Toll-like receptors (TLRs) coupled to intracellular signaling cascades function as central elements of innate immunity that control transcription of numerous pro-inflammatory genes. Two minor anionic phospholipids present in the pulmonary surfactant complex, palmitoyl-oleoyl-phosphatidyglycerol (POPG) and phosphatidylinositol (PI), antagonize the cognate ligand activation of TLRs 2 and 4. The lipids block recognition of activating ligands by the TLRs, either directly or via the TLR4 coreceptors CD14 and MD2. Antagonism of TLR activation results in inhibition of the initiating step of the pro-inflammatory signaling pathways. Evidence for this mechanism of action comes from direct binding studies between CD14 and MD2 with POPG and PI. Additional evidence for this mechanism of antagonism also comes from monitoring the reduction of protein phosphorylation events that characterize the intracellular signaling by activated TLRs. The pathogenesis of respiratory syncytial virus (RSV) and influenza A virus (IAV) have been linked to TLR4 activation, and we examined the action of POPG and PI as potential antagonists of the pathology of these viruses. Surprisingly, POPG and PI dramatically curtail infection, in addition to inhibiting inflammatory sequelae associated with RSV and IAV infections. The mechanism of action by the lipids is disruption of virus particle binding to host cell plasma membrane receptors, required for viral uptake. The antagonism of activation of TLRs and virus binding to the alveolar epithelium by resident constituents of the pulmonary surfactant system suggests that POPG and PI function in homeostasis, to prevent inflammatory processes that result in reductions in gas exchange within the alveolar compartment.

### Biological niche of the respiratory system

The lungs are uniquely situated at the interface between the external and internal environment, and in the alveolar compartment, where gas exchange occurs, a tissue layer of only 0.6 μm separates ambient air from the vascular compartment (1). Lung epithelia are continually exposed to pro-inflammatory airborne irritants, microbes, and microparticulates in the ∼10^4 liters of air inspired each day. Despite this daily onslaught of pro-inflammatory stimuli, the lungs remain relatively non-responsive to such insults, in healthy individuals. This hypo-responsive state is conferred by a variety of molecular systems that suppress inflammatory signaling cascades until their engagement is essential. Upon activation of inflammatory processes, as occurs with active bacterial or viral infections, there is a rapid infiltration of neutrophils and monocytes into the tissue, release of numerous inflammatory mediators, increased vascular permeability, and reduced efficiency of O_2/CO_2 exchange within the alveolar compartment. Fundamentally, the lungs exhibit a high threshold for engaging inflammatory processes, until their activation is essential, and this homeostatic balance prevents unwanted inflammation and reduced gas exchange in response to routine exposure to environmental stimuli, such as ambient levels of airborne LPS, and other microbial products. Elucidation of the molecular mechanisms by which the lungs suppress inflammation is critical not only for understanding the function and homeostasis of the organ, but also because it presents opportunities to amplify, or complement, such intrinsic processes for therapeutic benefit in inflammatory disease states.

### Pulmonary surfactant constituents

Pulmonary surfactant is a secreted, extracellular complex of lipids and proteins, which lines the alveolar compartment at the external air/tissue interface, and also plays an important role in regulating inflammatory processes within the lung (2–4). The surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) (5) constitute ∼10% of the complex. SP-A and SP-D are collectins (collagen domain containing lectins) located primarily within the alveolar hypophase, the thin layer of fluid that resides above the apical surface of the alveolar epithelium. SP-A and SP-D can regulate inflammatory processes by acting as opsonins, and by direct interaction with pathogens and Toll-like receptors (TLRs) (3, 6–9).

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1. The abbreviations used are: LPS, lipopolysaccharide; TLR, Toll-like receptor; DPPC, dipalmitoylphosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; POPG, palmitoyl-oleoyl-phosphatidyglycerol; POPC, palmitoyl-oleoyl-phosphatidylcholine; TNF, tumor necrosis factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; RSV, respiratory syncytial virus; IAV, influenza A virus; pfu, plaque-forming unit.
SP-B and SP-C are extraordinarily hydrophobic proteins that play important roles in regulating the transformation of lipids from their intracellular storage form within lipid-rich lamellar bodies in alveolar type 2 cells to their extracellular interfacial form at the external air/tissue interface (10). Phospholipids are the major constituents of pulmonary surfactant, with phosphatidylcholine being the most abundant lipid class and dipalmitoylphosphatidylcholine (DPPC) being the most abundant molecular species (11). DPPC plays a major role in respiratory mechanics by reducing surface tension at the air/tissue interface of the alveolar compartment (12). Absence of DPPC leads to alveolar collapse and greatly increases the effort required for breathing and oxygen exchange. Indeed, prior to the development of artificial pulmonary surfactants, newborn respiratory distress, which results when parturition precedes full lung maturity, was the leading cause of mortality in neonates in developed countries (13).

Immunoregulatory actions of minor pulmonary surfactant phospholipids

The anionic phospholipids phosphatidylglycerol (PG) and phosphatidylinositol (13) are minor constituents (~2–10% of total phospholipid) of the pulmonary surfactant complex, but their concentration within the alveolar hypophase is extraordinarily high (~5–10 mg/ml for PG and ~2 mg/ml for PI) and greatly exceeds that for any other tissue or mucosal surface (11). For decades the functions of PG and PI within the alveolar compartment remained enigmatic, but over the last several years elucidation of the actions of these lipids as regulators of innate immune processes has emerged (2).

In our earliest studies on this topic, we serendipitously discovered that liposomes composed of POPG were potent suppressors of LPS-induced activation of the innate immune receptor TLR4 (5). In contrast to the activity of POPG, a control phospholipid harboring the same fatty acids but a different polar head group, palmitoyl-oleoyl-phosphatidylcholine (POPC), was ineffective as a regulator of innate immunity (5). In a typical experiment either primary human alveolar macrophages, or human macrophage cell lines, are treated with LPS, and this elicits the production and secretion of pro-inflammatory mediators, such as TNFα, which are detected by ELISA (Fig. 1). TNFα is one of a large spectrum of pro-inflammatory molecules produced and released by cells in response to TLR activation (14). The intracellular signaling events for TLR4 are outlined in Fig. 1; they are well-known; and involve (in approximately sequential order) a pathogen-derived molecule binding to receptor, dimerization and a conformational change in the intracellular domain of the receptor, receptor interaction with MyD88, activation of IRAKs 1, 2, and 4, activation of TRAF6, phosphorylation, (inactivation) of IκBα, release of NF-κB from the complex with IκBα, nuclear import of NF-κB, activation of transcription of pro-inflammatory genes (e.g. TNFα, IL-6, and IL-8, among others), and new transcription of the gene for MKP1. A parallel arm of signaling cascades uses phosphorylation of MAP kinases (ERKs, JNK, and p38) and subsequent activation of transcription factor AP-1, which results in new transcription of cyclooxygenase 2 (COX2). TLR2 activation follows the same general scheme with Pam3Cys activating TLR2/1 heterodimers and MALP2 activating TLR2/6 heterodimers. For the TLR2 pathways, POPG and PI appear to block ligand recognition by the TLR heterodimers.
The activation of TLR4 by LPS depends upon three LPS interacting proteins: LPS-binding protein (LBP), cluster of differentiation 14 (CD14) (15), and lymphocyte antigen 96 (MD2) (16). LBP is a plasma protein that facilitates transport of LPS through aqueous compartments. CD14 is a PI-glycan–anchored peripheral plasma membrane protein that binds LPS. MD2, which also binds LPS, is a peripheral plasma membrane protein tethered to TLR4. A generally accepted model is that LBP transfers LPS to CD14, which subsequently transfers LPS to MD2. A multimolecular complex of CD14-LPS-MD2-TLR4 is required to induce the dimerization and conformational change to TLR4 that initiates the intracellular signaling cascade (14).

Mechanisms of action of surfactant phospholipids as antagonists of TLR4 and TLR2

When we probed the plasma membrane signaling pathway from LPS-activated TLR4, we discovered that all the downstream signaling events we interrogated (phosphorylation of p38, ERK, and JNK by phosphorylation and inactivation by phosphorylation of IκBα, and new synthesis of MKP-1. LPS (10 ng/ml) and POPG (200 μg/ml) were added to cultured U937 macrophages for 15–60 min, as indicated. Cell extracts were prepared at the indicated time points and subjected to solubilization with SDS-PAGE buffer and subsequently electrophoresed, transferred to nitrocellulose, and immunoblotted with antibodies recognizing the proteins, p38, ERK, and IκBα, and their phosphorylated variants, or MKP-1. A, B, the effects of LPS (10 ng/ml) and 200 μg/ml lipids (DPPC, dipalmitylophosphatidylglycerol (DPPG), dimyristoyl phosphatidylglycerol (DMPG), POPG, and PI) upon expression and secretion of TNFα in response to LPS challenge were measured by ELISA. A, Data are from Ref. 5 and are from 1 of 3 experiments. B, data are from three independent experiments, with duplicate samples in each experiment. Values shown are mean ± S.D. Significance: * corresponds to p < 0.05. C, D, direct concentration-dependent binding reactions between soluble human CD14 and solid phase POPG and POPC. Error bars in panels C and D correspond to mean ± S.E. for 3 experiments.

We also investigated the actions of POPG upon TLR2-dependent inflammatory pathways. TLR2 forms heterodimers with either TLR1 or TLR6 to recognize a variety of bacterial lipopeptides (14). The synthetic ligand Pam3Cys activates TLR2/1 complexes, and the synthetic ligand MALP-2 activates TLR2/6 complexes; both heterodimeric forms use essentially the same intracellular signaling cascades described above in Fig. 1 for TLR4 to induce inflammatory mediator production (17, 18). In our studies of TLR2 activation and its antagonism, we utilized mouse and human macrophages and monitored the expression and secretion of TNFα, the expression of cyclooxygenase 2, and the release of arachidonic acid, which serves as a marker for downstream eicosanoid synthesis (e.g. prostaglandins D and E and thromboxanes) (16, 17). POPG potently inhibited TLR2 activation and the linked downstream phosphorylation of p38, ERKs, and IκBα, induced expression of COX2, and arachidonic acid release. POPG was significantly more effective than dipalmitoyl-PC as an antagonist of TLR2 activation, indicating that the fatty acid composition of the phospholipid is an important element of lipid structure related to antagonistic
activity (17). Additional data in Fig. 2B reveal that dimyrristoyl PG and PI are nearly as potent as POPG.

**Structural plasticity of antagonistic surfactant phospholipids**

In an additional line of investigation we examined the effects of manipulating the structure of the glycerol moiety of the POPG head group, upon its activity as an antagonist of TLR2 and TLR4 (17). All the analogs we synthesized harbored the same fatty acids (palmitate and olate) as found in POPG. We synthesized 12 compounds that altered the head group glycerol moiety, which consists of three carbons and three hydroxyl groups, by changing 1) the number of aliphatic carbons from 0 to 5, 2) the number of hydroxyl substituents from 1 to 3, 3) the position of the hydroxyl substitutions, and 4) the branching of the head group aliphatic chain. We also synthesized an analog in which the head group sn-2-hydroxyl moiety was substituted with an amino group (17). For lipid antagonism of TLR2 activated by a MALP-2 ligand, POPG consistently had the lowest IC₅₀ and elimination of hydroxyl groups from the head group aliphatic chain greatly reduced the efficacy of the lipids (17). For lipid antagonism of TLR4 activated by LPS, nearly all the analogs were as effective as POPG with the exception of those with 3- or 4-carbon aliphatic chains and lacking hydroxyl substituents. Two additional structural features of analogs, which ablated lipid antagonism of both TLR2 and TLR4 activation, were complete deletion of the phospholipid head group distal to the phosphodiester (which creates phosphatidate) and introduction of an amino group in lieu of a hydroxyl group in the head group aliphatic chain. Comparison of IC₅₀ data for POPG and analog inhibition of TLR2 and TLR4 also demonstrated that inhibition of TLR2 requires about 10 times the lipid concentration required for antagonism of TLR4 (17). Such discrepancies in dose-response curves make it plausible to selectively inhibit TLR4 and spare TLR2 in vivo, by simply altering antagonist concentration.

**Secondary activation of TLR4 by respiratory viral infection**

The inhibitory activity of POPG upon TLR4 activation and signaling prompted us to investigate whether the lipid could also disrupt unexpected inflammatory processes dependent on this receptor that were revealed by genetic studies in mice. Surprisingly, one of these processes was the in vivo inflammatory response to respiratory syncytial virus (RSV) (15, 19, 20). RSV-elicited lung inflammation was an attractive target to interrogate for lipid antagonism for several reasons, including 1) nearly all children are infected with the virus before age 2, and it is the primary cause of newborn hospitalizations in the United States; 2) worldwide, the early life mortality because of RSV is very high in underdeveloped countries, and rivals that attributable to malaria (21); 3) there is no vaccine for the virus, and durable immunity does not develop following childhood infection (22); 4) the virus has been implicated as a significant cause of exacerbations of chronic lung diseases in adults, especially those with asthma and chronic obstructive pulmonary disease (COPD) (22).

Our initial studies with in vitro RSV infection using primary human bronchial epithelial cells and bronchial cell lines demonstrated the virus elicited robust production of the inflammatory mediators IL-6 and IL-8, and this process was markedly inhibited by POPG and PI, but not by the control lipid POPC (23–25). Unexpectedly, we also observed remarkable protection of cell cultures from virus-mediated cytopathology and lysis (23–25). These findings suggested the possibility of direct interactions between RSV and specific phospholipids. To test for interactions between the virus and lipids, we prepared phospholipid solid phases composed of either POPG or PI or POPC in microtiter wells. The solid phases were incubated with varying concentrations of RSV and then washed extensively, and bound virus was detected using anti-RSV antibodies and ELISA. These experiments revealed that POPG and PI bound virions in a concentration-dependent, high-affinity, and saturable reaction (23–25). In further studies we examined whether the lipid-binding reaction could interfere with virus attachment to epithelial cell surfaces. These latter experiments demonstrated that POPG and PI, but not POPC prevented the attachment of viral particles to the plasma membrane of epithelial cells (23–25).

**Antagonism of respiratory virus infections in mice**

To test the relevance and potential utility of the aforementioned in vitro lipid–protein interactions, we performed infection studies in mice and examined the effects of adding POPG by intranasal inoculation at the time of infection (23). Mouse tissues were analyzed 5 days after initiating the viral infection, when viral titers are near maximum. Histopathology of the lungs revealed that RSV infection produced marked inflammation of the airways and alveoli, with significant inflammatory cell infiltrates. However, mice treated with POPG at the time of infection showed scant evidence of infection or inflammation. Consistent with the histopathology, the cell populations recovered in lung lavage from RSV-infected mice contained high percentages and elevated absolute numbers of lymphocytes and neutrophils. In contrast, mice that were given POPG at the time of infection had normal cell numbers in their lung lavage, and only slightly increased percentages of lymphocytes and neutrophils. Most significantly, the viral burden in lung tissue was ~2 × 10³-fold higher in RSV-infected animals, compared with animals receiving RSV and POPG. These data, summarized in Fig. 3, provide compelling evidence that POPG and PI can protect mice from RSV infection. The inhibition of viral infection with POPG treatment was more potent than that for PI, but the turnover of PI was significantly slower than POPG (23–25). Such data suggest that the potency of POPG may be greater than that for PI; but the latter may be more persistent in the bronchoalveolar compartment and confer longer-lasting protection against the virus.

Examination of the turnover of POPG following intranasal inoculation in mice demonstrated distribution of the lipid throughout the bronchoalveolar compartment of lung, but a short half-life of ~45 min (24). Turnover of extracellular lipids is proportional to the respiratory rate, and in mice this rate is as high as 300/min. However in newborn humans (the population at highest risk for RSV infection in the United States) with a
respiratory rate of 25/min, the half-life of POPG is ~30 h, as determined by using stable isotope versions of the lipid added to synthetic pulmonary surfactant (26). The comparatively long turnover time of POPG in humans suggests that this lipid may remain at suitable levels to provide protection from RSV for significant periods of time. Similar studies with PI also demonstrated efficacy against RSV both in vitro and in vivo, and this lipid reduced the viral burden within the lungs by a factor of 30, measured after 5 days of infection (25). The turnover rate for PI in mice occurs with a half-life of ~6 h, which is eight times longer than that observed for POPG (25). There are currently no data available for PI turnover in humans receiving synthetic pulmonary surfactant, but the expectation is that the anti-inflammatory and antiviral actions of PI are longer lasting than those of POPG.

We have also determined the effects of POPG and PI upon cultured epithelial cells that were pre-infected with RSV before administration of lipids. These studies demonstrated that both lipids block the spread of RSV from infected to uninfected cells (24, 25). In one set of experiments, cultured cells were infected with RSV at low multiplicity of infection, for 24 h prior to addition of lipids. Quite remarkably, addition of POPG at concentrations from 0.2 to 1.0 mg/ml reduced the viral burden in the cultures by a factor of 10^5-10^6. These later experiments provide strong in vitro evidence to support the concept that sustained high concentrations of POPG, or PI, can profoundly reduce or even arrest the progression of an established RSV infection (25).

Our findings demonstrating the inhibition of RSV infection by POPG and PI also led us to ask if either infection by influenza A virus (IAV) or the subsequent inflammatory sequelae might also be affected by anionic surfactant lipids (2). This line of inquiry was also stimulated by reports that TLR4 activation contributes significantly to the pathogenesis of IAV infections (27). Our initial studies examined H3N2-IAV and its interactions with either cultured primary bronchial epithelial cells or MDCK cells, which are routinely used to propagate and titer IAVs. Infection of cultured bronchial epithelial cells with H3N2-IAV elicited robust production of IL-8 and this was almost completely suppressed by POPG, but not a control lipid, POPC. Examination of MDCK cultures revealed that by 36 h after infection, nearly all the cells were lysed in the absence of lipid, or the presence of a control lipid (POPC), but were completely protected from lysis by 1 mg/ml POPG. Immunoblotting analysis demonstrated that cultures infected with virus...
produced relatively large amounts of viral proteins, which were unaffected by the addition of the control lipid, POPC, but reduced ~90% by the addition of POPG. Analysis of viral mRNA levels by qRT-PCR also demonstrated a ~90% reduction in the mRNA for hemagglutinin by POPG (2).

We next focused upon the mechanisms by which POPG protects cells from IAVs (2). For these studies we again used solid phase phospholipids to quantify interactions between virions and POPG and POPC. Viral attachment to lipid solid phases was quantified using anti-IAV antibodies and ELISA. These experiments revealed that IAV binding to POPG was high affinity, concentration dependent, and saturable (2). Similar results were also found for IAV interactions with PI. In contrast, the interaction between viral particles and POPC was low affinity and nonspecific. We further explored the relevance of the POPG–virus interaction to the infection process by determining the effects of the lipids on viral adsorption to MDCK cells, at 8 °C, where endocytosis is minimal. These latter experiments demonstrated that POPG, but not POPC, blocked IAV attachment to the cell surface. In additional studies, we sought to determine whether POPG treatment could reverse the adsorption of virus after its attachment to the cell surface. For these latter studies, we incubated cells with virus at low temperature for 4 h prior to adding lipids at low temperature for an additional 4 h. After such preliminary incubations the cultures were shifted to 37 °C and infection was allowed to progress. Subsequently, viral replication was quantified by measuring viral proteins after 36 h. These “virus pre-incubation” experiments demonstrated that once the viral particles are bound to the cell surface they become refractory to inhibition of infection by POPG. These findings provide further evidence that the site of action of POPG precedes viral attachment to the cell surface.

We also used H1N1-IAV, for the purpose of determining the relationship between our in vitro studies and models of animal infection (2). Prior to conducting these studies we determined that the interaction of H1N1-IAV with POPG in solid phase binding assays was comparable to that of H3N2-IAV. In the studies summarized in Fig. 4, mice received an
intranosal inoculation of the virus (~100 pfu) along with 3 mg POPG, and the infection was carried out for 6 days. Analysis of lung histology revealed the presence of inflammatory infiltrates within the lungs in virus-infected animals and a relatively high histopathology score as shown in Fig. 4A. In contrast, the lungs of mice infected with virus and receiving POPG, had a histopathology score nearly equivalent to sham-infected animals. Consistent with the histopathology, the total cells recovered in lung lavage from “virus plus POPG”–treated mice were modestly elevated above control mice, and the numbers of neutrophils and lymphocytes were correspondingly low. Most importantly, the viral burden in lung tissue from mice receiving virus plus POPG, was <10% of that for mice receiving only virus. Collectively, these data demonstrate significant protection from the H1N1-IAV infection by POPG. Similar data were obtained when PI was used as the viral antagonist, as shown in Fig. 4B.

**Structural basis of lipid antagonism**

Currently, we do not know the fine structural details of how POPG and PI interact with TLRs and viruses. Our focus now is resolving these molecular interactions using crystallography and cryo-EM. Our current model is that the anionic lipids are acting as decoy ligands for TLRs and as decoy receptors for viral attachment proteins (e.g. hemagglutinins for IAV strains). Molecular models demonstrate clear structural similarities between the lipid A moiety of LPS and the anionic surfactant lipids. There are also significant structural similarities between sialic acid containing lipid receptors (e.g. the sialglycolipid GM1) for the hemagglutinin of H1N1-IAV and the anionic surfactant lipids. Resolution of these unanswered structural questions has the potential to reveal structural requirements for new classes of inhibitors for specific TLRs, RSV, and IAV, deduced from structural features of the relevant viral and cellular molecules.

**Suppression of respiratory virus infections as a means for curtailing pandemics**

Our findings identifying high-affinity inhibitory interactions between anionic pulmonary surfactant phospholipids and TLR2, TLR4, RSV, and IAVs demonstrate these lipids play an important role in regulating infectious as well as innate immune processes. The immunoregulatory activities of POPG and PI make sense in the context of their presence within the alveolar compartment, where homeostatic suppression of inflammation prevents unnecessary interference with the efficiency of gas exchange. This suppression prevents spurious recruitment and activation of neutrophils and other inflammatory cells which release a mixture of proteases, cytokines, and reactive oxygen species that not only dispatch pathogens but also create bystander damage to the alveolar epithelium (28). Likewise, the antiviral actions of these lipids can be viewed as playing an essential role in preventing the loss of alveolar gas exchange units resulting from the cytopathic effects of respiratory viruses. Our studies also suggest that POPG, PI and analogs of these lipids have significant potential for short-term prevention and therapeutic application for reducing rampant inflammatory processes and aggressive respiratory virus infections. Finally, the actions of POPG and PI as agents capable of preventing infection of naïve cells raises the possibility that these lipids can be applied to subjects with active infections, for the purpose of preventing the transmission of infectious viruses to uninfected individuals.

**Animal studies**

All studies performed with animals were reviewed and approved by the National Jewish Health Animal Care and Use Committee.

**Human subjects studies**

All studies performed with human derived primary cells were approved by the National Jewish Health Institutional Review Board and conform to the principles of the Declaration of Helsinki.

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