Neutrophil Spontaneous Death Is Mediated by Down-Regulation of Autocrine Signaling through GPCR, PI3K\(\gamma\), ROS, and actin

Neutrophil spontaneous death is mediated by down-regulation of autocrine signaling through GPCR, PI3Kγ, ROS, and actin

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Edited* by Solomon Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved January 7, 2010 (received for review November 4, 2009)

Neutrophil spontaneous apoptosis plays a crucial role in neutrophil homeostasis and the resolution of inflammation. We previously established Akt deactivation as a key mediator of this tightly regulated cellular death program. Nevertheless, the molecular mechanisms governing the diminished Akt activation were not characterized. Here, we report that Akt deactivation during the course of neutrophil spontaneous death was a result of reduced PtdIns(3,4,5)P3 level. The phosphatidylinositol lipid kinase activity of PI3Kγ, but not class IA PI3Ks, was significantly reduced during neutrophil death. The production of PtdIns(3,4,5)P3 in apoptotic neutrophils was mainly maintained by autocrinely released chemokines that elicited PI3Kγ activation via G protein–coupled receptors. Unlike in other cell types, serum-derived growth factors did not provide any survival advantage in neutrophils. PI3Kγ, but not class IA PI3Ks, was negatively regulated by gradually accumulated ROS in apoptotic neutrophils, which suppressed PI3Kγ activity by inhibiting an actin-mediated positive feedback loop. Taken together, these results provide insight into the mechanism of neutrophil spontaneous death and reveal a cellular pathway that regulates PtdIns(3,4,5)P3/Akt in neutrophils.

Akt | apoptosis | reactive oxygen species

Neutrophils are the most abundant cell type among circulating white cells and are the major players in the innate immune system. Neutrophils are terminally differentiated and normally have a very short lifespan (7–20 hr) in circulation and in tissue (1–4 days) (1). The daily turnover of human neutrophils is $0.8–1.6 \times 10^9$ cells per kg of body weight. The same number of neutrophils need to die to keep cellular homeostasis under physiologic condition.

Neutrophils die even in the absence of any extracellular stimuli; thus, this type of death is also called spontaneous death. It shares many features of classical apoptosis, such as cell body shrinkage, cellular crenation, exteriorization of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, vacuolated cytoplasm, mitochondria depolarization, nuclear condensation, and internucleosomal DNA fragmentation (2, 3). Neutrophil death can be modulated by various extracellular stimuli such as proinflammatory cytokines, cell adhesion, phagocytosis, red blood cells, and platelets. Under most conditions, neutrophils will be exposed to both pro- and antiapoptotic factors. The net effect on neutrophil death and survival reflects a balance between the activities of such factors. Constitutive neutrophil death is associated with up-regulation of death signaling and down-regulation of survival signaling. We recently reported that the activity of protein kinase B (PKB)/Akt, a well known prosurvival and antiapoptotic factor, decreases dramatically during the course of neutrophil death. Both PI3 kinase and Akt inhibitors enhance neutrophil death. Conditions delaying neutrophil death, such as treatment with GM-CSF, G-CSF, or IFN-γ, restore Akt activity. Neutrophils depleted of PTEN, a phosphatidylinositol 3′-phosphatase that negatively regulates Akt activity, live much longer than wild-type neutrophils (4, 5). However, the molecular mechanisms by which PtdIns(3,4,5)P3/Akt activity is down-regulated during neutrophil spontaneous death remain ill defined.

In the present study, we identified an autocrine signal pathway that is involved in the down-regulation of PtdIns(3,4,5)P3/Akt activity during neutrophil spontaneous death. Our data demonstrate that the activity of Akt in apoptotic neutrophils is mainly maintained by autocrinely released chemokines that elicit PI3Kγ activation via G protein–coupled receptors. Reactive oxygen species accumulated in apoptotic neutrophils, by blocking an actin-mediated positive feedback loop, serve as a physiological negative regulator of PI3Kγ and the subsequent PtdIns(3,4,5)P3 production and Akt activation.

Results

Akt Deactivation During the Course of Neutrophil Spontaneous Death Is a Result of Reduced PtdIns(3,4,5)P3 Level. We have demonstrated that Akt deactivation is a causal mediator of neutrophil spontaneous death, but the molecular mechanisms by which Akt activity is down-regulated have not been fully investigated (4). Akt activation relies on its membrane translocation mediated by its specific association with PtdIns(3,4,5)P3 on the plasma membrane. Only the Akt molecules on the plasma membrane can be phosphorylated and activated. The level of active Akt (phospho-Akt) drastically declines during neutrophil death, whereas total Akt does not change (Fig. 1 *A*–*C*), suggesting that the decrease of Akt activity is not a result of protein degradation. Akt membrane translocation and subsequent activation was previously thought to depend solely on concentrations of PtdIns (3,4,5)P3 in the membrane (6, 7). Recently, we demonstrated that two inositol phosphates, InsP7 and Ins(1,3,4,5)P4, compete for Akt-PH domain binding with PtdIns(3,4,5)P3 both in vitro and in vivo, providing another level of regulation for Akt membrane translocation and activation (8, 9). However, the levels of InsP7 and Ins(1,3,4,5)P4 are extremely low in unstimulated neutrophils, suggesting that the decreased Akt activation is likely caused by the decrease of PtdIns(3,4,5)P3 production (8) (Fig. S1). To confirm this, we measured the level of PtdIns(3,4,5)P3. Our results show that during the course of neutrophil death, levels of PtdIns(3,4,5)P3 decrease dramatically, whereas levels of PtdIns(4,5)P2, the substrate of PtdIns

Author contributions: Y.X., F.L., and H.R.L. designed research; Y.X. and F.L. performed research; Y.X., F.L., and H.R.L. analyzed data; and H.R.L. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0912717107/DCSupplemental.

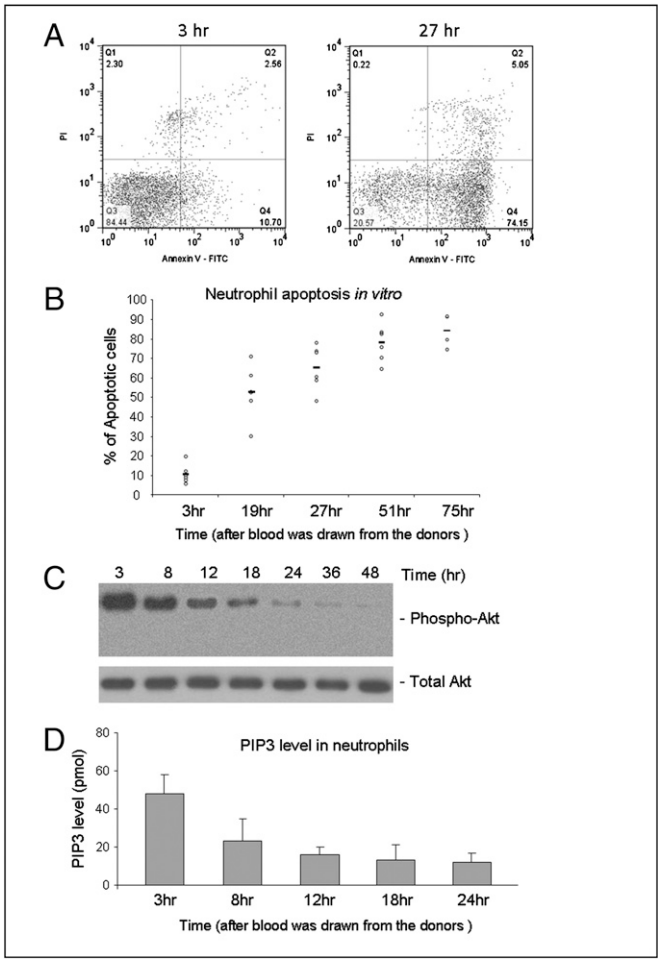

Fig. 1. PtdIns(3,4,5)P3 levels are down-regulated during the course of neutrophil spontaneous death. (*A*) FACS analysis of neutrophil spontaneous death. Q3, viable cell; Q4, early apoptotic cells; Q1+Q2, late apoptotic cells and necrotic cells. (*B*) Time course of neutrophil spontaneous death. All values represent mean ± SD of three separate experiments ($n > 3$). (*C*) The level of phosphorylation of endogenous Akt decreases during neutrophil spontaneous death. Neutrophils were cultured as described above. Protein extracts were resolved on SDS/PAGE. Total and phosphorylated Akt were detected by Western blot using anti-Akt and anti-phospho-Akt (Ser-473) antibodies as described (5). All samples were normalized to the amount of total Akt. Shown is the result of a representative experiment that was repeated three times. (*D*) Changes in the level of PtdIns(3,4,5)P3 during the course of neutrophil spontaneous death. At each indicated time points, cells (10^7 cells per data point) were collected and the amount of PtdIns(3,4,5)P3 in neutrophils was measured using a PIP3 Mass Strip kit (Echelon) following the protocol provided by the manufacturer. All values represent mean ± SD of three separate experiments.

(3,4,5)P3, do not change (Fig. 1*D*). This was detected in all of the blood donors we examined (more than five donors). The level of PtdIns(3,4,5)P3 declines by more than half in only 8 hr in culture (Fig. 1*D*).

Deactivation of PtdIns(3,4,5)P3/Akt Signaling During Neutrophil Spontaneous Death Is at Least Partially Caused by Reduced PI3Kγ Activity. The best established activator of Akt is PtdIns(3,4,5)P3 generated by PI3 kinase (6, 10). Conceivably, deactivation of PI3 kinase is responsible for Akt's deactivation. PtdIns(3,4,5)P3 level can also be regulated by the tumor suppressor PTEN and SHIP (SH2-containing inositol 5′-phosphatase), that converts PtdIns (3,4,5)P3 to PtdIns(4,5)P2 and PtdIns(3,4)P2, respectively (11, 12). So far, PTEN is the only phosphatidylinositol 3′-phosphatase identified in mammalian cells, and hematopoietic cell-specific SHIP (or SHIP1) is responsible for the majority of phosphatidylinositol 5′-phosphatase activity in neutrophils (ubiquitously expressed SHIP2 only plays a minor role in hematopoietic cells) (11–13). PTEN or/and SHIP might get activated during neutrophil spontaneous death, leading to down-regulation of Akt. Similar with what is discovered in the PTEN null neutrophils (4). Gardai et al. (14) reported that the half-life of neutrophils depleted of SHIP1 was also dramatically increased. To investigate the role of PTEN and SHIP in neutrophil spontaneous death, we measured the level of PTEN and SHIP1 in normal and apoptotic neutrophils. Because phosphatase activity can also be regulated by phosphorylation, we also assessed the levels of phosphorylated PTEN and SHIP1 in neutrophils using anti-phospho-SHIP and anti-phospho-PTEN antibodies. Our results show no convincing association between neutrophil spontaneous death and the reduction of PTEN or SHIP1 protein levels. The level of phosphorylated PTEN and SHIP1 also stayed essentially unaltered during the course of neutrophil spontaneous death (Fig. 2 *A* and *B*). These results suggest that the deactivation of Akt during neutrophil death may be caused by reduced PI3 kinase activity in apoptotic neutrophils.

PI3 kinase activity in neutrophils can be contributed by different isoforms. PI3Ks have been divided into three distinct classes (I, II, and III). Only the class I PI3Ks phosphorylate PtdIns(4,5)P2 to form PtdIns(3,4,5)P3. Class IA PI3Ks consist of a catalytic subunit, p110 (α, β, or δ) and an adaptor subunit, p85 (α or β), and are regulated by receptor tyrosine kinase stimulation. The only member of class IB is p110γ, which is associated with a p101 regulatory subunit and is regulated by G protein coupled receptors (6, 10, 15). All four class I PI3Ks are expressed in neutrophils. To determine which isoform(s) is responsible for neutrophil death associated-reduction of PtdIns (3,4,5)P3 production, we immunoprecipitated each isoform with their specific antibodies and examined the lipid kinase activity in immunoprecipitated samples (Fig. 2 *C–H*). All three isoforms of class IA PI3Ks were pulled down with a p85 antibody. The level of each isoform remained unaltered during the course of neutrophil death (Fig. 2*C*). The PI3 kinase activity in the immunoprecipitation pellets prepared from healthy and apoptotic neutrophils was also the same (Fig. 2 *D* and *E*). Interestingly, although the level of PI3K class IB (PI3Kγ) did not change during neutrophil death (Fig. 2*F*), the PI3 kinase activity of the immunoprecipitated enzyme decreased sharply. In 19 hr, the activity was reduced by nearly 80% (Fig. 2 *G* and *H*). Because PI3Kγ is the only isoform whose activity decreased, we conclude that deactivation of PtdIns(3,4,5)P3 signaling during neutrophil spontaneous death is mainly caused by the reduction of PI3Kγ activity. Akt phosphorylation and subsequent activation can also be regulated by many other factors such as mTOR, PDK1, PHLPP, PP2A, and PKC (1, 16). Their involvement in neutrophil spontaneous death cannot be completely ruled out. However, deactivation of PI3Kγ will lead to down-regulation of Akt; thus, it certainly plays an important role in deactivating Akt during neutrophil spontaneous death.

Neutrophil Spontaneous Death Is Mainly Regulated by G Protein–Coupled Receptors. The survival of most cell types requires various serum-derived growth factors such as IGF, G-CSF, PDGF, FGF, and TGF-β. These factors activate the class IA PI3K/Akt pathway via their specific membrane receptors. Although neutrophils are routinely cultured in medium containing 10% FBS, it is largely unknown whether these serum-derived growth factors and the neutrophil response to these factors play any role in regulating neutrophil death/survival. To answer this question, we measured the rate of neutrophil spontaneous death in serum-free medium. Surprisingly, serum deprivation did not exert any effect on the half-life of cultured neutrophils. At each time point examined, serum-starved neutrophils died at a similar rate as the

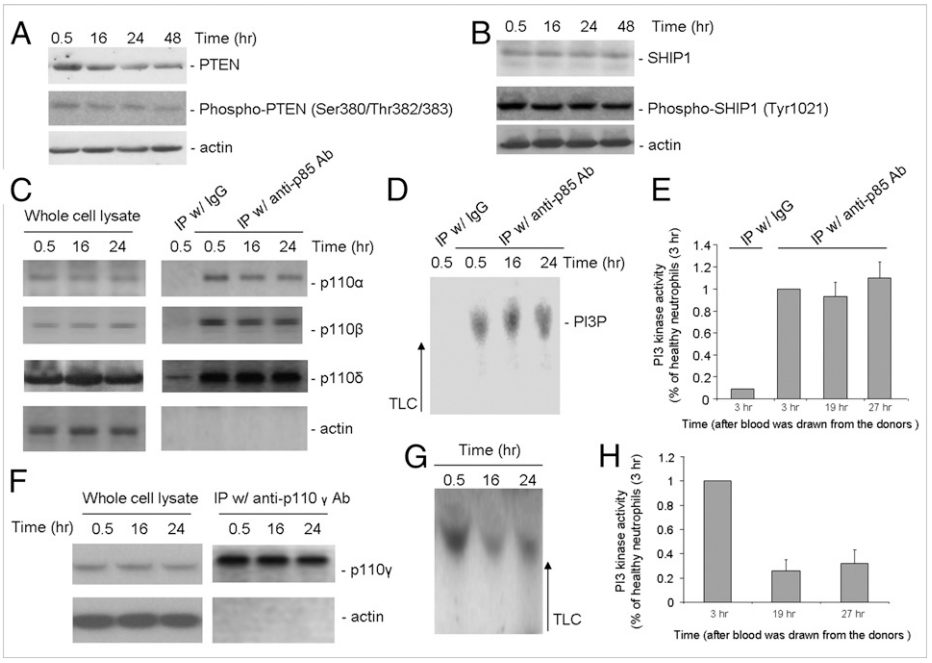

Fig. 2. Deactivation of PtdIns(3,4,5)P3/Akt signaling during neutrophil spontaneous death is a result of reduced PI3Kγ activity. (*A*) The level of PTEN in normal and apoptotic neutrophils. Shown is the result of a representative experiment that was repeated three times. (*B*) The level of SHIP in normal and apoptotic neutrophils. Total and phosphorylated SHIP were detected by Western blot using anti-SHIP and anti-phospho-SHIP antibodies, respectively (Cell Signaling). Shown is the result of a representative experiment that was repeated three times. (*C–E*) The enzymatic activity of PI3K class IA is not altered during neutrophil spontaneous death. (*C*) The three isoforms of PI3K class IA were pulled down with a PI3K p85 antibody (Upstate Biotechnology). Neutrophil whole-cell lysates and immunoprecipitated samples were blotted with indicated PI3K antibodies. Shown is the result of a representative experiment that was repeated three times. (*D*) PI3 kinase activity of the immunoprecipitated enzymes. Shown is the result of a reprehensive TLC plate. At each indicated time point, the kinase reaction was stopped and the lipids were extracted and analyzed by TLC. The positions of individual phosphatidylinositol were assigned from their migration distance matching those of corresponding authentic ^{32}P-labeled standards. The amount of ^{32}P-labeled PtdIns(3)P was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics). The PI3 kinase activity was expressed as the percentage of activity at time 3 hr (healthy neutrophils). (*E*) The data of the densitometric analyses are expressed as the percentage of the normal neutrophil control (3 hr). All values represent mean ± SD of three separate experiments. (*F* and *G*) The enzymatic activity of PI3K class IB (PI3Kγ) is down-regulated during neutrophil spontaneous death. (*F*) PI3Kγ was pulled down with a p110γ antibody (Upstate Biotechnology). Neutrophil whole cell lysates and immunoprecipitated samples were blotted with a rabbit polyclonal anti-p110 antibody. Shown is the result of a representative experiment that was repeated three times. (*G*) PI3 kinase activity of the immunoprecipitated enzymes. Shown is the result of a reprehensive TLC plate. (*H*) The data of the densitometric analyses are expressed as the percentage of the normal neutrophil control. All values represent mean ± SD of three separate experiments.

control cells cultured in the presence of serum (Fig. 3*A*). This result is consistent with the fact that class IA PI3Ks are not involved in regulating PtdIns(3,4,5)P3 signal during neutrophil death (Fig. 2).

In neutrophils, PtdIns(3,4,5)P3 signal can also be elicited by heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins)-coupled receptors. Chemokines bind receptors on cell membrane and induce the dissociation of a specific G protein into α and βγ subunits. Released βγ subunits are able to directly initiate activation class IB PI3K (PI3Kγ) (1, 6). We have shown that deactivation of PI3Kγ, but not class IA PI3Ks, was responsible for Akt deactivation during neutrophil death, suggesting the involvement of GPCR-mediated pathways in regulating neutrophil death. We explored the role of GPCR using a bacterial-derived toxin, pertussis toxin (PTX), which catalyzes ADP ribosylation of G proteins and thus suppresses their activation. We found a significant accelerated death in PTX-treated neutrophils (Fig. 3*B*). At 9 hr in culture, PTX-treated neutrophils showed nearly one fold higher death rate than untreated neutrophils. Similar results were obtained at 15 and 24 hr. The difference became nonsignificant at 48 hr, because most untreated neutrophils also became apoptotic.

Because serum deprivation did not affect the half-life of neutrophils, the factors leading to GPCR activation might be produced by the cultured neutrophils in an autocrine manner. To test this, we cultured neutrophils in serum-free medium and examined the secreted "GPCR activating activity" in the supernatants (Fig. 3*C*). Uniform treatment of freshly isolated neutrophils with chemokines or formyl-peptide (e.g., fMLP) elicits instant GPCR activation and elevation of PtdIns(3,4,5)P3 in the plasma membrane (17). We evaluated GPCR-elicited PtdIns (3,4,5)P3 signaling by measuring the level of endogenous Akt phosphorylation. Before chemoattractant stimulation, Akt phosphorylation was virtually undetectable in neutrophils (5). Upon stimulation, neutrophils showed maximum Akt phosphorylation at 2 min, which then declined marginally by 5 min. We used the level of Akt phosphorylation at 3 min after stimulation to assess GPCR activation. Our results showed that a large amount of "GPCR activating activity" was secreted and accumulated in the culture medium (Fig. 3*C*). Its ability to induce Akt phosphorylation was completely inhibited by PTX, further demonstrating that the activity of these secreted factors was indeed mediated by GPCR. Supporting this autocrine chemokine release mechanism, a significant amount of CXC chemokine IL8 was detected in the neutrophil culturing medium (Fig. 3*D*). IL8 can bind and activate G protein–coupled CXCR1 and CXCR2 receptors leading to activation of PI3Kγ and Akt (18). Thus, IL8 should be one of the neutrophil-released chemokines that support neutrophil survival. However, numerous chemokines can be produced by neutrophils; it is unlikely that IL8 will be the only one playing a role in neutrophil spontaneous death.

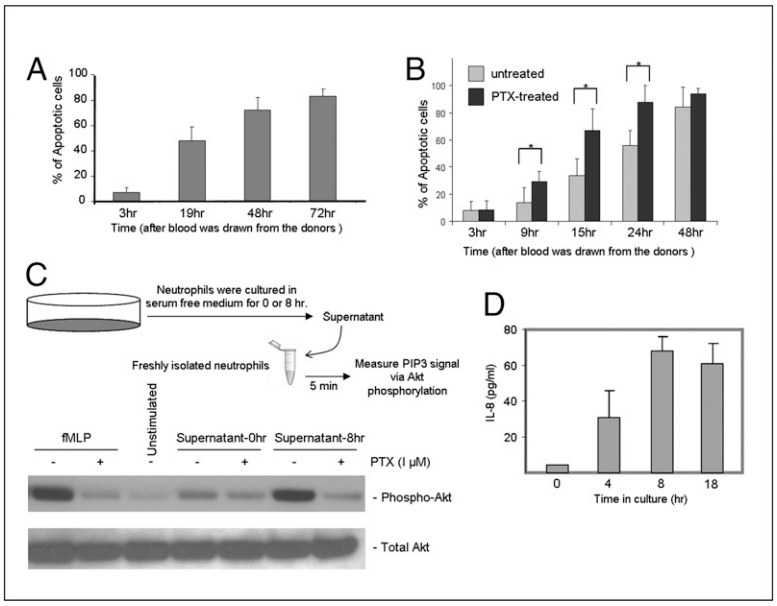

Fig. 3. Neutrophil spontaneous death is mainly regulated by G protein–coupled receptor. (*A*) Neutrophil spontaneous death in the absence of serum. Neutrophils were cultured in RPMI medium 1640 containing 1% BSA at a density of 2×10^6 cells per mL and maintained at 37 °C. (*B*) Pertussis toxin, an inhibitor of G protein, promotes neutrophil death. Freshly prepared neutrophils (3 hr after blood was drawn from healthy donors) were treated with 1 μM pertussis toxin (PTX) (Calbiochem) for indicated time. *, $P < 0.001$ versus untreated cells by Student's *t* test. Most PTX-treated and untreated cells became apoptotic after 48 hr in culture. (*C*) In vitro cultured human neutrophils release ligands of G protein–coupled receptor. Human primary neutrophils were cultured for indicated periods of time. Supernatants were harvested and used to stimulate freshly isolated neutrophils (for 3 min). Total and phosphorylated Akt were detected by Western blot using anti-Akt and anti-phospho-Akt (Ser-473) antibodies as described in Fig. 1. fMLP, a commonly used chemoattractant, was used as a positive control. Shown is the result of a representative experiment that was repeated three times. (*D*) Neutrophils release IL-8 chemokine. Human primary neutrophils were cultured for indicated periods of time. Supernatants were harvested, and IL-8 chemokine level was determined using an ELISA kit. Data are presented as means ± SD, $n \geq 3$ donors in each group.

Accumulation of Reactive Oxygen Species Is Responsible for Reduced PI3Kγ Activity in Apoptotic Neutrophils. A large amount of ROS are produced by NADPH oxidase in activated neutrophils to facilitate bacterial killing. Although many cellular functions are drastically reduced in apoptotic neutrophils, these cells are still capable of producing a large amount of ROS in response to chemoattractant stimulation (Fig. 4*A*). As a result, ROS accumulate in intracellular space during neutrophil spontaneous death (Fig. 4*B*). ROS has been recognized as one of the causal mediators of neutrophil death (1). Glutathione (GSH), an H_2O_2 scavenger, inhibits neutrophil death (19). Treatment with catalase, which reduces H_2O_2 to water, also delays apoptosis of normal neutrophils (20, 21). Pharmacological inhibition of intracellular NADPH oxidase has been shown to improve the survival of neutrophils (21–23). Consistent with this, the spontaneous death of neutrophils isolated from CGD patients decreases significantly compared to cells from healthy people (19, 20). Because PI3K/Akt is also a critical pathway involved in neutrophil death, we investigated whether accumulation of reactive oxygen species is responsible for reduced Akt activation in apoptotic neutrophils (Fig. 4*C*). GSH treated neutrophils showed nearly four times higher Akt phosphorylation than control neutrophils, at 19 hr in cultures. This enhancement was observed only when neutrophils were treated with compounds that can provide a long-term antioxidant effect such as GSH. Interestingly, *N*-acetyl-cysteine (NAC), another antioxidant, can only provide a short-term antioxidant effect in neutrophils (2–3 hr) and failed to reduce the death-associated Akt deactivation. In addition, no synergistic effect was observed when the two antioxidants were used together (Fig. 4*C*). Consistent with the fact that only PI3Kγ activity is reduced during neutrophil death, GSH treatment significantly elevated the kinase activity of PI3Kγ at both 16 and 24 hr in cultures; however, it did not exert any effect on the activity of class IA PI3Ks (Fig. 4*D*). This result also indicates that the inhibitory effect of ROS on PI3K may not be due to direct modification of the PI3 kinase catalytic domain, because all PI3K isoforms share very similar catalytic domain structure. Thus, the specific inhibition of PI3Kγ by ROS is most likely caused by suppression of cellular pathways leading to PI3Kγ activation in neutrophils.

ROS Inhibit Actin Polymerization in Neutrophils. We have shown that ROS is at least partially involved in Akt deactivation during neutrophil spontaneous death, and that Akt deactivation is mainly contributed by the reduction of PI3Kγ activity. Besides direct activation by GPCR, the only currently known mechanism leading to PI3Kγ activation is a positive feedback loop mediated by actin (24, 25). Neutrophils are polarized upon chemoattractant stimulation. It was reported that the highly localized PtdIns(3,4,5)P3 production in polarized neutrophils is mediated by a positive feedback loop which includes PI3K and actin polymerization. Amplification of the internal PtdIns(3,4,5)P(3) gradient, which was measured as the level of Akt phosphorylation, is markedly impaired by latrunculin or jasplakinolide, toxins that inhibit polymerization or depolymerization of actin, respectively (24, 25). Thus, we investigated whether ROS-induced Akt deactivation is mediated by suppression of chemoattractant-elicited actin polymerization in neutrophils. NADPH oxidase-mediated ROS production was suppressed using diphenyleneiodonium chloride (DPI), which is widely used as an NADPH oxidase inhibitor in neutrophils (Fig. S2). Upon stimulation with fMLP, F-actin levels increased dramatically in both DPI treated and untreated neutrophils within 1 min. Neutrophils treated with DPI always showed significantly higher F-actin levels compared to untreated neutrophils. F-actin levels were enhanced both before (0 sec) and after fMLP stimulation (at 30 sec and 3 min) (Fig. S2).

Inhibition of Actin Polymerization Aggravates Akt Deactivation and Accelerates Neutrophil Spontaneous Death. To directly examine the role of actin polymerization in regulating Akt activity and neutrophil spontaneous death, we inhibited chemoattractant-elicited actin polymerization with latrunculin which binds actin monomers near the nucleotide binding cleft and prevents them from polymerizing. As expected, latrunculin-treated neutrophils showed a more exaggerated Akt deactivation at each time point examined compared to WT neutrophils. However, the decreased was more pronounced at 3 hr in culture (6 hr after blood was drawn from the donors) when most neutrophils are still healthy (Fig. S3). Consistent with the much reduced Akt activation, the percentage of apoptotic cells increased by one-fold in the latrunculin treated neutrophils, at 12 hr in culture. The difference became nonsignificant at 48 hr, because most untreated neutrophils also became apoptotic (Fig. S3). It is noteworthy that disruption of F-actin in neutrophils leads to enhanced, instead of decreased, PtdIns(3,4,5)P3 signaling within the first 60-min

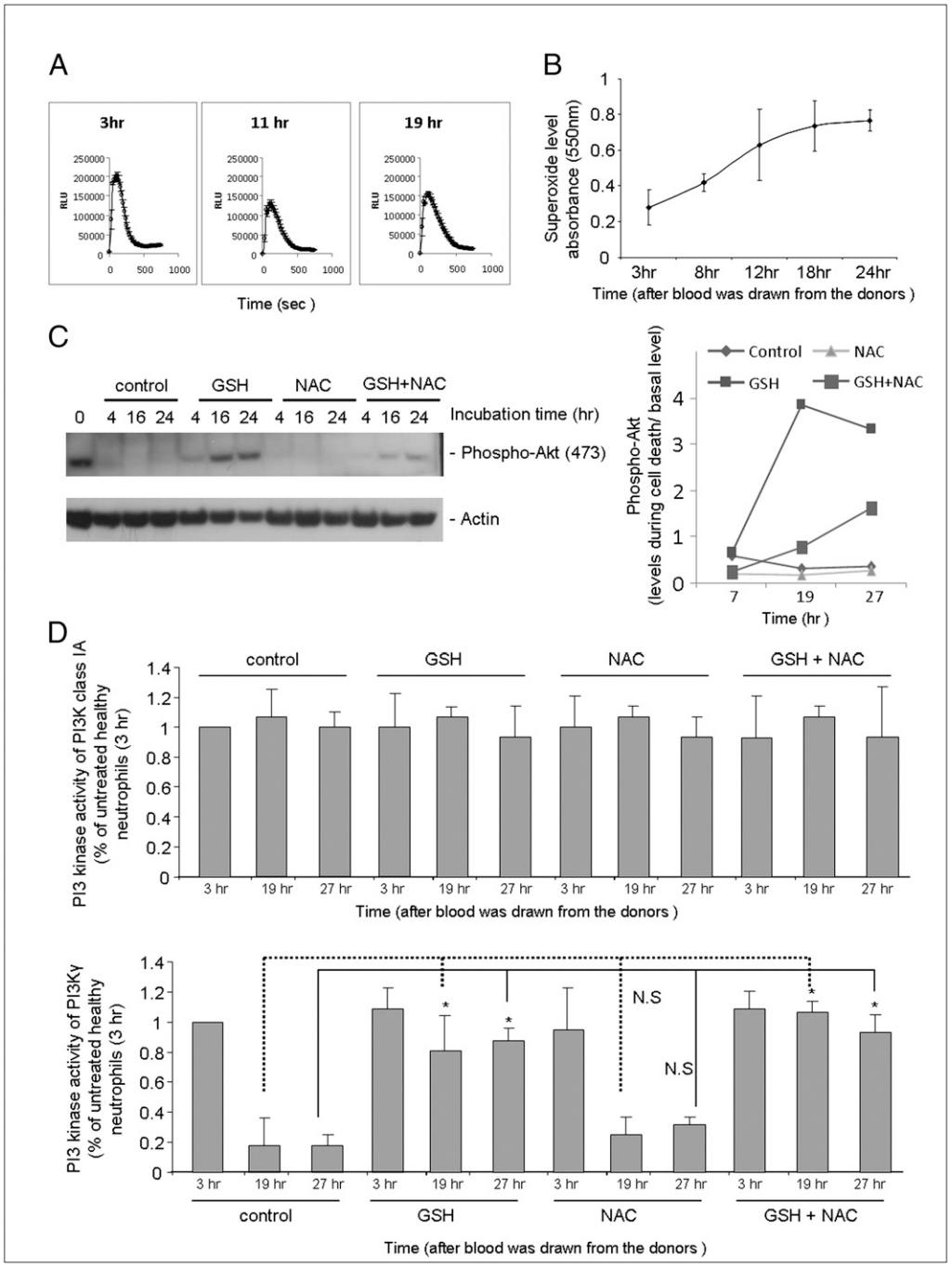

Fig. 4. ROS production is required for deactivation of PI3Kγ in neutrophil spontaneous death. (*A*) Aging neutrophils can still produce ROS. Human neutrophils were culture for indicated periods of time and stimulated with 100 nM fMLP (10^5 cells per 200 μL per well). ROS production was monitored in the presence of 50 μM isoluminol and 0.8 U of HRP in a luminometer at 37 °C. Chemiluminescence (arbitrary light units) was recorded (for 2 sec) at indicated time points after the addition of fMLP. Data are mean ± SD from one experiment representative of three. (*B*) Reactive oxygen species accumulate during the course of neutrophil spontaneous death. Human neutrophils (10^7 per data point) were cultured for indicated periods of time. The cells were then filter-lysed through two layers of 5-μm filter membrane, and the cytosolic ROS levels were assessed using cytochrome *c*. The absorbance (550 nm) represents the level of superoxide ion in each sample. All values were normalized to the number of intact cells (PI^- cells). Shown are means ± SD of three independent experiments. (*C*) Antioxidant reagents enhance the level of phosphorylation of endogenous Akt during neutrophil spontaneous death. Neutrophils were cultured in the presence of GSH (5 mg/mL) and/or NAC (0.2 mM) for indicated periods of time. Total and phosphorylated Akt were detected by Western blot as described (5). All samples were normalized to the amount of total Akt. basal level, level of phospho-Akt at time 3 hr. (*D*) Antioxidant reagents enhance the PI3 kinase activity of PI3Kγ but not PI3K class IA enzymes during the course of neutrophil spontaneous death. Neutrophils were cultured in the presence of indicated antioxidants as described above. The PI3 kinase activities of immunoprecipitated PI3K class IA and PI3Kγ were analyzed as described in Fig. 2. All values represent mean ± SD of three separate experiments. *, $P < 0.001$ versus untreated cells at the same point by Student's *t* test. N.S, not significant.

period. It is well known that disruption of cortical F-actin in neutrophils will augment degranulation and release of chemokines, which might be responsible for the early elevation of PtdIns(3,4,5)P3 signal in the treated cells.

Discussion

Neutrophil spontaneous apoptosis plays a crucial role in neutrophil homeostasis and the resolution of inflammation. We previously demonstrated that Akt deactivation is a key mediator of this tightly regulated cellular death program. In current study, we characterized an autocrine signal that controls Akt activity during neutrophil spontaneous death (Fig. S4). In apoptotic neutrophils, the activity of Akt is maintained by autocrinely released chemokines which elicits PI3Kγ activation via G protein coupled receptors. At the same time, PI3Kγ is negatively regulated by gradually accumulated ROS in apoptotic neutrophils, which suppress PI3Kγ activity by inhibiting an actin-mediated positive feedback loop. Taken together, these results provide insight into the mechanism of neutrophil spontaneous death and reveal a cellular pathway that regulates PtdIns(3,4,5)P3/Akt in neutrophils.

One surprising finding from this study is that neutrophil spontaneous death is completely independent of serum-derived growth factors. It is well known that growth factors such as IGF, G-CSF, PDGF, FGF, NGF, and TGF-β, are critical for the survival of most cell types such as neurons, fibroblasts, muscles cells, as well as premalignant and malignant cancer cells. These factors activate class IA PI3K/Akt pathway and the downstream pathways via their specific membrane receptors. In contrast, in neutrophils, the activation prosurvival Akt pathway is mainly maintained by autocrinely released chemokines. These chemokines bind to G protein coupled receptors and act through class IB PI3K (PI3Kγ). These results are consistent with previous reports that neutrophil apoptosis is enhanced in PI3Kγ deficient mice, where Akt activity is reduced (26, 27).

Materials and Methods

Measurement of PtdIns(3,4,5)P3 Levels in Normal and Apoptotic Neutrophils. Neutrophils were cultured in RPMI medium 1640 containing 10% heat-inactivated FBS at a density of 2×10^6 cells per mL and maintained at 37 °C. At each indicated time point, cells (10^7 cells per data point) were collected and lysed with 1 mL of ice-cold 1 M HCl. The lipids were extracted with 2 mL of chloroform:methanol (1:1) (9). After centrifugation at 1,500 ×*g* for 5 min, the lower organic phase was isolated and further extracted with 2 mL of methanol:1 M HCl (1:1). The lower phase was then isolated and dried under nitrogen gas. The dried lipid samples were resuspended in 12 μL of $CHCl_3$:MeOH:H_2O (1:2:0.8). The amount of PtdIns(3,4,5)P3 was measured by using a PIP3 Mass Strip kit (Echelon) according to the protocol provided by the manufacturer. The extracted lipids (10 μL) were spotted on the left side of the nitrocellulose strip. The PIP3 Strip was blocked with 5–10 mL of PBS–3% BSA per strip for 1 hr at room temperature and then incubated with 2.5 μL of PIP3 Detector (PIP3-specific Grp1 PH domain) in 5 mL of PBS + 3% BSA for 45 min. The standard curve for each strip contains 20, 15, 10, 5, 4, 2, 1, and 0.5 pmol of PtdIns(3,4,5)P3. Other methods are described in *SI Materials and Methods*.

Statistical Analysis. Values shown in each figure represent mean ± SD. Statistical significances were calculated with Student's *t* test. Differences were considered significant for $P < 0.005$.

ACKNOWLEDGMENTS. We thank Leslie Silberstein, John Manis, Li Chai, and people in the Joint Program in Transfusion Medicine for helpful discussions. H.R.L. is supported by National Institutes of Health Grants HL085100, AI076471, HL092020, and GM076084 and by a Research Scholar Grant from the American Cancer Society.

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Supporting Information

Xu et al. 10.1073/pnas.0912717107

SI Discussion

Constitutive neutrophil death is an important mechanism for modulating neutrophil homeostasis. Accelerated neutrophil death leads to a decrease of neutrophil counts (neutropenia), augments the chance of contracting bacterial or fungal infections, and impairs the resolution of such infections. However, delayed neutrophil death elevates neutrophil counts (neutrophilia), which is often associated with bacterial infection, myeloid leukemia, and acute myocardial infarction. Neutrophil death is also an essential cellular event for maintaining neutrophil number in infection and inflammation. Neutrophils are recruited to the infected tissues to engulf, kill, and digest invading microorganisms. However, the enzymes and reactive oxygen species (ROS) released by neutrophils can also damage the surrounding tissues. To prevent senescent neutrophils from releasing their toxic contents, these cells become apoptotic and are then recognized, engulfed, and cleared by professional phagocytes such as tissue macrophages. This safe clearance provides a mechanism of reducing the number of viable and activated neutrophils without releasing the potentially harmful enzymes and ROS, thereby facilitating the resolution of inflammatory response. Delayed death and clearance of neutrophils in tissues causes unwanted and exaggerated inflammation. Thus, the death program in neutrophils needs to be well controlled to provide a nice balance between their immune functions and their safe clearance (1–4).

In this study, we identified ROS as a key regulator of PI3Kγ in neutrophils. ROS have been implicated in a variety of cell death processes and are also recognized as one of the causal mediators of neutrophil spontaneous death. ROS accumulate during the course of neutrophil spontaneous death, and their pro-death activity is likely mediated by multiple pathways and mechanisms. ROS may lead to DNA alteration and trigger p53, which classically induces apoptosis following genotoxic injury (5, 6). Alternatively, ROS may directly alter the activity of intracellular signaling pathways involved in neutrophil death/survival such as NF-κB and MAPK (7–9). In neutrophils, it was also shown that death receptor clustering and the subsequent activation of caspase-8 are the results of ROS-dependent ceramide generation and may occur independently of Fas ligation in spontaneous death (10, 11). The cytotoxic free radical level can also be elevated by nitric oxide synthase (NOS)-mediated NO production. Exogenous nitric oxide and physiologically relevant NO donors, such as *S*-nitrosoglutathione, SIN-1, SNP, and GEA3162 significantly enhanced neutrophil apoptosis (12–16). Interestingly, high levels of ROS or reactive nitrogen species (RNS) inhibit caspase activity, indicating that an alternative caspase-independent death pathway may be involved in ROS-induced cell death (17, 18). It was reported that oxidative stress can trigger endonuclease G-mediated DNA fragmentation in the absence of caspase activity, providing a possible caspase-independent death pathway mediating ROS-induced neutrophil death (19).

The current study provides a mechanism by which ROS induce apoptosis in neutrophils, namely by inhibiting actin polymerization and subsequent amplification of the prosurvival PI3K/Akt pathway. How do ROS inhibit actin polymerization? In recent years, ROS has been identified as an important second messenger that can regulate intracellular signal transduction under a variety of physiological and pathophysiological conditions. During respiratory burst or oxidative stress, it is becoming increasingly clear that intracellular signal transduction gets altered (20–22). Such redox regulation of cell signaling involves modification of reactive thiols on specific cysteine residues of proteins, converting them from a reduced to an oxidized form (23–26). In recent years, an increasing number of thiol-containing proteins have been identified to use ROS as a mediator to regulate their function. Most importantly, many of these thiol modifications are reversible, ensuring that normal protein function can be restored upon release of oxidative stress or termination of oxidative burst. The major types of thiol modifications that have been shown to play an important redox dependent role include glutathionylation, sulfenic acid formation, nitrosylation, and disulfide bond formation. Many cellular targets such as protein tyrosine phosphatases, protein tyrosine kinases, integrins, and Ras, have been identified, and ROS could regulate actin polymerization indirectly by modulating these targeted signal molecules. Alternatively, ROS may also directly modify actin. Monomeric G-actin is a cytosolic protein that continuously polymerizes and depolymerizes from a filamentous F-actin polymer in an ATP-powered cycle (27). Numerous studies have shed light on the functionality and mechanisms underlying actin glutathionylation. In-vitro actin polymerization assays demonstrated that glutathionylated actin polymerizes inefficiently in comparison with unglutathionylated actin (24, 28, 29).

We identified a ROS-mediated intracellular mechanism that regulates actin polymerization and subsequent amplification of PtdIns(3,4,5)P3 signaling. Because actin is involved in a variety of cellular functions such as migration, polarization, and cell adhesion, it will be intriguing to see whether ROS also play a role in these cellular processes. In addition, it will be important to examine whether other downstream targets of PtdIns(3,4,5)P3, such as GSK3, BAD, PDK1, and Foxo, as well as the related cellular functions can also be regulated by ROS.

SI Materials and Methods

Human Primary Neutrophils. We isolated human primary neutrophils from discarded white blood cell filters (WBF2 filter; Pall Corporation), which were provided by the Blood Bank Lab at the Children's Hospital, Boston. Neutrophils were purified using a standard protocol. Briefly, erythrocytes were sedimented by adding an equal volume of dextran/saline solution (3% dextran T-500 in 0.9% NaCl) at room temperature for 25 min. The erythrocyte-depleted supernatants were then layered on Lymphocyte Separation Medium (1.077 g/mL Ficoll–Hypaque solution; Voigt Global Distribution) and centrifuged at $400 \times g$ at room temperature for 20 min. Contaminated erythrocytes in the neutrophil pellets were lysed after a brief (<30 sec) treatment with 0.2% NaCl. Neutrophils were then resuspended in RPMI medium 1640 containing 10% heat-inactivated FBS at a density of 4×10^6 cells per mL and maintained at 37 °C. The purity of neutrophils was >97% as determined by both Wright–Giemsa staining and FACS analysis with CD15 antibody. We routinely obtain about $1–3 \times 10^8$ neutrophils from one filter (450 mL of blood from a healthy donor). We have compared the neutrophils that we collected through filter with those obtained by vein puncture and stored in anticoagulant testing tubs, and found that the filtration method does not impair neutrophil function (e.g., chemotaxis and the time course of cell death). All blood is drawn from healthy blood donors.

PI3K Assays. Human primary neutrophils were cultured in 35-mm dishes at a density of 4×10^7 cells per mL per plate. At each time point, cells ($\sim 3 \times 10^7$ cells per data point) were lysed in 200 μL of lysis buffer (50 mM Tris·HCl pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate,

5 μg/mL aprotinin, 1 μg/mL leupeptin, 6 μg/mL chymostatin, 0.7 μg/mL pepstatin, 1 mM DFP, 1 mM PMSF). The samples were centrifuged for 10 min to sediment insoluble material. The supernatants were transferred to new tubes, and incubated with 5 μL of anti-PI3 kinase antibody (p85 antibody or p110γ antibody; Upstate Biotechnology) for 1 hr at 4 °C. Protein A-agarose beads (60 μL of 50% slurry) were added to each tube and incubated with mixing for another hour at 4 °C. Immunoprecipitated enzymes were collected by centrifuging for 5 sec and washed three times with freshly prepared reaction buffer (10 mM Tris·HCl pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.1 mM Na_3VO_4). The kinase assay was carried out at 30 °C for 15 min in a 50-μL reaction containing 10 mM $MgCl_2$, 10 μM ATP, 20 mM Hepes (pH 7.5), 20 μCi [γ-^{32}P] ATP, and 0.1 mg/mL phosphatidylinositol. The reaction was stopped with 100 μL of ice-cold 1 M HCl. The lipids were extracted with 2 mL of chloroform:methanol (1:1). After centrifugation at 1,000 rpm for 5 min, the lower organic phase was isolated and further extracted with 2 mL of methanol:1 M HCl (1:1). The lower phase was then isolated and dried under nitrogen gas. Silica gel 60 TLC plate (VWR) was prerun overnight with 1.2% potassium oxalate (Sigma) in dH_2O:methanol (3:2) and then dried and heat-activated in an oven (100 °C) for 3 min. The dried lipid samples were resuspended in 30 μL of chloroform:methanol (2:1) and 10 μL was spotted. TLC was performed using chloroform:acetone: methanol:acetic acid:dH_2O (30:12:10:9:6) as a mobile phase. After the solvent front reached the top, the plate was taken out, dried, and analyzed by antoradiography.

FACS Analysis of Neutrophil Spontaneous Death. Neutrophils were cultured for the indicated time and stained using an Annexin V detection kit (Caltag Laboratories) following a protocol provided by the manufacturer. FACS was performed using a FACSCanto II flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser. Ten thousand cells were collected and analyzed using BD FACSDiva software (Becton Dickinson).

Western Blot analysis. Neutrophils were kept at 4×10^6 cells per mL in the spontaneous death assay. At each indicated time point, neutrophils (4 million cells per data point) were spun down and lysed immediately with 100 μL of boiling protein loading buffer (Invitrogen). Samples were incubated at 100 °C for 5 min and transferred on ice. After a brief sonication (5–10 sec), 25 μL of lysate was used for Western blot analysis. For Western blotting, a 4–20% SDS/PAGE system (Invitrogen) was used for protein separation, and an ECL Western blotting kit (Amersham) was used for protein detection.

Release of IL-8 Chemokine by in Vitro–Cultured Neutrophils. Human primary neutrophils were cultured in 35 mm-dishes at a density of 4×10^7 cells per mL per plate for 30 min and washed with RPMI medium 1640–1% BSA three times. Cells were resuspended in 500 μL of RPMI 1640–1% BSA and then transferred to a 1.5-mL Eppendorf tube. Supernatants were collected at indicated times and secreted IL-8 chemokines was measured by an ELISA kit following a protocol provided by the manufacturer (R&D Systems).

NADPH Oxidase Activity Assay. Superoxide anions produced by NADPH oxidase were detected using isoluminol chemiluminescence (30). Neutrophils were resuspended at a density of 10^7/mL in HBSS (containing Ca^{2+}/Mg^{2+} salts) and kept on ice until use. A reaction mixture containing 20 μL of 0.5 mM isoluminol (TCI America), 10 μl of 80 U/mL horseradish peroxidase (Type XII; Sigma), 40 μL of cells, and 110 μL of HBSS (containing Ca^{2+}/Mg^{2+} salts) was added into each well of a 96-well Maxisorp plate (Nunc) and allowed to equilibrate to 37 °C for 4 min in a 1420 Wallac Victor^2 multilabel counter. A prestimulus luminescence reading was taken (for 2 sec). Twenty microliters of 10× concentrated fmlp was then added to the reaction mixture via the injection port of the luminometer and luminescence was recorded (for 2 sec) at fixed time intervals.

Measurement of Total ROS Level in Neutrophils. The levels of reactive oxygen species in neutrophils were assessed using a cytochrome-C assay as described (31). Briefly, freshly prepared human neutrophils (10^7 per data point) were cultured for indicated periods of time, washed, and resuspended in 1 mL of HBSS containing 1.5 mg/mL cytochrome *c*. The cells were then filter-lysed through two layers of 5-μm filter membrane. After 5 min at RT, cytochrome *c* reduction in each sample was detected by spinning-down cell debris and reading absorbance (at 550 nm) of the supernatant in a spectrophotometer. The absorbance represents the level of superoxide ion in each sample.

Quantification of F-Actin Levels. Human neutrophils were cultured at a density of 5×10^6/mL in RPMI/0.25% BSA. Cells (5×10^5) were stimulated with 100 μL of 200 nM fMLP in RPMI/0.25% BSA for 1, 3, or 5 min, fixed with 200 μL of 8% formaldehyde, and incubated on ice for 20 min. After preblocking overnight at 4 °C with 5% nonfat dry milk, cells were stained for 30 min with 0.13 μg/mL fluorescein phalloidin (Sigma) in PBS containing 0.1% Triton X-100 and 5% milk. Intensity of phalloidin-staining was analyzed using a FACSCalibur machine.

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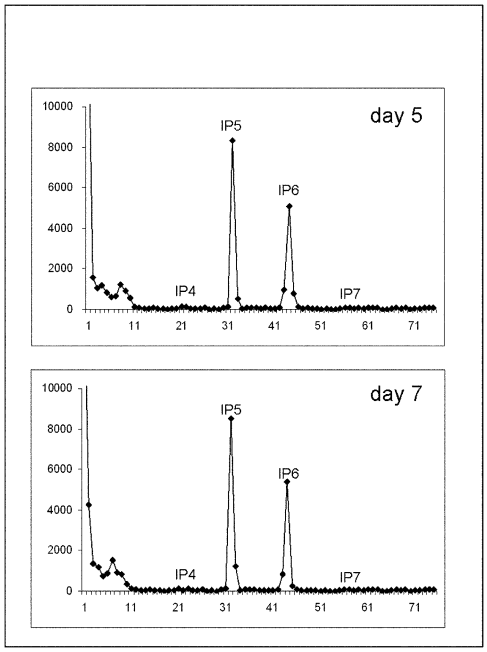

Fig. S1. The levels of InsP7 and Ins(1,3,4,5)P4 are extremely low in unstimulated neutrophils. HL60 cells were cultured in RPMI medium 1640 supplemented with 10% FBS and 4 mM glutamine. For differentiation, cells were plated at a density of 3 × 106 cells per mL and treated with DMSO (1.3% vol/vol) for indicated days. At day 5, most neutrophil-like differentiated HL60 cells [Jia Y, et al. (2007) *Immunity* 27:453–467] were healthy. At day 7, ~40–50% cells became apoptotic due to spontaneous death. [3H]inositol (100 μCi/mL) was added into the medium on day 3 to label endogenous inositol phosphates. Cells (1 mL or 3 × 106 cells) were lysed at indicated time points. The inositol phosphates were extracted and analyzed by HPLC as described [Luo HR, et al. (2003) *Cell* 114:559–572]. Peaks detected before 15 min are mixtures of various species of free inositol, InsP, InsP2, and InsP3. All of the data were normalized to the total amount of protein extracted from each sample.

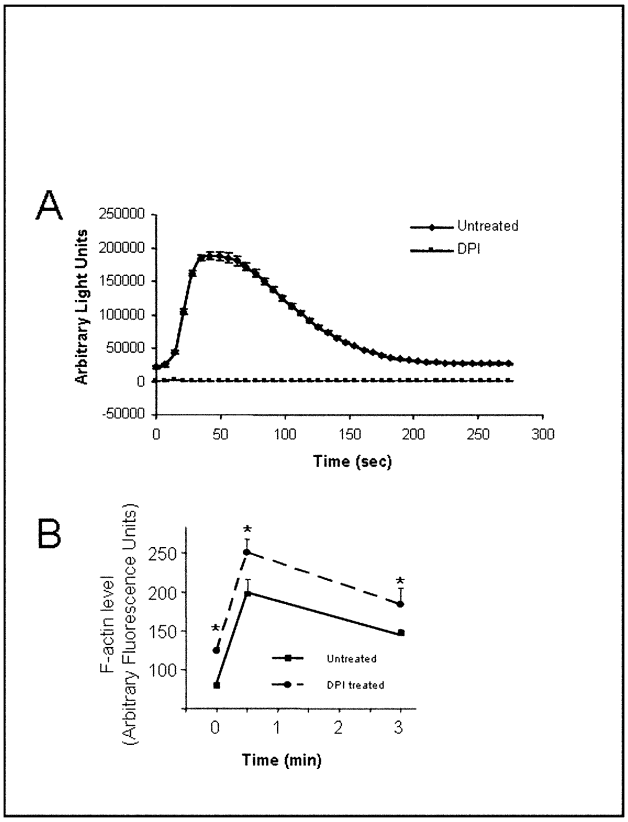

Fig. S2. ROS inhibit actin polymerization in neutrophils. (*A*) Chemoattractant-induced ROS production is suppressed by a specific pharmacological inhibitor of NADPH oxidase. Human blood neutrophils (5 × 105) were left untreated or treated with 50 μM diphenyliodonium chloride (DPI) for 30 min at 37 °C. Cells were then stimulated with 100 nM fMLP and ROS production was monitored in the presence of 50 μM luminol and 0.8 U of HRP in a luminometer at 37 °C. Chemiluminescence (arbitrary light units) was recorded (for 1 sec) at indicated time points. Data are mean ± SD (n = 3) from one experiment representative of three. (*B*) Actin polymerization in DPI-treated human neutrophils. Human neutrophils (0.5 × 106) treated with (or without) 50 μM DPI and then stimulated with 10 nM fMLP. Cells were fixed at specified time points, permeabilized, and stained with rhodamine-phalloidin as described in ref. 5. Stained neutrophils were then analyzed by fluorescence-activated cell sorting (FACS). Data are represented as mean of median fluorescence ± SD (n = 3) from one experiment representative of three.

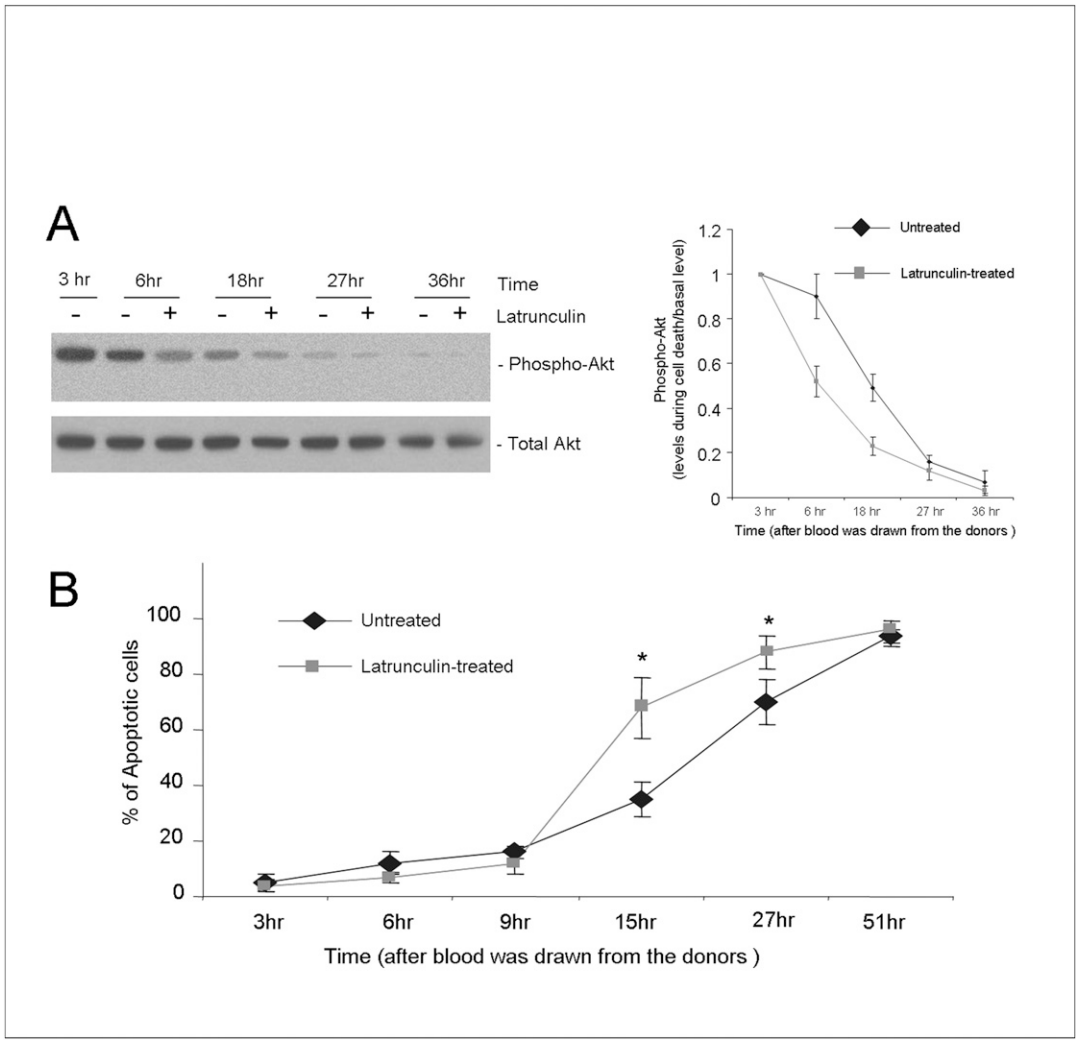

Fig. S3. ROS-induced Akt deactivation and neutrophil spontaneous death are mediated by actin. (*A*) Latrunculin A, an inhibitor of actin polymerization, reduces the level of phosphorylation of endogenous Akt during neutrophil spontaneous death. Neutrophils were cultured in the presence of 2 μM Latrunculin A for indicated periods of time. Protein extracts were resolved on SDS/PAGE. Total and phosphorylated Akt were detected by Western blot using anti-Akt and anti-phospho-Akt (Ser-473) antibodies as described (31). Relative amounts of phosphorylated Akt were quantified using NIH Image software. All samples were normalized to the amount of total Akt. Basal signal, level of phospho-Akt at time 3 hr. Data presented are the means (± SD) of three independent experiments. (*B*) Latrunculin A enhances neutrophil death. Freshly prepared neutrophils (3 hr after blood was drawn from healthy donors) were treated with 2 μM Latrunculin A (Calbiochem) for indicated periods of time. Cell viability was assessed by FACS analysis as described in Fig. 1. At least three separate experiments were carried out with a minimum of 100,000 cells counted per data point. Cell viability was determined as the ratio of live to total cell number. The results are the means of three independent experiments. Bars indicate mean ± SD. *, $P < 0.001$ versus untreated cells by Student's *t* test.

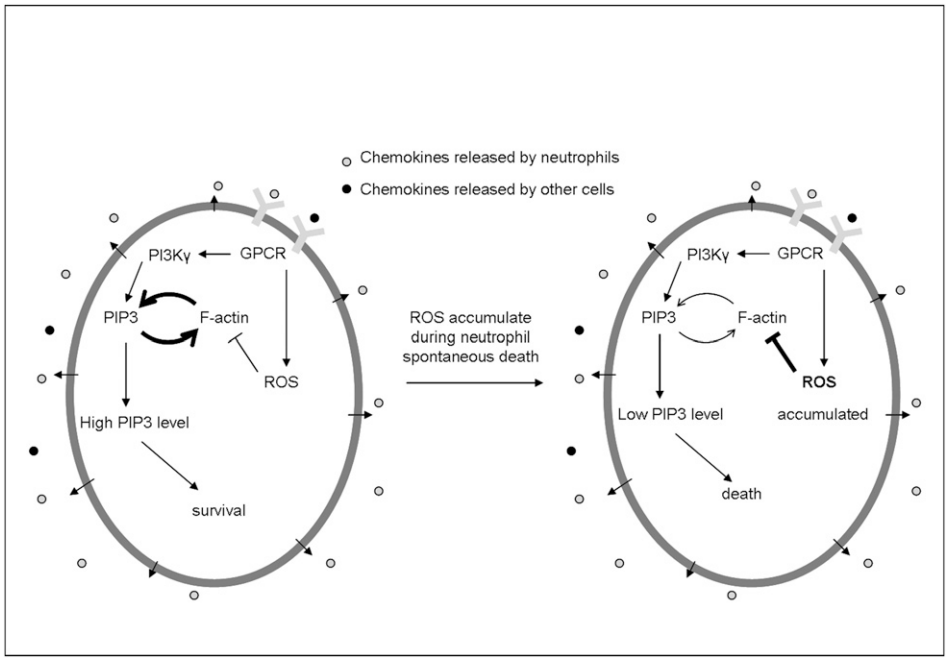

Fig. S4. Neutrophil spontaneous death is mediated by down-regulation of autocrine signaling through G protein–coupled receptors, PI3Kγ, ROS, and actin.