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Class IA Phosphoinositide 3-Kinase β and δ Regulate Neutrophil Oxidase Activation in Response to Aspergillus fumigatus Hyphae

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An effective immune response to the ubiquitous fungus Aspergillus fumigatus is dependent upon production of reactive oxygen species (ROS) by the NADPH oxidase. This is evidenced by the acute sensitivity of oxidase-deficient humans and mice to invasive aspergillosis. Neutrophils are recruited to the lungs shortly postinfection and respond by phagocytosing conidia and mediating extracellular killing of germinated hyphae in a ROS-dependent manner. However, the signaling mechanisms regulating the generation of ROS in response to hyphae are poorly understood. PI3Ks are important regulators of numerous cellular processes, with much recent work describing unique roles for the different class I PI3K isoforms. We showed by live-cell imaging that the lipid products of class I PI3Ks accumulated at the hyphal-bound neutrophil plasma membrane. Further, we used pharmacological and genetic approaches to demonstrate essential, but overlapping, roles for PI3Kβ and PI3Kδ in the ROS and spreading responses of murine neutrophils to Aspergillus hyphae. Hyphal-induced ROS responses were substantially inhibited by deletion of the common β2-integrin subunit CD18, with only a minor, redundant role for Dectin-1. However, addition of soluble algal glucans plus the genetic deletion of CD18 were required to significantly inhibit activation of the PI3K-effector protein kinase B. Hyphal ROS responses were also totally dependent on the presence of Syk, but not its ITAM-containing adaptor proteins FcRγ or DAP12, and the Vav family of Rac-guanine nucleotide exchange factors. These results start to define the signaling network controlling neutrophil ROS responses to A. fumigatus hyphae. * The Journal of Immunology, 2011, 186: 2978–2989.

Aspergillus fumigatus is a ubiquitous fungus whose infectious spores (conidia) are inhaled in the hundreds by most individuals on a daily basis. In healthy individuals, as well as in mice, low doses of inhaled conidia are phagocytosed by alveolar-resident macrophages and recruited neutrophils and are subsequently destroyed by the release of microbial substances into the phagosome. Some conidia, in particular after high inocula, escape immune detection, swell, and germinate into the invasive, hyphal form. Hyphae are too large to be phagocytosed and are instead targeted by neutrophils for extracellular killing, whereby neutrophils release reactive oxygen species (ROS) and granule contents onto their target (1, 2). The importance of ROS in these killing mechanisms is demonstrated by sufferers of chronic granulomatous disease (CGD), who carry genetic lesions in essential components of an ROS-generating NADPH oxidase complex and commonly present with life-threatening, invasive aspergillosis (1, 3). Further, neutrophils from CGD patients show impaired hyphal killing in vitro (4).

The neutrophil NADPH oxidase consists of a membrane-resident heme-containing flavocytochrome b$_{558}$, made up of gp91$^{phox}$ (NOX2) and p22$^{phox}$, together with the cytosolic subunits p67$^{phox}$, p47$^{phox}$, p40$^{phox}$, and the GTPase Rac (5). Upon stimulation of neutrophils with soluble agonists (e.g., inflammatory mediators) or particulate agonists (e.g., bacteria), these components assemble on plasma, phagocytic, or other intracellular membranes to form a functional NADPH oxidase complex. This complex transfers electrons from cytosolic NADPH across the membrane to molecular oxygen, to generate superoxide anions (O$_{2}$$^{-}$). O$_{2}$$^{-}$ is then quickly consumed by several reactions, including those converting it into other reactive species, collectively known as ROS (e.g., hydroxyl radical, H$_{2}$O$_{2}$, and HOCl) (6). The precise roles of ROS in pathogen killing are still debated, but they are likely to involve direct reactions with proteins and lipids, as well as the direct and indirect activation of proteases (2). The molecular mechanisms governing the assembly of the oxidase at the appropriate time and location are incompletely understood, but progress has been made in elucidating the roles of several signaling pathways, including the protein kinase C and ERK families of protein kinases and the PI3K family of lipid kinases (7).

The PI3K family of enzymes is subdivided into classes I, II, and III. Classes II and III family members are important in endosomal trafficking, autophagy, and phagosomal maturation and mediate their actions through the binding of phox homology (PX) and FabI/ YOTB/Vac/EEA1 domain-containing proteins to their sole lipid
product PtdIns(3)P. In contrast, class I PI3Ks catalyze the formation of the lipid second messenger PtdIns(3,4,5)P3, and its dephosphorylated derivative PtdIns(3,4)P2 and are important signal-transduction components of pathways downstream of a diverse array of cell-surface receptors, including those for growth factors, hormones, and Ags (8). Upon activation, class I PI3K lipid products mediate the recruitment and activation of pleckstrin homology (PH) domain-containing proteins, the best established of which is protein kinase B (PKB) (9). Class I PI3Ks are divided into two subfamilies: class IA, which consists of a heterodimer of a single homologous p110 catalytic subunit (α, δ, or β) and one of five related p50/p85 regulatory subunits, and class IB, which contains a single p110γ catalytic subunit and one of two p84 or p101 regulatory subunits (8, 9). PI3Kγ is classically activated by Gα-coupled G-protein coupled receptors through direct binding to Gβγ subunits and Ras (10). The class IA heterodimers are activated by recruitment, via their Src homology 2-domain-containing regulatory subunits, to phosphotyrosine residues in receptors or associated adaptor proteins. In neutrophils, the sequential activation of PI3Kγ and PI3Kδ has been identified as an important link in the regulation of the NADPH oxidase in response to soluble agonists acting through G-protein coupled receptors (e.g., FMLP and C5a), at least in part through the activation of guanine nucleotide exchange factors (GEFs) for Rac (11). However, the role of class I PI3Ks in oxidaode activation by other neutrophil receptors remains underexplored.

Upon recruitment to sites of infection, neutrophils engage various pathogen-associated molecular patterns via a broad range of pathogen-related receptors, including members of the TLR and C-type lectin receptor families, together with the β2-integrin Mac-1 (also known as complement receptor 3, αMβ2, CD11b/CD18) (2, 12). In particular, Dectin-1 was shown to bind β1-3-glucans on the surface of swollen A. fumigatus conidia and induce a neutrophil respiratory burst. Dectin-1 knockout (KO) mice are also susceptible to A. fumigatus infection (13). β2-integrins are shown to engage Syk-dependent signaling pathways for neutrophil oxidase activation in a manner dependent on the ITAM-bearing adaptor proteins Fcγ and DAP12, whereas Dectin-1 does so in an analogous manner via its own ITAM-like motif (12, 14).

We set out to investigate the contribution made by PI3Ks to neutrophil NADPH oxidase activation by A. fumigatus hyphae and to investigate the potential receptors and ITAM-based signaling pathways that may be involved in this response.

Materials and Methods

Reagents

Laminarin (from Laminaria digitata), human serum albumin (HSA), anti-HSA IgG, Congo Red, Tween 80, and Sabouraud’s agar were from Sigma-Aldrich. PI-103 was from Cayman Chemicals. All other reagents and inhibitors were as described (15).

Mouse strains

PI3Kγ−/− (yKO) (16), MyD88−/− (KO) (17), CD18−/− (β2KO) (18), P-Rex1−/− (KO) (19, 20), p40phox−/−/R50a−/− (21) (each on a mixed 129/Sv x C57BL/6d genetic background), PI3Kδ−/−/− (δKO) (22) (on a C57BL/6d background), Dectin-1−/−/− (KO) (23) (on a 129/Sv background), and Vav1−/−/NAVαν′ν'/Vav2−/−/Vav′2 (Vα1/23/3KO) (24) (mice on a B10.R background) have been described previously. Radiation chimeras with PI3Kβ−/−/−δKO/δKO/δKO (δKO/δKD); S. Kulkarni, C. Sitara, Z. Jakus, K. E. Anderson, G. Damoulakis, K. Davidson, M. Hirose, J. Juss, D. Oxley, T.A.M. Chessa, et al., submitted for publication) in the hematopoietic system were generated by adoptive bone-marrow transfer using Rag2−/− (19, 20), p40phoxR58A/R58A (21) (each on a mixed 129/Sv x 80/2335 backcross to B10.BR background) have been described previously. Radiation chimeras with PI3Kγ−/−/−DAP12−/− (F(Fe)KγKO/DAP12KO) (26, 27), Syk−/− (KO) (28, 29), or controls were generated as described and used in the laboratory of Attila Mocsai (Semmelweis University, Budapest, Hungary), according to protocols approved by the Semmelweis University Animal Experimentation Review Board. Where possible, mice were strain, age, or littermate matched to wild-type (WT) controls.

Preparation of mouse neutrophils

Murine bone-marrow neutrophils (BMNs) were purified as follows: bone marrow from femurs and tibias was dispersed in HBSS (without Ca2+/Mg2+) with 0.25% fatty acid-free BSA and centrifuged (1256 × g, 30 min, room temperature [RT]) over a discontinuous gradient composed of 62% and 55% Percoll in HBSS. Mature neutrophils were obtained from the interface (purity 75–90% by cytospin), and contaminating RBCs were removed by ammonium chloride lysis (130 mM NH4Cl, 5 mM KCl, 0.8 mM Na2HPO4, 0.175 mM KH2PO4 in salt buffer). Cells were washed twice in HBSS/BSA and resuspended in Dulbecco’s PBS (with Ca2+/Mg2+) containing 1 g/l glucose and 4 mM sodium bicarbonate (D-PBS). BMNs were purified from FeRγKO/DAP12KO and Syk KO chimeras as follows: whole bone marrow was dispersed in HBSS (without Ca2+/Mg2+) with 0.5% FCS, RBCs were removed by ammonium chloride lysis, and the cells were centrifuged (1200 × g, 30 min, RT) over a 62.5% Percoll gradient. Neutrophils were collected from the bottom of the Percoll layer, washed twice in HBSS/0.5% FCS, and resuspended in HBSS (with Ca2+/Mg2+) containing 20 mM HEPES. BMNs purified by either method produced ROS equivalently with all agonists tested. Cells were used immediately or were incubated with vehicle (DMSO; maximum 0.1%) or control the indicated inhibitors for 10 min at 37˚C prior to use.

Culturing of A. fumigatus

A. fumigatus Fresenius (American Type Culture Collection, cat. #90240) conidia were inoculated on Sabouraud’s agar in 175 cm2 flasks and grown for 7 d at 37˚C. Conidia were harvested by shaking with glass beads in PBS/0.05% Tween 80, filtered (40-μm cell strainer), washed twice, resuspended, enumerated, and stored at 4˚C. Fresh conidia were prepared weekly from cultures maintained at passages two through six.

For the measurement of ROS production using immobilized hyphae, 2 × 105 conidia in 200 μl RPMI 1640 medium were incubated for 18–22 h at 37˚C in a 96-well format in polystyrene plates (no. 23300, Berthold Technologies) or tissue-culture-treated plates (Nunc) for luminol and NBT-based assays, respectively. Where indicated, hyphae or corresponding empty wells were incubated in PBS or 100% FCS for 1 h at RT for blocking plastic surface. Prior to use, hyphae were washed twice with PBS using a 23 g needle for aspiration to minimize dislodgement. For ROS production using hyphae in suspension, freshly prepared conidia (1 × 106/ml) were grown on glass petri dishes (to minimize attachment to the surface) for 17 h at 37˚C. Hyphae were collected, washed (1500 × g), resuspended in D-PBS, and used. For imaging-based assays, hyphae were grown on 22 mm poly-l-lysine-coated cover slips, washed, and used. For Western blotting and immunoprecipitation assays, 5 × 106 conidia in 37˚C in modified RPMI 1640 in 100 μl HEPES/bicarbonate (to enrich for growth on larger plate surface) per well, in a six-well format, were grown into hyphae, washed, and used. More than 90% of conidia routinely germinated into >40-μm-long hyphae under all conditions.

Measurement of ROS production

Luminol-based chemiluminescence assays were performed as described (15). Light emission was recorded by a Berthold Microtiter-Plus luminometer for all the genotypes, with the exception of DAP12KO and Syk KO, which used a Berthold Mithras LB940 luminometer, both at 37˚C. Data output are expressed as relative light units per 106 cells by guest on November 8, 2017 http://www.jimmunol.org/ Downloaded from
well. TNF-α–potentiated adhesion assay was performed as described (32). Unopsonized zymosan A was prepared as per the manufacturer’s instructions (Invitrogen). Serum-opsonized Escherichia coli was prepared as described (15). A total of 5 × 10^5 BMNs was added per well for each assay. The phorbol ester PMA (300 nM), a receptor-independent oxidase stimulus, was used to demonstrate equivalent cell numbers and functional NADPH oxidase across genotypes within all experiments (data not shown).

**Fixed-cell imaging**

BMNs were incubated with hyphae in the presence or absence of NBT, supernatants were aspirated, and coverslips were washed with shaking to remove unattached cells and fixed in 4% paraformaldehyde. BMNs/hyphae were visualized by light transmission for NBT-formazan deposition or were visualized after blocking with anti-CD16/CD32 Ab (5 μg/ml; eBiosciences) and labeling with FITC-conjugated anti-Gr1 Ab (0.312 μg/ml; BD Biosciences). Hyphae exhibit marked autofluorescence at 500–525 nm when excited at 543 nm. Cells were visualized using an Olympus FV1000 confocal microscope and a 60× oil-immersion lens. Fluorescent BMNs were captured through the z-axis at 2 μm steps using Fluoview software. Five regions of interest per coverslip were imaged, and a minimum of 100 neutrophils was counted. Only cells attached to hyphae were scored and designated as “spread” when they exhibited a clear nonrounded morphology.

**Live-cell imaging**

BMNs were added to Congo Red-labeled (40 μg/ml) hyphae, and images were acquired on a spinning Nipkow disk Nikon-Eclipse confocal microscope equipped with an ultrasensitive EM-CCD camera (Andor iXon 897) using a Plan/Apo 100× oil-immersion objective (NA 1.4). A 488-nm laser line was used to excite GFP-expressing BMNs and Congo Red-labeled hyphae, with emission collected sequentially at 500–550 nm (GFP) and 580–653 nm (Congo Red). Videos were compiled from raw TIFF files in Velocity (Perkin Elmer) software, edited using ImageJ, and exported as Quicktime.mov format.

**Immunoprecipitation and Western blotting**

For immunoprecipitation experiments, 4 × 10^5 BMNs were incubated with hyphae in a six-well format at 37˚C, 6% CO_2 for the times indicated. Neutrophils/hyphae were lysed (10 mM Tris, 50 mM NaCl, 30 mM NaPi, 50 mM NaF, 1% Nonidet P-40, 1 mM NaVO_4, 1 mM PMSF, 1 mM β-glycerophosphate, 0.25 U/ml aprotinin and 10 μg/ml each leupeptin, antipain, and pepstatin A). and Vav1 was immunoprecipitated using rabbit anti-Vav1 polyclonal antisera (L126, gift from M. Turner, Babraham Institute) and protein G Sepharose. Immunoprecipitated protein from −potentiated adhesion assay was performed as described

**FIGURE 1.** Neutrophils release extracellular ROS in a hyphae-specific manner. A. Murine BMNs were incubated in the presence of luminol/HRP (Total), isoluminol/HRP (Extracellular) or luminol/SOD (Intracellular) and added (1 × 10^5 cells/well) to immobilized, unopsonized A. fumigatus hyphae; ROS production was determined. Error bars represent the mean ± SD of triplicate wells from one experiment, representative of four. B. Hyphae-coated or empty plastic wells were incubated in PBS (nonblock) or 100% FCS (FCS block) for 1 h. BMNs in luminol/HRP were added (1 × 10^5 cells/well), and chemiluminescence was recorded. Error bars represent the range of duplicate wells from a single experiment. C, BMNs in luminol/HRP (total) or luminol/ SOD (intracellular) were added (1 × 10^5 per well) to hyphae (2 × 10^5 per well) in suspension, and ROS production was determined. Error bars represent the range of duplicate wells from a single experiment. D, BMNs preincubated with DPI (5 μM) or vehicle in the presence of NBT were added to hyphae and incubated at 37˚C for 20 min. BMNs/hyphae were fixed, and differential interference contrast images were captured. Images, representative of three independent experiments, of single neutrophils interacting with hyphae are depicted. Note formazan deposits on hypha, with little intraneutrophil deposits, only in the absence of DPI. Original magnification ×60.
and added peroxidase, this assay gives a measure of extracellular ROS. Hyphae immobilized to the plate surface elicited a robust, sustained production of ROS from BMNs, of which >95% was extracellular (Fig. 1A). Consequently, all subsequent experiments using immobilized hyphae measured total ROS production (i.e., in the presence of luminol with added peroxidase). These ROS responses were dependent on the presence of neutrophils and hyphae (data not shown), could not be blocked by preincubation of the hyphal-coated plastic with FCS (Fig. 1B), and could be measured with hyphae in suspension (Fig. 1C). BMN dilution and time-course experiments determined a range of neutrophil concentrations and assay times within which the initial rate of ROS production was linear with respect to the number of neutrophils added (Supplemental Fig. 1A). Thus, a neutrophils/hyphae ratio of 1:2 and an assay time of 20 min was chosen for all subsequent experiments.

A second assay of oxidase activity, based on the reduction of NBT by superoxide, to form a dark blue formazan precipitate was also used. These precipitates can be visualized under the light microscope to provide information about the cellular localization of oxidase activity, and they can also be solubilized to yield population estimates of total superoxide formed. Formazan deposition was inhibited by the nonspecific NADPH oxidase inhibitor diphenyleneiodonium (DPI) and was restricted to the surface of hyphae in contact with neutrophils, with few intraneutrophil deposits (Fig. 1D) and with little escaping into the medium, suggesting neutrophils release ROS onto hyphae in a spatially restricted manner. For population assays, cell-dilution and time-course experiments determined that a neutrophils/hyphae ratio of 5:1 and an assay time of 1 h were within the linear range (Supplemental Fig. 1B); therefore, all subsequent experiments used these conditions.

**Neutrophil NADPH oxidase activation by A. fumigatus hyphae is dependent on the class IA PI3Ks (PI3Kβ and PI3Kδ)**

The general PI3K inhibitors wortmannin and PI-103 were used to investigate the involvement of PI3Ks in *A. fumigatus* hyphal-induced ROS. Wortmannin and PI-103 potently inhibited ROS production in a dose-dependent manner, with IC_{50} values ~3 and 85 nM, respectively, in the luminol assay, and 2.5 and 245 nM, respectively, in the NBT assay (Fig. 2A). Wortmannin and PI-103 exhibit broad, overlapping specificity across classes I, II, and III PI3Ks, but careful comparison between these dose curves and the known IC_{50} values for these inhibitors against various PI3Ks suggested that their most likely targets were class I PI3Ks (15, 33).

In agreement with this, BMNs from mice carrying a mutation in their p40phox PX domain (R58A), which prevents activation of the oxidase by the class II/III PI3K product PtdIns3P (15), exhibited no significant defect in ROS production measured in either assay (Fig. 2B).

We further investigated the potential involvement of class I PI3Ks in this response using a combination of isoform-selective catalytic site inhibitors and mouse genetics. Neutrophils purified from mice lacking the p110γ catalytic subunit (γKO) had similar ROS responses to WT neutrophils (Fig. 3A, 3B), suggesting that PI3Kγ does not play a unique, nonredundant role in this response. We were unable to effect acute, selective inhibition of PI3Kγ because the best available γ inhibitor, AS605240, exhibited marked off-target activity in γKO neutrophils (data not shown).

However, the hyphal ROS response in γKO neutrophils did not display an increased sensitivity to PI-103 (Fig. 3C), as would be expected should PI3Kγ be playing a redundant role (IC_{50} values of PI-103 for α, δ, β, and γ are 8, 48, 88, and 150 nM respectively). Thus, our data suggested that PI3Kγ does not play a major role in hyphal-induced ROS production.

We then used a selection of inhibitors to effect ≥85% isoform-selective inhibition of PI3Kβ (40 nM TGX221), δ (1 μM IC87114), or α/δ (2 μM YM024), or combinations thereof. The results of these experiments suggested that acute loss of δ or β activity alone resulted in minor reductions in the ROS response to hyphae, but combined inhibition of both isoforms substantially reduced this response (Fig. 3D). Further, the lack of any significant impact of YM024 versus IC87114 indicated that any role for PI3Kα must be minor. BMNs from mice lacking the p110δ γ catalytic subunits (γKO/δKO) displayed ~30% defect in the extent of ROS production; importantly, this defect was substantially increased to 80% by the addition of 100 nM TGX221 (Fig. 3E, 3F), a dose chosen to near maximally inhibit PI3Kβ but with some cross-inhibition of PI3Kδ (which is negated on a p110δ-deficient background). Further, neutrophils from mice lacking p110δ and carrying a kinase-dead (KD) knockin mutation in p110δ (δKO/δKD) also displayed a marked 80 ± 4.5% defect in this assay. The addition of a low dose of PI-103 (100 nM) to δKO/δKD neutrophils reduced this ROS response further, close to levels observed with 100 nM wortmannin, indicating a potentially minor

**FIGURE 2.** Neutrophil NADPH oxidase activation by hyphae is PI3K dependent but is largely independent of PtdIns(3)P. A. BMNs were preincubated in the presence of the indicated concentrations of wortmannin (wort), PI-103, or vehicle (DMSO), together with luminol/HRP (luminol) or NBT. BMNs (1 x 10^5 per well) were added to immobilized hyphae for luminol-based assays, chemiluminescence was recorded, and integrated light units over 20 min were plotted relative to DMSO control. BMNs (1 x 10^5 per well) were added to hyphae for NBT-based assays and incubated for 1 h, formazan deposits were solubilized, and OD at 655 nm was plotted relative to DMSO control. Data shown are means ± SD of two to five independent experiments, each with pooled BMNs from two mice per group. B, BMNs purified from mice with a point mutation in the PX domain (R58A) of p40phox or WT controls were assessed for ROS formation in response to hyphae using a luminol- or NBT-based assay, as described above. Representative kinetics of ROS production from luminol assay are shown (i), and integrated light units emitted over 20 min in luminol assay or formazan deposition over 1 h in NBT assay were measured and expressed relative to WT control (ii). Data are mean ± SD of triplicate wells from a single representative experiment (i) or mean ± SEM of at least three independent experiments, each performed with BMNs from one animal per group. p > 0.05.
role for PI3Kα in the absence of PI3Kβ and δ (Fig. 3G, 3H). Together, these results point to a major role for the class IA PI3Ks β and δ in supporting neutrophil ROS formation in response to hyphal recognition.

PI3Kβ and PI3Kδ regulate spreading of neutrophils on hyphae

When examined under the microscope, neutrophil attachment to A. fumigatus hyphae was usually followed by extensive spreading across and along the hyphal surface (Fig. 4A). This spreading was difficult to quantify because of the three-dimensional network of hyphal growth above the coverslip. Therefore, as a more qualitative measure, cells were scored as being spread when they exhibited a clear nonrounded phenotype. On average, ~60% of neutrophils attached to hyphae were spread within 20 min, whereas the addition of 3 μM PI-103 or 100 nM wortmannin completely prevented this (Fig. 4B). Moreover, genetic ablation of

FIGURE 3. Class IA PI3K regulates hyphae-induced NADPH oxidase. A through C, BMNs from PI3Kγ WT or KO mice were assessed for ROS production in response to hyphae, as described in Fig. 2. A, Kinetics from representative luminol-based assay, mean ± SD of triplicate wells. B, Total ROS production from luminol- and NBT-based assays; data are mean ± SEM of five independent experiments, each performed with BMNs from one animal per group. C, γWT or γKO BMNs were preincubated with the indicated concentrations of PI-103 or vehicle (DMSO) and added to hyphae for luminol-based assay. Total ROS production is expressed relative to DMSO-treated WT BMNs. Data are mean ± SEM of three independent experiments, each performed with BMNs from one animal per group. D, BMNs were preincubated with 40 nM TGX221 (221), 1 μM IC87114 (IC), 2 μM YM024 (YM), 100 nM wortmannin (Wm), or vehicle (DMSO) alone or in indicated combinations and then assessed for hyphal-induced ROS production, as described in Fig. 2, using luminol- or NBT-based assay. Data are mean ± SD of two or three independent experiments, each performed with pooled BMNs from two mice. E and F, BMNs from PI3KγKO or γWTWT mice were preincubated with 100 nM TGX221 or vehicle (DMSO). Representative kinetics (E) and total ROS production (F) are shown. Data are mean ± SD of triplicate wells from a single representative experiment (E) or mean ± SD of two independent experiments, each performed with BMNs from one animal per group (F). G and H, BMNs from PI3KβKO or βWTWT bone-marrow chimeras were preincubated with 100 nM PI-103 or vehicle (DMSO). Representative kinetics (G) and total ROS production (H) from luminol-based assay are shown. Data are mean ± SD of triplicate wells from a single representative experiment (G) or mean ± SEM of three independent experiments, each performed with BMNs from one animal per group (H). *p < 0.01.
PI3Kβ and δ activity also resulted in a 50% defect in spreading at 10 and 20 min (Fig. 4C). Therefore, class I PI3Ks are required for neutrophil spreading on hyphae; although PI3Kβ and δ clearly play a major role, there may be more redundancy between PI3K isoforms in spreading than the ROS response.

**Hyphae induce sustained activation of class I PI3K at the neutrophil–hyphal interface**

PKB seems to be invariably activated downstream of class I PI3Ks through PH domain-mediated recruitment to PtdIns(3,4,5)P3/PtdIns(3,4)P2 at the plasma membrane and subsequent phosphorylation on residues Thr308 and Ser473 (34). Ser473 phosphorylation levels were very low in unstimulated BMNs, but they markedly increased over 20 min of hyphal stimulation. Inhibition of PI3K with PI-103 (1 μM) completely abrogated hyphal-induced Ser473 phosphorylation (Fig. 5A).

Expression of a fusion protein of GFP with the PH domain of PKB (GFP-PH) has been used to determine the temporal and spatial regulation of class I PI3K in neutrophils (25). BMNs from GFP-PH-expressing mice were imaged during incubation with Congo Red-stained A. fumigatus hyphae using a spinning disk confocal microscope for rapid image acquisition in a single plane of focus, obviating confounding factors associated with widefield microscopy, such as cytosolic entrapment. Upon settling on coverslips, GFP-PH was diffusely cytosolic, but upon attachment to the hyphal filament or the parental conidial head (data not shown), it rapidly accumulated at the site of contact. GFP-PH exhibited sustained localization at the growing neutrophil–hyphal interface over ≥20–30 min (Fig. 5B, Supplemental Video 1). Occasionally, neutrophils attempted to engulf more than a single hypha (Supplemental Video 2), and GFP-PH accumulation was evident along neutrophil membranes interacting with both hyphae. In conclusion, there was rapid and prolonged synthesis of PIP3 in neutrophils adjacent to A. fumigatus hyphae.

**Hyphal-induced neutrophil oxidase activation is dependent on β2-integrins**

We attempted to identify upstream components of the signal-transduction pathway mediating neutrophil oxidase activation in response to A. fumigatus hyphae using previously established strains of genetically engineered mice.

Certain TLR family members were shown to recruit class I PI3Ks via the adaptor protein MyD88 (35); furthermore, deletion of the MyD88 gene renders otherwise healthy mice more susceptible to infection with A. fumigatus (36). In agreement with previous findings (37), BMNs from MyD88 KO mice exhibited a partial defect in ROS production to serum-opsonized Escherichia coli (Fig. 6A), whereas they showed similar hyphal-induced ROS responses to WT (Fig. 6Ai, 6Aii, 6Aiii), indicating that TLR signaling does not play a unique role in NADPH oxidase activation in these assays.

Dectin-1 is important in mediating neutrophil NADPH oxidase activation in response to unopsonized A. fumigatus conidia (13). However, the contribution of neutrophil Dectin-1 to the recognition of hyphae has not been formally addressed. As expected (23),

**FIGURE 4.** PI3Kβ and PI3Kδ are required for optimal neutrophil spreading on hyphae. WT BMNs preincubated with 3 μM PI-103, 100 nM wortmannin, or vehicle (DMSO) were added to hyphae and incubated at 37˚C for 20 min. Coverslips were washed, and cells were fixed and stained with FITC-conjugated anti-Gr1 Ab. Hyphae show autofluorescence at 500–525 nm when excited at 543 nm. A. Representative images depict neutrophil morphology in the absence (left panel) or presence (right panel) of PI3K inhibition. Scale bar, 10 μm. B. BMNs attached to hyphae were scored as “spread” when they exhibited a clear nonrounded morphology, otherwise they were scored as “unspread.” At least 100 neutrophils were counted per coverslip. Data are expressed as the percentage of total BMNs (spread or unspread) per coverslip. Error bars represent the SD of duplicate coverslips from one experiment, representative of two. C. PI3K βWT/WT or βKO6KD BMNs were incubated with hyphae for the times indicated, and coverslips were processed as in B. Data are expressed as the mean percentage of spread versus unspread BMNs ± SEM of three independent experiments, each performed in triplicate with BMNs from one animal per group. *p < 0.01, significantly fewer βKO6KD BMNs spread compared with βWT/WT BMNs at the 20 min time point.

**FIGURE 5.** Class I PI3K is activated at the neutrophil plasma membrane interacting with hyphae. A. BMNs preincubated with vehicle (DMSO) or 1 μM PI-103 were added to hyphae for the times indicated and lysed, and total lysates were probed for phospho-Ser473 PKB (anti-p–PKB) or Vav1, as loading control, by Western blotting. A single experiment, representative of four, is shown. B. BMNs from mice expressing the isolated PH domain of PKB fused to GFP (GFP-PH) were added to Congo Red-stained hyphae. Single frames from a single focal plane at the indicated time points are shown; the full video is available in the supplemental material. Arrow shows diffuse localization of GFP-PH reporter in BMN unattached to hyphae (i), whereas upon attachment (iv) and during engagement (vii), the reporter exhibits enrichment at the point of contact. Scale bar, 5 μm.
Dectin-1 KO BMNs had a severe defect in the ROS response to the yeast cell-wall particle zymosan (Fig. 6Bi). Surprisingly, however, Dectin-1 KO BMNs did not display a significant defect to hyphae (Fig. 6Bii, 6Biii). The β2-integrin family of cell surface receptors has been implicated in adhesion, chemotaxis, and phagocytosis (via opsonin-dependent and -independent modes) (38). BMNs from mice deficient for the β2 subunit (β2 KO) had severe defects in the ROS response to TNF-α–fibrinogen (Fig. 6Ci); moreover, they displayed an ~65% defect in ROS production to hyphae (Fig. 6Cii, 6Ciii).

We attempted to resolve whether there was any significant redundancy between β2-integrins and glucan receptors, such as Dectin-1, in hyphal ROS responses by investigating the effects of the reported Dectin-1 antagonist laminarin (39) (a soluble extract from the alga L. digitata that is predominantly composed of β-1,3-glucans) and β2-integrin–blocking Abs on neutrophils from β2 KO and Dectin-1 KO genetic backgrounds. The addition of laminarin effected a further 20% inhibition of hyphal ROS responses in BMNs from β2 KO mice, but it had no significant effect on Dectin-1 KO BMNs (Fig. 6Di, 6Dii). The addition of anti-β2 subunit-blocking Abs resulted in dose-dependent inhibition of hyphal ROS responses that asymptoted to a level of inhibition very similar to that seen in the β2 KO neutrophils (Supplemental Fig. 2). Further, addition of a single dose of blocking Ab caused a small, but significant (15–20%), further inhibition in Dectin-1 KO
BMNs compared with WT BMNs (Fig. 6Dii). Together, these data indicated that β2-integrins play a major role in the activation of the NADPH oxidase in response to hyphae, whereas Dectin-1 has only a minor, redundant role.

Hyphal-induced spreading and phosphorylation of PKB are relatively unaffected by deletion of β2-integrins or Dectin-1

BMNs isolated from β2 KO and Dectin-1 KO mice exhibited only minor, nonstatistically significant defects in spreading on hyphae (Fig. 7A, 7B). Consistent with this, loss of β2-integrins or Dectin-1 caused equivalently small defects in hyphal-induced phosphorylation of PKB (Fig. 7C–F). Thus, in contrast to activation of the NADPH oxidase, β2-integrins do not have a major unique role in the spreading of neutrophils on hyphae or the activation of class I PI3Ks, at least as assessed by PKB phosphorylation. Nevertheless, the addition of laminarin to BMNs from β2 KO mice effected a significant 75% reduction in phospho-PKB levels (Fig. 7C, 7E). However, β2-integrin-blocking Abs had little effect when combined with genetic deletion of Dectin-1 (Fig. 7D, 7F), suggesting that incomplete inhibition of β2-integrins on a Dectin-1 KO background still permits PI3K activation or, alternatively, laminarin may be inhibiting glucan receptors other than Dectin-1 that are required for PI3K activation. Taken together with the data described above, this suggested that β2-integrins and glucan receptors play a role in the activation of PI3K on hyphal recognition but that β2-integrins play a more exclusive role in the regulation of ROS production.

Syk is required for hyphal-induced neutrophil oxidase activation

β2-integrin–mediated neutrophil NADPH oxidase activation is dependent on the ITAM-containing adaptors FcRγ and DAP12 and the nonreceptor tyrosine kinase Syk in response to various adhesive surfaces (14). Furthermore, Syk is also activated by Dectin-1 and is required for NADPH oxidase activation downstream of this receptor in macrophages (40). Therefore, the potential involvement of an ITAM–Syk-signaling axis in response to A. fumigatus hyphae was addressed. To test this, BMNs were purified from mice that lacked FcRγ and DAP12 (FcRγKO/DAP12KO) or Syk, specifically in their hematopoietic compartment. FcRγKO/DAP12KO and Syk KO neutrophils had severe defects in immune complex-induced ROS production (Fig. 8Ai, 8Bii), as previously shown (26, 41). However, although FcRγKO/DAP12KO neutrophils exhibited no defect in ROS responses to hyphae (Fig. 8Aii, 8Aiii), Syk KO neutrophils had a profound 85–90% reduction in this response (Fig. 8Bii, 8Biii). Therefore, although Syk is required for hyphal-induced ROS formation, the ITAM-containing adaptor proteins known to regulate its activity in neutrophils are not.

Vav family of Rac GEFs are required for hyphal-elicted ROS production by BMNs

Rac is an essential component of the NADPH oxidase whose activation involves GTP/GDP exchange by GEF proteins (5, 6). P-Rex1 and Vav1/2/3 are the main GEFs previously established to be important in murine neutrophils for regulating Rac and the oxidase downstream of different receptors (20, 42, 43). P-Rex1 KO neutrophils had no defect in ROS production in response to hyphae (Fig. 9A), whereas Vav1/2/3 triple KO neutrophils had a near complete defect in this response (Fig. 9B).

The GEF activity of Vav is thought to be regulated by tyrosine phosphorylation and PtdIns(3,4,5)P3 binding to the PH domain of Vav (44–46). We detected a significant, but transient, increase in phosphorylation and PtdIns(3,4,5)P3 binding to the PH domain of Vav during hyphal engagement (Fig. 9C). This phosphorylation was partially inhibited by the addition of 1 μM PI-103, suggesting that class I PI3K regulation of hyphal-induced neutrophil ROS is dependent, at least in part, on the activation of Vav GEFs.

Discussion

Immunocompromised patients, particularly those with neutropenia, together with CGD sufferers who lack a functional NADPH oxidase, are particularly prone to invasive aspergillosis (1, 3). Fur-
thermore, mice deficient in gp91<sub>phox</sub> readily succumb to infection, highlighting the importance of neutrophil NADPH oxidase-derived ROS in the immune response to pulmonary <i>A. fumigatus</i> infection (47). In particular, although neutrophils and macrophages contribute to the removal of germinating conidia from the lung, neutrophils are thought to be the only immune cells capable of eliminating the hyphal form (1). In this study, we showed that class I PI3Ks are activated throughout engagement with hyphae in a spatially restricted manner along the interacting neutrophil plasma membrane; moreover, class IA PI3Ks are of critical importance in the activation of the neutrophil NADPH oxidase and an appropriate respiratory burst.

We used unopsonized hyphae in our experiments, because we found that serum opsonization had little effect on the kinetics or magnitude of ROS production (data not shown). Further, the validity of serum as an opsonizing factor for an organism whose usual route of infection is the lung is questionable. Other lung-restricted factors, such as surfactant proteins, have been suggested to play opsonin-like roles (48), although the contribution of these factors to the removal of germinating conidia from the lung is unclear, but they may involve another unidentified ITAM adaptor.

The protein tyrosine kinase Syk was absolutely required for murine neutrophils to mount an ROS response to hyphae. Surprisingly, however, deletion of the two major ITAM adaptors previously established to activate Syk in response to integrin engagement on neutrophils, FcRy and DAP12, had no effect on this response. Dectin-1 harbors an ITAM-like motif (so-called “hemiITAM”) that contains only a single consensus YxxI/L motif, and it was proposed that Syk may cross-ligate two Dectin-1 receptors (53). However, the means by which β2-integrins might mediate Syk activation in response to hyphae is unclear, but they may involve another unidentified ITAM adaptor or an ITAM-independent mechanism. Prior to the reports that ITAM-modulated signaling is important downstream of β2-integrins, the prevailing view was that these receptors, and the related β3-integrins, could activate Syk in a more direct manner. Syk was shown to bind β2- and β3-integrins in vitro, as well as to associate with these integrins in human platelets or CHO transfected cells, in a manner dependent on the Syk Src homology 2 domains but independent of their phosphorytosine-binding capability or integrin tyrosine phosphorylation (54, 55). Src-family kinases may activate Syk in these circumstances. The reasons for the disparity between the modes of signaling by β2-integrins in different settings is unclear, but they may be due to differential mechanisms of ligand-induced activation or differing interactions
with other receptors activated simultaneously. In this latter respect, our data indicated that, at the very least, a combination of β2-integrins and glucan receptors is responsible for activation of the neutrophil oxidase through Syk-dependent pathways.

In line with the involvement of protein tyrosine kinases in hyphal ROS responses, we found that class IA isoforms of PI3K, but not the class IB isoform, were required for this response. Further, a combination of pharmacological and genetic approaches defined a specific role for the β and δ isoforms, with an apparently more minor contribution from PI3Kα. Much recent work has focused on revealing unique roles for individual PI3K isoforms. In certain contexts, these unique roles may derive primarily from different relative levels of expression; thus, PI3Kδ is specifically expressed in hematopoietic cells, where it fulfills major nonredundant functions in T cell development and mast cell function (56, 57). However, this seems less likely in other contexts: for example, α and β isoforms are widely expressed, yet each was shown to be differentially regulated in response to certain receptors (e.g., PI3Kα downstream of the insulin receptor or PI3Kβ downstream of integrins in platelets) (58, 59). Mechanistic explanations for these individual roles remain elusive, particularly because each catalytic subunit is thought to nonspecifically associate with one of the same family of five potential p50-85 regulatory subunits. Some of the major problems in making progress in this area have been identifying the precise signaling complexes into which class IA PI3Ks are recruited and, in particular, the critical phospho-tyrosine residues to which, it is assumed, the regulatory subunits of the class IA PI3Ks are bound. Thus, further work needs to be done to understand how hyphal recognition directs the recruitment and activation of PI3Kβ and δ in neutrophils and what specific properties these two isoforms bring to the hyphal response.

We still have an incomplete understanding of how the PtdIns(3,4,5)P3 and PtdIns(3,4)P2 synthesized by class I PI3Ks regulate neutrophil spreading, movement, and activation of the NADPH oxidase; however, an important previously defined common component is the activation of Rac GTPases. We were unable to measure a consistent increase in Rac-GTP levels in response to hyphae using PAK-CRIB pull-down assays (data not shown).
possibly because it is difficult to achieve synchronized contact between neutrophils and hyphae in our assays. However, we found that Vav family GEFs were absolutely required for oxidase activation by hyphae and that tyrosine phosphorylation of Vav1, a surrogate of activation, was reduced by class I PI3K inhibition. This suggests that a PI3K-Vav-Rac link may provide at least some of the explanation for PI3K effects on neutrophil spreading and oxidase activation in response to hyphae. Vav GEFs have been implicated in neutrophil NADPH oxidase activation in response to FcγR, TLR, and β2-integrin ligation and so are important integrators of diverse signals for Rac activation (42, 43, 60). However, the relationship between class I PI3Ks and Vavs in these pathways is complex. P(3,4,5)P3 can bind to the PH-domain of Vavs and is important for certain Vav functions (45, 46), but a clear consensus on its effects on tyrosine phosphorylation and GEF activity is lacking, and so its influence is likely to be context dependent. Moreover, PI3K activation was shown to be reduced upon β2-integrin ligation of Vav1/3 doubly deficient neutrophils and ligation of Dectin-1 in microglial cells with suppressed Vav1 expression (61, 62), suggesting that PI3K and Vav proteins are part of a mutual feedback loop. In this respect, we found that neutrophils deficient in Vav proteins had a greater hyphal ROS defect than did combined class IA inhibition, suggesting that PI3K is not the sole regulator of Vav function in these responses.

Our work has started to define key components of the signal-transduction pathways that link neutrophil recognition of A. fumigatus hyphae to the activation of the NADPH oxidase. It is clear that β2-integrins play a major role in coordinating these pathways, which are absolutely dependent on Syk and Vav. We also identified major roles for the two class I PI3K isoforms, PI3Kβ and PI3Kδ, possibly in part through Vav regulation of Rac. However, further work needs to be done to establish the precise relationships between these signaling intermediates and how they are integrated into the greater signaling web that is created upon hyphal recognition. Thus, although β2-integrins and laminarin-sensitive receptors seem to act in a cooperative or redundant manner to control the activation of PI3K and spreading, β2-integrins play a substantial, nonredundant role in the regulation of the NADPH oxidase response in inverse to the invasive hyphal form of this pathogen.

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Disclosures

The authors have no financial conflicts of interest.

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