expression module (pACN; A. Plagge, Babraham Institute; Bunting et al, 1999) was inserted into the SnaBl site in the intron between exons 3-4 (Figure 1A). The TAC promoter is expected to operate in the testis and to drive Cre/Lox mediated deletion of this cassette on breeding of targeted chimeras, deletion is predicted to leave 59 bp of foreign DNA remaining in the intron. The final targeting vector was digested with DraI and SstI (in the 5’ polylinker) to remove excess pS-C3-Z vector sequence and used to transfect E14 129/Sv ES cells by the Gene Targeting Facility, Babraham Institute. Forty-eight hours after transfection, colonies were initially screened for homologous recombination using a 3’-Southern screen (probe 3’ to targeted sequence, EcoRI digest, 4.6-8.3 kb transition) and positive clones re-screened by a 5’-Southern (probe 5’ to targeted sequence, SpeI digest, 11.9-10.0 kb transition) and for single insertion of the Neo cassette (Neo probe, EcoRI/BglI and SpeI/ SpeI, 7kb). Two clones were taken forward for blastocyte injection and male chimeras from these mice were bred with female C57Bl/6 mice to yield approximately 525 and 100 bp of foreign DNA remaining in the intron. Deletion of the Neo cassette was confirmed by appropriate Southern and PCR analysis and separate p40phoxR58A/- heterozygotes on a mixed 129s/v/C57BL/6 background. Deletion of the p40phoxR58A/- cassette was confirmed by appropriate Southern and PCR analysis and separate p40phoxR58A/- colonies were created from each of the original targeted ES cell lines and housed under specific pathogen-free conditions in the SABU facility at the Babraham Institute. Genotyping of the mice was performed by PCR amplification of an approximately 850 bp region flanking exon 3 (to include the additional XhoI site) and subsequent diagnosis by susceptibility to the presence of the mutated sequence. This modified segment was sequenced and then reintroduced into the targeting vector.

Preparation of bone marrow-derived neutrophils
BMNs were prepared as previously described (Ellson et al, 2006). The final targeting vector was digested with DraI and SstI (in the 5’ polylinker) to remove excess pS-C3-Z vector sequence and used to transfect E14 129/Sv ES cells by the Gene Targeting Facility, Babraham Institute. Forty-eight hours after transfection, colonies were initially screened for homologous recombination using a 3’-Southern screen (probe 3’ to targeted sequence, EcoRI digest, 4.6-8.3 kb transition) and positive clones re-screened by a 5’-Southern (probe 5’ to targeted sequence, SpeI digest, 11.9-10.0 kb transition) and for single insertion of the Neo cassette (Neo probe, EcoRI/BglI and SpeI/ SpeI, 7kb). Two clones were taken forward for blastocyte injection and male chimeras from these mice were bred with female C57Bl/6 mice to yield approximately 525 and 100 bp of foreign DNA remaining in the intron. Deletion of the Neo cassette was confirmed by appropriate Southern and PCR analysis and separate p40phoxR58A/- heterozygotes on a mixed 129s/v/C57BL/6 background. Deletion of the p40phoxR58A/- cassette was confirmed by appropriate Southern and PCR analysis and separate p40phoxR58A/- colonies were created from each of the original targeted ES cell lines and housed under specific pathogen-free conditions in the SABU facility at the Babraham Institute. Genotyping of the mice was performed by PCR amplification of an approximately 850 bp region flanking exon 3 (to include the additional XhoI site) and subsequent diagnosis by susceptibility to the presence of the mutated sequence. This modified segment was sequenced and then reintroduced into the targeting vector.

Preparation of particulate stimuli
Carboxylate-modified latex beads (0.9 µm diameter) were cross-linked to sulphydryl-modified BSA and coated with an anti-BSA monoclonal antibody, as previously described (Cox et al, 1999) (IgG-latex beads). S. aureus were serum-opsonized by incubation in DPBS + with 10% mouse serum at 37°C with end-over-end mixing for 15 min followed by washing.

S. aureus phagocytosis assay
5 x 10^5 primed BMNs or differentiated GFP-iPX expressing PLB-985s were incubated in suspension at 37°C with 1 x 10^5 RITC-labelled, serum-opsonised S. aureus (Roosjakkers et al, 2005). After 5 min (PLB-985) or 30 min (BMN), samples were cytospun onto glass slides and fixed in 100% methanol containing 0.01% methylene-blue. In some experiments, samples were placed on ice for 5 min, to prevent further phagocytosis, and non-phagocytosed RITC-labelled S. aureus quenched with trypsin blue (1.2 mg/ml), prior to cyto spinning. Coverslips were placed over samples with Aquapolymount anti-fading solution (Poly-Science Inc) and GFP-positive phagosomes (PLB-985s) and phagocytosed bacteria were visualised by fluorescence microscopy and enumerated.

In vitro bacterial killing assays
Bacteria (S. aureus Wood 46) were subcultured at 37°C to logarithmic growth from an overnight culture. 4 x 10^7 bacteria were washed in DPBS +, and opsonised as detailed above. Opsonised bacteria (1.5 x 10^9) were added to 6.2 x 10^5 primed BMNs (2.5 x 10^5/µl) at 37°C with rapid orbital mixing. At the indicated times, 50 µl aliquots were removed to 950 µl ice cold Luria Broth (LB) containing 0.05% saponin. Samples were sonicated (Misonix, Sonicator 3000, output 1.5, 10s) to liberate intracellular bacteria and returned to ice. Suspensions were serially diluted and plated on LBagar to enumerate surviving bacteria. In some experiments, neutrophils were incubated for 5 min with 3 µM DPI or vehicle (DMSO) alone, prior to the addition of bacteria.

In vivo S. aureus survival assays
S. aureus (LS-1) were subcultured at 37°C to logarithmic growth from an overnight culture. Bacteria were washed and resuspended in injection-grade PBS at 2.5 x 10^9/ml. Three animals of each genotype per timepoint were injected intraperitoneally with 0.2 ml of bacterial suspension (5 x 10^9 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, 5%/ml heparin. Aliquots were diluted, sonicated, plated and bacteria were enumerated as for the in vitro killing assays.
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References


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