Innate immune responses are essential to host defense, yet if unchecked can lead to tissue injury and illness (1). PMN are the primary initial immune effectors of acute inflammation, and these cells use as many as fifty toxins for microbial killing in phagocytic vacuoles (2). Incomplete closure of phagolysosomes or aberrant extracellular release of reactive oxygen species (ROS), granule components, lipid mediators, hypochlorous acid, and other potentially toxic PMN products to surrounding tissues contribute to injury in several human diseases, including the devastating clinical entities of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) for which no disease-remitting therapy is currently available (3).

To prevent an overexuberant inflammatory response and limit damage to the host, PMN activation programs need to be tightly controlled (1). Select membrane-derived lipid mediators have recently been described as autacoid regulators of PMN functional responses in acute inflammation (4). One of these classes of antiinflammatory membrane lipids involves isoprenoid metabolism. Immune regulatory roles for isoprenoids are emphasized by the hyper-IgD and periodic fever syndrome that results from low polyisoprenyl phosphate (PIPP) levels secondary to defective mevalonate kinase activity (5). Recently, we identified a novel PIPP signaling pathway in PMN (6). One of its components is presqualene diphosphate (PSDP), present in freshly isolated human PMN in nanomolar quantities at baseline (i.e., 1.7 nmol/10^7 cells) (6). PMN exposure to the chemoattractant and secretagogue leukotriene B_4 (LTB_4) initiates transient activation of this PIPP signaling pathway with conversion of PSDP to its monophosphate form, presqualene monophosphate (PSMP). PSDP carries biological...
activity as a potent counterregulatory mediator that prevents ROS generation (6, 7). In sharp contrast, PSMP is >100-fold less active than PSDP for inhibition (6, 7). Thus, incoming positive signals for PMN (e.g., LTB4) initiate rapid degradation and inactivation of an inhibitory lipid signal (i.e., PSDP) coincident with cell responses (e.g., ROS generation). PSDP levels quickly return to baseline in a time frame that parallels cellular deactivation. Intracellular targets for PSDP to control PMN activity remain to be elucidated. Select PSDP structural mimetics are also active in vivo, dampening mouse responses to zymosan A–induced peritonitis (8).

In addition to PSDP remodeling, LTB4 also initiates phosphatidylinositol 3–kinase (PI3K) activation in PMN to promote NADPH oxidase assembly and ROS production (9, 10). Phosphoinositide signaling initiated by PI3Ks is a critical early event in PMN responses, such as phagocytosis (11) and chemotaxis (12), and contributes to ALI pathogenesis (13). Because LTB4 initiates PMN PI3K activation and PIPP remodeling, we hypothesized that these signaling events were related in the regulation of PMN responses. Here, we report that PI3K activity and PSDP remodeling are linked during PMN activation and deactivation with direct inhibition of PI3K by PSDP to limit PMN responses and lessen the severity of experimental lung inflammation.

RESULTS AND DISCUSSION
PSDP and PI3K regulate LTB4–triggered O2− release by human PMN
To determine if PSDP and PI3K regulate LTB4-stimulated responses, we exposed freshly isolated human PMN to a new structural PSDP mimic (Fig. 1 A) or a potent and specific inhibitor of PI3K activity (LY294002) before LTB4. A marked increase in the rate of O2− generation was observed within seconds after LTB4 addition that was transient, slowing considerably by 60 s (Fig. 1 B). The presence of either PSDP mimetic (100 nM) or PI3K inhibitor (3 μM) markedly blocked O2− generation. Exposure to LTB4 (10 min) induced 1.6 ± 0.3 nmol O2−/106 PMN (as compared with vehicle 0.5 ± 0.1 nmol O2−/106 PMN; P < 0.01). Both the PSDP mimetic and PI3K inhibitor led to >50% inhibition of LTB4–triggered O2− generation (0.8 ± 0.3 nmol/106 PMN and 0.7 ± 0.2 nmol/106 PMN, respectively; P < 0.05) (Fig. 1 C). The PSDP mimetic concentration in these experiments (i.e., 100 nM) was 1,000-fold lower than its critical micellar concentration (CMC) (<100 μM; Fig. 1, A and B). To verify that the observed inhibition was not secondary to micelle formation and sequestration of the lipid agonist LTB4, in the next experiments, there was pretreatment with the mimetic, a wash, and stimulation with LTB4. Although this extra step reduced total ROS generation in response to LTB4, the PSDP mimetic still led to a >50% decrease (71.2 ± 27.7% inhibition, n = 4). Together, these results indicate that human PMN activation by LTB4 is highly dependent on PI3K activity and can be inhibited by a new PSDP structural mimic.

Figure 1. Superoxide anion generation by human PMN is regulated by PI3K and PSDP. (A) Structure of PSDP and a new amido PSDP structural mimic (CS ChemDraw software). (B) Freshly isolated human PMN were exposed to the PSDP mimetic (100 nM) (●), a PI3K inhibitor (3 μM) (○), or vehicle (□) before LTB4 (100 nM) and O2− generation was determined. Results are representative for n = 3. (C) Total O2− generation was also determined for LTB4–activated PMN (10 min) in the presence or absence of the test compounds (mean ± SEM; n = 4 from separate PMN donors; *, P < 0.01 as compared with vehicle; **, P < 0.05 as compared with LTB4).

Relationship between PI3K activity and PSDP during PMN activation
To determine if PSDP remodeling and PI3K activation were related, we first examined their kinetics in human PMN after exposure to LTB4. Because G protein–coupled receptors can activate class IA and IB PI3Ks (10), we measured PI3K formation in vitro by members of these PI3K classes. After LTB4, p110γ–PI3K activity in PMN rapidly increased (within 5 s), reached a maximum rate of activity by 20 s, and then declined to basal levels within 30 s (Fig. 2 A, representative for n = 3). LTB4 also rapidly stimulated p85–PI3K activity in PMN, but at lower levels than p110γ–PI3K. By 5 s, significant decrements were also evident in total PSDP levels (7.9 ± 1.2%; P < 0.01) (see supplemental.
These results indicate that LTB₄-triggered activation of PMN PI3K and PSDP remodeling was concurrent and temporally overlapped with initiation of ROS generation. Although both of these signaling events were rapid in onset, the kinetics for PI3K activation and deactivation differed from the time course for total PSDP remodeling during the initial 30-s interval. To determine if PSDP directly interacted with PI3K as its activity decreased, PMN were exposed to LTB₄ for 30 s and p110γ–PI3K was immunoprecipitated from cellular materials. Lipid extracts were performed and analyzed by gas chromatography/mass spectrometry (GC/MS) (6). Selective ion monitoring at m/z 137 (2 isoprenoid units) revealed a unique peak at 18.1 min in LTB₄-exposed PMN extracts. MS spectral diagnostic ions (Fig. 2 C), namely m/z 567 [M⁺ − (H₂O)], 488 [M⁺ − (H₃PO₄)], 410 [M⁺ − (H₅P₂O₇)], 341 [M⁺ − (H₅P₂O₇) − 69], 205 [M⁺ − (H₃PO₄) − 69 − CH₃(C₂H₅)CHCH₂ × 2], 137 [C₇H₄], 97 [H₃P₂O₇], 81 [CH₃(C₂H₅)CH(CH₂)₂ − H⁺] and 69 [base peak; (CH₃)₂CCHCH₂], were consistent with authentic PSDP in the PI3K immunoprecipitated material.

Direct inhibition of p110γ–PI3K by PSDP
Because PI3K activity and PSDP remodeling were both early signaling events in PMN with interaction between PSDP and p110γ–PI3K, next we questioned if PSDP could directly regulate PI3K activity. Recombinant human (rh) p110γ–PI3K activity was determined by PIP₃ formation in vitro in the presence of PSDP, PSMP, or the PI3K inhibitor LY294002. PIP₃ formation was significantly decreased by PSDP (800 pmol) with 94.7 ± 5.3% inhibition (P < 0.001) and a PI3K inhibitor (500 pmol) with 46.7 ± 6.7% inhibition (P = 0.01) (Fig. 3 A). PSDP inhibited p110γ–PI3K in a concentration-dependent fashion (Fig. 3 B). In sharp contrast, PSMP (8–800 pmol) did not significantly impact p110γ–PI3K activity. The IC₅₀ for PSDP (38 pmol) had a stoichiometry with PI3K of 1:11601. These results indicate that PSDP is a potent direct inhibitor of p110γ–PI3K with a structure–activity relationship that suggests an important role for the diphosphate structure in PSDP’s action on p110γ–PI3K activity. The LTB₄-mediated PMN remodeling of PSDP corresponds to an ∼50 pmol change in PSDP/10⁶ PMN, a decrease that is within the concentration range for regulation of p110γ–PI3K activity (Fig. 3 B). After cell activation, the percent change in total PSDP (i.e., 28%) is similar to the change in phosphatidylinositol (17%) that occurs in activated PMN membranes (14). Collectively, our new findings indicate that receptor-mediated agonists for PMN remodel PSDP in time-frames and amounts consistent with functional impact on PI3K activity and cellular responses.

PSDP remodeling in vivo during tissue injury and inflammation
Because PI3K activity occupies a central role in regulating PMN activation during lung injury and inflammation (13), next we determined PSDP remodeling in vivo in mouse lungs during an experimental model of mild ALI secondary...
to aspiration of gastric acid (15), which is a common clinical event (3). To simulate acid aspiration, hydrochloric acid (HCl) (0.1 N, pH = 1.5) was selectively instilled into the animals’ left lungs (15). Lung PMN infiltration was maximal 12 h after HCl injury (14.2 ± 1.8 vs. 5.5 ± 0.8 × 10⁴ PMN/mg lung; P < 0.01) (Fig. 4 A). Expression of class IA and IB PI3Ks in mouse lungs were both increased at 2 and 12 h after HCl (Fig. 4 B). Lungs were removed and lipid extracts were prepared for PSDP determination. Of interest, despite increased PMN numbers, PSDP levels were significantly lower in the left lungs of HCl-injured mice (4.6 ± 0.3 μg PSDP vs. 9.0 ± 1.6 μg PSDP in control lungs; P < 0.02). These results indicate that experimental lung injury led to decrements in PSDP concomitant with increased PMN, suggesting an inverse relationship in vivo between lung PSDP and inflammation.

PSDP mimetic blocks PMN infiltration and PI3K activity
To determine if PSDP can block pulmonary inflammation and PI3K in vivo, we administered a PSDP structural mimetic (0.8 μg/mouse, i.v.) or vehicle 15 min before HCl instillation into the left main-stem bronchus. PSDP markedly reduced lung PMN 12 h after injury (Fig. 5 A). Tissue morphometry on LY-6G–stained histological sections (for identification of mouse PMN) revealed significant inhibition with the PSDP mimetic (46.8 ± 7.1% LY-6G staining [HCl] vs. 18.8 ± 5.8% LY-6G staining [HCl plus PSDP mimetic]; P < 0.05) (Fig. 5 B). In view of the prominent class IA PI3K lung expression that increased markedly after ALI (Fig. 4 B), we next determined class IA PI3K activity after acid injury in the presence or absence of the PSDP mimetic. HCl injury induced significant increases in lung PI3K activity in p85 immunoprecipitates (0.59 ± 0.17 PIP₃/mg lung with HCl vs. 0.05 ± 0.02 pmol PIP₃/mg lung with PBS; P < 0.05). Administration of the PSDP mimetic blocked the HCl–induced increase in class IA PI3K activity to near basal levels (0.05 ± 0.01 pmol PIP₃/mg lung; P < 0.05) (Fig. 5 C). Thus, PSDP can regulate PMN activation, tissue accumulation, and total PI3K activity in vivo during experimental acid-initiated ALI.

During acute inflammation, PI3Ks orchestrate several cellular responses for host defense, including PMN ROS generation (12). Befitting its central role in cell activation, several mechanisms are in place to restrain PI3K activity (16–18). Previous reports have suggested a link between decreased PIPP formation and increased PI3K (19, 20). Results presented here are the first to demonstrate direct inhibition of PI3K by a PIPP and inverse relationships between PI3K...
activity and PSDP levels both in vitro and in vivo. In addition, PSDP bound to p110γ–PI3K in activated PMN and potently inhibited rhp110γ-PI3K in vitro and a PSDP structural mimetic blocked PI3K activity in vivo. Together, these new findings support a signaling relationship between PI3K and PIPPs in the regulation of leukocyte functions during inflammation.

Pivotal regulatory properties have been ascribed to isoprenoids. For example, polyisoprenyl glycolipids form antigen complexes with CD1 to activate T cells (21), and cholesterol is critical to PMN cell membrane organization and polarization in response to chemotactic stimuli (22). Although PIPPs are appreciated as cholesterol biosynthetic intermediates, PSDP is also present in cells, such as human PMN, that cannot use it for cholesterol biosynthesis because they lack squalene cyclase and other mixed function oxidase activities (23). There are now several lines of evidence to support a role for PSDP as a counterregulatory signal in PMN functional responses. PSDP is also present in cells, such as human PMN, that cannot use it for cholesterol biosynthesis because they lack squalene cyclase and other mixed function oxidase activities (23). There are now several lines of evidence to support a role for PSDP as a counterregulatory signal in PMN functional responses. PSDP is also present in cells, such as human PMN, that cannot use it for cholesterol biosynthesis because they lack squalene cyclase and other mixed function oxidase activities (23).

Activated PMN contribute to the development and severity of tissue injury during inflammatory illnesses such as ALI. No specific therapy is currently available to modulate the inflammatory response to airway injury from acid and protect the lung in ALI. PI3K is an important enzyme in the proinflammatory PMN signaling program of ALI, as p110γ–PI3K deficiency dampens the severity of endotoxin-induced ALI (13). In addition to PMN, PI3Ks are also activated during ALI in lung-resident cells (27) and both endothelial cell and PMN PI3Ks contribute to PMN accumulation in the lung (28). Select lipid mediators, namely lipoxin A4 (LXA4), inhibit PI3K in structural cells (29). LXA4 signaling also promotes resolution of experimental ALI (15) and inhibits PMN activation by LTB4 in a postreceptor manner, in part by blocking PSDP remodeling (7). Here, PSDP levels were...
decreased in acid-injured lungs and a novel PSDP mimetic blocked PI3K, PMN ROS generation, and PMN accumulation in the lung. Because the PSDP mimetic was administered intravenously, regulation of cells other than PMN may have also contributed to the marked inhibition of leucocyte trafficking after acid injury.

In conclusion, the ability of PSDP and a new PSDP mimetic to directly inhibit PMN early intracellular activating signals, such as PI3K, and to lessen the inflammation associated with experimental ALI provides insight into new mechanisms for in vivo protection from excess PMN-driven inflammation and tissue injury. Together, our findings suggest that PIPP signaling pathways, and specifically PSDP, can serve as natural templates for the design of new therapeutic strategies in inflammatory diseases.

MATERIALS AND METHODS

Materials. PSDP and PSMP were isolated from human PMN or prepared by total organic synthesis (8). The bisphosphonate PSDP structural mimetic, tetraethyl presqualene carboxamido-methylene-diphosphonate was prepared from presqualene carboxylic acid. All synthetic compounds were characterized by NMR spectroscopy.

Human PMN incubations. Peripheral blood was obtained by venipuncture from healthy volunteers who denied taking any medications for at least 2 wk and had given written informed consent to a protocol approved by Brigham and Women’s Hospital’s Human Research Committee. PMN were isolated from whole blood as described previously (6). Freshly isolated PMN (1–5 × 10^6 PMN/ml HBSS plus 1.6 mM CaCl_2) were incubated (5 min, 37°C) in the presence of 3 μM LY294002, 100 nM PSDP mimetic, or vehicle (0.1% ethanol), then exposed to LT_B4 (100 nM) in the presence of 7 mg/ml cytochrome c. This concentration of cytochrome c. This concentration of cytochrome c was chosen because it initiates PMN NADPH oxidase assembly (7) and is similar to amounts measured in vivo at sites of acute inflammation (30). In some incubations, PMN in HBSS without calcium were exposed to PSDP mimetic or vehicle, pelleted by centrifugation (700 g, 3 min), and resuspended in HBSS plus 1.6 mM CaCl_2, without PSDP before the addition of agonist. Superoxide anion generation was determined (37°C) as superoxide dismutase-inhibitable cytochrome c reduction by monitoring (550 nm) at 5-s intervals in a continuously flowing water-jacketed cassette or after timed incubations. For PSDP identification, PMN (50–100 × 10^6 cells/ml) were activated (LT_B4, 100 nM, 30 s) before disruption by N_2 cavitation (350 psi, 20 min, 4°C). Remaining intact cells and nuclei were pelleted (500 g, 10 min, 4°C) and supernatants were used for immunoprecipitation with anti-p110 γ− selective antibody. After immunoprecipitation, PSDP and several related compounds were isolated from human PMN or prepared (60 μg in 100 μl of Kinase-Glo reagent and incubated an additional 10 min at room temperature. Incubations were stopped with 50 μl of Kinase-Glo reagent and incubated an additional 10 min at room temperature.

Luminescence was measured with a FLx800 microplate luminometer (BioTek Instruments, Inc.).

Experimental model of ALL. All animal protocols were approved by the Harvard Medical Area Animal Institutional Review Board. Acid (0.1 N HCl, pH = 1.5, 50 μl) was instilled intratracheally into the left lung of anesthetized mice (FVB, male, 10–12 wk; Charles River Laboratories) (15). A PSDP mimetic (0.8 μg in 100 μl 0.9% saline) or vehicle (1% ethanol) was administered by tail vein 15 min before HCl instillation. After 12 h, lungs were removed, prepared for MPO (15) or PI3K assay, or were fixed in IHC zinc buffer and paraffin embedded for immunostaining with LY-6G (1:50 dilution). Area and number of positively staining cells was measured with National Institutes of Health Image software and percentage of positive cells/area calculated.

Statistical analysis. Results are expressed as the mean ± SEM. Statistical significance of differences was assessed by Student’s t test and one-way analysis of variance. P < 0.05 was set as the level of significance.

Online supplemental material. Fig. S1 shows the CMC determination for the PSDP mimetic and related compounds. Further information on materials and experimental protocols are supplied as the supplemental Materials and methods. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052143/DC1.

The authors would like to acknowledge the contributions of Dr. C.N. Serhan to the development of the PSDP mimetic and for his critical review of the manuscript. We also thank Dr. M.A. Perrella and members of the Brigham and Women’s Hospital Lung Biology Center and Histopathology Core Laboratory for their assistance with the experimental models of ALL.

This work was supported in part by the NIH (HL68669 and NIDCR Specialized Research Center grant no. DE016191), and fellowships from La Fondation de la Recherche Médicale, Pfizer, and Uehara Memorial Research Foundation.

The authors have no conflicting financial interests.

Submitted: 24 October 2005
Accepted: 27 February 2006

REFERENCES


Submitted on March 31, 2017.


