

Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils

Alison M. Condliffe, Keith Davidson, Karen E. Anderson, Chris D. Ellson, Tom Crabbe, Klaus Okkenhaug, Bart Vanhaesebroeck, Martin Turner, Louise Webb, Matthias P. Wymann, Emilio Hirsch, Thomas Ruckle, Montserrat Camps, Christian Rommel, Shaun P. Jackson, Edwin R. Chilvers, Len R. Stephens, and Phillip T. Hawkins

It is well established that preexposure of human neutrophils to proinflammatory cytokines markedly augments the production of reactive oxygen species (ROS) to subsequent stimuli. This priming event is thought to be critical for localizing ROS to the vicinity of the inflammation, maximizing their role in the resolution of the inflammation, and minimizing the damage to surrounding tissue. We have used a new generation of isoform-selective phosphoinositide 3-kinase (PI3K) inhibitors to show that ROS production under

these circumstances is regulated by temporal control of class I PI3K activity. Stimulation of tumor necrosis factor- α (TNF- α)-primed human neutrophils with N-formyl-methionyl-leucyl-phenylalanine (fMLP) results in biphasic activation of PI3K; the first phase is largely dependent on PI3K γ , and the second phase is largely dependent on PI3K δ . The second phase of PI3K activation requires the first phase; it is this second phase that is augmented by TNF- α priming and that regulates parallel activation of ROS production. Surpris-

ingly, although TNF- α -primed mouse bone marrow-derived neutrophils exhibit superficially similar patterns of PI3K activation and ROS production in response to fMLP, these responses are substantially lower and largely dependent on PI3K γ alone. These results start to define which PI3K isoforms are responsible for modulating neutrophil responsiveness to infection and inflammation. (*Blood*. 2005; 106:1432-1440)

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Introduction

Neutrophils are critical components of the immune system and have a vital role in combating bacterial and fungal infections.¹ A key weapon in the neutrophil armory is the so-called “respiratory burst,” the generation of reactive oxygen species (ROS) by a multicomponent oxidase complex.^{2,3} Patients with chronic granulomatous disease (CGD) caused by defective expression of active oxidase components experience recurrent, life-threatening infections.⁴ The role of ROS in fighting infections is complex. ROS are involved in the killing process directly through the damaging actions of oxygen radicals and their halogenated derivatives and indirectly through the activation of phagosomal proteases.⁵⁻⁷ It is also becoming apparent that ROS may regulate the neutrophil lifespan, modify the extracellular matrix through which the neutrophils migrate, and modulate the function of other cells participating in the inflammatory response.⁸⁻¹²

Given the potential for self-damage, a key feature of the inflammatory response is to confine ROS generation in time and

space to areas where it is required. One way in which this is thought to occur is through a form of signal integration in which prior exposure to local proinflammatory factors is necessary for maximal activation by subsequent oxidase-triggering signals.¹³⁻¹⁷ One of best studied examples of this “priming” phenomenon is the ability of tumor necrosis factor- α (TNF- α), a cytokine released primarily by macrophages, to dramatically augment the oxidase response to bacterially derived peptides (eg, N-formyl-methionyl-leucyl-phenylalanine [fMLP]) or components of the complement cascade (C5a). Indeed it is thought that failure to limit priming of the oxidase may play a key role in pathologic conditions in which inflammation is not effectively resolved, such as in acute respiratory distress syndrome (ARDS) or joint involvement in rheumatoid arthritis.

The neutrophil oxidase is regulated by the combined action of several intracellular signaling pathways, including those driven by phosphoinositide 3-kinase (PI3K), phospholipase C (PLC)/Ca²⁺/

From the Inositide Laboratory and Laboratory of Lymphocyte Signalling and Development, Babraham Institute, Cambridge, United Kingdom; Celltech R&D Ltd, Berkshire, United Kingdom; Ludwig Institute for Cancer Research, London, United Kingdom; Department of Biochemistry and Molecular Biology, London, United Kingdom; Department of Biochemistry and Molecular Biology, London, United Kingdom; Centre of Biomedicine, Department of Clinical and Biological Science, University of Basel, Basel, Switzerland; Department of Genetics, Biology and Biochemistry, University of Torino, Torino, Italy; Sero Pharmaceutical Research Institute, Geneva, Switzerland; Australian Centre for Blood Diseases, Monash University, Victoria, Australia; and Respiratory Medicine Division, Department of Medicine, University of Cambridge School of Clinical Medicine, Addenbrooke's and Papworth Hospitals, Cambridge, United Kingdom.

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The online version of the article contains a data supplement.

An Inside *Blood* analysis of this article appears in the front of this issue.

Reprints: Phillip T. Hawkins, The Inositide Laboratory, Babraham Institute, Babraham Research Campus, Cambridge CB2 4AT, UK; e-mail: phillip.hawkins@bbsrc.ac.uk.

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protein kinase C (PKC), phospholipase D (PLD), phospholipase A₂ (PLA₂), and p38/Erk.^{2,3} Presumably, this diversity reflects the need for the oxidase to respond to multiple families of cell-surface receptors (eg, receptors for Fc, integrins, lipopolysaccharide, chemokines, cytokines, and bacterial fragments) that function through different proximal signal-transducing elements such as heterotrimeric G proteins or protein tyrosine kinases. It is clear from the use of specific catalytic site inhibitors and certain mouse knockouts that the PI3K signaling pathway is important for the mechanisms by which G_i-coupled receptors regulate ROS production in neutrophils—for example, downstream of receptors for fMLP, C5a, platelet-activating factor (PAF), histamine, LTB₄, and adenosine triphosphate (ATP).¹⁸⁻²¹ Moreover, we have previously described the ability of TNF- α to potentiate PI3K activation in response to fMLP, suggesting it may be a mechanism that underlies oxidase priming by this cytokine.^{22,23}

Neutrophils contain members of each of the currently defined families of PI3K—classes I, II, and III. The class I family of PI3Ks is responsible for cell-surface receptor-generated PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ and is thought to play the major role in the regulation of the oxidase.²⁴ Class I PI3Ks are subdivided into class IA and class IB based on the nomenclature of their p110 catalytic subunits (α , β , γ , δ) and mode of regulation; hence, PI3K γ is subdivided into class IB, and PI3K α , β , and δ are subdivided into class IA.²⁴ The class IB enzyme has a p101 regulatory subunit and is activated by G-protein-coupled receptors. The class IA enzymes have p55-85 regulatory subunits and are classically activated by tyrosine kinase-coupled receptors.²⁴ Recent studies using mouse PI3K γ knockouts have defined the class IB isoform as the major player in fMLP-driven PtdIns(3,4,5)P₃ synthesis and ROS production.¹⁹⁻²¹ However, there is an apparently contradictory body of work implying the involvement of class IA rather than class IB PI3Ks in the activation of the oxidase by fMLP in human neutrophils.²⁵⁻²⁷ Furthermore, our studies describing the effects of TNF- α on the fMLP-stimulated formation of PtdIns(3,4,5)P₃ in human neutrophils suggested a potentiating effect at later, rather than earlier, times of fMLP stimulation,²² implying a level of PI3K complexity in the response. In light of these observations, we set out to use several recently developed isoform-selective PI3K inhibitors and mouse PI3K “knockout” models to assess the contribution of the various class I PI3Ks to TNF- α priming of the fMLP oxidase response in human and mouse neutrophils.

Materials and methods

The PI3K inhibitors TGX-221 (patent WO-2004016607), AS-252424 (5-[5-(4-fluoro-2-hydroxy-phenyl)-furan-2-ylmethylene]-thiazolidine-2,4-dione; synthesis described in the Supplementary Materials, available on the *Blood* website [see the link at the top of the online article]), IC87114²⁷ (patent WO-0181346), and YM-024 (compound 24 in Table 6; patent EP1277738) were synthesized solely for the purposes of this study. Human TNF- α (hTNF- α) was from R&D (Minneapolis, MN); fMLP, horseradish peroxidase (HRP), luminol, cytochrome *c*, and Hanks balanced salt solution (HBSS) were from Sigma (Dorset, United Kingdom).

Mouse strains

The PI3K γ ^{-/-}, PI3K δ ^{D910A/D910A}, and PI3K δ ^{-/-} mouse strains used in this study have been described previously.^{21,28,29} These strains were bred on a C-57BL/6 background for at least 8 generations. The PI3K γ ^{-/-}, δ ^{-/-} strain was created by interbreeding PI3K γ ^{-/-} and PI3K δ ^{-/-} mice on a mixed C-57BL/6, 129/Sv background. In all experiments in which these strains of mice were used, they were compared with appropriate 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 License PPL 80/1875.

Preparation of neutrophils

Human neutrophils (at least 95% purity by cyto-spin) were isolated from the peripheral blood of healthy volunteers by centrifugation over plasma/ Percoll gradients, as previously described.³⁰ Murine bone marrow was dispersed in HBSS (without Ca²⁺ and Mg²⁺) with 0.25% fatty acid-free bovine serum albumin (BSA) (HBSS/BSA) and was centrifuged (1256g, 30 minutes, room temperature) over discontinuous gradients composed of 81%, 62%, and 55% Percoll in HBSS. Mature neutrophils were obtained from the 55%/62% interface (purity, 75%-85% by cyto-spin), and contaminating red blood cells were removed by ammonium chloride lysis; cells were washed twice in HBSS/BSA.

Measurement of [³²P]phosphoinositides in [³²P]Pi-prelabeled neutrophils

Neutrophils (4 × 10⁷ human; 7 × 10⁷ mouse) were washed twice in inorganic phosphate (Pi)-free HBSS and incubated with [³²P]Pi (6 mCi [222 MBq]; PBS13; Amersham Pharmacia, Bucks, United Kingdom) and TNF- α (200 U/mL and 500 U/mL for human and mouse cells, respectively) in 2 mL Pi-free HBSS for 65 minutes at 37°C. Cells were washed and resuspended in a final volume of 2.4 mL Pi-free HBSS; 150- μ L aliquots were warmed to 37°C in the presence of 10 μ L PI3K inhibitors (no more than 0.01% dimethyl sulfoxide [DMSO]) for 5 minutes and then challenged with 10 μ L fMLP (100 nM and 10 μ M final concentrations for human and mouse cells, respectively) for various times. Incubations were terminated, and the lipids were extracted, deacetylated, and quantified by anion-exchange chromatography, as described previously.^{31,32} The data represent disintegrations per minute (dpm) in the appropriate fractions corrected between experiments to 1 × 10⁵ dpm GroPIns(4,5)P₂ in the average control samples.

Measurement of neutrophil oxidative burst activity

Neutrophils (2 × 10⁶/mL human; 10⁷/mL mouse) were incubated in HBSS with TNF- α at 37°C for 25 minutes before the addition of PI3K inhibitors for another 5 minutes (as described in the previous paragraph). Cells were stimulated with fMLP (as described in the previous paragraph) in the presence of cytochrome *c* (1.2 mg/mL final), and the optical density of the supernatants was determined by spectrophotometry (550 nm). To obtain data on the kinetics of the oxidative burst, measurements were also obtained using luminol-dependent chemiluminescence. One hundred microliters of neutrophils (4 × 10⁶/mL human; 5 × 10⁶/mL mouse) were primed with TNF- α and preincubated with PI3K inhibitors (as described in the previous paragraph); 100 μ L luminol (1 μ M final concentration) and HRP (62.5 U/mL final concentration) in HBSS were added, and 150- μ L aliquots were transferred to a prewarmed 96-well luminometer plate. Light emission was recorded by a Berthold MicroLumat Plus luminometer (Berthold Technologies, Hartfordshire, United Kingdom) (data output is in relative light units per second); fMLP was added through the injection port.

Immunoprecipitation and PI3K activity measurement

Human neutrophils (2 × 10⁷/mL) were primed with TNF- α (200 U/mL) or HBSS for 30 minutes at 37°C. Cells were treated with diisopropyl fluorophosphate (DIFP) (7 μ M, 5 minutes, RT), pelleted, and resuspended at 4 × 10⁷/mL in HBSS. Aliquots (0.25 mL each) were equilibrated to 37°C and stimulated with fMLP (1 μ M) for 30 seconds, unless otherwise indicated. Reactions were terminated by the addition of 1 mL ice-cold phosphate-buffered saline (PBS)/0.5 mM orthovanadate; cells were pelleted and lysed in 1 mL ice-cold lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% NP-40, 10% glycerol, 1 mg/mL fatty acid-free BSA, 0.5 mM orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], and 10 μ g/mL each of leupeptin, antipain, pepstatin A, and aprotinin). Antiphosphotyrosine antibody-directed immunoprecipitation of PI3K activity was performed with 25 μ g FB2 (Cancer

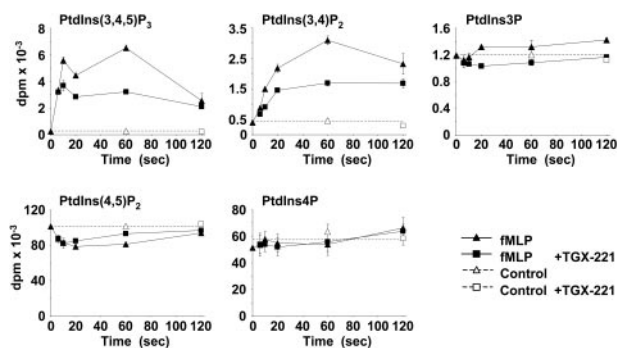


Figure 1. Time course of changes in phosphoinositide levels during fMLP stimulation of TNF- α -primed human neutrophils. Human neutrophils were labeled with [32 P]Pi in the presence of 200 U/mL TNF- α , as described in "Materials and methods." Neutrophils were washed, warmed for 5 minutes at 37°C in the presence or absence of 0.3 μ M TGX-221, and then challenged with 100 nM fMLP or its vehicle for the times indicated. Incubations were terminated, lipids were extracted, and [32 P] phosphoinositides were quantified as described in "Materials and methods." Data are mean \pm SD ($n = 3$ -5) collected from separate preparations of neutrophils (where not shown, errors lie within the symbols).

Research UK, London, United Kingdom), and PI3K activity was assayed as described previously.³³

Results

Measurement of PI3K activity in TNF- α -primed, fMLP-stimulated human neutrophils

We measured the levels of [32 P]-phosphoinositides in [32 P]Pi-prelabeled, TNF- α -primed human neutrophils stimulated with 100 nM fMLP (Figure 1). The low basal levels of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ and the dramatic increase in their concentrations on exposure to fMLP are consistent with previous work describing fMLP stimulation of class I PI3K activity, catalyzing the conversion of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃, followed by 5-phosphatase-linked conversion to PtdIns(3,4)P₂.^{32,34} The significantly higher basal level of [32 P]PtdIns3P and the smaller increase on fMLP stimulation are consistent with previous reports of negligible effects of fMLP on class III or class II catalyzed conversion of PtdIns to PtdIns3P.³² The transient decrease in [32 P]PtdIns(4,5)P₂ on fMLP stimulation is consistent with competing effects of consumption (by PI3K and PLC) and resynthesis of this lipid (by PI4P5K), as previously reported.³²

The fMLP-stimulated synthesis of PtdIns(3,4,5)P₃ in TNF- α -primed human neutrophils is biphasic, with levels peaking approximately 10 seconds and 60 seconds after the addition of fMLP (Figure 1). We have previously shown that the size of this latter peak of PtdIns(3,4,5)P₃ accumulation is dependent on prior exposure to TNF- α (5- to 6-fold reduced in the absence of TNF- α),²² suggesting significant complexity in the response. We sought to define the roles of the various class I PI3K isoforms under these conditions by using several recently developed isoform-selective, catalytic site inhibitors. The origins and structures of the compounds chosen for this study are given in "Materials and methods" and Figure S1, and their reported PI3K selectivities in vitro are given in Table 1. We confirmed the relative selectivity of these compounds by assessing their IC₅₀ against the in vitro lipid kinase activities of recombinant PI3K α , β , δ , and γ in identical assays (Table S1); given that each of these compounds acts as a competitive ATP site inhibitor and that the concentration of ATP used in our assays differed from that of others, the data are in good

Table 1. Inhibition of PI3K activity in vitro

	IC ₅₀ , μ M			
	α	β	γ	δ
TGX-221	5	0.007	3.5	0.1
IC87114	> 100	75	29	0.5
YM-024	0.30	2.65	9.07	0.33
AS-252424	1.07	> 20	0.035	> 20

The IC₅₀s for inhibition of the different catalytic subunits of class I PI3Ks by the compounds used in this study are listed. Chemical structures of the inhibitors are shown in Figure S1. Data have been drawn from the appropriate patent and publication information (see "Materials and methods"); concentrations of ATP used in the relevant in vitro PI3K assays were TGX-221 (100 μ M), IC87114 (200 μ M), YM-024 (2 μ M), and AS-252424 (each PI3K isoform was assayed at its respective K_m for ATP—that is, 89 μ M, 70 μ M, 23 μ M, and 59 μ M for the α , β , γ , and δ isoforms, respectively). We have also generated analogous data comparing each of these compounds in the same in vitro assay at 2 μ M ATP (Table S1).

agreement with those presented for these compounds in the relevant patent applications.

The effect of 0.3 μ M TGX-221 on the time course of [32 P]phosphoinositide accumulation in fMLP-stimulated, TNF- α -primed human neutrophils is shown in Figure 1. At this concentration, TGX-221 has a negligible effect on the increasing phase of the first peak of PtdIns(3,4,5)P₃ accumulation (6-second time point; Figure 1) but a substantial effect on the second peak of PtdIns(3,4,5)P₃ accumulation (60-second time point; Figure 1). Concentration-response curves for the effects of TGX-221 on PtdIns(3,4,5)P₃ accumulation after 6- or 60-second stimulation of TNF- α -primed cells are shown in Figure 2; effects of TGX-221 on the other lipids measured are described in Figure S2. The results clearly indicate that TGX-221 inhibits the second phase of PtdIns(3,4,5)P₃ accumulation more potently than the first phase.

Concentration-response curves for the effects of IC87114 and YM-024 on fMLP-stimulated accumulation of PtdIns(3,4,5)P₃ at 6

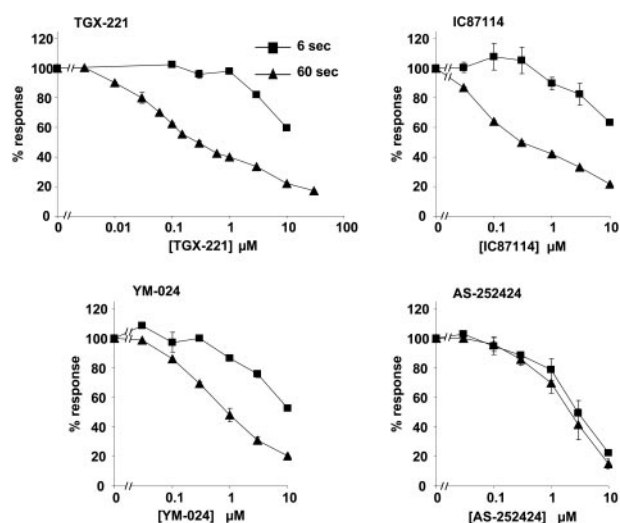


Figure 2. Effects of PI3K inhibitors on PtdIns(3,4,5)P₃ accumulation in fMLP-stimulated, TNF- α -primed human neutrophils. Human neutrophils were labeled with [32 P]Pi in the presence of 200 U/mL TNF- α , as described in "Materials and methods." Neutrophils were washed and warmed for 5 minutes at 37°C with the indicated concentration of inhibitor and then challenged with 100 nM fMLP for either 6 seconds or 60 seconds. Incubations were terminated, and [32 P]phosphoinositides were quantified as described in "Materials and methods." Data shown are for the accumulation of PtdIns(3,4,5)P₃ expressed as a percentage of the value with no inhibitor; data are mean \pm SD or range ($n = 2$ -5) collected from separate preparations of neutrophils (where not shown, errors lie within the symbols). Analogous dose curves describing the effects of the inhibitors on the other [32 P] phosphoinositides are shown in Figure S2.

and 60 seconds are also shown in Figure 2 and on other phosphoinositides in Figure S2. The relative lack of effect of TGX-221, IC87114, and YM-024 on the levels of PtdIns(4,5)P₂ and PtdIns4P suggest that, at the concentrations tested, these compounds have few nonspecific effects on other neutrophil phosphoinositide kinases. The effects of TGX-221 on basal PtdIns3P levels suggest this compound may inhibit a class III PI3K with an IC₅₀ of approximately 1 μM (Figure S2A). The lag in fMLP-stimulated accumulation of PtdIns(3,4)P₂ means that the 6-second data for this lipid is a composite of roughly equal proportions of basal and stimulated synthesis (Figure S2C) and, hence, is difficult to interpret. The results for PtdIns(3,4,5)P₃ accumulation, however, allow several clear conclusions to be drawn. TGX-221, IC87114, and YM-024 each inhibit the second phase of fMLP-stimulated PtdIns(3,4,5)P₃ accumulation more potently than the first, and there is multicomponent inhibition of the second phase (Figure 2). Thus, more than one isoform of PI3K is involved in the synthesis of PtdIns(3,4,5)P₃ downstream of fMLP stimulation in TNF-α-primed cells. Comparison of the effects of TGX-221, IC87114, and YM-024 on fMLP-stimulated accumulation of PtdIns(3,4,5)P₃ at 60 seconds with the respective IC₅₀ against PI3Kδ (Table 1) suggests 50% to 60% of this accumulation is dependent on the δ isoform. The identity of the other PI3Ks contributing to PtdIns(3,4,5)P₃ accumulation at 60 seconds is more ambiguous, but the data suggest limits of no more than 15% for PI3Kβ, 15% for PI3Kα, and 30% for PI3Kγ. By contrast, PtdIns(3,4,5)P₃ accumulation at 6 seconds cannot be significantly dependent on the α, β, or δ isoform (lack of sensitivity to inhibition by YM-024, TGX-221, and IC87114, respectively, at concentrations relevant to inhibition of these isoforms) and, hence, must be largely dependent on PI3Kγ.

To confirm the contribution of PI3Kγ activity to PtdIns(3,4,5)P₃ accumulation downstream of fMLP stimulation in TNF-α-primed cells, we investigated the effects of a recently developed PI3Kγ-selective inhibitor, AS-252424 (Figures 2, S2). In contrast to the other inhibitors used, AS-252424 inhibited both phases of PtdIns(3,4,5)P₃ accumulation in parallel (Figure 2). The IC₅₀ for this inhibition is substantially greater than that predicted by our in vitro PI3K assays (Table 1), but this is likely to reflect the poor cellular accessibility of this compound (M.C., personal communication, January 2005). Given the greater than 15-fold difference between the in vitro IC₅₀ for AS-252424 inhibition of PI3Kγ and that for PI3Kδ, these results indicate that the second, largely PI3Kδ-dependent phase of PtdIns(3,4,5)P₃ accumulation requires the prior PI3Kγ-dependent phase of PtdIns(3,4,5)P₃ accumulation.

Precedent suggests that class I PI3Ks (α, β, δ) are activated downstream of receptors by a tyrosine kinase-based transduction mechanism, with tyrosine phosphorylation of YXXM motifs in an adaptor protein leading to subsequent SH2 domain-mediated recruitment and activation of the p85/p110α, β, and δ PI3K heterodimers.²⁴ Therefore, we measured the presence of PI3K activity associated with antiphosphotyrosine-directed immunoprecipitates isolated from fMLP-stimulated neutrophils. We could indeed measure a TNF-α-dependent, fMLP-stimulated accumulation of PI3K activity in antiphosphotyrosine immunoprecipitates (Figure 3A), albeit with a time course that did not correlate precisely with PtdIns(3,4,5)P₃ accumulation in intact cells (Figure 3B). The sensitivity of the in vitro PI3K activity captured in the antiphosphotyrosine immunoprecipitates to PI3K inhibitors indicates that this activity is largely PI3Kδ (Figure 3C).

We investigated the effects of 2 inhibitors with specificity toward Src and Tec family protein tyrosine kinases PP1²⁶ and LFM-A13,³⁵ respectively, on PtdIns(3,4,5)P₃ accumulation in re-

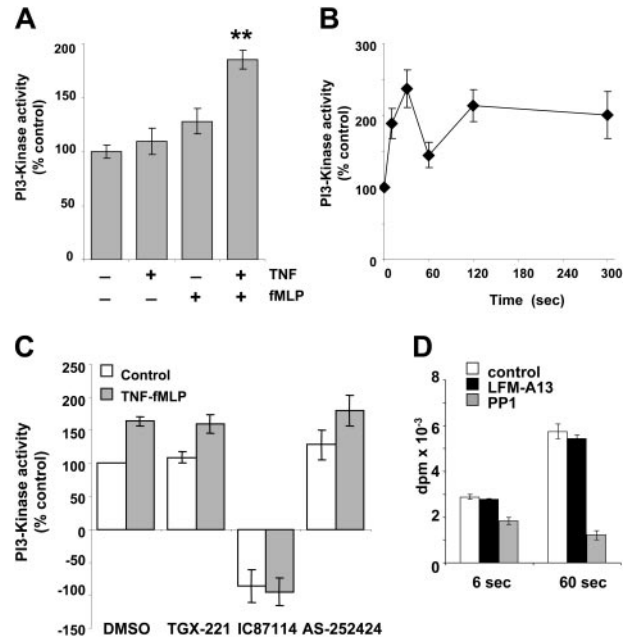


Figure 3. Activation of PI3K by tyrosine kinases in human neutrophils. (A-C) Human neutrophils (1×10^7) were primed with 200 U/mL TNF- α for 30 minutes (unless otherwise indicated), then challenged with 1 μ M fMLP or vehicle for 30 seconds. Detergent-soluble lysates were immunoprecipitated with antiphosphotyrosine antibody, and immunoprecipitates were assayed for PI3K activity, as described in "Materials and methods." Data shown are expressed as a percentage of basal activity and are mean \pm SEM of at least 3 experiments performed in at least duplicate. ** $P < .001$ compared with no TNF/fMLP; Student t test. Where appropriate, PI3K inhibitors (15 nM TGX-221, 1 μ M IC87114, 80 nM AS-252424) or DMSO controls were preincubated with immunoprecipitates for 5 minutes before measurement of PI3K activity. (D) Human neutrophils were labeled with [³²P]Pi in the presence of 200 U/mL TNF- α , as described in "Materials and methods." Washed cells were warmed for 5 minutes at 37°C with either 25 μ M LFM-A13, 20 μ M PP1, or their vehicle (0.01% DMSO) and then challenged with 100 nM fMLP for either 6 or 60 seconds. Incubations were terminated, and [³²P] phosphoinositides were measured as described. Data shown are for the levels of [³²P]PtdIns(3,4,5)P₃ (mean \pm SD; $n = 3$) from a single preparation of neutrophils.

sponse to fMLP in TNF-α-primed cells. PP1 inhibited PtdIns(3,4,5)P₃ accumulation at 60 seconds more than at 6 seconds (Figure 3D), with little effect on the levels of PtdIns3P, PtdIns4P, and PtdIns(4,5)P₂ (data not shown), suggesting a Src family kinase may be involved in class IA PI3K recruitment in this response.

Measurement of oxidase activity in TNF-primed, fMLP-stimulated human neutrophils

fMLP stimulation of human neutrophils leads to the production of ROS by the neutrophil oxidase complex. Extracellular release of ROS was measured by luminometry, which, in the presence of HRP and luminol, provides a rate measure of H₂O₂ production or by cytochrome *c* reduction, which measures the accumulated production of O₂⁻ (the direct product of the oxidase). Prior exposure of human neutrophils to TNF-α caused approximately 10-fold priming of the fMLP-stimulated oxidase, as previously described²² (Figure 4D). We investigated the sensitivity of the fMLP-stimulated ROS response in TNF-α-primed cells to PI3K inhibitors (Figure 4). Similar sensitivities were obtained with both types of ROS assay (AS-252424 could not be used in the luminometry measurements because of its strong coloration). For each of the 4 PI3K inhibitors, the concentration curves describing inhibition of ROS production correlated closely with those describing the inhibition of PtdIns(3,4,5)P₃ accumulation at 60 seconds rather than at 6 seconds of fMLP stimulation. Thus, ROS production

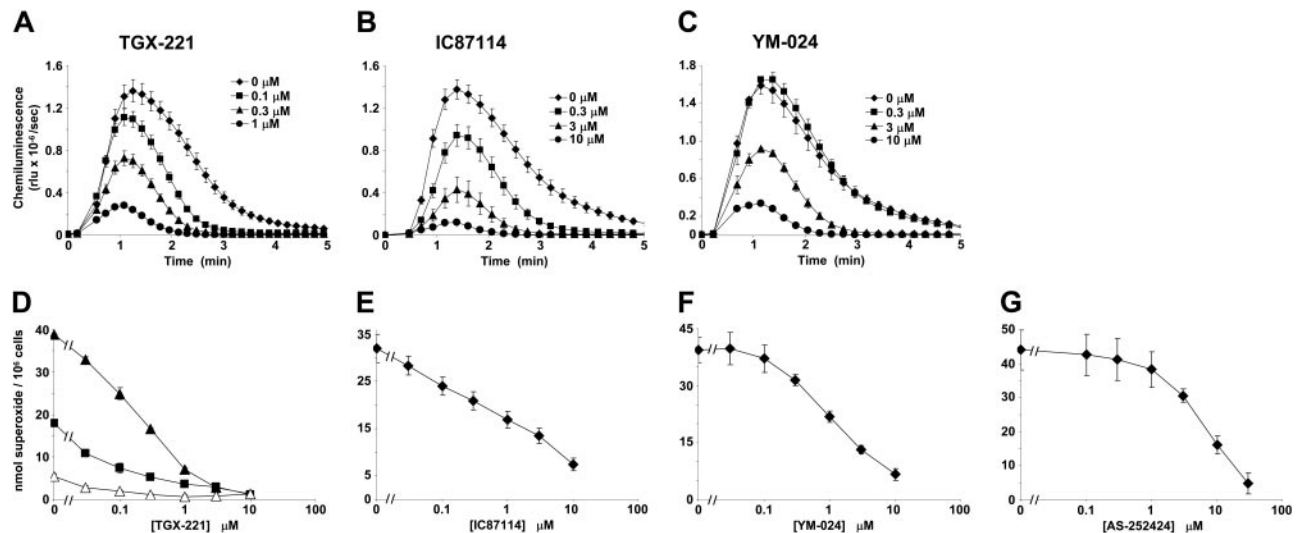


Figure 4. Effects of PI3K inhibitors on oxidase activity in fMLP-stimulated, TNF- α -primed human neutrophils. (A-C) Human neutrophils were primed with 200 U/mL TNF- α and were preincubated with 0 to 10 μ M TGX-221 (A), IC87114 (B), and YM-024 (C) in the presence of luminol and HRP, as described in "Materials and methods." Cells (3×10^5 per well) were stimulated with fMLP (100 nM) and light emission (relative light units [rlu]) recorded at 10-second intervals using a Berthold MicroLumat Plus luminometer. All incubations were performed in duplicate or triplicate, and data (mean \pm SEM or range) from 1 representative experiment of 2 to 5 experiments are shown. (D) Human neutrophils primed with 200 U/mL TNF- α (\blacktriangle and \blacksquare) or vehicle (\triangle) were pretreated with either TGX-221 at the indicated concentrations (\blacktriangle and \triangle) or TGX-221 in the presence of 1 μ M IC87114 (\blacksquare), and the oxidative burst in response to fMLP (100 nM) was quantitated by reduction of cytochrome c. Data represent mean \pm range of 2 separate experiments, with measurements performed in duplicate. (E-G) Human neutrophils primed with 200 U/mL TNF- α were preincubated with 0 to 30 μ M IC87114 (E), YM-024 (F), or AS-252424 (G), and the oxidative burst in response to fMLP (100 nM) was quantitated by the reduction of cytochrome c. Data are mean \pm SEM of 3 separate experiments, with measurements performed in duplicate.

correlated with the second phase of fMLP-stimulated PtdIns(3,4,5) P_3 accumulation across TNF- α priming and PI3K inhibition.

We attempted to resolve the relative contribution of PI3K β to the oxidase response by investigating parallel concentration curves for inhibition by TGX-221 in the presence or absence of 1 μ M IC87114 (a concentration of IC87114 predicted to inhibit at least 70% PI3K δ). These data revealed a sensitivity of approximately 20% of the oxidase response to concentrations of TGX-221 in the range predicted to inhibit PI3K β (Figure 4D). Further experiments with a range of different donors, however, suggested the contribution of PI3K δ compared with PI3K β to ROS production was variable (PI3K δ , 50%-70%; PI3K β , 5%-20%; data not shown).

Measurement of PI3K activity in TNF- α -primed, fMLP-stimulated mouse neutrophils

We next measured fMLP-stimulated changes in the levels of [32 P]-phosphoinositides in [32 P]Pi-labeled, TNF- α -primed mouse bone marrow-derived neutrophils (Figures 5, S3). Mouse neutrophils showed a similar biphasic pattern of [32 P]PtdIns(3,4,5) P_3 accumulation in response to fMLP, but the magnitude of this accumulation was some 5-fold less (relative to basal levels of [32 P]PtdIns(4,5) P_2) than the analogous response in human neutrophils. Other marked differences were observed between mouse and human data. Mouse neutrophils showed a relatively poor fMLP-stimulated accumulation of [32 P]PtdIns(3,4) P_2 , had a higher ratio of basal PtdIns(4,5) P_2 to PtdIns4P, and had a more pronounced fMLP-stimulated accumulation of PtdIns4P than human cells. We performed similar experiments with bone marrow-derived neutrophils prepared from mice lacking p110 γ ($\gamma^{-/-}$), mice with 110 δ replaced with a catalytically inactive allele ($\delta^{D910A/D910A}$), and mice lacking p110 δ and p110 γ ($\gamma^{-/-}$, $\delta^{-/-}$). Neutrophils from each strain of mouse had remarkably similar relative levels of [32 P]-PtdIns3P, -PtdIns4P, and -PtdIns(4,5) P_2 and fMLP-stimulated increases in [32 P]-PtdIns4P (Figure S3).

fMLP-stimulated PtdIns(3,4,5) P_3 synthesis was similar in TNF- α -primed neutrophils derived from the bone marrow of wild-type and PI3K $\delta^{D910A/D910A}$ mice (Figure 5). The initial PtdIns(3,4,5) P_3 accumulation at 6 seconds was identical within the error of measurement, and PtdIns(3,4,5) P_3 accumulation at 60 seconds was only reduced 10% to 15% in $\delta^{D910A/D910A}$ neutrophils. In contrast, neutrophils from PI3K $\gamma^{-/-}$ and PI3K $\gamma^{-/-}$, $\delta^{-/-}$ mice had dramatically reduced fMLP-stimulated PtdIns(3,4,5) P_3 responses, though a

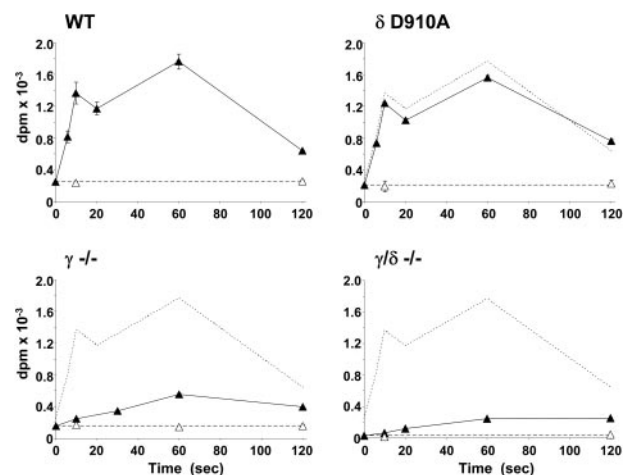


Figure 5. Time course of changes in the levels of PtdIns(3,4,5) P_3 in fMLP-stimulated, TNF- α -primed mouse neutrophils. Neutrophils derived from the bone marrow of the indicated strains of mice were labeled with [32 P]Pi in the presence of 500 U/mL TNF- α , as described in "Materials and methods." Neutrophils were washed, warmed to 37°C over 5 minutes, and challenged with 10 μ M fMLP (\blacktriangle) or vehicle (\triangle) for the indicated times. Dotted lines without symbols indicate values for fMLP-stimulated wild-type (WT) cells. Incubations were terminated, and [32 P] phosphoinositides were quantified as described in "Materials and methods." Data shown are for the levels of [32 P] PtdIns(3,4,5) P_3 (mean \pm SD; n = 3 collected from separate preparations of neutrophils; where not shown, errors lie within the symbols). Analogous data showing the levels of the other [32 P] phosphoinositides measured are described in Figure S3.

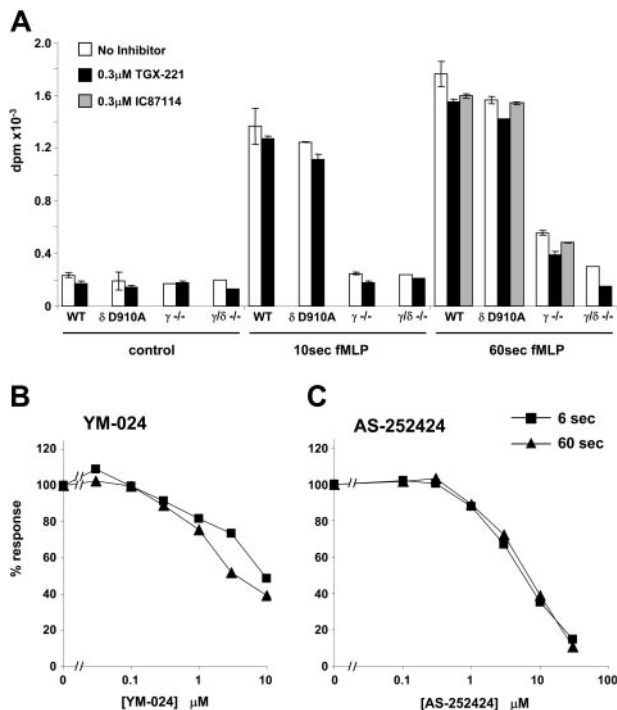


Figure 6. Effects of PI3K inhibitors on PtdIns(3,4,5)P₃ accumulation in fMLP-stimulated, TNF- α -primed mouse neutrophils. Bone marrow neutrophils derived from the indicated strains of mice were labeled with [³²P]Pi in the presence of 500 U/mL TNF- α , as described in “Materials and methods.” Neutrophils were washed, warmed to 37°C over 5 minutes in the presence of the indicated concentration of inhibitor, and stimulated with 10 μ M fMLP or vehicle for the indicated times. Incubations were terminated, and [³²P]-phosphoinositides were quantified as described in “Materials and methods.” Data shown are for the levels of [³²P] PtdIns(3,4,5)P₃ (mean \pm SD or range, n = 2-3, collected from separate preparations of neutrophils).

significant, slowly developing PtdIns(3,4,5)P₃ accumulation amounting to approximately 15% to 20% of the wild-type response at later times could be measured (Figure 5).

The results demonstrating a primary role for PI3K γ in fMLP-stimulated PtdIns(3,4,5)P₃-accumulation are consistent with previous, less detailed measurements of PtdIns(3,4,5)P₃ accumulation in neutrophils from PI3K γ ^{-/-} mice.¹⁹⁻²¹ The results, however, from the PI3K δ ^{D910A/D910A} mice are surprising, given previous indications that neutrophils from PI3K δ ^{-/-} mice have significantly reduced PtdIns(3,4,5)P₃-responses to fMLP.³⁶

We sought confirmation of the precise role of PI3K δ in mouse neutrophil PtdIns(3,4,5)P₃ responses by investigating the effects of TGX-221, IC87114, and YM-024. At concentrations relevant to PI3K δ inhibition, each of these drugs failed to inhibit fMLP-stimulated PtdIns(3,4,5)P₃ responses in TNF- α -primed cells by more than approximately 15% (Figure 6), in line with the experiments using the genetically modified mice. In contrast, the PI3K γ -selective inhibitor AS-252424 inhibited fMLP-stimulated PtdIns(3,4,5)P₃ accumulation at 6 seconds and 60 seconds in parallel, with an IC₅₀ similar to that seen in the analogous experiments with human neutrophils (compare Figures 6 and 2). This is consistent with a dominant role for PI3K γ in both phases of the mouse neutrophil PtdIns(3,4,5)P₃ response. The relative lack of involvement of PI3K δ in the mouse fMLP response cannot be explained on the basis of p110 δ expression levels, which are similar in human and mouse neutrophils (Figure S4). There are also no reported compensatory changes in the expression of other class I PI3K catalytic subunits in PI3K γ ^{-/-} or PI3K δ ^{D910A/D910A} mice.^{21,28}

Measurement of oxidase activity in TNF- α -primed, fMLP-stimulated mouse neutrophils

We measured the activation of the neutrophil oxidase in mouse bone marrow-derived neutrophils in experiments similar to those performed on human neutrophils. The scale of ROS production to fMLP, the PKC agonist phorbol myristate acetate (PMA), and opsonized zymosan were all substantially lower (approximately 10-fold, based on neutrophil numbers) in mouse neutrophils than in human neutrophils (data not shown). The degree of TNF- α priming of subsequent responses to fMLP or C5a were also lower in mouse neutrophils, in the 3- to 4-fold range compared with 8- to 12-fold in human cells. Furthermore, mouse neutrophils were relatively more responsive to fMLP when first isolated and lost this sensitivity in the absence, but not in the presence, of TNF- α during subsequent incubation at 37°C for 30 minutes, leading to a substantial effect of TNF- α when assayed at this point. In contrast, human neutrophils were relatively unresponsive to fMLP alone when first isolated. They gained sensitivity when subsequently incubated with TNF- α . The time course of fMLP-stimulated ROS production in TNF- α -primed mouse neutrophils was also different from that in human cells (compare Figures 7A and 4A). In mouse cells, ROS production was essentially complete by 1 minute, whereas maximal ROS production in the human cells occurred between 1 and 2 minutes after the addition of fMLP (averaged over at least 20 experiments, mean durations of mouse and human oxidase bursts were 1.2 minutes and 3.3 minutes, respectively).

fMLP stimulated similar oxidase activation in TNF- α -primed neutrophils derived from wild-type or PI3K δ ^{-/-} mice (Figure 7A; similar results were obtained with PI3K δ ^{D910A/D910A} mice; data not shown). In contrast, oxidase activation in response to fMLP in TNF- α -primed neutrophils from PI3K γ ^{-/-} or PI3K γ ^{-/-}/ δ ^{-/-} mice

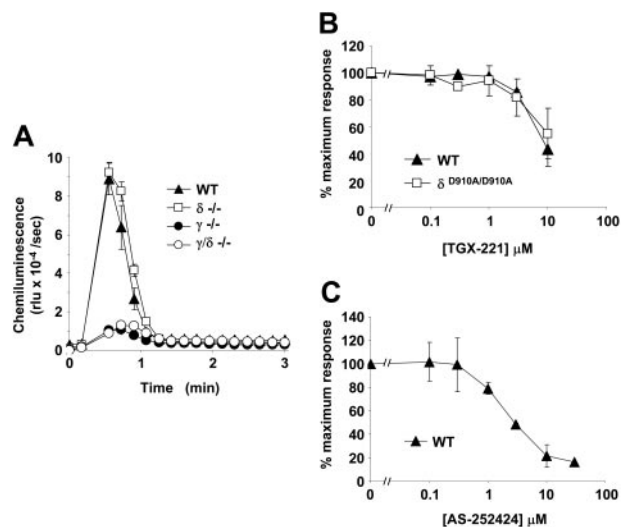


Figure 7. Oxidase activity in fMLP-stimulated, TNF- α -primed mouse neutrophils. (A) Bone marrow neutrophils obtained from the indicated strains of mice were prepared in parallel, primed with 500 U/mL TNF- α , and preincubated with luminol and HRP, as described in “Materials and methods.” Cells (3.75×10^5 per well) were stimulated with 10 μ M fMLP, and chemiluminescence was recorded using a Berthold MicroLumatPlus luminometer. Incubations were performed in duplicate, and data (mean \pm range) from 1 of 2 representative experiments are shown. (B) Experiments were performed as described in panel A except that cells were incubated with the luminol and HRP in the presence of 0 to 30 μ M TGX-221. Data represent the accumulated light emission over 3 minutes expressed as a percentage of the value with no inhibitor (mean \pm SEM from 3 separate experiments). (C) Wild-type murine neutrophils (5×10^6 /point, performed in triplicate) were primed with 500 U/mL TNF- α and preincubated for 5 minutes with 0 to 30 μ M AS-252424 before stimulation with 10 μ M fMLP. Oxidative burst activity was quantitated by a reduction of cytochrome c. Data represent mean \pm SEM from 3 separate experiments.

was inhibited by more than 80% (Figure 7A). PMA stimulated similar oxidase responses in all 4 genotypes tested (data not shown). These results are consistent with previously published defects in oxidase activation to G-protein-coupled agonists in PI3K $\gamma^{-/-}$ neutrophils.¹⁹⁻²¹

We investigated the sensitivity of the primed fMLP-stimulated oxidase response in mouse neutrophils to PI3K inhibitors. Concentration-response curves for inhibition of ROS formation by TGX-221 (Figure 7B), IC87114 (Figure S5), and YM-024 (Figure S5) were all shifted to substantially higher concentrations compared with the analogous experiments in human neutrophils. Furthermore, the concentration-response curves for TGX-221 and IC87114 were similar in wild-type and PI3K $\delta^{D910A/D910A}$ neutrophils (Figures 7B, S5). These results all point to a relatively small role for PI3K δ in the mouse oxidase response to fMLP, consistent with a minor role for this isoform in PtdIns(3,4,5)P₃ accumulation at 60 seconds. In contrast, the inhibition of fMLP-stimulated ROS production by AS-252424 was similar to that observed in human neutrophils (Figure 7C), supporting a key role for PI3K γ in the mouse in PtdIns(3,4,5)P₃ accumulation and in activation of the oxidase.

Discussion

Stimulation of unprimed human neutrophils with fMLP resulted in a rapid pulse of PtdIns(3,4,5)P₃ accumulation, peaking at approximately 6 to 10 seconds, and in minimal activation of ROS production. Pretreatment of human neutrophils with TNF- α resulted in additional, fMLP-dependent accumulation of PtdIns(3,4,5)P₃ at approximately 60 seconds and substantial, parallel activation of ROS production.^{22,23} Specific pharmacologic inhibition of this later pulse of PtdIns(3,4,5)P₃ by isoform-selective PI3K inhibitors correlated with ROS production, indicating PtdIns(3,4,5)P₃ accumulation at later times (and PtdIns(3,4)P₂ derived from it) is essential, and indeed of high control strength, for the pathways by which fMLP regulates the oxidase. There are now several suggestions for signaling pathways by which PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ may regulate oxidase activity, including PtdIns(3,4,5)P₃/PtdIns(3,4)P₂-regulated guanine exchange factors (GEFs) for rac (P-Rex1; vav),³⁷ PtdIns(3,4,5)P₃/PtdIns(3,4)P₂-regulated phosphorylation of p47^{phox} through atypical PKCs or p21-activated kinase (PAK),^{2,3} and direct binding of PtdIns(3,4)P₂ to p47^{phox} itself.³⁸ There is still no clear assessment, however, of the relative importance of these mechanisms to ROS production *in vivo*.

Our data indicate that the initial phase of PtdIns(3,4,5)P₃ synthesis on the addition of fMLP to human neutrophils is entirely dependent on PI3K γ . fMLP receptors transduce their signals through direct activation of G_i heterotrimeric G-proteins, leading to substantial release of G α_i and G $\beta\gamma$ subunits. G $\beta\gamma$ subunits have been shown to elicit direct and substantial activation of PI3K γ *in vitro*,³⁹ providing a plausible mechanism for rapid, PI3K γ -dependent synthesis of PtdIns(3,4,5)P₃. The small GTPase Ras also activates PI3K γ directly through binding to its p110 catalytic subunit, though the activations measured so far are relatively modest.⁴⁰ fMLP receptors are known to stimulate guanosine triphosphate (GTP) loading of Ras⁴¹; therefore, it is also plausible that Ras may play a role in this response.

The second phase of fMLP-stimulated PtdIns(3,4,5)P₃ accumulation is augmented by pretreatment with TNF- α and is driven largely by class IA PI3Ks. Our data suggest PI3K δ is the predominant (50%-60%) PI3K activity in this phase of the response, with smaller contributions from PI3K β (15%) and possibly from PI3K α (15% or less). It is unclear whether it is simply the timing of this second phase of PtdIns(3,4,5)P₃ accumu-

lation that is critical to oxidase activation or whether its source is also critical. Close comparison of the inhibition curves for PtdIns(3,4,5)P₃ synthesis compared with ROS production suggest the oxidase must recognize PtdIns(3,4,5)P₃ generated by PI3K δ and β , but the shallower curve for IC87114 inhibition of ROS production compared with that for TGX-221 (and compared with their analogous inhibition curves for PtdIns(3,4,5)P₃ synthesis) suggests the oxidase may recognize PtdIns(3,4,5)P₃ generated by the γ -isoform less effectively. However, given the error in the measurements and the relatively small, direct contribution of the γ isoform to PtdIns(3,4,5)P₃ synthesis at 60 seconds, additional experiments will be needed to clarify this. A striking feature of this second phase of class IA-driven PtdIns(3,4,5)P₃ accumulation is that it is significantly dependent on the activity of PI3K γ ; this idea that PtdIns(3,4,5)P₃ may amplify its own accumulation is also thought to be relevant to understanding the "feed forward" loops in signaling neutrophil migration.⁴²

The mechanism by which class IA PI3Ks are activated by fMLP receptors and also the point of intervention of TNF- α signaling is unknown. In principle, TNF- α could be activating the relevant PI3Ks or inhibiting the relevant phosphatases (parallel changes in PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ suggest the phosphatase would have to be a 3-phosphatase of the phosphatase and tensin homolog [PTEN] family). Whatever the point of action of TNF- α , precedent would suggest a tyrosine kinase-based signaling pathway is important at some level for switching on the class IA PI3K.²⁴ There is significant conflict in the literature concerning the contribution that tyrosine kinase-based signaling pathways make to PtdIns(3,4,5)P₃ and O₂⁻ production elicited by fMLP, though our data suggest some of this confusion is likely to arise from the extent of priming, controlled or otherwise, in the various studies.^{19-21,25-27,35,43-45} There is also a substantial body of work describing the activation of Src-family tyrosine kinases downstream of fMLP receptors in human neutrophils⁴⁶ and several suggestions that they are important for the activation of PI3K and O₂⁻ production.^{26,47} Our data offer circumstantial support of the idea that there is recruitment of PI3K δ to phosphotyrosine-containing complexes downstream of fMLP stimulation, that this is dependent on TNF- α priming, and that Src-family kinases may be involved, but more work must be done to establish the relevance of these observations to the accumulations of PtdIns(3,4,5)P₃ measured.

Our observations that class IA PI3Ks do not play a major role in PtdIns(3,4,5)P₃ production and oxidase activation in response to fMLP in TNF- α -primed mouse neutrophils are surprising because of our results in the analogous experiments in human neutrophils and in light of a recent report suggesting there is a substantial role for PI3K δ in fMLP-stimulated PtdIns(3,4,5)P₃ synthesis in mouse neutrophils.³⁶ Both studies are internally consistent with respect to the effect of a PI3K δ inhibitor (IC87114) and the PI3K $\delta^{-/-}$ mouse strain used; at present, we cannot understand this discrepancy. Our results do suggest that mouse bone marrow-derived neutrophils have a superficially similar, biphasic PtdIns(3,4,5)P₃ response to fMLP when compared with human neutrophils; however, the magnitude of this response is much lower in the mouse cells. It is surprising that PI3K γ is the major isoform responsible for both phases of PtdIns(3,4,5)P₃ production in murine cells; multiple receptors, multiple mechanisms of PI3K regulation (eg, G $\beta\gamma$ vs ras), potential regulation of PtdIns(3,4,5)P₃ hydrolysis, and potential secretion of further active agents all provide plausible mechanisms for obtaining temporal differences in PtdIns(3,4,5)P₃ accumulation driven through this single isoform.

There are also significant differences in fMLP-stimulated ROS production in TNF- α -primed mouse and human neutrophils. In human cells, maximal production of ROS occurs between 1 and 2 minutes after the addition of fMLP, and there is substantial accumulation of class IA PI3K-derived PtdIns(3,4,5)P₃/PtdIns(3,4)P₂. In mouse cells there is a much smaller and shorter burst of ROS production and a much lower PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ accumulation. We do not understand the mechanistic basis for this difference. The basis may be related to the different ways in which oxidase activation is organized in mouse and human cells. However, it is also plausible that our findings resulted from differences in the state of differentiation or in the cellular environment between the pool of "reserve" neutrophils in the bone marrow and those in the circulation. Only small differences in ROS production have been measured between these 2 populations of neutrophils in the mouse.⁴⁸ The basis may also be attributed to a difference in the relative physiologic importance of the particular agonists we have chosen for the mouse and human cells. These are obviously important points to resolve, but, whatever the cause of the differences between our results with mouse and human neutrophils, they raise a cautionary note in using the mouse assays as a model for human neutrophil behavior, particularly with regard to testing the potential efficacy of PI3K inhibitors in PtdIns(3,4,5)P₃ and ROS production.

Given the established importance of the class I PI3K signaling pathway in many aspects of human disease, particularly in cancer and inflammation, the pharmaceutical industry is showing great interest in developing isoform-selective PI3K inhibitors.^{49,50} The

results presented here and elsewhere^{27,51} suggest that it may indeed be possible to generate compounds with the appropriate selectivity and, furthermore, that the complexity of PI3K responses may afford useful operating windows for specificity in the inhibition of a particular cellular response. The results presented here suggest PI3K γ is a "master" PI3K downstream of fMLP receptors in human neutrophils and that inhibition of this isoform will inhibit all PtdIns(3,4,5)P₃/PtdIns(3,4)P₂-dependent responses to this agonist; however, inhibition of PI3K δ may inhibit only a subset of these responses (eg, ROS production). Similar levels of complexity have been revealed recently in the roles of the PI3K γ and PI3K δ isoforms in the cooperation between immunoglobulin E (IgE) and adenosine receptors in the regulation of mast cell secretion.^{52,53} It will be interesting to establish whether this level of PI3K complexity exists in many other contexts of cellular regulation.

Acknowledgments

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