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Phosphoinositide 3-kinase γ required for lipopolysaccharide-induced transepithelial neutrophil trafficking in the lung

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ABSTRACT: Phosphoinositide 3-kinase γ(*PI3Kγ*) is a critical mediator of directional cell movement. Here, we sought to characterise the role of *PI3Kγ* in mediating the different steps of polymorphonuclear leukocyte (PMN) trafficking in the lung.

In a murine model of lipopolysaccharide (LPS)-induced lung injury, PMN migration into the different lung compartments was determined in *PI3Kγ* gene-deficient (*PI3Kγ*$^{-/-}$) and wild-type mice. Bone marrow chimeras were created to characterise the role of *PI3Kγ* on haematopoietic *versus* nonhaematopoietic cells. A small-molecule *PI3Kγ* inhibitor was tested *in vitro* and *in vivo*.

PMN adhesion to the pulmonary endothelium and transendothelial migration into the lung interstitium was enhanced in *PI3Kγ*$^{-/-}$ mice. However, transepithelial migration into the alveolar space was reduced in these mice. When irradiated PI3Kγ$^{-/-}$ mice were reconstituted with bone marrow from wild-type mice, migratory activity into the alveolar space was restored partially. A small-molecule *PI3Kγ* inhibitor reduced chemokine-induced PMN migration *in vitro* when PMNs or epithelial cells, but not when endothelial cells, were treated. The inhibitor also reduced LPS-induced PMN migration *in vivo*.

We conclude that *PI3Kγ* is required for transepithelial but not for transendothelial migration in LPS-induced lung injury. Inhibition of *PI3Kγ* activity may be effective at curbing excessive PMN infiltration in lung injury.

KEYWORDS: Acute lung injury, chemotaxis, inflammation, polymorphonuclear leukocytes, transmigration

Recruitment of polymorphonuclear leukocytes (PMNs) to inflamed tissues is an essential requirement of the innate immune response but can lead to organ damage when excessive and uncontrolled. In the lung, excessive PMN infiltration can result in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), a condition that can follow pneumonia, acid aspiration, major trauma or sepsis, and causes ~75,000 deaths per year in the USA alone [1]. Depletion of PMNs curbs experimental lung damage [2] but is not desirable in most patients because it induces impaired host defence. Although the pathogenicity of PMNs in ALI/ARDS has been impressively demonstrated, molecular mechanisms underlying PMN trafficking in the lung remain poorly understood [3]. This may explain why, to this day, there is no strategy for the modulation of PMN infiltration in humans and no therapy available for ALI/ARDS beyond mechanical ventilation and other supportive approaches [4]. The mortality of ARDS remains high at 35–40% [5].

Pulmonary infiltration with inflammatory leukocytes is initiated by activation of circulating PMNs, resulting in altered mechanical properties and enhanced migratory activity [6]. Initial contact between PMNs and pulmonary endothelium requires adhesion molecules in some ARDS models [7], but not in others [8]. Once activated PMNs adhere to the pulmonary capillaries, additional steps are required to initiate transendothelial migration into the lung interstitium and transepithelial migration into the alveolar space, including activation of chemokine receptors and structural rearrangement of adhesion molecules [9]. Cytoskeletal reorganisation of PMNs, and endothelial and epithelial cells is a prerequisite to facilitate directional movement of leukocytes to the lung.

The family of class I phosphoinositide 3-kinases (*PI3Ks*) are isoforms of heterodimeric lipid-modifying proteins that are involved in the regulation of numerous cell functions, including cell growth, proliferation, adhesion, motility and

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survival [10]. *PI3Kγ* is a class IB PI3K, consisting of a p110γ catalytic subunit and a 101-kD regulatory subunit (p101). *PI3Kγ* signalling is found downstream of different receptor types, including G protein-coupled chemokine receptors. Activation of chemokine receptors leads to the release of the G protein subunit βγ that associates with the p110 adaptor protein and initiates translocation of *PI3Kγ* to the cell membrane, where it mediates the phosphorylation of posphatidylinositol (PI) 3,4-bisphosphate to PI 3,4,5-trisphosphate [11]. PI 3,4,5-trisphosphate is an essential mediator of cell orientation and directional cell movement [12], thus making *PI3Kγ* a promising target in leukocyte-dependent inflammatory diseases [13].

Involvement of *PI3Kγ* in ALI has been implicated but study results have been ambiguous. In a model of ventilator-induced lung injury, *PI3Kγ* gene-deficient ($PI3K\gamma^{-/-}$) mice exhibited improved lung mechanics and reduced formation of hyaline membranes while release of chemotactic cytokines in the lung was unaltered [14]. In the same model, others demonstrated an attenuation in the activation of nuclear factor (NF)-κB in inflammatory cells and a decrease in the release of inflammatory cytokines in mice pretreated with a nonselective PI3K inhibitor [15]. In contrast, $PI3K\gamma^{-/-}$ mice were more susceptible to acute lung injury induced by intraperitoneal administration of *Escherichia coli* [16] or intratracheal application of pneumococcal virulence factor pneumolysin [17]. Pretreatment with the nonselective PI3K inhibitor wortmannin increased serum levels of pro-inflammatory cytokines and increased mortality in another sepsis model [18]. In different models of ALI, PMN recruitment and infiltration into the lungs of $PI3K\gamma^{-/-}$ mice was found to be attenuated [19], increased [16] or similar [17] to wild-type mice. It is important to recognise that, in all these studies, single steps of PMN trafficking in the lung were not differentiated.

Functional expression of *PI3Kγ* in endothelial cells has recently been demonstrated and suggested to mediate selectin-dependent adhesion of leukocytes [19]. Whether *PI3Kγ* on pulmonary microvascular endothelial or epithelial cells is involved in adhesion or transmigration is unknown.

The current study was designed to elucidate the role of *PI3Kγ* for the different steps of PMN trafficking in the lung, *i.e.* recruitment from the peripheral blood and adherence to the pulmonary capillaries, transendothelial migration into the lung interstitium, and transepithelial migration into the alveolar space. We used gene-deficient mice and a selective small-molecule inhibitor to block *PI3Kγ* function *in vitro* and *in vivo*. We created bone marrow chimeras to study *PI3Kγ* effects on haematopoietic *versus* nonhaematopoietic cells. Our results demonstrate a specific role of *PI3Kγ* in transepithelial neutrophil migration during ALI that might help to interpret conflicting results from previous studies.

MATERIALS AND METHODS

Mice

Wild-type male C57Bl/6 mice were obtained from Jackson Labs (Bar Harbor, ME, USA). Breeder pairs of *PI3Kγ* gene-deficient mice ($PI3K\gamma^{-/-}$, C57Bl/6 background) were provided by D. Wu at the University of Connecticut (Farmington, CT, USA). Mice were bred, and deletion of the p110 subunit of *PI3Kγ* was confirmed by PCR [20]. Wild-type littermates ($PI3K\gamma^{+/+}$) served as control animals. All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia (Charlottesville, VA, USA). Mice were 8–12 weeks of age.

Differential blood cell counts

Increased blood cell counts in gene-deficient mice with targets that alter cell transmigration have been described [21] and will influence the analysis of migratory activity. To reveal possible differences between the different groups of mice, baseline differential blood counts were performed in $PI3K\gamma^{+/+}$ and $PI3K\gamma^{-/-}$ mice using an automatic analyser (Hemavet 850 FS; CDC Technologies, Oxford, CT, USA).

Generation of chimeric mice

Chimeric mice were generated by transferring bone marrow between $PI3K\gamma^{+/+}$ and $PI3K\gamma^{-/-}$ mice as described previously [22]. Briefly, recipient mice were lethally irradiated in two doses of 600 rad each (separated by 4 h). This regimen results in >90% donor-derived neutrophils at 6 weeks of reconstitution. Bone marrow from donor mice was harvested from both femora and tibiae, and ~5 million cells were injected intravenously into recipient mice. Bone marrow transplantation (BMT) was performed in four groups of mice: 1) bone marrow from $PI3K\gamma^{-/-}$ into $PI3K\gamma^{+/+}$ (chimeric mice express *PI3Kγ* on nonhaematopoietic cells only); 2) bone marrow from $PI3K\gamma^{+/+}$ into $PI3K\gamma^{-/-}$ (chimeric mice express *PI3Kγ* on haematopoietic cells only); 3) bone marrow from $PI3K\gamma^{-/-}$ into $PI3K\gamma^{-/-}$; and 4) bone marrow from $PI3K\gamma^{+/+}$ into $PI3K\gamma^{+/+}$. Mice in groups 3 and 4 served as negative and positive controls for possible radiation effects. Chimeric mice were used for experiments 6 weeks after BMT.

Small-molecule PI3Kγ inhibitor

We evaluated the small-molecule *PI3Kγ* inhibitor AS-605240 (5-quinoxalin-6-ylmethylene-thiazolidine-2,4-dione) (Merck Serono, Geneva, Switzerland) [23] for its efficiency to block PMN transmigration *in vitro* and *in vivo*. AS-605240 selectively inhibits *PI3Kγ* enzymatic activity, *PI3Kγ*-mediated downstream signalling and chemotaxis [23]. Stock solutions were prepared in 0.5% carboxymethyl cellulose (CMC) and 0.25% Tween 20 in saline and used at indicated concentrations.

In vitro transendothelial migration

To test whether inhibition of neutrophil PI3Kγ is important in regulating migration, we conducted *in vitro* transmigration studies with PMNs and pulmonary endothelial cells (PECs) so that we could treat the cell types separately with AS-605240. PECs were harvested from wild-type male C57Bl/6 mice using a positive immunomagnetic selection for CD31 (Mec 13.3) (EasySep® Biotin Selection Kit; StemCell Technologies, Vancouver, BC, Canada). PECs were cultured in DMEM (D-valine instead of L-valine; Chemikon, Phillipsburg, NJ, USA) with 10% of fetal bovine serum (FBS), 20 mM HEPES, 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), and 50 $\mu g \cdot mL^{-1}$ endothelial cell growth supplement (Sigma Co., St. Louis, MO , USA). Purity of PECs was confirmed by staining for von Willebrand factor (Abcam, Cambridge, MA, USA) and CD31 and their uptake of fluorescein isothiocyanate-labelled acetylated low-density lipoprotein (Biomedical Technologies Inc., Stoughton, MA, USA). Magnetic immunoseparation

yielded a >90% pure endothelial cell culture. Endothelial cells were plated on fibronectin-coated filters in a Transwell system (6.5 mm diameter, 3.0 μm pore size; Corning Inc., Corning, NJ, USA) and grown until confluent (72 h). Medium was replaced with phenol-free DMEM with 1% FBS 2 h before the experiment. Filters without endothelial cells served as negative controls.

PMNs from C57Bl/6 or *PI3Kγ*$^{-/-}$ mice were isolated from bone marrow using a three layer Percoll gradient (78, 66, and 54%) as previously described [9]. This technique yielded a cell purity of >90%. PMNs, endothelial cells or both were incubated with AS-605240 at 15 μM for 30 min. This concentration has been previously shown to significantly reduce monocyte chemotactic protein-1-induced migration of mouse monocytes [23]. Negative controls were treated with vehicle only (CMC 0.5% and Tween 20 0.25% in saline). For the final 15 min, PMNs were labelled with calcein AM (5 μM; Molecular Probes, Carlsbad, CA, USA) and washed twice. Filters were moved to outer wells containing 400 μL of phenol-free DMEM with or without chemokine (CXC motif) ligand (CXCL)2/3 (macrophage inflammatory protein-2, 200 ng·mL^{-1}; PeproTech Inc., Rocky Hill, NJ, USA). 2.5×10^5 PMNs were plated on filters with or without endothelial cells. Filters were incubated for 2 h at 37°C and fluorescence was measured in the bottom wells (excitation 485 nm; emission 530 nm).

In vitro *transendothelial and transepithelial migration of human cells*

PMNs from healthy donors were isolated by a two-layer Percoll gradient (72% and 63%) as previously described [24]. The purity of the resulting cell population was >95%. Human A549 pulmonary epithelial cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI containing 10% FBS, 1% epithelial cell growth supplement, and 1% penicillin/streptomycin solution. 100,000 epithelial cells were seeded on the collagen-coated undersurface of inverted Transwell filters and allowed to adhere for 2 h at 37°C in a humidified 5% CO_2 incubator. Nonadherent cells were removed, filters were moved to wells containing culture medium, and cells were incubated for 72 h until a confluent monolayer was formed [25]. PMNs, A549 cells or both were incubated with AS-605240 at 15 μM for 30 min, and migratory activity was determined as described above. Negative controls were treated with vehicle only (CMC 0.5% and Tween 20 0.25% in saline). In additional experiments, human pulmonary microvascular endothelial cells (HPMECs) (ScienCell Research Laboratories, Carlsbad, CA, USA) were plated on fibronectin-coated filters in a Transwell system, and transmigration of human PMNs was assessed as described above.

Murine model of ALI

Up to four mice were exposed to aerosolised lipopolysaccharide (LPS) in a custom-built cylindrical chamber (20 × 9 cm) connected to an air nebuliser (MicroAir; Omron Healthcare, Vernon Hills, IL, USA). LPS from *Salmonella enteritidis* (Sigma Co.) was dissolved in 0.9% saline (500 μg·mL^{-1}) and mice inhaled LPS for 30 min. As previously shown, this mimics several aspects of ALI, including PMN recruitment into all compartments of the lung, increase in vascular permeability [26], release of chemokines and disruption of the pulmonary architecture [27]. Control mice were exposed to saline aerosol.

In vivo *inhibition of PI3Kγ*

To evaluate PMN migration *in vivo*, wild-type and *PI3Kγ*$^{-/-}$ mice were intraperitoneally injected with AS-605240 1 h prior to LPS exposure. The inhibitor was used at a concentration of 50 mg·kg^{-1} as previously suggested [23]. Control mice received vehicle only (CMC 0.5% and Tween 20 0.25% in saline).

PMN trafficking in the lung

PMN recruitment into the different compartments of the lung (pulmonary vasculature, interstitium, alveolar airspace) was assessed as previously described [26]. Briefly, 24 h after LPS exposure (peak of LPS-induced accumulation of PMNs in the bronchoalveolar lavage fluid (BALF)), intravascular PMNs were labelled by an *i.v.* injection of Alexa 633-labelled GR-1. After 5 min, mice were euthanised and nonadherent PMNs were removed from the pulmonary vasculature by flushing 10 mL of PBS at 25 cmH_2O through the spontaneously beating right ventricle. BALF was withdrawn and lungs were removed, minced and digested in the presence of excess unlabelled anti-GR-1 to prevent possible binding of the injected antibody to extravascular PMN. A cell suspension was prepared by passing the digested lungs through a 70 μm cell strainer (BD Falcon, Bedford, MA, USA). Total cells in BALF and lung were counted and percentage of PMNs determined by flow cytometry. In the BALF, PMNs were identified by their typical appearance in the forward/sideward scatter and their expression of CD45 (clone 30-F11), 7/4 (clone 7/4), and GR-1 (clone RB6-8C5). In the lung, the expression of GR-1 was used to distinguish intravascular (CD45+7/4+GR-1+) from interstitial (CD45+7/4+GR-1-) PMNs, which were not reached by the injected antibody. In all experiments, isotype control antibodies were used to set the gates.

Cytospins of BALF

Cytospins of BALF from wild-type and *PI3Kγ*$^{-/-}$ mice harvested 24 h after LPS exposure were prepared using a cytocentrifuge (Thermo Shandon, Waltham, MA, USA). Cytospun cells were stained (Diff-Quick staining; IMEB Inc., San Marcos, CA, USA), air dried and coverslipped.

Pulmonary microvascular permeability

Pulmonary microvascular permeability in wild-type and *PI3Kγ*$^{-/-}$ mice was determined by measuring extravasation of Evans blue dye [28]. Evans blue (20 mg·kg^{-1}; Sigma-Aldrich, St Louis, MO, USA) was injected intravenously 30 min prior to euthanasia. Lungs were perfused with cold PBS through the spontaneously beating right ventricle to remove intravascular dye. Lungs were removed and Evans blue was extracted as previously described [29]. The absorption of Evans blue was measured at 620 nm and corrected for the presence of haem pigments: $A620(\text{corrected}) = A620 - (1.426 \times A740 + 0.030)$ [30]. Extravasated Evans blue was determined in the different animal groups 6 h after LPS (peak of LPS-induced increase in microvascular permeability) or saline inhalation and calculated against a standard curve (microgrammes Evans blue dye per gramme lung). In additional experiments, wild-type mice were pretreated with AS-605240 (50 mg·kg^{-1} *i.p.*) and microvascular permeability was determined.

BALF protein

We measured LPS-induced accumulation of protein in the BALF of wild-type mice as an indicator of epithelial permeability. 6 h after LPS, protein in the BALF was determined by a colorimetric method against a standard curve according to the manufacturer's direction (bicinchoninic acid; Thermo Scientific, Rockford, IL, USA). Some mice were pretreated with AS-605240 (50 mg·kg^{-1} *i.p.*).

Statistical analysis

Statistical analysis was performed with JMP Statistical Software (version 7.0; SAS Institute Inc., Cary, NC, USA). Differences between the groups were evaluated by one way ANOVA followed by a *post hoc* Tukey test. Data were presented as mean±SD and $p<0.05$ was considered statistically significant.

RESULTS

Blood counts

To reveal potential PMN count alterations in the *PI3Kγ*$^{-/-}$ mice, baseline differential blood counts were determined using an automatic analyser. No differences in PMN counts were detected between wild-type and *PI3Kγ*$^{-/-}$ mice. However, monocyte counts were elevated in *PI3Kγ*$^{-/-}$ mice ($0.6\pm0.3\times10^3$ μL^{-1} *versus* $0.3\pm0.2\times10^3$ μL^{-1}; $p<0.05$; table 1).

PI3Kγ regulates transepithelial PMN transmigration into the lung

We used a flow cytometry-based method to detect PMNs in the different compartments of the lung of wild-type and *PI3Kγ*$^{-/-}$ mice. PMNs were identified by their typical appearance in the forward/side scatter and their expression of CD45 and 7/4 (fig. 1a). In the lung, we defined intravascular PMNs by their additional expression of GR-1+ (fig. 1b). In the BALF, all PMNs were identified by their expression of CD45, 7/4 and GR-1 (monoclonal antibodies added after harvesting) (fig. 1c). At baseline (no LPS), all PMNs in the lung were intravascular (fig. 1b left panels, 7/4+ and GR-1+, right upper square). LPS inhalation induced transendothelial migration into the lung interstitium as confirmed by the occurrence of GR-1- PMNs (fig. 1b, right panels, right lower square). In the BALF, no PMNs were detected at baseline (fig. c, left panels). Baseline PMN counts in lung interstitium and BALF did not differ between wild-type and *PI3Kγ*$^{-/-}$ mice; however, *PI3Kγ*$^{-/-}$ mice demonstrated a higher PMN accumulation in the pulmonary microvasculature (fig. 2a).

LPS inhalation induced significant PMN recruitment into all compartments of the lung of wild-type and *PI3Kγ*$^{-/-}$ mice (figs 1 and 2). LPS-induced PMN accumulation in the pulmonary circulation was significantly higher in *PI3Kγ*$^{-/-}$ compared with wild-type mice at 24 h after LPS ($2.2\pm0.6\times10^6$ *versus* $1.1\pm0.3\times10^6$; $p<0.05$; fig. 2a). In addition, PMN migration into the interstitium was significantly higher in *PI3Kγ*$^{-/-}$ mice ($2.4\pm0.4\times10^6$ *versus* $1.5\pm0.4\times10^6$; $p<0.05$; fig. 2b). Despite higher PMN counts in the lung tissue (intravascular and interstitial), PMN migration into the alveolar space (BALF; fig. 2c) was reduced in *PI3Kγ*$^{-/-}$ mice ($1.1\pm0.2\times10^6$ *versus* $2.4\pm0.6\times10^6$; $p<0.05$), suggesting that *in vivo*, *PI3Kγ*$^{-/-}$ is required for transepithelial but not for transendothelial migration in the lung. Reduced PMN counts in the alveolar airspace of *PI3Kγ*$^{-/-}$ was confirmed by cytospin of BALF (fig. 2e).

PMN trafficking in chimeric mice

To characterise the role of *PI3Kγ* on haematopoietic and nonhaematopoietic cells, we created chimeric mice by transferring bone marrow between wild-type and *PI3Kγ*$^{-/-}$ mice. LPS-induced PMN migration in control mice that received bone marrow from mice of the same genotype was similar to wild-type (positive control group) or *PI3Kγ*$^{-/-}$ (negative control group) mice, respectively (fig. 3). In mice that expressed *PI3Kγ* on nonhaematopoietic cells only, transepithelial migration into the BALF was significantly reduced ($0.8\pm0.2\times10^6$ *versus* $2.4\pm0.5\times10^6$; $p<0.05$; fig. 3c). The reduction was to a level similar to mice of the negative control group (bone marrow of *PI3Kγ*$^{-/-}$ into *PI3Kγ*$^{-/-}$ mice; $0.8\pm0.2\times10^6$ *versus* $0.9\pm0.2\times10^6$; nonsignificant). Consistent with a defect in transepithelial migration, intravascular and interstitial PMN counts were elevated in these mice (fig. 3a and b). It is possible that neutrophils get ''backed up'' in the intravascular and interstitial compartment when their transepithelial migration is impaired in *PI3Kγ*$^{-/-}$ mice. When *PI3Kγ*$^{-/-}$ mice were reconstituted with bone marrow from wild-type mice, transepithelial migration was only partially restored ($1.6\pm0.3\times10^6$ *versus* $2.4\pm0.5\times10^6$ in mice that express *PI3Kγ* on all cells; $p<0.05$; fig. 3c). Intravascular and interstitial PMN counts in *PI3Kγ*$^{-/-}$ mice reconstituted with bone marrow from wild-type mice were not different from wild-type mice reconstituted with bone marrow of wild-type mice, but significantly less than in mice of the negative control group or in *PI3Kγ*$^{-/-}$ mice. This finding supports the hypothesis that *PI3Kγ* on nonhaematopoietic cells is involved in transepithelial migration of PMNs.

TABLE 1 Baseline cell counts

	PI3Kγ$^{+/+}$	*PI3Kγ*$^{-/-}$	p-value
Leukocytes ×10^3 μL^{-1}	8.8±3.8	11.3±4.9	0.22
Neutrophils ×10^3 μL^{-1}	1.6±0.6	2.2±0.9	0.08
Lymphocytes ×10^3 μL^{-1}	6.8±3.2	8.7±4.5	0.28
Monocytes ×10^3 μL^{-1}	0.3±0.2	0.6±0.3	<0.05
Neutrophils %	18.9±4.2	19.5±4.9	0.76
Lymphocytes %	76.4±4.1	73.9±4.9	0.23
Monocytes %	3.6±1.8	5.1±1.9	0.10

Data are presented as mean±SD of eight mice, unless otherwise stated. Baseline differential cell counts were performed in phosphoinositide 3-kinase gene-deficient (*PI3Kγ*$^{-/-}$) and wild-type (*PI3Kγ*$^{+/+}$) mice using an automatic analyser.

AS-605240 inhibits in vitro transmigration

To evaluate its efficiency to inhibit chemokine-induced PMN migration *in vitro*, we incubated PMNs from wild-type C57Bl/6 or *PI3Kγ*$^{-/-}$ mice with the small-molecule *PI3Kγ* inhibitor AS-605240 (15 μM), and allowed them to migrate through a Transwell filter. Migratory activity of *PI3Kγ*$^{-/-}$ PMNs was significantly reduced compared with wild-type PMNs. AS-605240 reduced CXCL2/3-stimulated migration of wild-type but not of *PI3Kγ*$^{-/-}$ PMNs by >60% ($p<0.05$ *versus* untreated control) (fig. 4a), confirming a specific effect of AS-605240 on this subtype of PI3K.

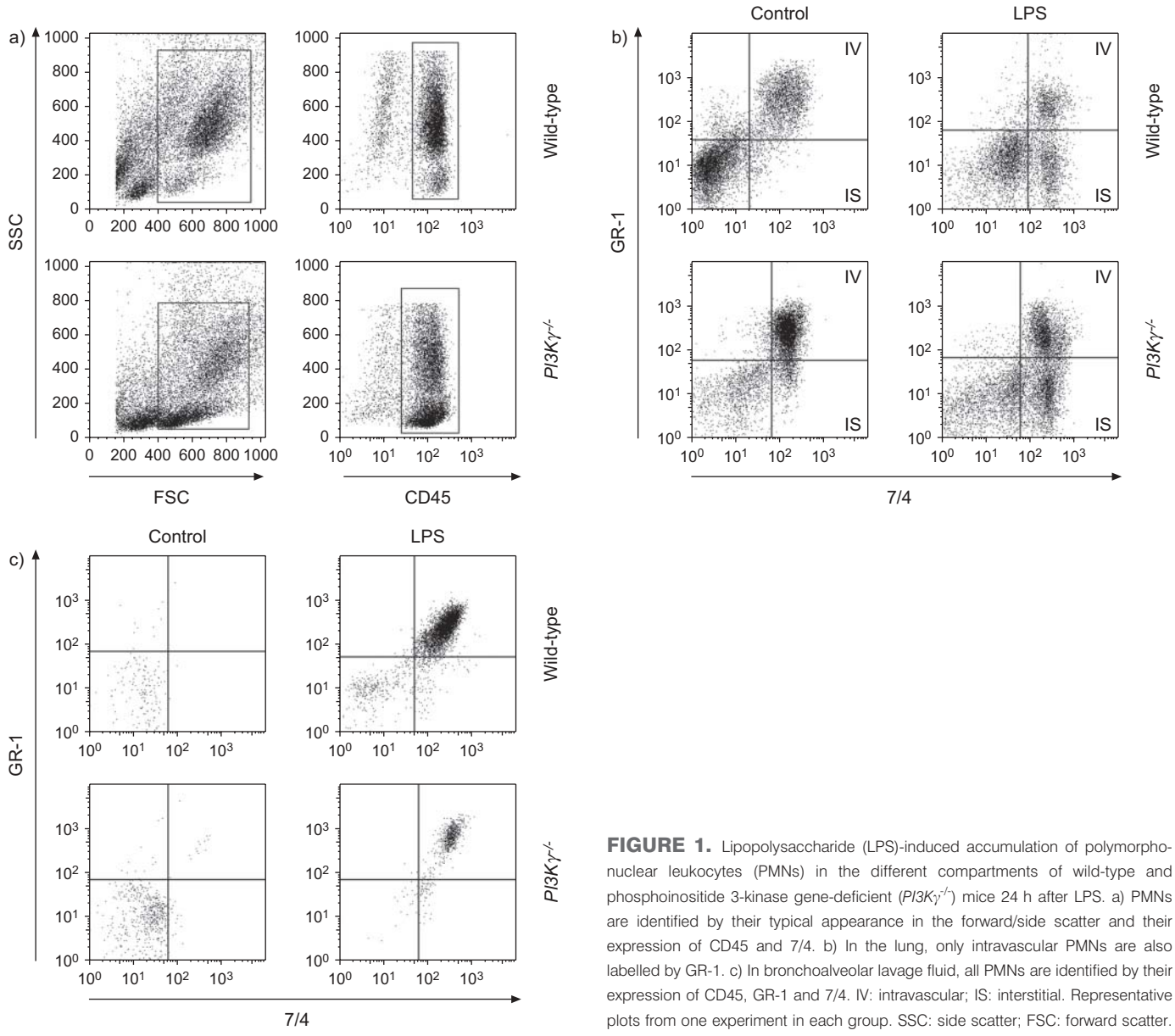

FIGURE 1. Lipopolysaccharide (LPS)-induced accumulation of polymorphonuclear leukocytes (PMNs) in the different compartments of wild-type and phosphoinositide 3-kinase gene-deficient (*PI3Kγ*$^{-/-}$) mice 24 h after LPS. a) PMNs are identified by their typical appearance in the forward/side scatter and their expression of CD45 and 7/4. b) In the lung, only intravascular PMNs are also labelled by GR-1. c) In bronchoalveolar lavage fluid, all PMNs are identified by their expression of CD45, GR-1 and 7/4. IV: intravascular; IS: interstitial. Representative plots from one experiment in each group. SSC: side scatter; FSC: forward scatter.

Next, we sought to determine the effect of AS-605240 on PECs *versus* PMNs. PECs were grown to confluence, and CXCL2/3-induced transendothelial PMN migration was measured. PMNs, PECs or both cell types were pretreated with the *PI3Kγ* inhibitor as indicated.

CXCL2/3-stimulated migration through the endothelial layer was significantly reduced when PMNs were pretreated with AS-605240 (>50% reduction; $p<0.05$ *versus* untreated control; fig. 4b). No effect was observed when PECs were pretreated with the *PI3Kγ* inhibitor. When both PMNs and PECs were pretreated simultaneously, migration was similar to wells in which only PMNs were pretreated, indicating that *PI3Kγ* in PMNs but not in endothelial cells is required for chemokine-induced endothelial transmigration (fig. 4b).

Our *in vivo* experiments implicated a distinct role of *PI3Kγ* for the transepithelial migration. We therefore hypothesised that blocking *PI3Kγ* in A549 cells would reduce transepithelial PMN migration *in vitro*. CXCL2/3-induced transepithelial migration was significantly reduced when PMNs were pretreated with AS-605240, similar to the transendothelial migration (46% reduction; $p<0.05$ *versus* untreated control; fig. 4c). When *PI3Kγ* was blocked in A549 cells alone, PMN migration was reduced by 26% ($p<0.05$ *versus* untreated control). This was in contrast to our findings with endothelial cells where blocking *PI3Kγ* did not affect migration and supports our hypothesis that epithelial *PI3Kγ* is involved in PMN trafficking in the lung. Blocking *PI3Kγ* in A549 cells and PMNs did not further decrease migration, indicating that *PI3Kγ* on PMNs limits *PI3Kγ*-dependent trafficking in our system. This is in line with our *in vivo* findings (fig. 3c).

To reveal potential species differences with respect to PI3K-dependent transmigration of PMNs, we repeated the *in vitro* transmigration assays with HPMECs. In analogy to our

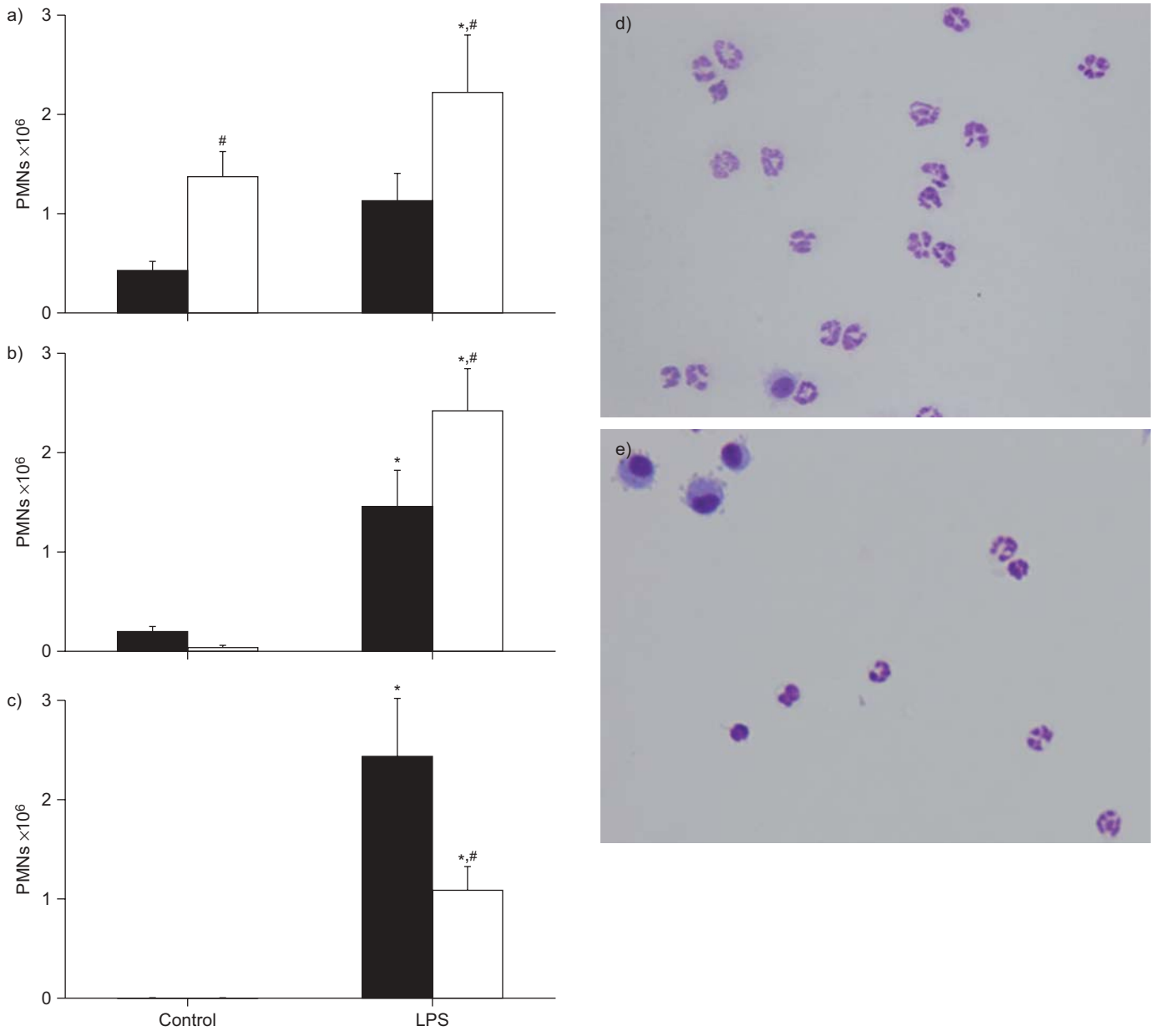

FIGURE 2. Lipopolysaccharide (LPS)-induced migration of polymorphonuclear leukocytes (PMNs) into the different lung compartments of wild-type (■) and phosphoinositide 3-kinase gene-deficient ($PI3K\gamma^{-/-}$) (□) mice. Accumulation of PMNs in a) the vasculature, b) the lung interstitium and c) the bronchoalveolar space were analysed. Cytospins of LPS-exposed bronchoalveolar lavage fluid in d) wild-type and e) $PI3K\gamma^{-/-}$ mice are shown to illustrate quantitative data. Values are presented as mean±SD of five experiments. *: $p<0.05$ *versus* negative control without LPS; #: $p<0.05$ *versus* wild-type mice within the same treatment group (±LPS).

findings with murine cells, inhibition of PI3K in HPMEC did not affect PMN migration (fig. 4d), suggesting that both species are comparable.

Effects of AS-605240 on in vivo *transmigration*

Next, we sought to determine the effects of AS-605240 (50 $mg \cdot kg^{-1}$) on LPS-induced PMN migration *in vivo*. Wild-type and $PI3K\gamma^{-/-}$ mice received AS-605240 30 min prior to LPS exposure. After 24 h, accumulation of PMNs in the different compartments of the lung was determined by flow cytometry. In wild-type mice, LPS-induced influx of PMNs into the BALF was significantly reduced by the pretreatment with AS-605240 ($1.6\pm0.3\times10^6$ *versus* $2.6\pm0.6\times10^6$; $p<0.05$; fig. 5). The inhibitor did not reduce recruitment of PMNs to the pulmonary vasculature or transendothelial migration into the interstitium. In addition, the inhibitor exhibited no effects on LPS-induced PMN migration in $PI3K\gamma^{-/-}$ mice, supporting its specificity for $PI3K\gamma$.

Microvascular permeability and BALF protein

Disturbance of endothelial integrity and efflux of protein-rich fluid into the lung tissue is one of the critical events in the early development of ARDS that accompanies PMN infiltration. We therefore determined the role of $PI3K\gamma$ in LPS-induced

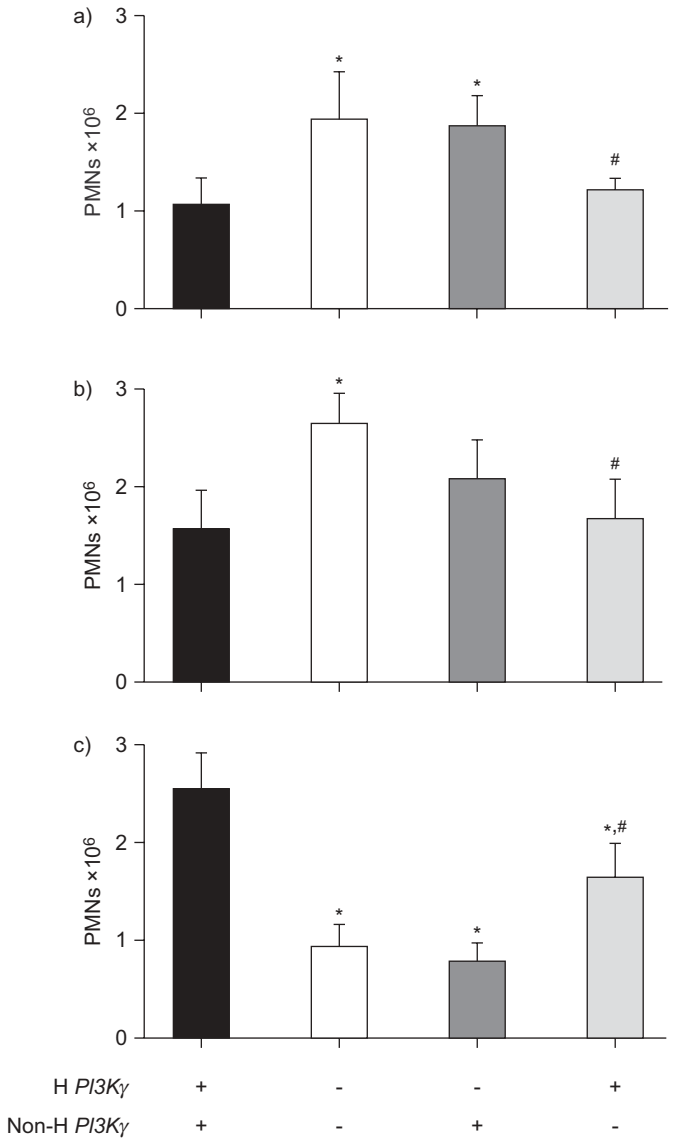

FIGURE 3. Haematopoietic (H) and nonhaematopoietic (non-H) cell phosphoinositide 3-kinase γ (*PI3Kγ*) participation in lipopolysaccharide-induced polymorphonuclear leukocyte (PMN) trafficking in the lung. Accumulation of PMNs in a) the vasculature, b) the lung interstitium and c) the bronchoalveolar space were analysed in chimeric mice (grey bars). Values are presented as mean±SD of five experiments. *: $p<0.05$ *versus* positive control (bone marrow transfer between wild-type mice: ■); #: $p<0.05$ *versus* negative control (bone marrow transfer between *PI3Kγ* gene-deficient mice: □).

microvascular permeability assessed by the extravasation of Evans blue and protein accumulation in the alveolar space as indicators of endothelial and epithelial permeability, respectively. LPS induced a significant increase in microvascular permeability in wild-type (394±33 *versus* 151±11 $\mu g \cdot g^{-1}$ lung; $p<0.05$) and *PI3Kγ*$^{-/-}$ mice (568±153 *versus* 279±97 $\mu g \cdot g^{-1}$ lung; $p<0.05$; fig. 6a). Although both baseline and LPS-induced microvascular permeability tended to be higher in *PI3Kγ*$^{-/-}$ mice, differences were not significant. Pretreatment with AS-605240 did not prevent LPS-induced microvascular permeability in wild-type or *PI3Kγ*$^{-/-}$ mice. In addition, LPS-induced protein efflux into the alveolar space was not affected by inhibition of PI3Kγ (fig. 6b). This suggests a distinct role of PI3Kγ for cell trafficking in our model.

DISCUSSION

The present study was designed to characterise the role of *PI3Kγ* in the distinct steps of PMN trafficking in the lung. In a murine model of ALI/ARDS, *PI3Kγ* was required for the transepithelial migration of PMNs from the lung interstitium into the alveolar airspace, while adhesion to and migration through the pulmonary endothelium remained unaffected in the absence of *PI3Kγ*. Transmigration was mainly dependent on *PI3Kγ* on bone marrow-derived cells, although *PI3Kγ* on nonhaematopoietic cells contributed to the transepithelial migration of PMNs. The small-molecule *PI3Kγ* inhibitor AS-605240 reduced PMN migration *in vitro* and PMN infiltration into the lung *in vivo*.

The key role of *PI3Kγ* in migration of leukocytes to inflamed tissue has led to several experimental studies that sought to identify the effects of *PI3Kγ*-involving pathways in ALI, a disease that is largely characterised by the infiltration of inflammatory cells. Consistent with the hypothesis that inhibition of *PI3Kγ* attenuates ALI, PURI *et al.* [19] found that LPS-induced PMN migration into the BAL was almost completely abolished in *PI3Kγ*$^{-/-}$ mice [19]. *PI3Kγ*$^{-/-}$ PMNs in the lungs exhibited diminished activation of NF-κB and expression of pro-inflammatory chemokines interleukin-1β and tumour necrosis factor (TNF)-α [31]. Similar results were found in a model of ventilator-induced lung injury where blocking PI3K with a nonselective PI3K inhibitor reduced nuclear translocation of NF-κB and the release of interleukin-6 and macrophage inflammatory protein-2 in the lung [15]. In a similar model, *PI3Kγ*$^{-/-}$ mice exhibited less lung damage as assessed by respiratory mechanics and the formation of hyaline membranes [14]. In that study, effects of the *PI3Kγ* pathway were independent of the release of chemotactic chemokines, but the authors observed an increased apoptotic activity in pulmonary cells of *PI3Kγ*$^{-/-}$ mice while cell necrosis was reduced in these mice.

The role of the *PI3K* pathway in mediating apoptosis is well established [32]. However, the role of apoptosis in the pathophysiology of inflammatory diseases remains controversial. Increased apoptosis, particularly in lymphoid tissue, contributes to immune suppression and organ failure that occurs during sepsis [33]. Conversely, apoptosis, in contrast to necrosis, generally does not produce inflammation and tissue damage [34]. In lung injury, cell necrosis rather than apoptosis is associated with an inflammatory response and inversely correlates with lung function [35]. In addition, *PI3Kγ*-dependent pathways seem notably important for the integrity of the alveolar epithelium [36], consistent with our finding that *PI3Kγ* was mediating the epithelial but not endothelial barrier function in the lung. BONNANS *et al.* [37] identified an endogenous PI3K inhibitory pathway that is initiated by the production of presqualene diphosphate (PSDP). In acid-induced lung injury, PSDP is suppressed and *PI3Kγ* activity increased. Consequently, pretreatment with a PSDP analogue reduced acid-induced PMN infiltration and lung tissue damage.

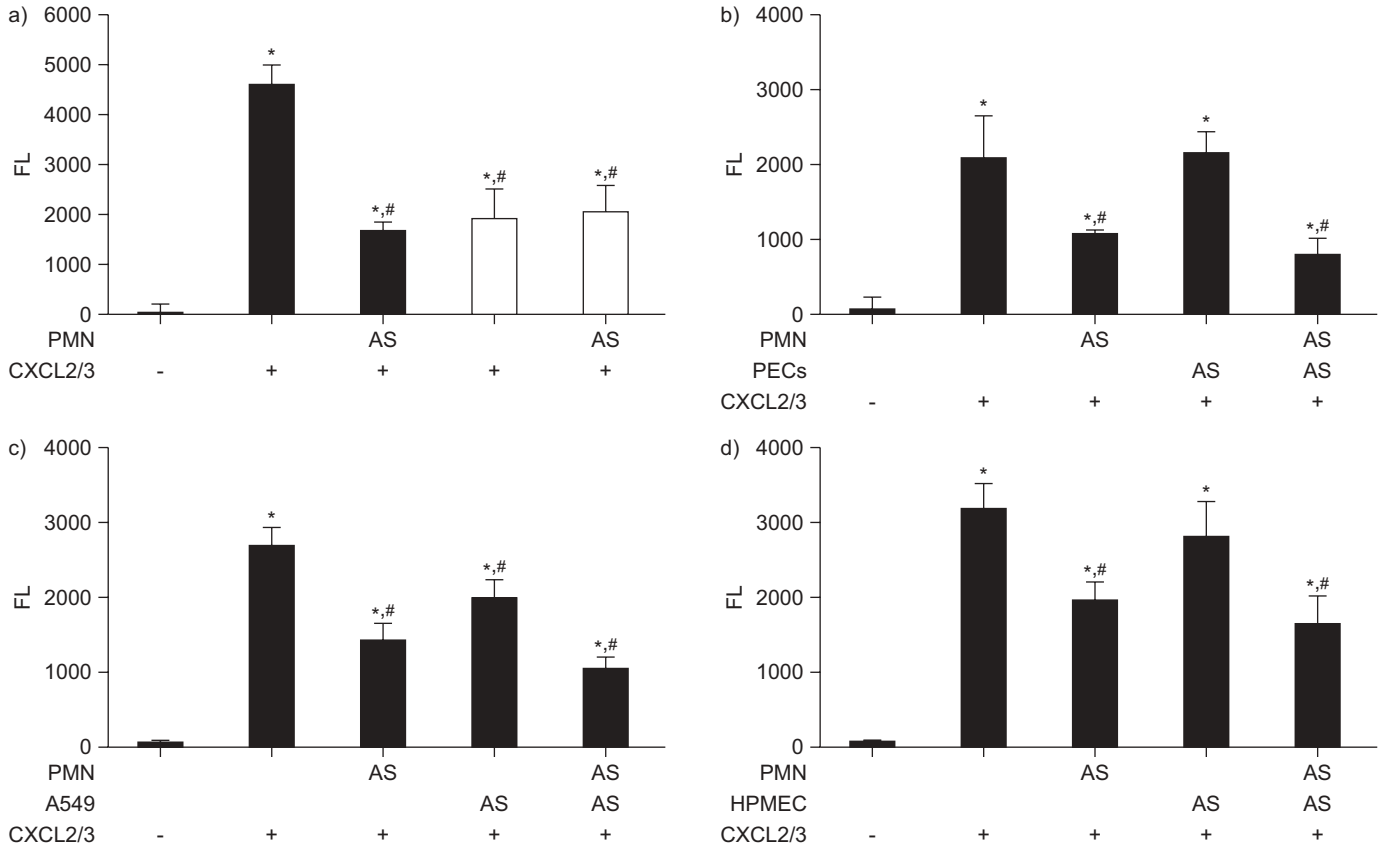

FIGURE 4. The effect of the phosphoinositide 3-kinase γ (*PI3Kγ*) inhibitor AS-605240 (AS) on chemokine-induced transmigration was evaluated *in vitro*. Transmigration across a) a Transwell filter alone or b) a confluent layer of cultured murine pulmonary endothelial cells (PECs), c) human A549 cells or d) human pulmonary microvascular endothelial cells (HPMECs) in a Transwell filter was significantly reduced when polymorphonuclear leukocytes (PMNs) were pretreated with AS-605240. No effect was seen when PECs or HPMECs were pretreated with the *PI3Kγ* inhibitor. However, blocking *PI3Kγ* in A549 cells reduced migration significantly. Data are presented as mean±SD from three separate experiments (each in duplicate). ■: wild-type; □: *PI3Kγ* gene deficient. FL: fluorescence. *: $p<0.05$ *versus* negative control without chemokine; #: $p<0.05$ *versus* positive control without inhibitor.

However, beneficial effects of *PI3Kγ* inhibition in ALI did not remain indisputable. Not inhibition, but activation of PI3K-dependent pathways was found to promote lung epithelial repair *in vitro* induced by Fas-induced apoptosis [38] or mechanical injury [39]. In *E. coli*-induced sepsis, pulmonary PMN accumulation and microvascular permeability was pronounced in *PI3Kγ*$^{-/-}$ mice and associated with increased expression of CD47 and β_3-integrins [16]. Consistent with our findings, the authors observed increased PMN counts in the lung interstitium by using morphometric analyses and suggested that upregulation of the CD47-associated β_3-integrin complex led to increased adhesion of PMNs within the extracellular matrix and accumulation of PMNs in the lung interstitium. Transepithelial migration into the BALF was not determined in that study. In endotoxaemic mice, nonspecific PI3K inhibition led to a state of hypercoagulation, increased release of cytokines and, most notably, increased mortality [40]. In addition, anti-inflammatory effects of lipoic acid or glucan phosphate, both stimulating the PI3K pathway, were abolished when PI3K signalling was blocked [41], indicating that the PI3K pathway is a physiological inhibitor of inflammation in endotoxaemia and sepsis. In a model of *S. pneumoniae*-induced lung inflammation, bacterial clearance was significantly reduced when *PI3K* signalling was inhibited, most likely due to a defect in respiratory burst and insufficient production of reactive oxygen species [17]. In addition, *PI3Kγ*$^{-/-}$ mice failed to sufficiently recruit monocytes into the lung while PMN trafficking remained unaffected, confirming cell-specific effects of *PI3K* signalling observed by others [42].

The activation of multiple PI3K-dependent pathways with opposing effects might be one explanation for the apparent discrepancies seen in lung injury in different studies [14, 16, 17, 40, 43]. It is also important to mention that so far, the use of nonselective PI3K inhibitors such as wortmannin or LY294002 hampered the validation of the *PI3Kγ* pathway as a therapeutic target.

Signalling of endothelial PI3K is known to mediate cell migration, vascular permeability and angiogenesis [44, 45] and has, therefore, been implicated as a promising target in various malignant diseases [46]. However, involvement of endothelial *PI3Kγ* in inflammatory responses has been controversial. *PI3Kγ* was not required for TNF-induced upregulation of NF-κB in human umbilical vein endothelial cells (HUVECs) [47]. Others, however, demonstrated *PI3Kγ*-dependent NF-κB binding to the intercellular adhesion molecule

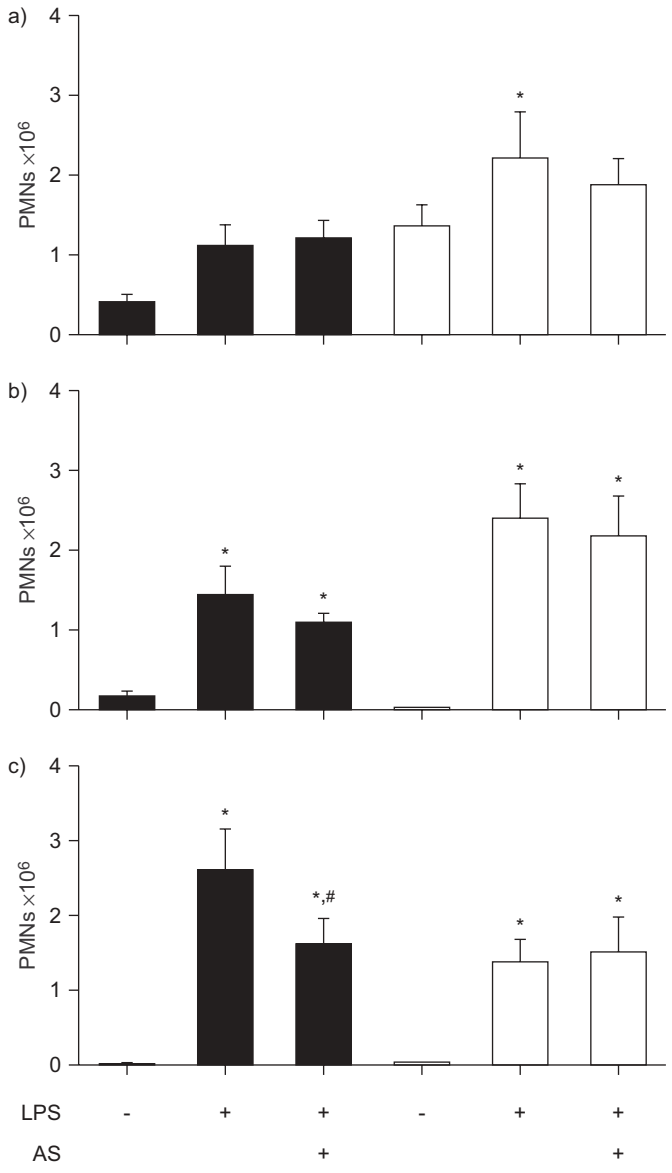

FIGURE 5. Effect of the phosphoinositide 3-kinase γ (*PI3Kγ*) inhibitor AS-605240 (AS) on lipopolysaccharide (LPS)-induced polymorphonuclear leukocyte (PMN) migration into the different compartments of the lung. Accumulation of PMNs in a) the vasculature, b) the lung interstitium and c) the bronchoalveolar space of wild-type (■) and *PI3Kγ* gene-deficient (*PI3Kγ*$^{-/-}$) (□) mice were analysed. Mice were pretreated 30 min prior to LPS exposure. AS-605240 significantly inhibited PMN migration in wild-type mice. No effect was seen in *PI3Kγ*$^{-/-}$ mice. Data are presented as mean±SD of five experiments. *: $p<0.05$ *versus* negative control without LPS; #: $p<0.05$ *versus* LPS without *PI3Kγ* inhibitor.

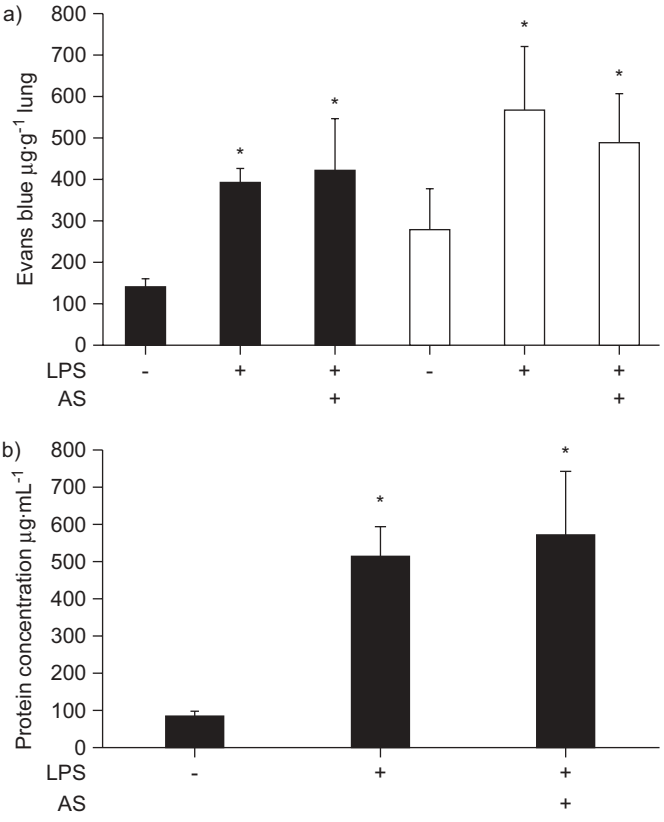

FIGURE 6. Lipopolysaccharide (LPS) inhalation induced a significant increase in microvascular permeability in wild-type (394±33 *versus* 151±11 µg·g^{-1} lung; $p<0.05$; ■) and phosphoinositide 3-kinase γ (*PI3Kγ*) gene-deficient (*PI3Kγ*$^{-/-}$) mice (568±153 *versus* 279±97 µg·g^{-1} lung; $p<0.05$; □) as assessed by the extravasation of Evans blue (a). Pretreatment with AS-605240 (AS) did not reduce LPS-induced microvascular permeability in wild-type mice. In addition, inhibition of *PI3Kγ* did not affect LPS-induced protein efflux into the bronchoalveolar lavage fluid (b). Data are presented as mean±SD from four experiments. *: $p<0.05$ *versus* negative control within the same group (wild-type or *PI3Kγ*$^{-/-}$ mice, respectively).

(ICAM)-1 promoter in pulmonary microvascular endothelial cells that was required for static adhesion of PMNs [48]. Chemokine-induced leukocyte adhesion was reduced in *PI3Kγ*$^{-/-}$ mice and in lethally irradiated wild-type mice that had been reconstituted with bone marrow from *PI3Kγ*$^{-/-}$ mice [49]. Interestingly, impairment of adhesion was not as severe when *PI3Kγ*$^{-/-}$ deletion was confined to bone marrow-derived cells (50% *versus* 80% reduction), underlining a contribution of nonleukocyte *PI3Kγ*. In our model, *PI3Kγ*-deficiency led to a three-fold increase in intravascular PMNs in the lungs. Several reasons may have contributed to this discrepancy. 1) SMITH *et al.* [49] tested the role of *PI3Kγ* in P-selectin-dependent adhesion. In our model, adhesion to the pulmonary microcirculation is P-selectin-independent (unpublished observation). 2) Cell trafficking in the systemic circulation differs substantially from the pulmonary microcirculation. Adhesion in the small pulmonary capillaries occurs largely independent of adhesion molecules and chemokines. 3) SMITH *et al.* [49] found that *PI3Kγ* was essential to keep leukocytes attached to post-capillary venules within a period of 60 s. In the lung, PMNs reside for a much longer time before they are released back to the circulation or migrate into the lung (1–2 h) [26]. Short time effects have not been investigated in the present study. 4) It is important to recognise that accumulation of PMNs in the pulmonary circulation is directly related to the migratory activity of these cells. Reduced migration into the alveolar space will increase numbers of PMNs in upstream compartments, *i.e.* interstitium and intravascular space.

In addition, endothelial but not leukocyte *PI3Kγ* mediated TNF-α-induced PMN adhesion to cremaster muscle venules,

and nonleukocyte *PI3Kγ* contributed to LPS-induced migration of PMNs into the BALF [19]. E-selectin-mediated adhesion of PNMs to cremaster muscle venules was almost completely abolished when *PI3Kγ* was absent on endothelial cells [19]. Others confirmed a role for *PI3Kγ* in chemokine-induced PMN transmigration but did not observe *PI3Kγ*-dependent adhesion and rolling [50]. In the present study, compartmentalisation of PMN trafficking in the lung revealed that adhesion to and transmigration through the pulmonary endothelium did not require *PI3Kγ*. Consistent with these findings, we found no effects when PECs were treated with AS-605240 *in vitro*. Although migratory activity was reduced when PI3Kγ was blocked in PMNs, the inhibitory effect of AS-605240 on neutrophil migration through an endothelial monolayer was comparable to that seen without monolayer. These studies indicate that PECs do not significantly contribute to PI3Kγ-mediatied PMN migration. In contrast, blocking PI3Kγ in human pulmonary epithelial cells significantly reduced PMN migration in an *in vitro* transmigration system (fig. 4c). This supports our hypothesis of a distinct role of epithelial PI3Kγ in pulmonary leukocyte trafficking.

In addition, *PI3Kγ*$^{-/-}$ mice had significantly higher PMN counts in the intravascular space than wild-type mice (figs 1b and 2a). This increased availability of intravascular neutrophils may have contributed to increased migration of PMNs through the endothelial barrier in *PI3Kγ*$^{-/-}$ mice. However, transepithelial migration into the alveolar airspace was significantly reduced when *PI3Kγ* was absent. The defect was prominent in *PI3Kγ*$^{-/-}$ mice and remained when *PI3Kγ* function on leukocytes was restored. The mechanisms that link nonleukocyte *PI3Kγ*-signalling to the recruitment of inflammatory cells are not fully understood, but PI3K-dependent activation of adhesion molecules appears to be involved. In HUVECs, cytokine-induced expression of ICAM-1 and vascular cell adhesion molecule-1 involves PI3K-signalling [51]. Others, however, demonstrated that PI3K rather suppressed the expression of adhesion molecules on endothelial cells [52]. ICAM-1 is a critical mediator in LPS-induced lung injury [7]. It is worth mentioning that ICAM-1 on alveolar and bronchial epithelium significantly contributes to inflammatory leukocyte recruitment to the lung [53]. PI3K deletion may reduce epithelial ICAM-1 expression and result in disturbed transepithelial migration that has been observed in our study. Additional mechanisms of nonleukocyte PI3K-signalling in inflammation include activation of heat shock protein 70 [54] and release of reactive oxidant species [48].

In summary, our study reveals a differentiated role of *PI3Kγ* signalling in LPS-induced PMN trafficking in the lung. Our findings point to a specific role of *PI3Kγ* for the transepithelial migration into the alveolar space that involves *PI3Kγ* on nonhaematopoietic cells. A small-molecule *PI3Kγ* inhibitor effectively reduced PMN transmigration but did not reduce LPS-induced microvascular permeability. Further investigations are required to determine its therapeutic potential in ALI.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

A statement of interest for T. Rückle can be found at www.erj.ersjournals.com/misc/statements.dtl

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