Cigarette smoke (CS) and nicotine delay neutrophil spontaneous death via suppressing production of diphosphoinositol pentakisphosphate

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Diphosphoinositol pentakisphosphate (InsP7), a higher inositol phosphate containing energetic pyrophosphate bonds, is beginning to emerge as a key cellular signaling molecule. However, the various physiological and pathological processes that involve InsP7 are not completely understood. Here we report that cigarette smoke (CS) extract and nicotine reduce InsP7 levels in aging neutrophils. This subsequently leads to suppression of Akt deactivation, a causal mediator of neutrophil spontaneous death, and delayed neutrophil death. The effect of CS extract and nicotine on neutrophil death can be suppressed by either directly inhibiting the PtdIns(3,4,5)P3/Akt pathway, or increasing InsP7 levels via overexpression of InsP6K1, an inositol hexakisphosphate (InsP6) kinase responsible for InsP7 production in neutrophils. Delayed neutrophil death contributes to the pathogenesis of CS-induced chronic obstructive pulmonary disease. Therefore, disruption of InsP6K1 augments CS-induced neutrophil accumulation and lung damage. Taken together, these results suggest that CS and nicotine delay neutrophil spontaneous death by suppressing InsP7 production and consequently blocking Akt deactivation in aging neutrophils. Modifying neutrophil death via this pathway provides a strategy and therapeutic target for the treatment of tobacco-induced chronic obstructive pulmonary disease.

In tobacco smoking-induced chronic obstructive pulmonary disease (COPD), chronic inflammation of the small airways and the lung parenchyma leads to fixed narrowing of small airways and alveolar wall destruction (emphysema) (1–4). The chronic inflammatory infiltrate is characterized by augmented numbers of alveolar leukocytes, including both B and T lymphocytes. However, the adaptive immune system may not be essential to the development of pulmonary emphysema in response to chronic tobacco exposure, because emphysema can still be induced in SCID mice, which lack functional lymphocytes (5). This finding indicates that innate inflammatory cells, such as neutrophils and macrophages, may play an essential role in the pathogenesis of COPD. Massive accumulations of neutrophils are frequently found in the bronchoalveolar lavage fluid (BALF) of patients with both stable and acute exacerbations of COPD (1–4). The enzymes and reactive oxygen species (ROSs) released by neutrophils can damage the surrounding tissues. Therefore, abnormal accumulation of neutrophils in the small airways and alveoli is likely to be a major contributor to the irreversible lung damage seen in tobacco-induced COPD (1–4).

The accumulation of neutrophils in the lungs can be due to increased neutrophil recruitment, or suppression of the clearance of apoptotic neutrophils by tissue macrophages (6, 7). In addition to these mechanisms, delayed spontaneous programmed cell death (apoptosis) of neutrophils can contribute to elevated numbers, contributing to unwanted and exaggerated inflammatory responses. Neutrophils are terminally differentiated cells, which normally have a very short lifespan (6–7 h in blood and 1–4 d in tissue), resulting in a rapid daily turnover (0.8–1.6×10^9 cells per kg of body weight). They readily undergo apoptosis, and only apoptotic neutrophils can be recognized, engulfed, and cleared by macrophages.

A reduction in neutrophil spontaneous death has been detected in tobacco-induced COPD patients (8). Some of the chemical constituents of cigarette smoke (CS), such as nicotine and acrolein, have been shown to directly delay neutrophil spontaneous death (9–11), providing a mechanism for the massive accumulation of neutrophils in the lungs of smoke-induced COPD patients. Here, we investigate the mechanism by which CS reduces neutrophil spontaneous death.

We previously established deactivation of the phosphatidylinositol 3,4,5 trisphosphate [PtdIns(3,4,5)P3]/Akt pathway as a causal mediator of neutrophil spontaneous death (12). Akt is a well known cellular survival signal. Akt activity decreases dramatically during the course of neutrophil spontaneous death. In the current study, we demonstrate that both CS extract (CSE) and nicotine significantly suppress Akt deactivation in aging neutrophils, and thus delay neutrophil spontaneous death. Inhibition of PtdIns(3,4,5)P3/Akt signaling prevents CSE- and nicotine-induced delay of neutrophil spontaneous death. In addition, we demonstrate that the CSE- and nicotine-induced suppression of Akt deactivation is mediated by inhibition of diphosphoinositol pentakisphosphate (InsP7) production in aging neutrophils, establishing a mechanism by which CS regulates PtdIns(3,4,5)P3 signaling in neutrophils. Taken together, we conclude that CS- and nicotine-induced delay of neutrophil spontaneous death is a result of inhibition of InsP7 production, and the subsequent blockage of Akt deactivation, in aging neutrophils.

Results

CSE and Nicotine Block Akt Deactivation and Delay Neutrophil Spontaneous Death. We used an in vitro assay to assess the effect of CSE and nicotine on neutrophil spontaneous death. The number of neutrophils undergoing spontaneous death was quantified using FACS analysis. We used Annexin V, an anticoagulant protein with high affinity and selectivity for phosphatidylserine (PS), to detect PS exteriorization, and propidium iodide (PI), a membrane impermeable dye, to monitor cell membrane integrity. Consistent with previous reports (8–11), CSE and nicotine significantly reduced neutrophil spontaneous death. The most significant effects were observed at 36 h and 54 h (Fig. S1).

We recently established that deactivation of the PtdIns(3,4,5)P3/Akt pathway, a well known survival signal, is a causal mediator of neutrophil spontaneous death (12). Therefore, we examined whether CSE- and nicotine-induced reductions in neutrophil spontaneous death are a result of blockage of Akt deactivation. Akt is recruited to the plasma membrane through specific binding to PtdIns(3,4,5)P3. Only Akt molecules on the plasma membrane can be phosphorylated and activated by two phosphatidylinositol-dependent protein kinases (PDKs), thus Akt phosphorylation has been widely used as an indicator of Akt activation. During the course of neutrophil death, levels of phospho-Akt decreased significantly, whereas levels of total Akt remained unaltered. CSE and nicotine dramatically inhibited this neutrophil death-associated Akt dephosphorylation (Fig. 1A). We next examined cellular Akt activity by measuring glycogen synthase kinase-3β (GSK-3β), a substrate of Akt, as a downstream marker of functional Akt activation. Levels of phospho-GSK-3β declined during neutrophil death with a time course similar to the decline in levels of phospho-Akt. CSE and nicotine significantly suppressed this decline (Fig. 1B). Finally, we directly measured Akt protein kinase activity in neutrophil cell lysates using an in vitro assay, and observed that the kinase activity of Akt was significantly increased in neutrophils treated with CSE or nicotine (Fig. 1C). Taken together, our results demonstrate that CSE- and nicotine-induced inhibition of neutrophil spontaneous death is associated with up-regulation of PtdIns(3,4,5)P3/Akt signaling.

The cellular effect of nicotine is mediated by specific receptors. We next examined whether nicotinic acetylcholine receptors (nAChRs) are involved in nicotine-induced delay of neutrophil death. nAChRs are primarily known for their action as ligand-gated ion channels that transduce action potentials across neuronal synapses. However, various nAChRs have also been detected on hematopoietic cells, including neutrophils (13, 14). Nicotinic acetylcholine receptors consist of five molecular subunits (α1–10, β1–4, δ, ε, and γ) that surround a central pore through which ions pass. To date, 17 nAChR subunits have been

identified, which are divided into three functional classes: muscle subunits (α1, β1, δ, ε, and γ), neuronal subunits (α1–6 and β2–4) that form in pairwise αβ combinations, and subunits α7–9 that can form homomeric nAChRs. We used specific nAChR inhibitors to reveal which subunits are involved in nicotine-induced up-regulation of Akt phosphorylation during neutrophil death. Benzoquinonium dibromide (nonselective), MG 624 (α7 specific), and methyllycaconitine (α7 specific) inhibited nicotine-induced up-regulation of Akt phosphorylation. Dihydro-β-erythroidine hydrobromide (α4 specific), ACV 1 (α9α10 specific), α-Conotoxin PIA (α6 specific), α-Conotoxin AuIB (α3β4 specific), dihydro-β-erythroidine hydrobromide (α4 specific), and α-Conotoxin EI (α1β1γδ specific) were essentially ineffective inhibitors of nicotine-induced up-regulation of Akt phosphorylation (Fig. 1D). These results suggest that nicotine-induced delay of neutrophil death is indeed mediated by nAChRs, in particular those composed of α7 subunits.

CS contains over 4,000 chemicals. Surprisingly, CSE-induced up-regulation of Akt phosphorylation in aging neutrophils could be partially suppressed by the AChR antagonists benzoquinonium dibromide, MG 624, or methyllycaconitine, suggesting that CSE-induced delay of neutrophil death is at least partially mediated by the nicotine in CS (Fig. 1 E and F).

Effects of CSE and Nicotine on Neutrophil Spontaneous Death Can be Suppressed by Inhibitors of the PtdIns(3,4,5)P3/Akt Pathway. We next suppressed PtdIns(3,4,5)P3/Akt signaling using pharmacological inhibitors. First, we used two newly developed Akt inhibitors, SH5 and SH6, to suppress Akt signaling in neutrophils. Because PI3-kinase is upstream of Akt, and inhibition of PI3-kinase is associated with deactivation of Akt, we also treated neutrophil cultures with two PI3-kinase inhibitors, LY294002 and wortmannin. Treatment with all of these drugs markedly reduced Akt phosphorylation without altering total Akt levels (Fig. 2A) and promoted the death of CSE-treated neutrophils measured by FACS analysis (Fig. 2B). Because the inositol phosphates InsP7
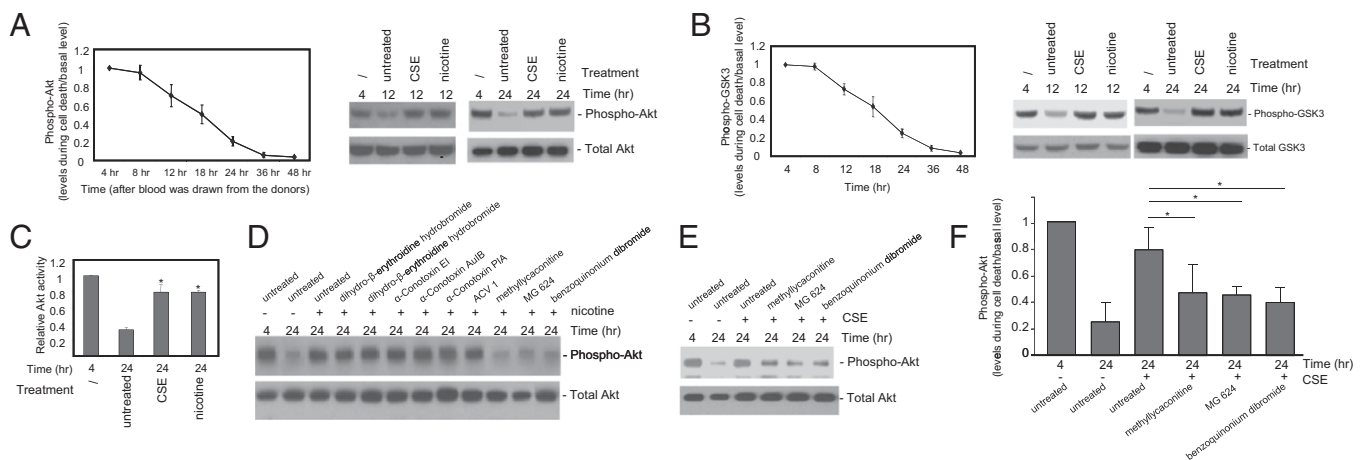
Fig. 1. CS and nicotine suppress Akt deactivation during neutrophil spontaneous death. Human neutrophils were cultured in the presence of CSE or nicotine for indicated period, as described in Fig. S1. (A) Protein extracts were resolved on SDS/PAGE. Total and phosphorylated Akt were detected by Western blot using anti-Akt and anti-phospho-Akt (Ser473) antibodies, respectively. Relative amounts of phosphorylated Akt were quantified using NIH Image software as described (26). All samples were normalized to the amount of total Akt. "Basal signal" refers to the level of phospho-Akt at time "4 h." Data presented are the means (±SD) of three independent experiments. (B) GSK-3β phosphorylation in intact neutrophils. GSK-3β is one of the targets of Akt and its phosphorylation was detected using phospho-GSK-3β (Ser9) antibody (Cell Signaling). (C) In vitro Akt protein kinase activity. Data presented are the means (±SD) of three independent experiments. *P < 0.001 versus untreated cells (24 h) by Student t test. (D) Human neutrophils were cultured with nicotine (1 μM) in the absence or presence of benzoquinonium dibromide (10 μM), MG 624 (0.5 μM), methyllycaconitine (10 nM), dihydro-β-erythroidine hydrobromide (1 μM), ACV 1 (50 nM), α-Conotoxin PIA (10 nM), α-Conotoxin AuIB (50 nM), dihydro-β-erythroidine hydrobromide (5 μM), or α-Conotoxin EI (50 nM) for the indicated time period. Total and phosphorylated Akt were assessed as described above. (E) Human neutrophils were cultured with CSE (2%) in the absence or presence of benzoquinonium dibromide (10 μM), MG 624 (0.5 μM), or methyllycaconitine (10 nM). Total and phosphorylated Akt were assessed as described above. (F) Relative amounts of phosphorylated Akt were quantified using NIH Image software as described above. All samples were normalized to the amount of total Akt. "Basal signal" refers to the level of phospho-Akt at time "4 h." Data presented are the means (±SD) of three independent experiments. *P < 0.001 by Student t test.
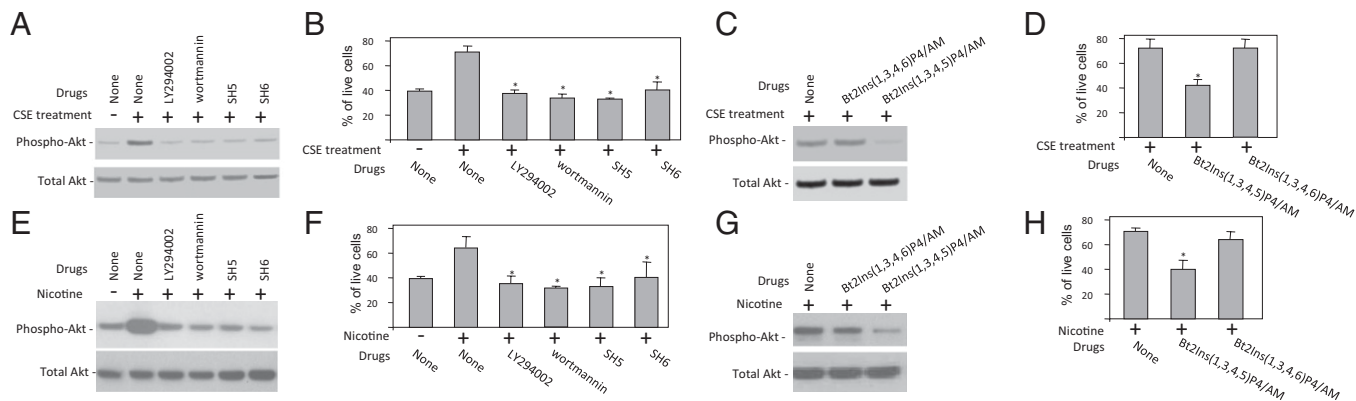
Fig. 2. Inhibiting Akt activity reverses CSE- and nicotine-induced delay of neutrophil spontaneous death. (*A*) Deactivation of Akt by PI3-kinase or Akt inhibitors. Freshly prepared human neutrophils were treated with 20 μM LY294002, 50 nM wortmannin, 10 μM SH5, or 10 μM SH6 in the presence or absence of 2% CSE for 15 h. Akt activity (phosphorylation) was assessed by Western blotting analysis as described above. (*B*) Cell viability was assessed by FACS analysis 34 h after blood was drawn from the donors. The results are the means of three independent experiments. Bars indicate mean (±SD). *$P < 0.001$ versus cells treated with CSE alone by Student *t* test. (*C* and *D*) Deactivation of Akt was achieved by treatment with Bt2Ins(1,3,4,5)P4/AM. Neutrophils were cultured in 50 μM Bt2Ins(1,3,4,5)P4/AM or Bt2Ins(1,3,4,6)P4/AM (as a control), and Akt phosphorylation and cell viability were assessed as described above. (*E–H*) Human neutrophils were cultured in 1 μM nicotine. All experiments were conducted as described in *A–D*.

and Ins(1,3,4,5)P4 compete with PtdIns(3,4,5)P3 for binding to the pleckstrin homolog (PH) domain, and thereby attenuate the membrane translocation of PH-domain-containing proteins (15–17), we also tried to suppress PtdIns(3,4,5)P3/Akt signaling using inositol phosphates. However, inositol phosphates, such as Ins(1,3,4,5)P4 and InsP7, are highly hydrophilic molecules and cannot passively cross the plasma membrane. Intracellular levels of Ins(1,3,4,5)P4 or InsP7 can therefore not be raised by simply adding the compounds to the culture medium. To circumvent this problem, we used a membrane-permeable derivative of Ins(1,3,4,5)P4, D-2,6-di-O-butyryl-myo-inositol 1,3,4,5-tetrakis-phosphate octakisacetoxymethyl ester [Bt2Ins(1,3,4,5)P4/AM]. Bt2Ins(1,3,4,5)P4/AM diffuses into cells, and is subsequently hydrolyzed by ubiquitous, nonspecific cellular esterases to release Ins(1,3,4,5)P4. Bt2Ins(1,3,4,5)P4/AM significantly inhibited Akt phosphorylation in CSE-treated neutrophils, in comparison with Bt2Ins(1,3,4,6)P4/AM, which was used as a negative control (Fig. 2*C*). We then examined the effect of Bt2Ins(1,3,4,5)P4/AM on neutrophil death. We observed that Bt2Ins(1,3,4,5)P4/AM, but not Bt2Ins(1,3,4,6)P4/AM, dramatically promoted the death of CSE-treated neutrophils (Fig. 2*D*). The same results were seen in neutrophils treated with nicotine (Fig. 2 *E–H*). All these results further indicate that CSE- and nicotine-induced inhibition of neutrophil spontaneous death is mediated by up-regulation of PtdIns(3,4,5)P3/Akt signaling.

CSE and Nicotine Inhibit Neutrophil Spontaneous Death-Associated Reduction of Akt Membrane Translocation, but Do Not Change the Level of PtdIns(3,4,5)P3 on the Membrane. We showed that the level of active Akt (phospho-Akt) drastically declined during neutrophil death, whereas CSE and nicotine suppressed this decline (Fig. 1). Because the total amount of Akt remained the same during this process, the increase in Akt activity is not as a result of up-regulation of total protein levels. Akt activation relies on membrane translocation mediated by its specific association with PtdIns(3,4,5)P3 on the plasma membrane. Only Akt molecules on the plasma membrane can be phosphorylated and activated. Accordingly, we next investigated whether Akt membrane translocation was altered in neutrophils treated with CSE or nicotine. We used the PH domain of Akt (PH$_{Akt}$) fused with green fluorescent protein (PH$_{Akt}$–GFP) as a marker for PH domain translocation. The construct expressing PH$_{Akt}$–GFP fusion protein was transfected into mouse primary neutrophils using a nucleofection system. Akt membrane localization and subsequent activation are essential for neutrophil survival; therefore, a significant amount of PH$_{Akt}$–GFP was detected on the plasma membrane in healthy

neutrophils. Akt membrane localization drastically declined during the course of neutrophil death, whereas CSE or nicotine partially restored Akt membrane localization (Fig. 3 *A* and *B*). However, unexpectedly, treatment with CSE or nicotine did not directly alter the amount of PtdIns(3,4,5)P3 in aging neutrophils (Fig. 3*C*), indicating that their effect on Akt activation may be mediated by another cellular mechanism.

CSE and Nicotine Reduce the Intracellular Level of InsP7, a Negative Regulator of PtdIns(3,4,5)P3 Signaling. We recently discovered that two inositol phosphates, InsP7 and Ins(1,3,4,5)P4, compete with PtdIns(3,4,5)P3 for PH-domain binding and suppresses PH-domain translocation, providing a mode of regulation for PtdIns(3,4,5)P3/Akt signaling (15–17). We therefore examined whether the levels of InsP7 and Ins(1,3,4,5)P4 were altered in neutrophils treated with CSE or nicotine. Primary neutrophils have a short life span of 24–48 h, which makes this system unsuitable for labeling endogenous inositol high phosphates (InsP6, InsP7, and InsP8) with [^{3}H]inositol. Incorporation of [^{3}H]inositol into these inositol phosphates would not reach plateau values until 3–4 d after the addition of [^{3}H]inositol. Thus, to detect InsP6 and InsP7, we used a human promyelocytic cell line, HL60, which can be induced to differentiate toward morphologically mature neutrophils (dHL60) by incubation with compounds such as dimethyl sulfoxide (DMSO) or retinoic acid. Consistent with the previous results (17), Ins(1,3,4,5)P4 was the major isoform of InsP4 present in neutrophils. Its level was undetectable in unstimulated neutrophils, and it remained undetectable in untreated, CSE-treated (2% vol/vol), or nicotine-treated aging neutrophils (Fig. S2). Interestingly, a significant amount of InsP7 was detected in unstimulated neutrophils (16). Treatment with CSE or nicotine reduced InsP7 levels in neutrophils (Fig. 3 *D* and *E*). These results strongly suggest that InsP7, rather than Ins(1,3,4,5)P4, is the major negative regulator of PtdIns(3,4,5)P3 signaling in CSE or nicotine-treated aging neutrophils.

InsP6K1-Mediated InsP7 Production Is a Key Mediator of CSE- and Nicotine-Induced Delay of Neutrophil Death. InsP7 arises from pyrophosphorylation of InsP6, the most abundant inositol phosphate in mammalian cells. The enzymes that catalyze the synthesis of InsP7 comprise a family of InsP6 kinases (InsP6K) including InsP6K1, InsP6K2, and InsP6K3 (18, 19). InsP6K1 and InsP6K2 are highly expressed in neutrophils, whereas InsP6K3 is essentially undetectable. In neutrophils, InsP6K1 and InsP6K2 isoforms have nonredundant roles. InsP6K1 seems to be equally distributed in the nucleus and cytoplasm, whereas InsP6K2 appears to be almost

Fig. 3. CSE and nicotine up-regulate Akt membrane translocation via inhibiting InsP7 production. (*A* and *B*) CSE and nicotine up-regulate Akt membrane translocation in aging neutrophils. (*A*) Membrane translocation of PH$_{Akt}$–GFP in aging neutrophils. Murine bone marrow derived neutrophils were isolated, transfected with an PH$_{Akt}$–GFP construct, and then cultured in the absence or presence of CSE (2%) or nicotine (1 μM). The confocal fluorescent images of PH$_{Akt}$–GFP were captured at each of the indicated time points. Each experiment was repeated at least three times and virtually the same results were obtained each time. Representative fluorescence images are shown. (*B*) Quantitative analysis of membrane localization of PH$_{Akt}$–GFP. The average membrane fluorescence intensities were measured with ImageJ software as described (15). Presented are mean membrane fluorescence intensities in arbitrary units (AU). *$P < 0.001$ (Student *t* test). (*C*) Treatment with CSE or nicotine did not alter the amount of PtdIns(3,4,5)P3 in aging neutrophils. Human neutrophils were isolated and then cultured in the absence or presence of CSE (2%) or nicotine (1 μM).

PtdIns(3,4,5)P3 levels were assessed at each of the indicated time points. Data are from three experiments (mean ± SD). N.S., not statistically significant. (*D* and *E*) Treatment with CSE or nicotine significantly reduced InsP7 levels in aging neutrophils. (*D*) H^3-Inositol labeled dHL60 cells were cultured in the absence or presence of CSE (2%) or nicotine (1 μM) for indicated time periods, and the levels of inositol phosphates were analyzed with HPLC. Inositol phosphates were identified by their coelution with the standards of H^3-inositol phosphates. The data were normalized to the total amount of protein extracted from the same samples. Shown are the peaks of InsP6 and InsP7. (*E*) Total amount of InsP7 produced by indicated cells. Data shown are mean (±SD) of three experiments. *$P < 0.001$ versus untreated cells by Student *t* test.
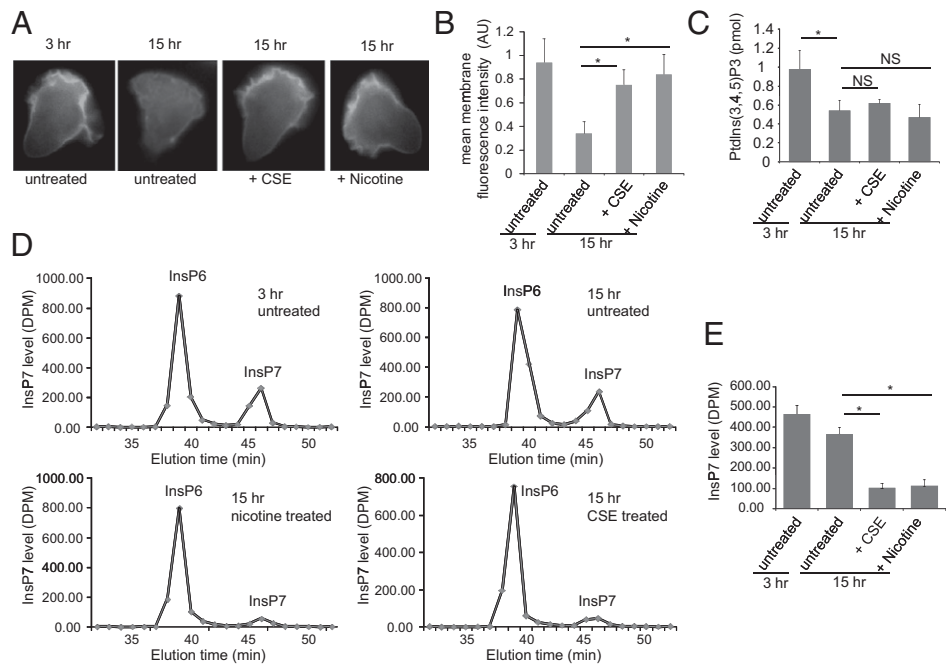
exclusively nuclear (20). In a recent study, we reported that PtdIns(3,4,5)P3/Akt signaling in neutrophils is mainly regulated by InsP6K1 (16). To further investigate the role of InsP7 in regulating PtdIns(3,4,5)P3 signaling in neutrophil spontaneous death, we elevated the intracellular level of InsP7 by overexpressing InsP6K1 in HL60 cells, in which specific genes can be easily overexpressed (Fig. 4*A*). Consistent with our previous results (16), we detected significantly more InsP7 in HL60 cells overexpressing InsP6K1, whereas a control construct or a "kinase-dead" mutant of InsP6K1 had no effect. The augmented InsP7 production was also detected in InsP6K1-overexpressing cells treated with CSE or nicotine, and this up-regulation remained during spontaneous death (Fig. 4*B*). Consequently, Akt phosphorylation (Fig. 4*C*) and activation (Fig. 4*D*) was significantly suppressed in dHL60 cells overexpressing InsP6K1, but not in those overexpressing the kinase-dead InsP6K1 mutant, indicating that InsP6K1-mediated conversion of InsP6 to InsP7 is essential for the suppression of PtdIns(3,4,5)P3 signaling. We next examined the effect of InsP6K1 overexpression on the spontaneous death of CSE- or nicotine-treated neutrophils. Consistent with the reduction in Akt activation, the CSE- and nicotine-induced delay of neutrophil death was abolished in cells overexpressing InsP6K1. More than 65% of neutrophils overexpressing the control construct or the kinase-dead mutant of InsP6K1 survived after 36 h in cultures containing CSE or nicotine, whereas less than 35% neutrophils overexpressing the active InsP6K1 survived under the same conditions (Fig. 4*E*). Finally, the effect elicited by InsP6K1 overexpression could be completely reversed by treating cells with TNP [N(2)-(m-(trifluoromethy)lbenzyl) N(6)-(p-nitrobenzyl)purine], a selective inhibitor of InsP6K (Fig. 4 *F* and *G*). Taken together, these results further confirm that reduced InsP7 production is responsible for CSE- and nicotine-induced delay of neutrophil death.

Disruption of InsP6K1 Augments CS-Induced Neutrophil Accumulation and Lung Damage. Alterations in neutrophil death will lead to changes in neutrophil accumulation at sites of inflammation. It

has been well documented that CS-induced delay of neutrophil spontaneous death plays a critical role in neutrophil accumulation and lung damage in smoke-induced COPD patients (9–11). We explored the role of InsP6K1 in regulating neutrophil accumulation and lung damage using a CS-induced COPD animal model (Fig. 4 *H–J*). Neutrophil accumulation in inflamed lungs was assessed by measuring neutrophil numbers in BAL fluid (Fig. 4*H*). Very few neutrophils were detected in the lungs of unchallenged mice. The number of neutrophils in BALF increased dramatically in response to CS, reaching 2 × 10^5 after 6 wk of exposure. InsP6K1 KO mice showed a dramatic increase in CS-induced neutrophil recruitment, with nearly 3.8 × 10^5 neutrophils present in BALF after 6 wk (Fig. 4*H*). Lung inflammation is always associated with significant cytokine and chemokine release. Accordingly, we measured the level of several cytokines/chemokines, including TNF-α, IL-1β, IL-6, Macrophage inflammatory protein 2 (MIP-2), and Keratinocyte Chemoattractant (KC) in the inflamed lungs of both WT and InsP6K1 KO mice. In BALF collected at 6 wk, the concentrations of all five cytokines/chemokines were significantly increased in the InsP6K1 KO mice (Fig. 4*I*). Dramatic increases in lung neutrophils and hyperinflammation leads to aggravated lung damage, which is usually accompanied by vascular leakage. We consistently detected augmented pulmonary edema formation, measured as total BALF protein level, in the lungs of InsP6K1 KO mice (Fig. 4*J*). Taken together, these results confirmed that disruption of InsP6K1 can augment CS-induced neutrophil accumulation and lung damage.

Discussion

InsP7, a higher inositol phosphate containing energetic pyrophosphate bonds (18, 21), has recently been established as a key signaling molecule in eukaryotic cells (22–25). In mammalian cells, InsP7 has been implicated in several cellular functions, including vesicular trafficking and exocytosis (26, 27), apoptosis (28–31), insulin disposition (32), insulin sensitivity, and weight gain (33). We recently showed that InsP7 also represents a
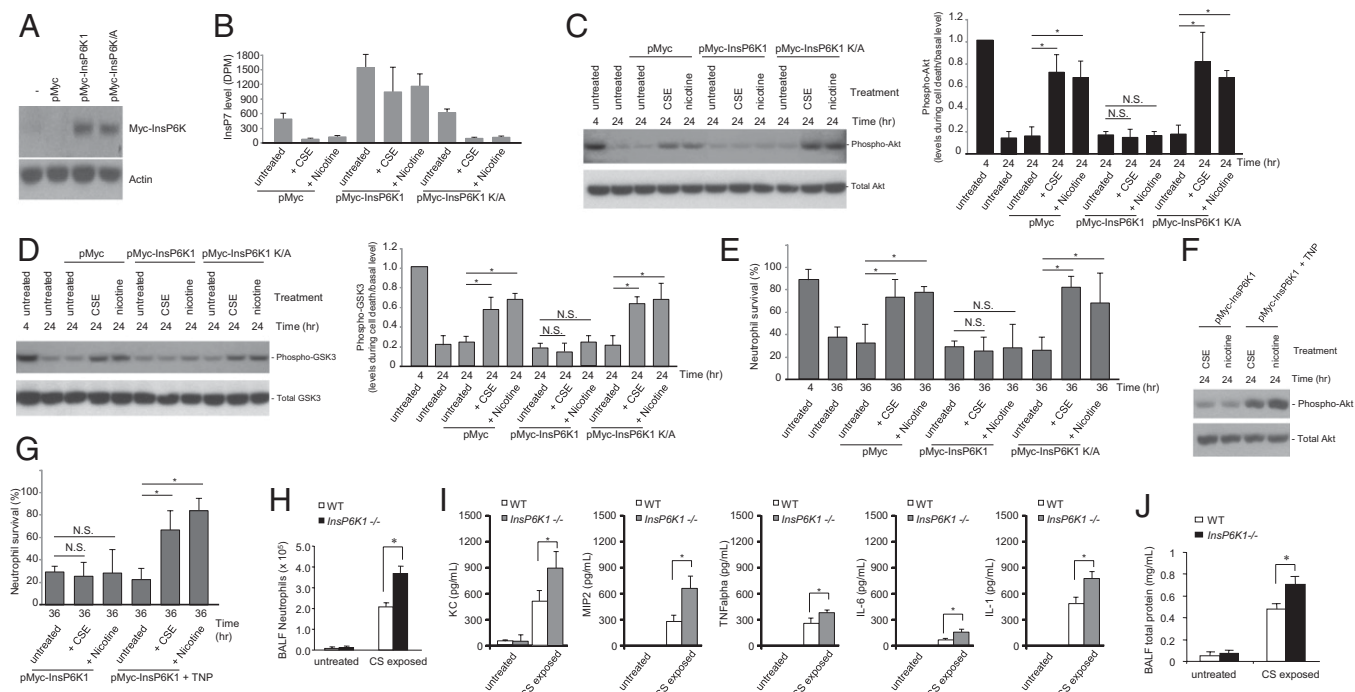
Fig. 4. Overexpression of InsP6K1 suppresses the delay of neutrophil spontaneous death induced by CSE or nicotine. (*A*) Expression of Myc-tagged InsP6K1 and the kinase-dead form InsP6K1 (InsP6K1-K/A mutant) in dHL60 cells transfected with indicated construct. (*B*) The levels of InsP7 in cells transfected with indicated constructs were analyzed by HLPC analysis. Data shown are mean ± SD of three experiments. (*C*) The level of phospho-Akt was reduced in cells over-expressing InsP6K1. Differentiated HL60 cells were cultured in the absence or presence of CSE (2%) or nicotine (1 μM) for the indicated amount of time. Akt phosphorylation was detected by Western blotting as described above. Relative amounts of phosphorylated Akt were quantified using NIH Image. Data shown are mean (±SD) of three experiments. *P < 0.001 versus untreated cells by Student t test. (*D*) The level of phospho-GSK-3β was reduced in cells overexpressing InsP6K1. GSK-3β phosphorylation was detected using phospho-GSK-3β (Ser9) antibody as described above. Data shown are mean (±SD) of three experiments. *P < 0.001 versus untreated cells by Student t test. (*E*) Overexpression of InsP6K1 suppressed the delay of neutrophil spontaneous death induced by CSE or nicotine. Neutrophil death was analyzed as described in Fig. 2. Data shown are mean (±SD) of three experiments. *P < 0.001 versus untreated cells by Student t test. N.S., not statistically significant. (*F* and *G*) Pharmacological inhibition of InsP6K activity reversed the effect elicited by InsP6K1 overexpression. (*F*) The level of phospho-Akt was elevated in TNP treated (10 μM, 2 h) dHL60 cells overexpressing InsP6K1. (*G*) Treatment with TNP reversed the effect elicited by InsP6K1 overexpression. Data are mean (±SD) of three independent experiments. N.S., not statistically significant. *P < 0.001 versus untreated cells by Student t test. (*H–J*) Disruption of InsP6K1 augments CS-induced neutrophil accumulation and lung damage in a murine CS-induced COPD model. (*H*) Disruption of InsP6K1 augments CS-induced neutrophil accumulation. WT or InsP6K1 KO mice were exposed to five cigarettes (about 2 h) per day and 5 d per week for a total of 6 wk. Shown are neutrophil numbers in BALF. The total number of cells in the lungs were counted using a hemocytometer. Differential cell counts were conducted on cytospin preparations stained with a modified Wright–Giemsa stain. Neutrophils were recognized by their lobular or segmented nuclei. The percentage of pulmonary neutrophils in the whole population (%PMN) was determined accordingly. Total number of pulmonary neutrophils (#PMN) recruited was calculated as follows: [#PMN] = [cell density] × volume × [%PMN]. All data are presented as mean (±SD), n ≥ 4 mice in each group. *P < 0.05, **P < 0.01 versus WT. (*I*) Disruption of InsP6K1 elevates CS-induced proinflammatory cytokine/chemokine production in the inflamed lungs. BALF chemokine and cytokine levels were determined using ELISA. (*J*) BALF total protein level. Protein accumulated in the inflamed lung was measured using a protein assay kit. The standard curve was constructed using BSA. Data are mean (±SD) of three independent experiments. *P < 0.001 versus WT mice by Student t test.

mechanism for controlling optimal PtdIns(3,4,5)P3/Akt pathway activation in neutrophils. As a key signaling molecule, PtdIns(3,4,5)P3 exerts its function by mediating protein translocation via binding to their PH domains. Akt contains a PH domain that specifically binds PtdIns(3,4,5)P3. The PtdIns(3,4,5)P3-mediated membrane translocation of Akt is essential for its phosphorylation and activation, and was previously thought to be dependent solely upon the concentration of PtdIns(3,4,5)P3 in the membrane. 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 (15). The amount of InsP7 in neutrophils is tightly regulated. There is substantial InsP7 in unstimulated cells, which prevents neutrophil hyperactivation and ensures an optimal cellular inflammatory response. The cellular concentration of InsP7 rapidly decreases after stimulation with chemoattractants, which allows the induction of sustained PtdIns(3,4,5)P3 signal in responding neutrophils (16). Reducing InsP7 production via InsP6K1 disruption augments chemoattractant-elicited PtdIns(3,4,5)P3 signaling in neutrophils.

Consequently, these neutrophils have greater phagocytic and bactericidal ability, and amplified NADPH oxidase-mediated production of ROSs. In the current study, we show that CSE and nicotine can also reduce InsP7 production and augment Akt signaling in aging neutrophils (Fig. S3). The mechanism by which InsP7 level is down-regulated in nicotine-treated neutrophils is largely unknown. It likely involves activation of inositol pyrophosphate phosphatase and/or deactivation of InsP6 kinase. Our results indicate that the nicotine-elicited effect is mediated by α7-containing nAChRs. Interestingly, it has been reported that nicotine-induced activation of nAChR can elicit Akt hyperactivation in many other cell types, including airway epithelial cells, adrenal chromaffin cells, bone-marrow-derived dendritic cells, neurons, and certain cancer cells (34, 35). It is possible that some of these effects may also be due to nicotine-induced reduction of InsP7 production.

CSE- and nicotine-induced reduction of InsP7 production leads to augmented Akt signaling and delayed neutrophil spontaneous death. Intriguingly, our previous study revealed that disruption of InsP6K1 abolishes chemoattractant-elicited InsP7 production, but does not alter the rate of neutrophil spontaneous

death (16). It appears that disruption of InsP6K1 does not affect Akt activation in aging neutrophils (Fig. S4). One explanation could be elevated ROS production in InsP6K1-deficient neutrophils. We recently identified ROS as negative regulators of PtdIns(3,4,5)P3/Akt signaling (36). Thus, disruption of InsP6K1 not only reduces InsP7 level, but also elevates ROS levels, resulting in unaltered Akt activation. Nicotine stimulation reduces InsP7 levels in aging neutrophils, with no effect on ROS production; in fact, a previous study has suggested that nicotine may even inhibit ROS production in neutrophils (37). As a result, treatment with nicotine significantly enhances Akt activity during neutrophil spontaneous death.

Materials and Methods

Animal Care. Mice aged 8–14 wk were used in this study. All procedures involving mice were approved and monitored by the Children's Hospital Boston Institutional Animal Care and Use Committee. *InsP6k1⁻/⁻* mice were generated as described (16). The corresponding WT littermates were used as paired controls for *InsP6k1⁻/⁻* mice.

Neutrophil Spontaneous Death. Neutrophils were cultured for indicated time and stained using an Annexin V Detection kit (Caltag Laboratories) (12). FACS was performed using a FACSCanto II flow cytometer (Becton Dickinson). The annexin V and propidium iodide (PI) double negative cells were defined as

live cells. The related methods, such as isolation of neutrophils, FACS analysis, Western blotting, Akt kinase assay, preparation of CSE, measurement of PtdIns(3,4,5)P3 levels, nucleofection, PH domain membrane translocation, and measurement of inositol phosphates, are described in detail in *SI Materials and Methods*.

CS-Induced COPD. WT or InsP6K1 KO mice were placed in an 18-L chamber and exposed to CS generated from 3R4F research cigarette (University of Kentucky, Lexington). They were exposed to five cigarettes (about 2 h) per day, 5 d per week for a total of 6 wk. Mice were euthanized 1 h after the last exposure to CS. Preparation of BALF, differential cell counting, and measurement of cytokine and total protein levels in BALF are described in detail in *SI Materials and Methods*.

Statistical Analysis. Values shown in each figure represent mean ± SD. Statistical significances were calculated with the Student t test. Differences were considered significant for P values less than 0.005.

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