CD18-dependent activation of the neutrophil NADPH oxidase during phagocytosis of *E. coli* or *S. aureus* is regulated by Class III but not Class I or II PI3Ks

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Short Title: Regulation of the oxidase by Class III PI3K

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ABSTRACT
Phagocytosis and activation of the NADPH oxidase are important mechanisms by which neutrophils and macrophages engulf and kill microbial pathogens. We investigated the role of PI3K signalling pathways in the regulation of the oxidase during phagocytosis of *S.aureus* and *E.coli* by mouse and human neutrophils, a mouse macrophage-like cell line and a human myeloid-like cell line. Phagocytosis of these bacteria was promoted by serum, independent of serum-derived antibodies, and almost totally abolished in mouse neutrophils lacking the β2-integrin common chain, CD18. A combination of PI3K isoform-selective inhibitors, mouse knock-outs and RNA-interference indicated CD18-dependent activation of the oxidase was independent of Class I and II PI3Ks, but substantially dependent on the single Class III isoform (Vps34). Class III PI3K was responsible for the synthesis of PtdIns(3)P on both *S.aureus*- and *E.coli*-containing phagosomes. The use of mouse neutrophils carrying a knock-in mutation in the PX domain of their p40phox oxidase subunit indicated that PtdIns(3)P binding to the p40phox-PX domain is important for oxidase activation in response to both *S.aureus* and *E.coli*. This interaction does not however, account for all the PI3K sensitivity of these responses, particularly the oxidase response to *E.coli*, suggesting that additional mechanisms for PtdIns(3)P-regulation of the oxidase must exist.
INTRODUCTION

Phagocytic leukocytes such as neutrophils and macrophages play several key roles in the innate immune system. One of their most important functions is to engulf small bacterial and fungal pathogens by the process of phagocytosis, subsequent formation of intracellular phagosomes and the destruction of the internalised pathogen.¹ This process is co-ordinated into a whole body response to infection through antigen presentation to the adaptive immune system and the tailored release of inflammatory mediators.

Phagocytosis and the maturation of the phagosome are regulated by an extensive signalling web downstream of several activated cell surface receptors. The receptors employed depend upon the nature of the prey, the extent to which the surface of the prey has been coated with host-derived opsonins (fragments of complement and/or antibodies) and soluble factors generated at the site of inflammation (cytokines, chemokines and bacterially-derived products such as formylated peptides and endotoxin).² Under most physiological circumstances several receptors will be cooperatively involved, including the major classes of ‘phagocytic’ receptors essential for regulating the process of internalisation (such as β₂-integrins, FcRs and scavenger receptors), together with cytokine-, G-protein-coupled-, and pattern recognition-receptors which modulate the host cells response to phagocytosis (reviewed in Underhill and Ozinsky²). Particularly important in the absence of a prior or effective response from the adaptive immune system are antibody-independent phagocytic mechanisms, the clearest example of which is the deposition of complement fragments (C3b/C3bi) on the surface of bacteria by the alternative pathway of
complement fixation and ligation of these fragments by the β2-integrin CD11b/CD18 (also known as CR3 or Mac-1). Thus, mice lacking CD18 (the common chain of β2-integrins) are more susceptible to bacterial infections,3 and patients deficient in CD11b/CD184 or possessing mutations in CD18,5 suffer recurrent and spontaneous microbial infections from prevalent but normally benign strains of bacteria, including strains of *S.aureus* and *E.coli*.

During engagement and internalisation, phagocytes employ several mechanisms to ensure effective killing of pathogens, including the use of antimicrobial peptides, broad spectrum proteases and the production of reactive oxygen species (ROS). The generation of ROS by the NADPH oxidase complex is thought to play an important role in both direct and indirect killing of several species of bacteria and fungi.6 Sufferers of chronic granulomatous disease (CGD) carry genetic lesions in essential components of this NADPH oxidase complex, and present with recurring, life-threatening infections, commonly of Staphylococcus, Salmonella or Aspergillus origin.7,8 The complex is comprised of two membrane bound proteins gp91phox and p22phox, and four cytosolic components p40phox, p47phox, p67phox and the GTPase-Rac.6 In response to inflammatory or phagocytic stimuli, these components assemble on the plasma and/or phagocytic membrane to form the functional NADPH complex which transfers electrons from NADPH on the cytosolic face of the membrane to molecular oxygen on the extracellular/phagosomal face, producing superoxide anions and other superoxide-derived ROS.

Most attempts to unravel the signalling mechanisms involved in the regulation of the NADPH oxidase have used simplified model systems designed to isolate the
contribution of individual receptors; these have included the presentation of inert particles coated with single opsonins (e.g. IgG or C3bi) or the use of soluble, inflammatory stimuli (e.g. fMLP or C5a). These studies have indicated that there is a complex signalling web between receptors and the assembly of an active oxidase, involving several established signalling pathways. Key targets of these pathways are guanine nucleotide exchange on Rac2, leading to productive complex formation between GTP-Rac2, p67\textsuperscript{phox} and gp91\textsuperscript{phox} and also phosphorylation of the C-terminus of p47\textsuperscript{phox}, leading to productive complex formation between p47\textsuperscript{phox}, p67\textsuperscript{phox} and p22\textsuperscript{phox}\textsuperscript{6}. Moreover, whilst activation of certain receptor classes can, in isolation, initiate sufficient signalling to activate the oxidase, robust activation normally requires co-operative interaction between more than one receptor type. Thus ligation of the major phagocytic complement receptor, CD11b/CD18 by C3bi-coated particles is sufficient to induce phagocytosis but does not induce substantial activation of a plasma membrane or phagosomal NADPH oxidase without co-stimulation with other receptor systems e.g. TLRs, cytokine receptors or low level stimulation of Fc\gammaRs\textsuperscript{9,10}.

There are now several lines of evidence which suggest that phosphoinositides synthesised by PI3Ks play important roles in regulating efficient assembly and activation of the oxidase in different contexts of cell stimulation.\textsuperscript{11,12} Class IA PI3Ks (PI3K\alpha,\beta and \delta) and Class IB PI3K (PI3K\gamma) play key roles in the signal transduction pathways downstream of a variety of protein tyrosine kinase and Gi-coupled cell surface receptors, respectively.\textsuperscript{13,14} Upon receptor activation, Class I PI3Ks synthesise the messenger lipids PtdIns(3,4,5)P\textsubscript{3} and PtdIns(3,4)P\textsubscript{2} in the plasma membrane and co-ordinate the recruitment and activation of several protein effectors, the best established of which possess PH-domains with high specificity for binding one or
both head groups of these lipids (e.g. PKB, BTK, GRP1 and ARAP3). Relevant here, both Class IA and IB PI3Ks play important roles in the activation of the NADPH oxidase in cytokine-primed neutrophils stimulated by soluble chemoattractants. Further, Class IA PI3Ks are important in the phagocytosis and concomitant oxidase burst stimulated by engagement of macrophages with large IgG-opsonised particles, with PtdIns(3,4,5)P$_3$ accumulating transiently on the nascent phagosomal cup, and disappearing rapidly upon phagosomal closure. The precise roles that PtdIns(3,4,5)P$_3$ and/or PtdIns(3,4)P$_2$ play in the signalling pathways to oxidase activation under these circumstances is still unclear but likely to include PtdIns(3,4,5)P$_3$-activation of RacGEFs (P-Rex1, DOCK2 and Vav1-3) and PtdIns(3,4)P$_2$-binding to p47$^{phox}$. The involvement of Class I PI3Ks in non-antibody mediated phagocytosis and oxidase activation is undefined.

Class III PI3K (also known as Vps34) synthesises the messenger lipid PtdIns(3)P in internal membrane compartments of the endosomal/lysosomal system. PtdIns(3)P is accepted to play a major regulatory role in these membranes by influencing the localisation and activity of several protein effectors possessing PtdIns(3)P-specific, FYVE and PX domains. PtdIns(3)P has also been shown to accumulate dramatically on phagosomal membranes subsequent to closure and separation from the plasma membrane. In one model system, in which non-phagocytic CHO cells were rendered competent to phagocytose IgG-opsonised particles via heterologous expression of Fc$\gamma$RIIA, phagosomal PtdIns(3)P-accumulation was blocked by anti-Class III PI3K antibodies. In all other examples of phagosomal PtdIns(3)P synthesis however, the source of this lipid is unknown. In this regard, it has been suggested that PtdIns(3)P can also be produced in plasma membrane/endocytic vesicles by
sequential dephosphorylation of Class I derived PtdIns(3,4,5)P$_3$ via specific 5- and 4-phosphatases$^{25}$ or, by one of the three still poorly characterised Class II PI3Ks (PI3KinaseC2α, β and γ).$^{26}$

PtdIns(3)P plays an important role in phagosomal maturation and pathogen destruction$^{20,27,28}$ and, indeed, some virulent strains of bacteria are thought to evade host defences by interfering with its metabolism e.g. *Mycobacterium tuberculosis*.$^{29}$ The molecular details by which PtdIns(3)P exerts these effects are still largely undefined, although the binding of PtdIns(3)P to the PX domain of p40$^{phox}$ has recently been shown to play an important role in phagosomal oxidase activation during engulfment of serum-opsonised *S.aureus* and IgG-opsonised particles.$^{27,30,31}$ The extent to which this interaction is important in other contexts of NADPH oxidase activation is unknown.

We set out to establish the relative involvement of Class I, II and III PI3Ks in non-antibody-dependent phagocytosis, phagosomal PtdIns(3)P accumulation, and NADPH oxidase activation in response to uptake of a gram-positive *S.aureus* and a gram-negative *E.coli* by both mouse and human neutrophils, and a mouse macrophage cell line.
Materials and Methods:

Materials

fMLP, luminol, murine GM-CSF and human serum were from Sigma-Aldrich. Murine and human TNF-α were from R&D Systems. Dulbecco’s PBS with Ca^{2+} and Mg^{2+} was from Sigma (D8662). Tissue culture reagents were from Invitrogen. Class I PI3K isoform-selective inhibitors were as previously described. All buffer components were from Sigma-Aldrich and were endotoxin free or low endotoxin, as available.

Mouse strains

PI3K_δ_D910A/D910A, PI3KC2β_−/−, Fcγ_−/−, mice (each on a C57BL/6J background) and CD18_−/−, PI3Kγ_−/−, p40_−/−, and gp91_−/− mice (each on a mixed 129/Sv, C57BL/6J background) have been described previously. p40_−/− mice were created through interbreeding of p40_−/− mice, as described in Supplementary Methods. In all experiments, mice were compared with appropriate age and strain matched wild-type controls. Animals were housed in the small animal barrier unit (SABU) at the Babraham Institute. This work was approved by Home Office Project Licence PPL 80/1875.

Preparation of cells

RAW264.7 cells stably expressing a GFP-tagged probe for PtdIns(3)P (GFP-PX-Raw) were maintained at 37°C in a 5%CO₂ humidified incubator and harvested by centrifugation (1000 x g, 5min, RT) and resuspension in Dulbecco’s PBS with Ca^{2+} and Mg^{2+}, 1g/l glucose, 4mM sodium bicarbonate (DPBS⁺).
Human neutrophils were isolated from the peripheral blood of healthy volunteers (REC Approval number 06/Q0108/165), and mature mouse neutrophils isolated from bone marrow, as described previously.\(^\text{16}\) Purity was determined by cytospin and REASTAIN Quick-Diff (Reagena) staining, and were at least 95% (human) or 70-85% (mouse) pure. After washing, neutrophils were resuspended in DPBS\(^+\). Use of human blood for this study has been approved by the NHS Research Ethics Committee.

Preparation of Mouse Serum

Mouse serum was isolated from either C57BL/6J (normal) or antibody-deficient RAG2/\(\gamma_c\)\(^\text{37}\) mice, and prepared as previously described.\(^\text{31}\) Where indicated, serum was heat-inactivated at 56°C for 60min, prior to opsonisation of bacteria. Antibody depletion of serum was performed by incubating 250\(\mu\)l of normal mouse serum with 125\(\mu\)l protein G sepharose (45min, 4°C, mixing end-on-end).

Preparation of Bacterial Strains

Bacteria (\textit{S.aureus} Wood 46 and \textit{E.coli} E2348169) were subcultured at 37°C to logarithmic growth from overnight cultures. Bacteria were washed in DPBS\(^+\) and opsonised by incubation in DPBS\(^+\) with 10% serum (mouse or human as appropriate) at 37°C with end-over-end mixing for 15min, followed by washing in DPBS\(^+\). Opsonised bacteria were resuspended in DPBS\(^+\) with 10% serum at 5x10\(^6\)/ml. Non-opsonised bacteria were prepared as above in the absence of serum. For some assays, bacteria were labelled with 20\(\mu\)g/ml RITC (Sigma) prior to opsonisation.\(^\text{27}\)

Measurement of ROS Production
Neutrophils (6.25x10^6/ml) were primed with TNFα (200U/ml human, 1000U/ml mouse) and GM-CSF (100ng/ml) for 1hr, 37°C with occasional gentle mixing. Where indicated, primed neutrophils, or GFP-PX-RAW cells were pre-incubated with PI3K inhibitors or vehicle control (DMSO, 0.1%) for 10min prior to stimulation. Rate kinetics of intracellular ROS production were measured using a luminol-based assay in polystyrene 96-well plates (no:23300, Berthold Technologies Ltd.) as described previously. Briefly, 5x10^5 cells in DPBS+ were incubated with luminol (150µM) for 3min, 37°C. Cells were then added manually to pre-warmed bacteria (final ratio 1:20), and measurement started immediately. Assays were conducted without HRP and thus represent intracellular ROS production (addition of 18.75U/ml HRP revealed negligible extracellular ROS production). Light emission was recorded by a Berthold Microlumat Plus luminometer (Berthold Technologies). Data output is relative light units per second (RLU/s) or total RLU integrated over the indicated periods of time.

RNAi knockdown of Class III PI3K
RNAi was performed using Expression Arrest™ pSM2 retroviral shRNAmir (Open Biosystems), employing non-silencing shRNAmir retroviral control (RHS1707) and two independent Vps34RNAi constructs (RMM1766-96740308, RHS1766-96881245) as per manufacturer instructions. Optimal viral titre of 1:1 dilution in media supplemented with 5µg/ml polybrene was added to GFP-PX-RAW cells seeded into 6-well dishes at 20% confluency. Viral supernatant was removed after 24h, and targeted cells selected with 3µg/ml puromycin. Cells were expanded for at least two weeks, replacing media every second day. For maximal superoxide responses, puromycin selection was relieved for three days prior to analysis.
In a number of experiments GFP-PX-RAW cells, adhered to glass coverslips, were incubated with RITC-labelled, serum-opsonised bacteria for 30 min. Cells were washed, and fixed in 4% paraformaldehyde and mounted as previously described. GFP-positive endosomes and/or phagosomes, and phagocytosed bacteria were visualised using a Zeiss LSM 510 META point-scanning confocal microscope. GFP-accumulation of specific regions of interest (endosomes, phagosomal membrane) or average intensity of three 3µm regions of the cell (cytosol) was quantitated using LSM 510 Image browser software.

Western Blotting
Neutrophils or GFP-PX-RAW cells (5 x 10^5) were sonicated into 1x SDS-loading buffer, subjected to SDS-PAGE, transferred, and blotted for PI3KC2β (611342; BD Transduction Laboratories), Vps34 (38-2100; Zymed), p67^{phox} (07-502, Upstate Biotechnology), p47^{phox} (07-500, Upstate Biotechnology), p40^{phox} (07-501, Upstate Biotechnology) and/or β-actin (Clone AC-15 A5441, Sigma). Signal was detected by ECL and quantified using Aida Image Analyser 2.2.
RESULTS:

Neutrophil ROS production in response to phagocytosis of serum-opsonised *S.aureus* and *E.coli* requires complement receptors but is antibody-independent.

Rate measurements of intracellular ROS production were performed in human peripheral neutrophils and mouse bone marrow derived-neutrophils, using a luminol-based chemiluminescence assay. Under the conditions described here, detection of ROS by this assay is abolished by the general flavocytochrome inhibitor diphenyleneiodonuim (DPI) and absent in mouse neutrophils derived from *gp91^phox-/-* mice, indicating the ROS detected are derived from the neutrophil NADPH oxidase complex (Figure S1). This assay was chosen because of its sensitivity and accuracy but is compromised by its dependency on peroxidase activity in the vicinity of the superoxide anions being measured (during phagocytosis this is provided by enzymes like myeloperoxidase). For this reason, key observations were also confirmed, albeit with lower fidelity, using an assay based on direct, O$_2^-$-catalysed reduction of soluble tetrazolium salts to form insoluble formazan deposits (nitroblue tetrazolium (NBT) assay, see Supplementary Methods).

Incubation of TNFα/GM-CSF-primed human neutrophils with *S.aureus* or *E.coli* resulted in substantial ROS generation, peaking between 15-20min, (Figure 1Ai,iii). Similar kinetics of ROS generation were observed in mouse neutrophils, although overall production was nearly an order of magnitude lower (Figure 1Aii,iv). In the absence of neutrophil priming, the rates of ROS generation induced by either *S.aureus* or *E.coli* were slower (peaking at around 30min), although reaching equivalent maximal levels (data not shown). Most of our analyses were subsequently performed
with primed neutrophils to more closely mimic the physiological context but, key observations were repeated with unprimed cells where specifically noted.

Opsonisation of \textit{S.aureus} or \textit{E.coli} with 10% serum (human or mouse as appropriate) substantially increased both the rate and total production of ROS in both human and mouse neutrophils (Figure 1A). The main serum-derived opsonin receptors of neutrophils are FcγRs, which engage IgGs, and a subgroup of β2-integrins which bind complement fragments.\textsuperscript{2} Depletion of antibody from serum prior to bacterial opsonisation in either human or mouse neutrophil ROS responses; or the use of antibody-deficient mouse serum in mouse neutrophil ROS responses had no significant effect (Figure 1B, and data not shown). Furthermore, ROS responses to serum-opsonised \textit{S.aureus} and \textit{E.coli} were equivalent in neutrophils derived from wild type (WT) mice, or mice lacking the γ-chain of their activating FcγRs (FcRγ\textsuperscript{−/−}, Figure 1B). These results suggest that the \textit{S.aureus}- and \textit{E.coli}-induced ROS responses are independent of IgG- FcγR signalling.

Heat-inactivation of serum effectively abolished (>95% inhibition) serum-enhanced ROS production (H-I, Figure 1B), suggesting a role for heat-labile complement factors in these bacterial responses. Both phagocytosis (data not shown) and ROS production (Figure 1B) were reduced by over 95% in neutrophils derived from mice lacking the β2-integrin subunit of their major complement receptor (CD18\textsuperscript{−/−}), while responses to the soluble ligand fMLP remained unaffected. These results support an important role for complement opsonisation in the serum-enhanced responses to \textit{S.aureus} and \textit{E.coli}. Additionally, since the loss of CD18 reduced ROS responses to
below the level induced by unopsonised bacteria, β2-integrins appear to act as phagocytic receptors for these bacteria in the absence of serum-derived opsonins.

PI3Ks are involved in \textit{S.aureus}- and \textit{E.coli}-induced neutrophil ROS responses. 

Human or mouse neutrophils were pre-incubated with increasing concentrations of the general PI3K inhibitor wortmannin, prior to measurement of ROS responses to serum-opsonised \textit{E.coli} or \textit{S.aureus}. Low concentrations of wortmannin inhibited ROS generation in both human and mouse neutrophils in response to either bacterium (Figure 2A), demonstrating strong PI3K involvement. There were, however, some significant differences in the sensitivities of the individual responses to wortmannin; ROS production to \textit{E.coli} in both human and mouse neutrophils was more sensitive to wortmannin than equivalent responses to \textit{S.aureus} and, as noted previously,\textsuperscript{27} there was a significant wortmannin-insensitive component of the ROS response to \textit{S.aureus} in mouse neutrophils (Figure 2Aiii). These differences in wortmannin sensitivity were preserved in the absence of serum-opsonisation, suggesting their origin is not directly connected to complement-dependent signalling (data not shown).

Importantly, wortmannin did not inhibit phagocytosis of opsonised \textit{S.aureus} or \textit{E.coli} in either human or mouse neutrophils (Figure S2i), consistent with previous reports that phagocytic uptake of small particles is relatively insensitive to PI3K inhibition.\textsuperscript{38} Thus, the wortmannin-sensitive, PI3K-dependent components of these bacterial responses are downstream of phagocytosis and most likely reflect direct involvement in the signalling pathways to NADPH oxidase activation.
*S. aureus*- and *E. coli*-induced neutrophil ROS responses are substantially independent of Class I PI3K activity

Wortmannin displays similar potency towards all reported Classes of PI3K (with the exception of Class II PI3KC2α)\(^3\) and thus is a poor tool to distinguish between them. Hence, we assessed the role of Class I PI3Ks in *S. aureus*- and *E. coli*-induced ROS production by using recently characterised, isoform-selective Class I PI3K inhibitors and mouse Class I PI3K models.

Pre-treatment of human or mouse neutrophils with isoform-selective inhibitors for p110\(\alpha\) (YM-024, IC50 18nM),\(^1\) p110\(\beta\) (TGX221, IC50 7nM),\(^4\) or p110\(\delta\) (IC87114, IC50 500nM)\(^,4\) at concentrations up to and beyond their reported IC50s, and which we have previously demonstrated to effectively inhibit fMLP-induced ROS production in human neutrophils,\(^1\) failed to significantly inhibit ROS generation induced by serum-opsonised *S. aureus* or *E. coli* (Figure 2A). The p110\(\gamma\) inhibitor (AS252424, IC50 1\(\mu\)M)\(^,1\) demonstrated no inhibition at concentrations up to 1\(\mu\)M (colouration at concentrations above 1\(\mu\)M affected the luminol-based assay). ROS responses to *S. aureus* and *E. coli* in neutrophils derived from mice lacking the p110\(\gamma\) catalytic subunit (p110\(\gamma^{\text{c−}}\)),\(^,3\) or possessing a kinase-dead version of p110\(\delta\), p110\(\delta^{D910A/D910A}\) (p110\(\delta^{D910A}\))\(^,3\) (Figure 2B) were normal.

To investigate whether there is any functional redundancy amongst Class I PI3K isoforms in these responses, we also examined the effects of combining inhibitors (at concentrations approximately 10-20 times greater than their IC50s against their individual targets), using mouse neutrophils from a WT or p110\(\gamma^{\text{c−}}\) background. Paired combinations of 0.1\(\mu\)M TGX221, 3\(\mu\)M IC87114 and 3\(\mu\)M YM-024, in either
the presence or absence of p110γ, had no significant effect of ROS responses in either primed (Figure 2C) or unprimed (Figure S3) neutrophils to either *S.aureus* or *E.coli*. Pre-treating p110γ−/− neutrophils with all three inhibitors prior to addition of bacteria, resulted in a minor reduction in ROS generation compared to untreated, WT neutrophils (Figure 2C), but this was less than 15% of the inhibition induced by low concentrations of wortmannin.

Taken together, these results suggest that *S.aureus*- and *E.coli*-induced ROS responses in both mouse and human neutrophils, under primed or non-primed conditions, are largely independent of Class I PI3Ks.

*S.aureus*- and *E.coli*-induced neutrophil ROS responses are substantially independent of Class IIβ PI3K activity

Class IIα and β PI3K isoforms are widely expressed, Class IIγ is limited to liver, breast, prostate and salivary glands. The α and β isoforms differ greatly in their sensitivity to wortmannin, with Class IIβ displaying inhibition at 2-30nM (similar to Class I and III PI3Ks), while the α isoform requires concentrations greater than 400nM for significant inhibition. Given the observed wortmannin sensitivity of the *E.coli*- and *S.aureus*-induced ROS production described above (Figure 2A, IC50s 10-30nM), it would appear that they do not involve Class IIα.

The potential role of Class IIβ PI3K was investigated using both primed and unprimed neutrophils derived from mice lacking this isoform (Figure 3A and data not shown). *S.aureus*- and *E.coli*-induced ROS responses were the same in Class IIβ−/− and WT neutrophils (Figure 3B). Moreover, the wortmannin sensitivities of these responses
were similar for both genotypes (data not shown) confirming the lack of involvement of the Class IIβ PI3K isoform in the wortmannin-sensitive component of the ROS responses to *S. aureus* and *E. coli*.

Class III PI3K is required for maximal ROS responses to *S. aureus* and *E. coli* in model cell lines

A single Class III PI3K (Vps34) exists, which directly phosphorylates PtdIns to generate PtdIns(3)P. Deletion of this enzyme in mice has not been described and there are no available Class III-selective PI3K inhibitors. Fully differentiated neutrophils are non-dividing and difficult to manipulate genetically. We therefore decided to assess the role of Class III PI3K in both phagosomal PtdIns(3)P and ROS production in response to *S. aureus* or *E. coli* by an RNAi-mediated knockdown strategy in a model cell line. For most of our analyses, we chose a previously described mouse macrophage-like cell line, RAW264.7, stably expressing a GFP-tagged reporter for PtdIns(3)P (the isolated PX domain of p40phox; GFP-PX-RAW).

GFP-PX-RAW cells demonstrated clear DPI-sensitive, ROS responses to both *S. aureus* and *E. coli* (Figure S1 and 4A) although, as detected by the luminol-depndant chemiluminescence assay, these responses were considerably smaller and more transient than those seen in either human or mouse neutrophils (compare with Figure 1A). This is probably due, in part, to the relative lack of phagosomal peroxidases in RAW cells, since more equivalent ROS responses were seen between mouse neutrophils and RAW cells using the NBT reduction assay (data not shown). As with the neutrophil responses, both the rate and magnitude of this ROS production increased with serum-opsonisation (Figure 4A) and this effect was independent of
antibodies but sensitive to serum heat-inactivation (data not shown). Further, ROS responses to both *S. aureus* and *E. coli*, but not phagocytosis (Figure S2ii), were inhibited by wortmannin (IC50 15nM, Figure 4Aiii) and insensitive to Class I PI3K-selective inhibitors (Figure 4B). Thus, the GFP-PX-RAW cells employed in this study exhibit similar characteristics with respect to *S. aureus-* and *E. coli*-induced ROS responses to human and mouse neutrophils.

GFP-PX-RAW cells were infected with retroviruses expressing shRNAi directed against two separate regions of Class III PI3K (Vps34.1 and Vps34.2) and a non-silencing control (CON). Western blot analysis demonstrated significant knockdown of Class III PI3K expression in both Vps34shRNAi cell populations (Figure 5A, Vps34.1, 32%, and Vps34.2, 20% of control expression). Phagocytosis of *S. aureus* and *E. coli* by Vps34shRNAi and control GFP-PX-RAW cells were equivalent (Figure S2iii). However, ROS generation in Vps34shRNAi cell populations in response to either *S. aureus* or *E. coli* were significantly impaired compared to CONshRNAi populations (Figure 5B), indicating a significant role for Class III PI3K in these responses. These reductions in ROS responses measured by luminol-dependant chemiluminescence were mirrored by parallel reductions in phagosomal ROS measured by the NBT assay (data not shown), suggesting they are driven by lowered NADPH oxidase activity and not peroxidase availability.

We also attempted to confirm some of the key observations made in the GFP-PX-RAW cells using the human myeloid-derived cell line, PLB-985. Both phagocytosis and ROS production in differentiated PLB-985 cells (0.5% *N,N*-dimethylformamide in cell media, for 6 days, see Supplementary Methods) challenged with *S. aureus* or
*E.Coli* showed very similar sensitivities to wortmannin and Class I PI3K inhibitors as the equivalent responses in RAW cells and neutrophils described above (data not shown). Furthermore, we were able to generate populations of differentiated PLB-985 cells with modest reductions in the expression of Class III PI3K using lentiviral-mediated delivery of control- and Vps34-directed shRNAi (Figure S4A) and these populations exhibited parallel reductions in ROS responses to *S.aureus* and *E.Coli* (Figure S4B), supporting a conserved role for Class III PI3K in their regulation.

Class III PI3K is required for PtdIns(3)P synthesis on *S.aureus* and *E.coli* phagosomes. As previously described, basal distribution of the GFP-PX, PtdIns(3)P-probe in GFP-PX-RAW cells was partially cytosolic and partially distributed in vesicular, endosomal structures. Uptake of *S.aureus* or *E.coli* resulted in strong phagosomal accumulation of the GFP-PX probe, which could be completely inhibited by wortmannin pre-treatment (data not shown). Quantitation of over 100 phagocytic events demonstrated that around 75% of *S.aureus* taken up by the cells during the first 7min were surrounded by GFP-positive phagosomes.

The distributions of the GFP-PX probe in CON- or Vps34shRNAi cells, in the absence (Figure 6A) or presence (Figure 6B) of RITC-labelled *S.aureus*, were carefully quantified by confocal microscopy (Figure 6C). In the absence of bacteria, CONshRNAi cells displayed similar cytosolic and vesicular distributions of the GFP-PX probe compared to uninfected GFP-PX-RAW cells (data not shown). In contrast, Vps34shRNAi cells exhibited an approximately 50% reduction in the ratio of vesicular to cytosolic GFP-fluorescence (Figure 6Ci), indicating knockdown of Class III PI3K expression results in effective reduction of vesicular PtdIns(3)P levels.
In the presence of *S. aureus*, CONshRNAi cells showed the expected, dramatic accumulation of GFP-PX probe around *S. aureus*-containing phagosomes and associated drop in cytosolic GFP-fluorescence (Figure 6B and data not shown). In marked contrast, Vps34shRNAi cells showed much reduced accumulation of the GFP-PX probe around the phagosome with less than 20% of engulfed, RITC-labelled *S. aureus* surrounded by a GFP-positive phagosome (Figures 6B, 6Cii). Similar results were observed in Vps34shRNAi cells following phagocytosis of serum-opsonised, RITC-labelled *E. coli* (data not shown).

These results strongly suggest that Class III PI3K plays a key role in regulating the accumulation of PtdIns(3)P in phagosomes created by uptake of serum-opsonised *S. aureus* or *E. coli*.

PtdIns(3)P binding to the PX domain of p40\textsuperscript{phox} contributes to the regulation of neutrophil ROS production in response to *S. aureus* and *E. coli*. The results described above suggest that Class III PI3K-generated PtdIns(3)P is responsible for most, perhaps all, of the wortmannin-sensitive component of phagosomal ROS produced in response to uptake of serum-opsonised *S. aureus* and *E. coli*. Previous studies have demonstrated a role for direct interaction of PtdIns(3)P with the core NADPH oxidase component p40\textsuperscript{phox} in several contexts of ROS production, including uptake of *S. aureus* by mouse neutrophils.\textsuperscript{27,30,43-45} We therefore sought to establish the extent to which the effects of Class III-generated PtdIns(3)P on oxidase regulation may be explained by this interaction.
ROS production was measured in neutrophils derived from mice carrying a homozgyous mutation in the PX domain of p40\textsuperscript{phox} (p40\textsuperscript{phoxR58A/R58A}), which prevents high affinity binding to PtdIns(3)P. This mutation did not affect NADPH oxidase subunit expression, in contrast to significantly reduced p67\textsuperscript{phox} expression when p40\textsuperscript{phox} is absent (Figure 7A).\textsuperscript{31} While ROS responses to fMLP were only mildly inhibited by the R58A mutation (<15%, Figure 7Cii), ROS generated in response to either \textit{S.aureus} or \textit{E.coli} was substantially reduced (50% and 30% overall inhibitions respectively, Figure 7B,Ci). As previously noted, in a comparison between \textit{S.aureus} responses in p40\textsuperscript{phoxR58A/-} versus p40\textsuperscript{phox+/-} neutrophils,\textsuperscript{27} this R58A-sensitivity represents a large proportion (approximately 70%) of the wortmannin-sensitive component of the \textit{S.aureus} response. However, the R58A-sensitive component of the ROS response to \textit{E.coli} represented a much smaller proportion (approximately 30%) of the wortmannin-sensitive response. This clearly suggests there are likely to be additional p40\textsuperscript{phox} PX domain-independent roles for PtdIns(3)P in the regulation of phagosomal ROS production. These additional roles are unlikely to include the relatively trivial explanation that PtdIns(3)P regulates delivery of peroxidase-containing granules to the phagosome, affecting the peroxidase–dependant luminol assay, since analogous reductions in ROS production were seen using the NBT assay (Figure S5).
DISCUSSION

Phagocytosis of *S. aureus* or *E. coli* by both human and mouse neutrophils *in vitro* induced a robust, intracellular ROS response. Serum-opsonisation increased the initial rate of these responses approximately two-fold. This effect was independent of serum-derived antibodies and probably dependent on the deposition of heat-labile, complement factors. In mouse neutrophils, both phagocytosis and ROS production, in the presence or absence of serum, were dependent on CD18, the β2-integrin common chain and independent of the FcR γ-chain. There is now strong *in vivo* evidence that CD18-dependent mechanisms are critical for the efficient removal and destruction of bacteria by our innate immune system, including strains of *S. aureus* and *E. coli.*

CD18-dependent phagocytosis of *S. aureus* and *E. coli* was insensitive to inhibition by wortmannin at concentrations expected to inhibit Class I, Class IIβ and Class III PI3Ks. This differs with a significant body of work indicating Class I PI3Ks play an important role in the phagocytosis of IgG-opsonised particles, particularly when the particle is large. Several lines of evidence now suggest that this difference may relate to the different extents to which Class I PI3K-dependent actin rearrangement and membrane extension is employed during these distinct modes of phagocytosis. However, the CD18-dependent ROS production in response to both *S. aureus* and *E. coli*, in both human and mouse neutrophils, was potently inhibited by wortmannin indicating that, whilst wortmannin-sensitive PI3Ks do not regulate phagocytosis under these conditions, they are involved in the signalling pathways regulating activation of the NADPH oxidase.
The insensitivity of the *S.aureus* and *E.coli* ROS responses, in both human and mouse neutrophils, to a variety of Class I PI3K isoform-selective inhibitors, used either alone, in combination, or together with mouse models lacking PI3Kγ or δ activity, indicate they are very substantially independent of all Class I PI3K activity. This is a surprise, given the clear importance of this signalling pathway in both GPCR and FcR driven activation of the oxidase in neutrophils and macrophages.\textsuperscript{16-18,49,50} It is also a surprise given that integrin mediated adhesion and spreading on a matrix stimulates the neutrophil oxidase via a Class I PI3K-dependent mechanism\textsuperscript{51} (data not shown). Recently it has been shown that CD18-dependent signalling to the oxidase during matrix adhesion requires the FcR γ-chain.\textsuperscript{52} Thus, there appears to be a significant and interesting difference in the signalling pathways used by CD18-integrins with respect to the use of both the FcR γ-chain and Class I PI3Ks depending on the context in which they are operating i.e. adhesion versus phagocytosis. It is also important to note that there is currently a great deal of interest within the pharmaceutical sector in developing novel therapies based on isoform-selective, Class I PI3K inhibitors, particularly anti-PI3Kα agents in oncology and anti-PI3Kγ anti-PI3Kδ agents in inflammation.\textsuperscript{39,53-55} The results presented here suggest there is no a priori reason to predict these agents will inhibit non-antibody mediated phagocytosis and killing of bacterial pathogens.

ROS production in response to *S.aureus* or *E.coli* was normal in Class IIβ\textsuperscript{−/−} neutrophils, effectively ruling out this enzyme in these responses, at least in the mouse. This leaves the single Class III PI3K isoform as the most likely candidate for a PI3K mediating the wortmannin sensitivity of ROS responses to these bacteria. shRNAi-mediated reduction in the expression of Class III PI3K in RAW 264.7 cells
substantially reduced both phagosomal accumulation of a GFP-PtdIns(3)P reporter and ROS production in response to both serum-opsonised *S.aureus* or *E.coli*. In addition, shRNAi-mediated reduction in the expression of Class III PI3K in differentiated PLB-985 cells also caused parallel reductions in ROS responses to these bacteria. Furthermore, the similar sensitivities of ROS responses to serum-opsonisation, wortmannin and Class I-selective PI3K inhibitors between RAW cells, PLB-985 cells, mouse and human neutrophils, together with the data described above, provides strong evidence that Class III PI3K indeed plays a major role in CD18-dependant ROS responses to *S.aureus* and *E.coli* in neutrophils.

Previous work has implicated Class III PI3K in phagosomal PtdIns(3)P production during FcR-mediated uptake of IgG-latex beads in CHO cells heterologously expressing FcγRIIa, suggesting that Class III PI3K may be a common route for PtdIns(3)P synthesis in phagosomes generated by distinct uptake pathways. The mechanisms coupling phagocytic receptors to activation of Class III PI3K are unknown but could in principle involve direct recruitment of Class III PI3K from the cytosol to the formed phagosome or indirect recruitment via the docking of Class III PI3K-enriched endosomal compartments. Good circumstantial evidence suggests that the small GTPase Rab5 may be involved in the recruitment mechanism since it is known to be both a key player in early phagosomal maturation and to bind directly to Class III PI3K.

Given the number of effectors in intracellular trafficking that specifically bind PtdIns(3)P, blocking its synthesis by reducing the expression of Class III PI3K could, in principle, reduce phagosomal ROS responses by several direct or indirect
routes. Previous work has suggested that the binding of PtdIns(3)P to the PX domain of p40phox is important for regulation of the oxidase in several different contexts of cell stimulation. \(^\text{27,30,43-45}\) We had previously created a mouse strain carrying an R58A knock-in mutation in the PX domain of p40phox to directly assess the significance of this interaction but, unfortunately, high rates of lethality amongst p40phoxR58A/R58A embryos forced a comparison between p40phoxR58A/− and p40phox+/− neutrophils. \(^\text{27}\) However, further breeding against a C57BL/6J background has now enabled us to create matched cohorts of p40phoxR58A/R58A and p40phox+/+ mice and allowed a direct comparison of neutrophil ROS responses between them. Results using these neutrophils strengthen our previous conclusion that PtdIns(3)P binding to the PX domain of p40phox can largely explain the wortmannin-sensitivity of the ROS response to \(S.\text{aureus}\) but, surprisingly, also indicate that this interaction cannot effect more than approximately 30% of the wortmannin-sensitive ROS response to \(E.\text{coli}\). This then provides a natural explanation for why the ROS responses to these two bacteria differ in their wortmannin sensitivity and brings sharply into focus the notion that PtdIns(3)P must also regulate ROS formation via p40phox PX domain-independent routes that are yet to be elucidated, these might include for example PtdIns3P-dependant membrane fusion events required for optimal NADPH oxidase assembly and activation.
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Authorship and Conflict of Interest Statements:

K.E.A.: Designed and performed research, analysed and interpreted data, wrote the manuscript
K.B.: Performed Research
K.D.: Performed Research
T.C.: Performed Research
S.K.: Performed Research
G.J.: Contributed vital reagents
A.S.: Contributed vital reagents
K.S.-K.: Contributed vital reagents
O.R.: Contributed vital reagents
L.R.S.: Designed experiments, analysed and interpreted data, wrote the manuscript
P.T.H.: Designed experiments, analysed and interpreted data, wrote the manuscript

The authors declare they have no competing financial interests
REFERENCES:


27. Ellson C, Davidson K, Anderson K, Stephens LR, and Hawkins PT. PtdIns3P binding to the PX domain of p40phox is a physiological signal in NADPH oxidase activation. EMBO J. 2006;25:4468-4478.


Figure legends:

Figure 1. ROS generation in human and mouse neutrophils in response to *S.aureus* and *E.coli*.

(A) Human peripheral (i, iii), and mouse bone marrow derived (ii, iv) neutrophils were prepared, primed with TNFα and GM-CSF and pre-incubated with luminol as described in “Materials and Methods”. Cells (5 x 10^5/well) were added to 1 x 10^7 unopsonised (open triangles) or serum-opsonised (closed squares) *S.aureus* (i, ii) or *E.coli* (iii, iv), or incubated in the absence of bacteria (open diamonds) and chemiluminescence recorded using a Berthold Microlumat Plus luminometer. Incubations were performed in duplicate, and data (mean ± range) from one experiment representative of three are shown, and are expressed as relative light units/sec (RLU/s).

(B) Primed bone marrow neutrophils derived from C57BL/6J mice (WT) or mice lacking either the β2-integrin common chain CD18 (CD18^-/-), or the Fc receptor γ chain (FcRγ^-/-) were incubated with *S.aureus* or *E.coli* opsonised with 10% serum generated from C57BL/6J (normal) or RAG2/^γC^-/- (Ab deficient) mice. Where indicated, normal serum was depleted of antibody prior to opsonisation, by incubation with protein G sepharose, or heat-inactivated (H-I) at 56°C for 1hr. Cells (5 x 10^5/well) were added to 1 x 10^7 serum-opsonised *S.aureus* (black), *E.coli* (grey), or 10µM fMLP (hatched bars), and light emission measured over 40min as described in Figure 1A. ROS production in response to *E.coli* opsonised with antibody depleted serum was not determined (N.D). All experiments were performed in duplicate and data (mean ± SEM) are accumulated light emission for a combination of at least two
experiments, expressed as a percentage of the response in WT mouse neutrophils to 10% normal serum-opsonised bacteria.

**Figure 2. Effect of general and Class I PI3K isoform selective inhibitors on *S.aureus* and *E.coli* induced ROS formation in neutrophils.**

(A). Primed human (i, ii) and mouse (iii, iv) neutrophils were pre-incubated for 10min with wortmannin (black squares), TGX-221 (light grey circles), IC87114 (grey diamonds), YM-024 (black triangles) or AS252424 (grey squares) as indicated, in the presence of luminol as described in “Materials and Methods”. Cells were added to serum-opsonised *S.aureus* (i, iii) or *E.coli* (ii, iv), and ROS responses measured over 40 min, as described in Figure 1. Data (mean ± SEM, n ≥ 3) are accumulated light emission, expressed as a percentage of response in the absence of inhibitor (DMSO control).

(B). Primed bone marrow neutrophils, derived from wild type mice (WT, black) or mice lacking p110γ (p110γ−/−, dark grey), or expressing a kinase-dead version of p110δ (p110δD910A, light grey) were prepared and ROS generation in response to *S.aureus* or *E.coli* measured as described in Figure 1. Data are mean ± SEM from at least two independent experiments performed in duplicate, and expressed as a percentage of response from neutrophils derived from wild type mice.

(C). Primed bone marrow neutrophils derived from WT or PI3Kγ−/− mice were pre-incubated with either 100nM wortmannin, 0.1µM TGX221 (TGX), 3µM YM-024 (YM), or 3µM IC87114 (IC) as indicated, alone or in combination, for 10min prior to addition of serum-opsonised *S.aureus* (black) or *E.coli* (grey). ROS generation was
measured as described in (A). Data are mean ± SEM, n≥6 and are expressed as a percentage of WT untreated responses.

Figure 3. Effect of absence of PI3KC2β on neutrophil ROS responses to *S.aureus* and *E.coli*.

(A). Bone marrow derived neutrophils from PI3KC2β *+/+, +/−, and −/−* animals were sonicated into SDS-sample buffer, subjected to SDS-PAGE, and immunoblotted for PI3KC2β as described in “Materials and Methods”. PI3KC2β is indicated by the arrow.

(B). Primed bone marrow neutrophils derived from C57BL/6J WT (black diamonds) and PI3KC2β−/− (grey squares) mice were prepared as described in “Materials and Methods” and ROS production in response to serum-opsonised *S.aureus* (i) and *E.coli* (ii) measured, in the presence of luminol, as described for Figure 1. Open symbols (black diamonds-WT, grey squares- PI3KC2β−−) represent neutrophil ROS generation in the absence of bacterial addition. Shown are mean ± range from duplicate measurements of one representative experiment of three. Insets show average ± SEM accumulated light emission over a 40min measurement period for combined data, expressed as a percentage of the wild type neutrophil responses.

Figure 4. Characterisation of ROS responses in GFP-PX-RAW cells in response to *S.aureus* and *E.coli*.

RAW264.7 cells expressing a GFP-PX probe for PtdIns(3)P (GFP-PX-RAW cells) were prepared as described in “Materials and Methods”. (A). Cells (5 x 10^5/well)
were added to 1 x 10^7 unopsonised (open triangles) or serum-opsonised (closed squares) \textit{S.aureus} (i) or \textit{E.coli} (ii), and chemiluminescence recorded as described in Figure 1. Open diamonds represent ROS generation in the absence of bacterial addition. Incubations were performed in duplicate, and data (mean ± range) from one representative experiment of three are shown, and are expressed as relative light units/sec (RLU/s). (iii) GFP-PX-RAW cells were pre-incubated with 0-100nM wortmannin in the presence of luminol as described in “Materials and Methods”. Cells (5 x 10^6/well) were added to 1 x 10^7 serum-opsonised \textit{S.aureus} (black squares) or \textit{E.coli} (grey triangles), and light emission measured over 40min as described in Figure 1. All incubations were performed in duplicate and data from at least three experiments (mean ± SEM, n ≥ 6), are accumulated light emission, expressed as a percentage of response in the absence of wortmannin.

(B). GFP-PX-RAW cells were pre-incubated for 10min at 37°C with either DMSO vehicle control or, 100nM wortmannin, 0.1µM TGX221, 3µM YM-024, 3µM IC87114 as indicated, alone or in combination, or in the presence of 1µM AS252424, prior to addition of serum-opsonised \textit{S.aureus} (black) or \textit{E.coli} (grey). ROS generation was measured as described in Figure 1. Data are mean ± SEM, n≥6 and are expressed as a percentage of WT untreated responses.

\textbf{Figure 5. Effect of RNAi-mediated knock-down of Class III PI3K expression on ROS responses to \textit{S. aureus} and \textit{E. coli} in GFP-PX-RAW cells.}
GFP-PX-RAW cells were infected with retroviral constructs directing expression of non-silencing control shRNAi (CON), or two independent Class III PI3K shRNAi constructs (Vps34.1, Vps34.2), as described in “Materials and Methods”.

(A). shRNAi treated GFP-PX-RAW cells were harvested, sonicated into SDS-sample buffer, subjected to SDS-PAGE and immunoblotted for Class III PI3K and, as a loading control, actin.

(B) Control (black diamonds) and two Vps34 (open squares and triangles) shRNAi GFP-PX-RAW cell populations were harvested, incubated with luminol, and ROS generation measured over time in response to serum-opsonised *S.aureus* (i) or *E.coli* (ii) as described in Figure 1. Data shown are mean ± range for duplicate measurements in one representative experiment of three. (iii). Total accumulated superoxide production for control and Vps34shRNAi GFP-PX-RAW cells in response to *S.aureus* and *E.coli* from four independently derived cell populations, was calculated over a 20min measurement period. Data are mean ± SEM.

**Figure 6. Effect of RNAi-mediated knock-down of Class III PI3K expression on endosomal and phagosomal PtdIns(3)P accumulation.**

GFP-PX-RAW cells expressing non-silencing control (CON) or independent Class III PI3K (Vps34.1, Vps34.2) shRNAi were adhered to glass coverslips and incubated in the absence (A), or presence (B) of RITC-labelled, serum-opsonised *S. aureus* as described in “Materials and Methods”. Samples were fixed, mounted, and GFP-positive endosomes (A) and phagosomes (B) visualised on a Zeiss LSM 510 META point-scanning microscope. Shown are images from a single 1µm confocal plane.
(C). GFP-endosomal and cytosolic accumulation (i) and GFP-positive phagosomes (ii) from cells described in (A) and (B) respectively, were quantified from 6 x 1µm z-section confocal images using LSM 510 software. Data are mean ± SEM for at least 20 cells, or 100 phagocyte events in control (black) and Vps34.1 (dark grey), Vps34.2 (light grey) shRNAi treated cell, and are expressed as endosomal GFP accumulation as a ratio of cytosolic GFP accumulation in (i) or GFP-positive phagosomes expressed as a percentage of total phagosomes in (ii).

Figure 7. Effects of R58A mutation in p40phox on mouse neutrophil ROS responses.

(A). Bone marrow neutrophils derived from wild type (p40phox+/+), p40phoxR58A/R58A, or p40phox−/− animals were sonicated into SDS-sample buffer, subjected to SDS-PAGE and immunoblotted for p40phox, p47phox and p67phox as described in “Materials and Methods”.

(Bi,ii) and (Ci). Primed bone marrow neutrophils from p40phox+/+ (black diamonds) and p40phoxR58A/R58A (grey triangles) mice were prepared and pre-incubated with luminol in the absence (solid symbols) or presence (open symbols) of 100nM wortmannin, as described in “Materials and Methods”. Cells (5 x 10⁵/well) were added to 1 x 10⁷ serum-opsonised S.aureus (Bi) or E.coli (Bii), and light emission measured over 40min as described in Figure 1. (Bi,ii). Open circles represent ROS production in the absence of bacteria for p40phox+/+ (black) and p40phoxR58A/R58A (grey) neutrophils. Rate measurements of ROS production (mean ± range) from a single experiment representative of three. (Ci) Accumulated light emissions over the 40min
measurement period combined from three individual experiments and expressed as a percentage of WT responses (means ± SEM).

(Cii). Primed bone marrow neutrophils from p40^{phox+/-} and p40^{phoxR58A/R58A} mice were incubated, in the presence of luminol and HRP (18.75 U/ml), with fMLP (10µM final) and ROS production measured as described in Figure 1, except light emission was recorded for three minutes. Data presented are accumulated light emissions from three independent experiments (mean ± SEM).
Figure 1.

A.

*Staphylococcus aureus* Human Neutrophils

*Escherichia coli* Human Neutrophils

Mouse Neutrophils

![Graphs showing ROS production over time for *S. aureus* and *E. coli* in human and mouse neutrophils.]

B.

![Bar graph showing ROS production in different genotypes and serum conditions.]

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Serum</th>
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<td>WT</td>
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<td>Ab depleted</td>
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<tr>
<td>CD18+</td>
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Figure 2.
Figure 3.

A.

\[
\text{PI3KC2}^\beta \quad +/+ \quad +/- \quad -/-
\]

B.

**S. aureus**

- **i)**
- \[\text{ROS production (RLU/sec x 10^7)}\]
- \[\text{Time (min)}\]

**E. coli**

- **ii)**
- \[\text{ROS production (RLU/sec x 10^7)}\]
- \[\text{Time (min)}\]
Figure 4.

A.

![Graph showing ROS production over time for S. aureus and E. coli](image)

B.

![Bar graph showing ROS response to different treatments](image)
Figure 5.

A.

<table>
<thead>
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<th>Vps34.2</th>
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<tr>
<td>Actin</td>
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</table>

B.

\[\text{ROS production (RFLUx10^{-2}/sec)}\]

\(\text{Time (min)}\)

\[\text{CON} \quad \text{Vps34.1} \quad \text{Vps34.2}\]

\[\text{ROS production (%control response)}\]

\(\text{S.aureus} \quad \text{E.coli}\)
Figure 6.

A. CON   Vps34.1   Vps34.2

B. GFP-PX   DIC
          CON
          RITC   MERGED
          1
          2

C. i) GFP-endoosomal vesicles (% total)
    CON   Vps34.1   Vps34.2
    ii) GFP-positive endosomes (% total phagosome)
     CON   Vps34.1   Vps34.2
Figure 7.

A.

$\begin{array}{ccc}
+/- & R58A/R58A & -/- \\
p67_{phox} & \text{band} & \text{band} \\
p47_{phox} & \text{band} & \text{band} \\
p40_{phox} & \text{band} & \text{band}
\end{array}$

B.

i) $S.\text{aureus}$

ii) $E.\text{coli}$

C.

i) $S.\text{aureus}$

ii) $E.\text{coli}$
CD18-dependent activation of the neutrophil NADPH oxidase during phagocytosis of *E.coli* or *S.aureus* is regulated by Class III but not Class I or II PI3Ks


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