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RESEARCH ARTICLE

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitors reduce vascular inflammation *in vitro* and *in vivo*

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Background and Purpose: Endothelial cells play central roles in increasing vascular permeability and leukocyte recruitment. Therapeutic approaches for treating endothelial cell barrier dysfunction to reduce unwanted fluid accumulation in tissues are limited. Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) enzymes are implicated in signalling inflammatory endothelial permeability and leukocyte recruitment. We investigated the ability of PI3K inhibitors to influence cutaneous oedema formation and neutrophil accumulation.

Experimental Approach: We used cultured endothelial cells to determine the effects of inflammatory mediators on permeability and vascular leakage, and a murine model of vascular inflammation in mouse skin *in vivo*. The effects of inflammatory mediators that induce vascular leakage and neutrophil accumulation (TNFα, IL-1β and C5a) were examined, with the neuropeptides substance P and α-CGRP used as controls. The ability of PI3K inhibitors to modulate inflammatory responses was studied.

Key Results: A broad spectrum PI3K inhibitor (PI-103) and a selective inhibitor of the class 1A p110α catalytic subunit (BYL-719/alpelisib) inhibited endothelial morphological changes and permeability induced by TNFα and IL-1β *in vitro*. *In vivo*, oedema and neutrophil accumulation induced by TNFα and IL-1β, but not by the complement fragment C5a, is inhibited by BYL-719, whereas PI-103 blocks effects of all three mediators. Neither influences the acute oedema formation induced by neuropeptides.

Conclusions and Implications: Selective p110α inhibition of vascular inflammation may provide a novel therapeutic pathway for limiting adverse tissue swelling.

Abbreviations: HMVEC-Ds, human dermal microvascular endothelial cells; VE-cadherin, vascular endothelial-cadherin.

Moreover, the limited effect of BYL-719 on C5a-mediated responses implies that this innate component of the immune response will continue to provide essential defence activity during p110α blockade.

KEYWORDS
endothelial cell, inflammation, neutrophil, oedema formation, phosphoinositide-3 kinase inhibitors

1 | INTRODUCTION

Endothelial cells (ECs) that line the postcapillary venules become permeable to fluids, plasma proteins and leukocytes, in response to inflammatory mediators (Pober & Sessa, 2007; Shasby et al., 1982). This can be detrimental in many inflammatory conditions, including cancer (Coussens & Werb, 2002), acute respiratory distress system (Bhatia & Moochhala, 2004) and heart failure (Clark & Cleland, 2013). There are few effective targeted drugs that act to reduce the vascular leakage, which can be fatal (Phoenix et al., 2022).

The leakage mainly occurs via paracellular EC junctions (Vandenbroucke et al., 2008). EC cell–cell junctions include adherens junctions and tight junctions, which act together with the associated actin cytoskeleton to regulate endothelial permeability. Adherens junctions contain the transmembrane cell adhesion molecule vascular endothelial (VE)-cadherin, which is linked to the actin cytoskeleton via a protein complex associated with its cytoplasmic tail, including **β-catenin** and α-catenin (Andriopoulou et al., 1999; Dejana, 2004). Inflammatory stimuli lead to an increase of vascular permeability via phosphorylation-driven VE-cadherin/catenin complex destabilization (Wessel et al., 2014). This is accompanied by actin and myosin filament contraction, which further disrupts junctions (Yamada et al., 2005). The VE-cadherin–catenin-actin complex is also modified to allow transient opening of endothelial junctions during leukocyte extravasation.

Multiple signalling proteins regulate EC junctions and neutrophil accumulation including **phosphoinositide 3-kinases (PI3Ks)** (Durrant & Hers, 2020; Leevers et al., 1999), which are composed of three main classes (I–III) (Cain et al., 2012; Puri et al., 2004). Class I are the best characterized subfamily of PI3Ks and are heterodimers composed of a regulatory domain (p85 or p101/p84) and a catalytic domain (p110α, p110β, p110γ and p110δ) (Cantley, 2002). **p110α (PI3Kα)** and **p110β (PI3Kβ)** are widely expressed, including in ECs, whereas expression of **p110γ (PI3Kγ)** and **p110δ (PI3Kδ)** is more restricted, for example, to immune cells such as neutrophils, ECs and some cancer cells (Cain et al., 2012). Neutrophil-expressed isotypes especially PI3Kγ have been linked to microvascular permeability in mice (Hao et al., 2019). However, PI3Kγ and PI3Kδ are also expressed in ECs (Cain et al., 2010; Fruman et al., 2017). **PI-103** is an effective antitumour agent, but its relatively broad specificity is associated with toxic side effects (Raynaud et al., 2007). PI-103 is also an **mTOR** inhibitor, and it is possible that combined PI3K/mTOR inhibitors may have additional antitumour and vascular effects (Vilchez et al., 2017), although inhibitors of mTOR signalling did not affect EC permeability *in vitro* (Cain et al., 2012). By comparison, more selective PI3K p110α inhibitors have been developed (Fritsch et al., 2014; Furet et al., 2013). The p110α-selective inhibitor **BYL-719 (also known as alpelisib, Piqray®)** was evaluated in the SOLAR-1 clinical trial and is now approved for the treatment of cancer (Andre et al., 2019; Dao et al., 2022). It has an IC_{50} in the nM range, whereas its IC_{50} for affinity for p110β, p110γ and p110δ is >50 times less, and it has no activity for mTOR or related kinases. Moreover, its IC_{50} or K_d value for a wide panel of other kinases is >50 fold lower than for p110α; for many, it has no activity (Fritsch et al., 2014).

We have previously investigated the roles of the four class I p110 catalytic subunits in **TNFα**-induced EC responses *in vitro* and identified p110α as the main isotype involved in increased endothelial

What is already known

- The PI3K isotype p110α mediates some TNFα-induced responses in cultured endothelial cells.
- PI3K inhibitors have anti-inflammatory effects, but their mechanisms of action are unclear.

What does this study add

- A broad-spectrum PI3K inhibitor blocked oedema and transendothelial migration to all neutrophil-dependent mediators tested.
- A p110α-selective inhibitor inhibited proinflammatory cytokine-induced leakage (TNFα, IL-1β), but not that to C5a.

What is the clinical significance

- Defining how PI3K 110α affects inflammatory oedema formation could be important for potential therapeutic treatment.
- This may include conditions where vascular leakage in inflammatory and cardiovascular disease is damaging.

permeability and leukocyte transendothelial migration (Cain et al., 2010). TNFα can activate neutrophils (Tecchio et al., 2014), and amplify inflammation (Johnson et al., 2013) involving neutrophil-dependent oedema formation *in vivo* (Wedmore & Williams, 1981; Zarban et al., 2023). PI-103 is effective at reducing endothelial permeability and leukocyte transendothelial migration *in vitro* (Cain et al., 2012). However, the role of p110 proteins *in vivo* is poorly investigated.

Here, we have evaluated the ability of PI-103 and the selective p110α inhibitor (BYL-719) to reduce endothelial cell permeability and inflammatory oedema formation. The objective was to learn how PI3Ks contribute to vascular inflammation and whether selective inhibition of p110α offers advantages as a potential therapy, compared with a less selective inhibitor. Their effect on ECs was studied *in vitro* and extended to an *in vivo* murine cutaneous inflammation model, which also involved the measurement of neutrophil accumulation. The experimental design included study of the effects of the two inhibitors on TNFα and **IL-1β** responses in addition to those of the chemotactic complement fragment **C5a** and the neuropeptides **substance P (SP)** and **alpha calcitonin gene-related peptide (α-CGRP)**. Both PI3K inhibitors markedly reduced oedema formation and neutrophil accumulation *in vivo*, but our results revealed differences between the inhibitors depending on the stimulus.

2 | METHODS

2.1 | *In vitro* primary cell culture

Pooled primary human umbilical vein endothelial cells (HUVECs, Lonza, Slough, UK) or human dermal microvascular endothelial cells (HMVEC-Ds, Lonza, Slough, UK) were grown on plasticware coated for 1 h with human fibronectin (Sigma-Aldrich, Gillingham, UK; 10 μg·ml^{-1}) in Dulbecco's phosphate buffered solution (DPBS) in a humidified incubator at 37°C with 5% CO_2. Cells were grown in endothelial cell basal medium-2 (EBM-2, Lonza) with endothelial cell growth supplement-2 (EGM-2) for HUVECs and microvascular EGM2 (EGM2-MV) for HMVEC-Ds. Cells were used up to passage 5.

2.2 | Indirect immunofluorescence (IF) assay and confocal imaging

ECs (5×10^4 cells per well) were plated on μ-slide eight-well chambers (Ibidi) coated with 10 μg·ml^{-1} fibronectin for 24 h, followed by treatment with either 10 ng·ml^{-1} of TNFα or IL-1β (R&D Systems, Abingdon, UK) for 18 h. ECs were then treated with different concentrations of each inhibitor for 1 or 6 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X 100 in Dulbecco's phosphate buffered solution followed by blocking with 3% bovine serum albumin (BSA) with 0.05% Tween-20. Fixed samples were incubated with mouse antihuman VE-cadherin antibody (BD Biosciences, #555289 Wokingham, UK; RRID:AB_395707) and Alexa Fluor 488 goat anti-mouse secondary antibody (Life Technologies, Thermo Fischer Scientific), then stained with Alexa Fluor 546-conjugated phalloidin (to label F-actin; Life Technologies) and **DAPI** (to label nuclei; Life Technologies). The cells were mounted with fluorescence mounting medium (Dako).

A Zeiss LSM510 confocal laser-scanning microscope with a Plan-Apochromat 20× DIC M27 objective and ZEN software was used to acquire images of fluorescently stained ECs. Images were taken at a resolution of 512 × 512 pixels using an average of two or four frames. The range finder function in the ZEN software was used to adjust the gain and offset to avoid saturation and high background in images. Images in each experiment were acquired using the same gain and offset settings. Quantification of cell nuclei (average number per image), spread area in square micrometre and cell elongation (the ratio between the *x*-plane (width) and *y*-plane (length) of the cell), was calculated using ImageJ software (NIH). For junctional analysis, fluorescence was calculated per field by using the VE-cadherin–antibody-stained channel, thresholding the image to create an even intensity stain and quantifying its intensity (Cain et al., 2010) using the software analysis options of ImageJ. For each treatment, a minimum of five fields were quantified (∼20 cells per field) per experiment, and data shown represent the mean of at least three independent experiments. For analysis, where data were normalized to control, a control section with typical morphology was chosen. This section was set to 100%, and all other sections were compared with it. The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.3 | Permeability *in vitro* assay

ECs were seeded in the upper compartment of transwell filter inserts (pore size 0.4 μm, 12 mm diameter, polyester membrane, Corning) that were previously coated with 10 μg·ml^{-1} fibronectin for 1 h. The lower compartments of the transwell chambers were filled with 1 ml EGM-2 (phenol red-free) followed by 18-h treatment with either TNFα or IL-1β (0.6 nM) or C5a (50 nM). Cells were then treated with different concentrations of each PI3K inhibitor (0.1–10 μM) for the last hour of the incubation period. After a 1 h incubation with the inhibitors, fluorescein isothiocyanate (FITC)-dextran (40 kDa, 0.1 mg·ml^{-1}; Sigma-Aldrich) was added to the upper compartment for 1 h. Fluorescence (ex: 485 nm; em: 535 nm) was measured with a fluorescence plate reader (CLARIOstar. BMG Labtech) and data were expressed as relative changes compared to control levels (Cain et al., 2012). All experiments were performed in technical duplicates and results shown are the mean of at least three independent experiments. The clarity of these *in vitro* results and the fact that they were performed to support *in vivo* results meant we limited the n number to 3 independent experiments only.

2.4 | *In vivo* dorsal skin oedema inflammation model

CD1, male, wildtype (WT) mice (30–35 g, 8–10 weeks; Charles River, UK) were obtained and acclimatized in a climatically controlled environment and allowed food and water *ad libitum*. Experiments were conducted according to the U.K. Animal studies and are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020), with local ethics approval from King's College London. The mice were kept under a 12-h light:12-h dark lighting regime at 21 ± 1°C and 45–65% humidity in Tecniplast 1284 Euro standard type 2 H-Temp Polysulfone (PSU) conventional cages with up to 5 mice per cage. They were allowed a normal mouse diet and water, without any restrictions. Mice displayed normal growth and behavioural characteristics and were weighed prior to procedures. Inhibitors (dissolved in DMSO with final concentration of <5% in saline) or their vehicles were given as pretreatments for 30 min intraperitoneally (i.p.). Group sizes were of at least five and randomly allocated to treatment. Experiments were carried out in a blinded manner as far as possible.

Procedures were performed under anaesthesia with 5% and 2% isoflurane for induction and maintenance respectively with 1% O_2 in an adapted and previously published method (Sawyer et al., 2011; Zarban et al., 2023). Following pretreatment with the inhibitors, Evans blue dye (Sigma, 5 mg·kg^{-1} i.v.) was administered. This was used as a biomarker of plasma extravasation, due to its high affinity for albumin. The dorsal skin was shaved, and sites were marked out according to a balanced site pattern. Intradermal (i.d.) injections of test agents or controls were given with a 27G needle, 50 µl per site and areas of response zones were further marked out. This was a blinded study and injections were randomly allocated (Zarban et al., 2023). TNFα and IL-1β were purchased from Novus Bio (Colorado, USA). C5a (full peptide) was from R&D Systems, Substance P, SP from Sigma-Aldrich UK and human α-CGRP from AnaSpec Inc., Fremont, USA. These agents were solubilized in Tyrode's solution (in mmol·L^{-1}: NaCl 137; KCl 2.7; $MgCl_2$ 0.5; NaH_2PO_4 0.4; $NaHCO_3$ 11.9; glucose 5.6), which was used as the vehicle control (non-injected and sham sites were also recorded). Mice were allowed to recover for the experimental time required (up to 4 h), re-anaesthetized and killed by cervical dislocation. See Figure S7 for a schematic. Samples were taken from these experiments for future analysis of oedema formation and also in the same samples for neutrophil accumulation (as Zarban et al., 2023). When needed body temperature was maintained with a heating blanket, maintained at 37°C.

Oedema formation in the mouse model was quantified for each site using the following equation: oedema volume (mm^3) = (π/6) × length × width × depth (measuring three planes of view) from each of the i.d. sites and calculated to obtain volume (mm^3), as Zarban et al. (2023).

2.5 | Myeloperoxidase assay

The same skin samples, as described above, were analysed for **myeloperoxidase (MPO)** measurements (as below), using an experimental design that has been recently published and reflects the expected level and site of neutrophil accumulation (Zarban et al., 2023). This technique was refined to allow the measure of oedema formation and neutrophil accumulation in the same samples allowing us to reduce the number of animals used (Zarban et al., 2023).

Areas of treatment from the dorsal skin were collected (16 mm diameter), weighed and snap-frozen in liquid N_2, then stored at −80°C. Skin samples were homogenized in 500 µl of homogenization buffer per half skin sample (0.1-M NaCl, 0.02-M $NaPO_4$ pH 4.7, 0.015-M EDTA, 0.5% HTAB) and lysed using QIAGEN TissueLyser II at 30 Hz for 5 min, three times, cooling in between. Skin samples were centrifuged at 13,000*g* for 15 min at 4°C and the supernatant was collected. Reactions were performed in 96-well plates at room temperature. Neutrophil accumulation was measured by comparing MPO activity in extracts from that in MPO standards and compared with that at skin sites (µg·mg^{-1} skin site). H_2O_2 oxidation of TMB Blue (tetramethylbenzidine, Bionostics, Skybio, Wyboston, Beds, UK) was used to determine MPO activity. Absorbance (OD) was read at 620 nm using a spectrophotometer and a standard curve was plotted of OD against MPO in the standard samples. Protein (BSA) concentrations were also measured in an identical fashion to standardize MPO measurements, where OD was read at 700–750 nm, thus acquiring the quantification of MPO/protein in each condition. This is justified as an index of neutrophil accumulation as described by Zarban et al. (2023).

2.6 | Neutrophil depletion assay and flow cytometry

Neutrophil depletion was achieved with one dose of monoclonal anti-Ly6G antibody (clone 1A8, BioXCell,25 mg·kg^{-1} i.p.; RRID:AB_2566317) 24 h prior to carrying out the dorsal skin oedema inflammation model. Equivalent doses of monoclonal anti-trinitrophenol (clone 2A3, BioXCell, trinitrophenol AB_1107769, which is not present in mammals) were administered to control mice. At 4 h after the dorsal skin model, mice were terminally anaesthetized and whole blood collected via cardiac puncture for flow cytometric analysis of neutrophils. The heparinized blood (1:100 stock heparin) was incubated with the following antibodies: anti-Ly6G-BV785 (1:200, Biolegend, Cat.127645; RRID:AB_2566317), anti-Ly6C BV605 (1:200, Biolegend, Cat. 128035; RRID:AB_2562352) and anti-CD45-APC-Cy7 (1:200, Biolegend, Cat. 103115; RRID:AB_312980). Samples were assessed in a LSRFORTESSA flow cytometer (BD Biosciences, Oxford, UK) and analysed using FlowJo software 9.7.5 (Ashland, USA).

2.7 | Treatments and inhibitors

PI3K inhibitors were dissolved to their stock concentration in 100% DMSO (dimethyl sulfoxide) and diluted in buffer for use. Final DMSO concentrations for *in vitro* studies were all less than 0.5% DMSO in buffer. Similarly, for *in vivo* experiments, drugs were dissolved in DMSO at a final concentration of <5% in saline.

2.8 | Data analysis

All data are from at least three independent experiments and *in vivo* experiments were of at least n = 5. Statistical significance was performed using GraphPad Prism version 7.0 software. Graphs were plotted as mean ± standard error of the mean (SEM) with appropriate post-hoc tests (Sidak's or Tukey's) post-hoc were conducted only if F achieved $P < 0.05$ and there was no significant variance in homogeneity. A threshold of $P < 0.05$ was considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2025).

2.9 | Materials

Details of other materials and suppliers are provided in the Supporting Information (Table S1).

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander et al., 2023a, 2023b, 2023c).

3 | RESULTS

3.1 | PI3K inhibition attenuates effects of proinflammatory cytokines on ECs

We chose to study the effects of PI3K inhibition in HUVECs first, following our previous studies in HUVECs to investigate the roles of class I PI3Ks in TNFα responses *in vitro* (Cain et al., 2010, 2012). We quantified the cell numbers from nuclei for the BYL-719 1-h pretreatment groups for both TNFα and the IL-1β groups. Results (mean ± SD, n = 3 with five replicates for each) are as follows for TNFα groups:- Control 112 ± 5.9, TNFα 68.5 ± 7.5, TNFα + BYL-719 (1 μM) 59.6 ± 7.2, TNFα + BYL-719 (3 μM) 72.7 ± 4.0, TNFα + BYL-719 (10 μM) 82 ± 9 cells per image. Thus, TNFα treatment revealed a decrease in cell nuclei, in keeping with a loss of viability, but this trend was not further observed with addition of BYL-719. This trend was not observed with the IL-1β groups. Results are as follows for IL-1β groups: control 140.3 ± 29, IL-1β 130.3 ± 30.6, IL-1β + BYL-719 (1 μM) 100.7 ± 8.3, IL-1β + BYL-719 (3 μM) 153.3 ± 18.9, IL-1β + BYL-719 (10 μM) 140 ± 23.8 cells per image.

The main objective was to test whether the addition of either a non-selective PI3K inhibitor (PI-103) or a p110α (PI3Kα)-selective inhibitor (BYL-719) altered either TNFα- or IL-1β-induced morphological changes. Inhibitor treatment for 1 h induced a concentration-dependent reversal of TNFα- or IL-1β-induced decreases in cell area and increases in elongation in HUVECs. Both BYL-719 and PI-103 at 3 μM reversed the responses by approximately 80% whilst a 10-μM dose inhibited the effect of both TNFα and IL-1β (Figure 1a–h). The 3-μM dose was selected for further studies.

We examined whether treatment of ECs with inhibitors for 6 h had a greater effect on cell morphology changes than 1 h. This could implicate sustained effects. For HUVECs, BYL-719 or PI-103 treatment for 6 h showed a trend to increased inhibitory effects on TNFα and IL-1β responses (Figure S1). The microvasculature is the primary site of increased vascular permeability, so we also investigated human dermal microvascular endothelial cells (HMVEC-Ds). Like HUVECs, TNFα and IL-1β induced a decrease in HMVEC area and increase in elongation, which showed a reversal trend with 1 and 6 h of inhibitor treatment, that was greater with 6-h inhibitor treatment in some cases (Figure S2).

TNFα has previously been shown to reduce VE-cadherin localization to EC cell–cell junctions, which is associated with increased permeability (Cain et al., 2010, 2012; Herwig et al., 2013; McKenzie & Ridley, 2007). Consistent with this, we observed a trend towards a decrease in VE-cadherin at cell–cell junctions in both HMVEC-Ds and HUVECs (Figure 2, Figure S3). Treatment with inhibitors for 1 h only had a small effect on VE-cadherin, whereas after a 6 h incubation the effects of TNFα and IL-1β showed a strong trend to complete reversal to resemble VE-cadherin localization in control untreated cells (Figure 2c–f, Figure S3), indicating the time dependency of this effect. The findings are in keeping with knowledge that PI3Ks can influence VE-cadherin activity (Tzima et al., 2005). A limitation is that we did not test whether total VE-cadherin levels were affected (e.g. by western blotting), although we have previous shown that p110α depletion or PI-103 treatment did not alter total VE-cadherin levels in TNFα-treated HUVECs (Cain et al., 2010, 2012), and thus, it is unlikely that they changed in these experiments. As previously observed, actin stress fibres were increased after stimulation with TNFα (McKenzie & Ridley, 2007) and were significantly inhibited by the PI3K inhibitors.

3.2 | Characterization of oedema-inducing mediators *in vivo*

We utilized the mouse skin multiple site dorsal skin assay, adapted from previous works (Sawyer et al., 2011, Zarban et al., 2023). Experiments were designed to investigate the effect of the cytokines TNFα and IL-1β that are traditionally considered to mediate oedema formation and neutrophil accumulation *in vivo*. We included the complement chemotactic fragment C5a that also induces oedema formation and a combination of sensory neuropeptide peptides (substance P and α-CGRP) that induce acute oedema formation independent of neutrophil involvement (Brain & Williams, 1985). Consistent with our previous observations, both C5a and the neuropeptide combination induced oedema formation after 30 min (Figure 3a); with C5a also inducing neutrophil accumulation after 30 min (Figure 3b). By contrast, TNFα and IL-1β did not induce oedema formation or neutrophil accumulation after 30 min (Figure 3a). Neutrophil accumulation was

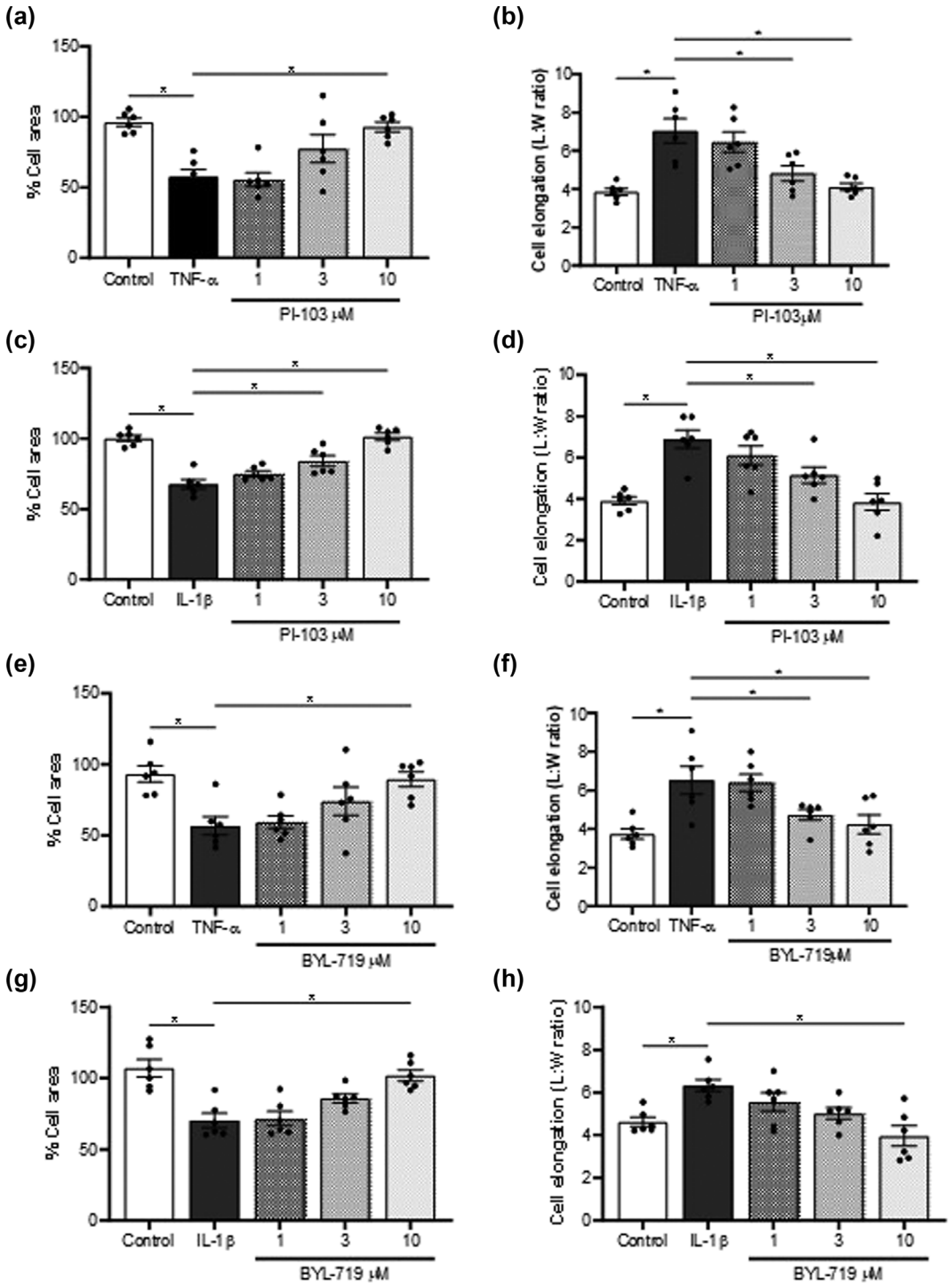

FIGURE 1 Endothelial cell morphological changes induced by inflammatory mediators and the inhibition of these effects by PI3K inhibitors. The effect of PI-103 (a–d) and BYL-719 (e–h) on HUVEC cell area (left) and elongation (right) in the presence of TNF-α or IL-1β (0.6 nM) treatment. HUVECs were initially treated with TNF-α or IL-1ß (0.6 nM) for 18 h, followed by 1 h treatment with the relevant PI3K inhibitor at three concentrations: 1, 3 and 10 μM. Data are presented as mean ± SEM, one-way ANOVA plus Tukey's test; n = 6 independent experiments, five images/condition assayed in duplicates and 10–20 cells/image; *$P < 0.05$ between control and either TNF-α or IL-1β and between TNF-α or IL-1β and different drug concentration treatments.

assessed by measuring levels of the major neutrophil-derived enzyme MPO. At 4 h, both TNFα and IL-1β induced neutrophil accumulation and oedema formation (Figure 3c,d). Of note, by 4 h, the oedema-inducing effect of the neuropeptide combination of SP and α-CGRP had diminished, but the effect of C5a was more sustained, in terms of both oedema formation and MPO activity.

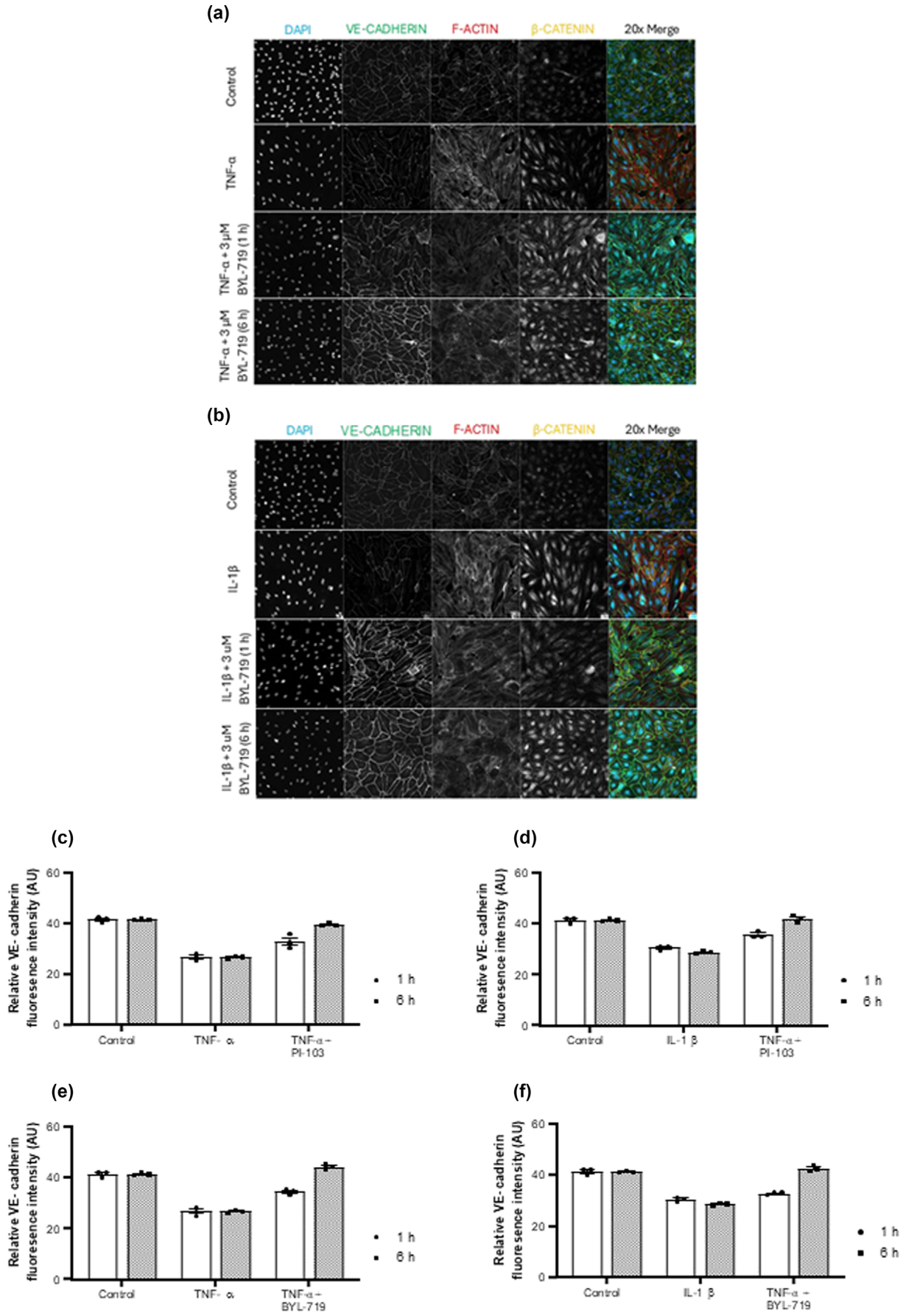

FIGURE 2 Legend on next page.

FIGURE 2 Time-dependent changes in intracellular VE-cadherin staining after PI3K inhibitor treatment. Representative confocal images showing HMVEC-Ds alone, cells treated with (a) TNF-α (0.6 nM) for 18 h or (b) IL-1β (0.6 nM) and then with BYL-719 (3 μM for 1 or 6 h). Cells were then fixed, permeabilized and stained for nuclear staining by DAPI, and for VE-cadherin, F-actin with phalloidin and β-catenin at 20× magnification. Data are mean ± SEM; n = 3 independent experiments, five images/condition in duplicates and 10–20 cells/image. The effect (trend) of PI-103 (b,c) and BYL-719 (d,e) on relative levels of VE-cadherin in the presence of TNF-α or IL-1β (0.6 nM) treatment is shown. HMVEC-Ds initially treated with TNF-α or (0.6 nM) for 18 h, followed by 1 or 6 h PI3K inhibitor treatment at 3 μM.

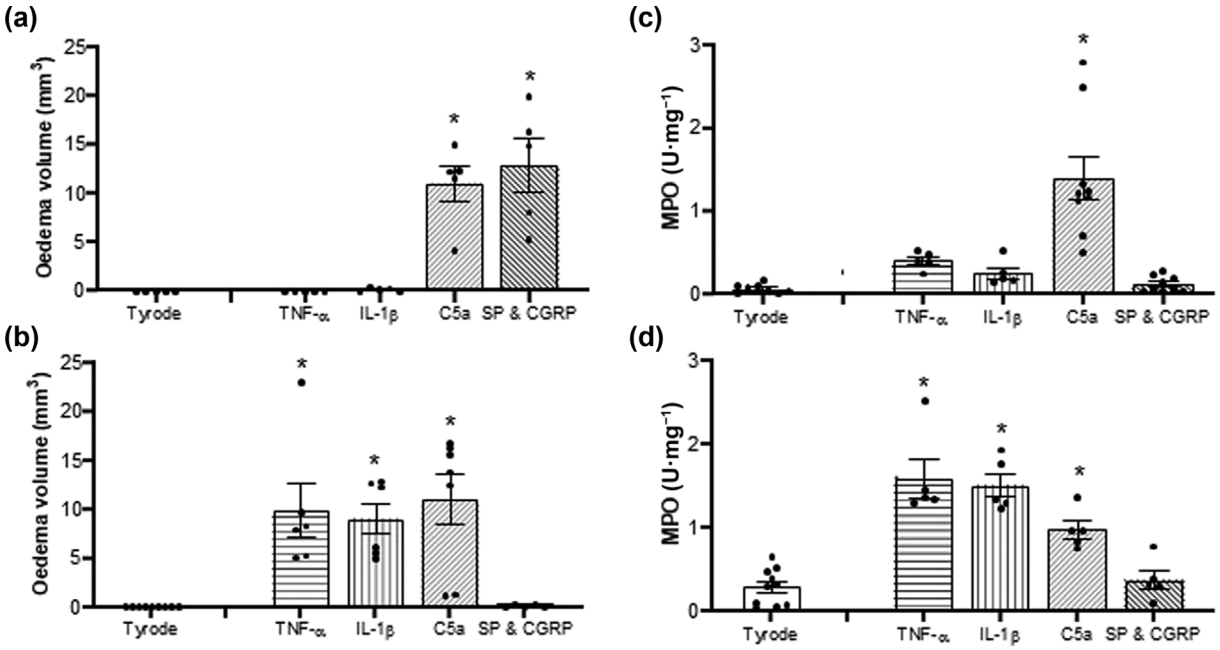

FIGURE 3 Characterization of oedema-inducing mediators and neutrophil accumulation mice at 1 and 4 h *in vivo*. Mice were with Evan's blue dye (i.v. oedema marker), followed by intradermal injections of Tyrode (control), TNF-α (0.6 pmol), IL-1β (0.6 pmol), C5a (30 pmol), SP (300 pmol) and α-CGRP (20 pmol) per 50 μl for 30 min (a,b) or 4 h (c,d). Data are mean ± SEM, one-way ANOVA plus Tukey's test; n = 5–9 independent experiments with duplicate sites; $^*P < 0.05$, between vehicle control (Tyrode) and treatments.

3.3 | PI3K inhibitors differ in their effects on TNFα and IL-1β but not C5a responses

Having established optimal conditions for oedema formation and neutrophil accumulation for the panel of stimuli, we then investigated the effects of the two PI3K inhibitors on these responses. The effects of the neuropeptides were not inhibited by either PI3K inhibitor (Figure S4), providing a valuable control, showing that the microvasculature could function normally. We then investigated the PI3K inhibitors or vehicles on TNFα, IL-1β and C5a responses simultaneously in the same mouse over 4 h. Whilst there was clear oedema formation in vehicle-treated (control) mice (Figure 4a,b, Figure S5), PI-103 reduced both neutrophil accumulation (MPO) and oedema after 4 h for all three stimuli (Figure 4a,b, Figure S5). BYL-719, on the other hand, reduced MPO and oedema production only in the presence of TNFα and IL-1β but did not affect either neutrophil accumulation or oedema formation induced by C5a (Figure 4c,d, Figure S5).

To decipher the specific role of ECs in PI3K-dependent permeability, we used a permeability assay *in vitro*. HMVEC-Ds (Figure 5) or HUVECs (Figure S6) were treated with either TNFα (0.6 nM), IL-1β (0.6 nM) or C5a (50 nM) for 18 h. All three mediators increased endothelial permeability. The nonselective inhibitor, PI-103, produced a concentration-dependent reduction in permeability of all mediators including C5a (Figure 5a–c, Figure S6a–c). By comparison, BYL-719 (0.1–10 μM) produced a dose-dependent inhibition of EC permeability induced by both TNFα and IL-1β but did not affect permeability induced by C5a (Figure 5d–f, Figure S6d–f).

3.4 | Influence of neutrophil depletion on oedema responses

We designed *in vivo* experiments to determine if the selective p110α inhibitor is acting primarily on neutrophils or ECs. To investigate this, we depleted circulating neutrophils with an antibody targeting Ly6G (Daley et al., 2008). Mice were administered either control IgG or anti-Ly6G blocking antibody at 25 mg·kg^{-1} i.p. at 24 h prior to the dorsal skin assay. Flow cytometry analysis revealed >95% of Ly6G^{hi} neutrophils were depleted from mouse blood 24 h after treatment with anti-Ly6G antibody (Figure 6), as Zarban et al. (2023).

The acute SP and α-CGRP-induced oedema formation (when given at 3.5 h for the last 30 min of the experiment) was independent

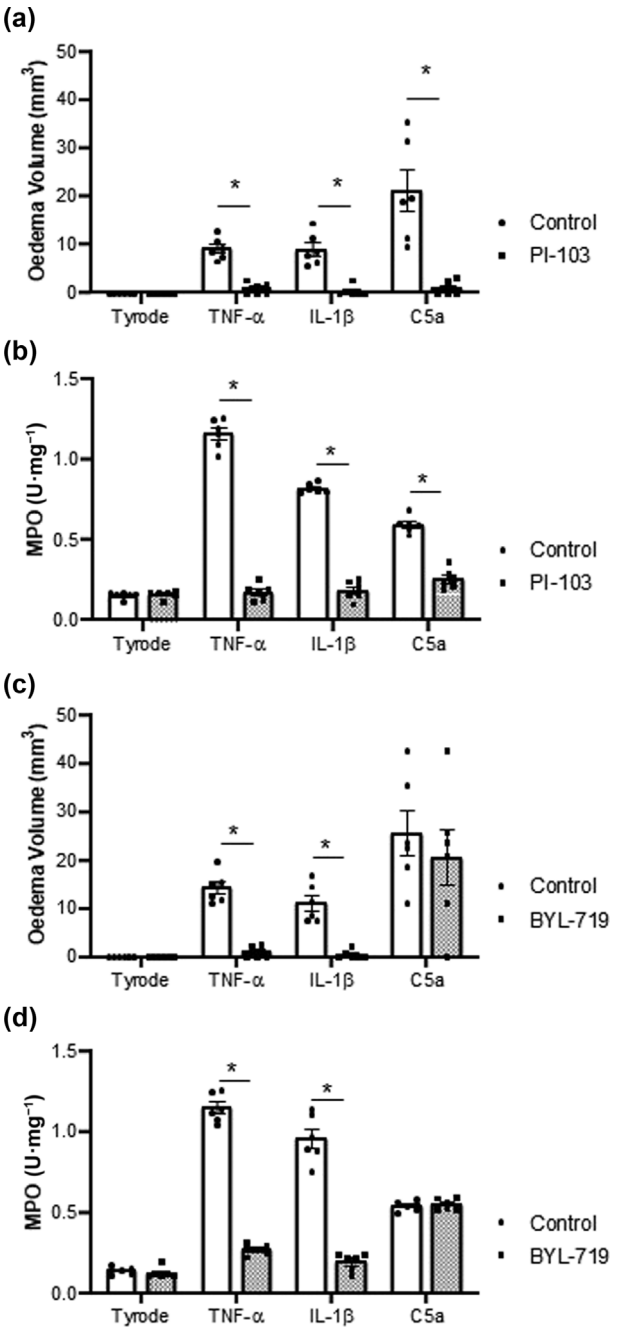

FIGURE 4 Effect of PI3K inhibitors on inflammatory and oedema-inducing mediators *in vivo*. Mice were pretreated for 1 h with either PI-103 (0.09 mmol·kg^{-1} i.p., a,b) or BYL-719 (0.11 mmol·kg^{-1} i.p., c,d) after which Evan's blue dye (i.v., oedema marker), followed by intradermal injections of Tyrode (control), TNF-α (0.6 pmol), IL-1β (0.6 pmol) and C5a (30 pmol) per 50 μl, for 4 h. Data are mean ± SEM, two-way ANOVA plus Tukey's test; n = 6 independent experiments with duplicate sites; *P < 0.05, between i.p. control and PI3K inhibitor treatments.

of neutrophils (Figure 7a), since neutrophil depletion did not prevent oedema formation. This also revealed that neutrophil depletion did not affect endothelial function. Neutrophil depletion abrogated the levels of MPO measured, consistent with the major source of MPO being neutrophils (Bradley et al., 1982; Zarban et al., 2023) (Figure 7b). Consequently, neutrophil-depleted mice did not induce oedema formation in response to TNFα, IL-1β or C5a, confirming that neutrophils are required for the oedema formation induced by all three of these agents *in vivo*. This indicates that the effect of the PI3K inhibitors is primarily on the neutrophil-mediated responses *in vivo*.

4 | DISCUSSION

This investigation has revealed that selective p110α (PI3Kα) inhibition of vascular inflammation may provide a novel therapeutic approach for limiting adverse oedema formation and neutrophil accumulation induced by the inflammatory cytokines TNFα and IL-1β. We identify that the broad spectrum PI3K inhibitor (PI-103) and the selective PI3K p110α inhibitor (BYL-719) inhibit permeability induced by the pro-inflammatory cytokines TNFα and IL-1β *in vitro* and *in vivo*. PI-103 also blocks the effects of the chemotactic chemokine C5a, whereas BYL-719 does not alter the responses to C5a. The lack of inhibitory effect of BYL-719 on C5a-mediated responses and additionally on acute neuropeptide responses implies that these innate components of the immune response will continue to provide essential defence activity against infection during p110α blockade. This may be beneficial in limiting adverse effects associated with removing the total immune response.

We previously chose HUVECs to investigate the roles of class I PI3Ks in TNFα responses *in vitro*, using siRNAs and small molecule inhibitors to determine their effects on endothelial morphology, cell–cell junctions, endothelial permeability and leukocyte transendothelial migration (Cain et al., 2010; Cain et al., 2012). In these studies, only p110α depletion reduced TNFα-induced responses, whereas depletion of other p110 isotypes or treatment with a p110δ-selective inhibitor **IC-87114** had no effects. We therefore chose to use a p110α-selective inhibitor, BYL-719, rather than other isotype-selective inhibitors. We also initially used HUVECs in this study to compare the effects of TNFα and IL-1β on endothelial morphology, investigating the same concentrations and timepoints that had been optimized previously in these *in vitro* experiments (Cain et al., 2010; McKenzie & Ridley, 2007). TNFα and IL-1β each decreased HUVEC spread area and increased cell elongation at 18 h after treatment. We note that TNFα showed a trend in reducing cell nuclei/numbers, possibly due to induction of apoptosis or lack of cell proliferation/adhesion. However, numbers did not further change in the presences of the PI3K inhibitors, suggesting that they did not show a trend to further reduce cell viability. We chose to treat cells with inhibitors for the final 1 h of cytokine treatment (Cain et al., 2012). The effect of TNFα on cell elongation is consistent with our observations, correlating with the alignment of stress fibres along the longest cell axis (Cain et al., 2010; Millán et al., 2010), but the effects of IL-1β on endothelial morphology has not previously been reported to our knowledge. Here, we also found similar effects of the inhibitors on HMVEC-Ds, chosen as these are taken from microvascular blood vessels that would be the site of leukocyte emigration and increased permeability

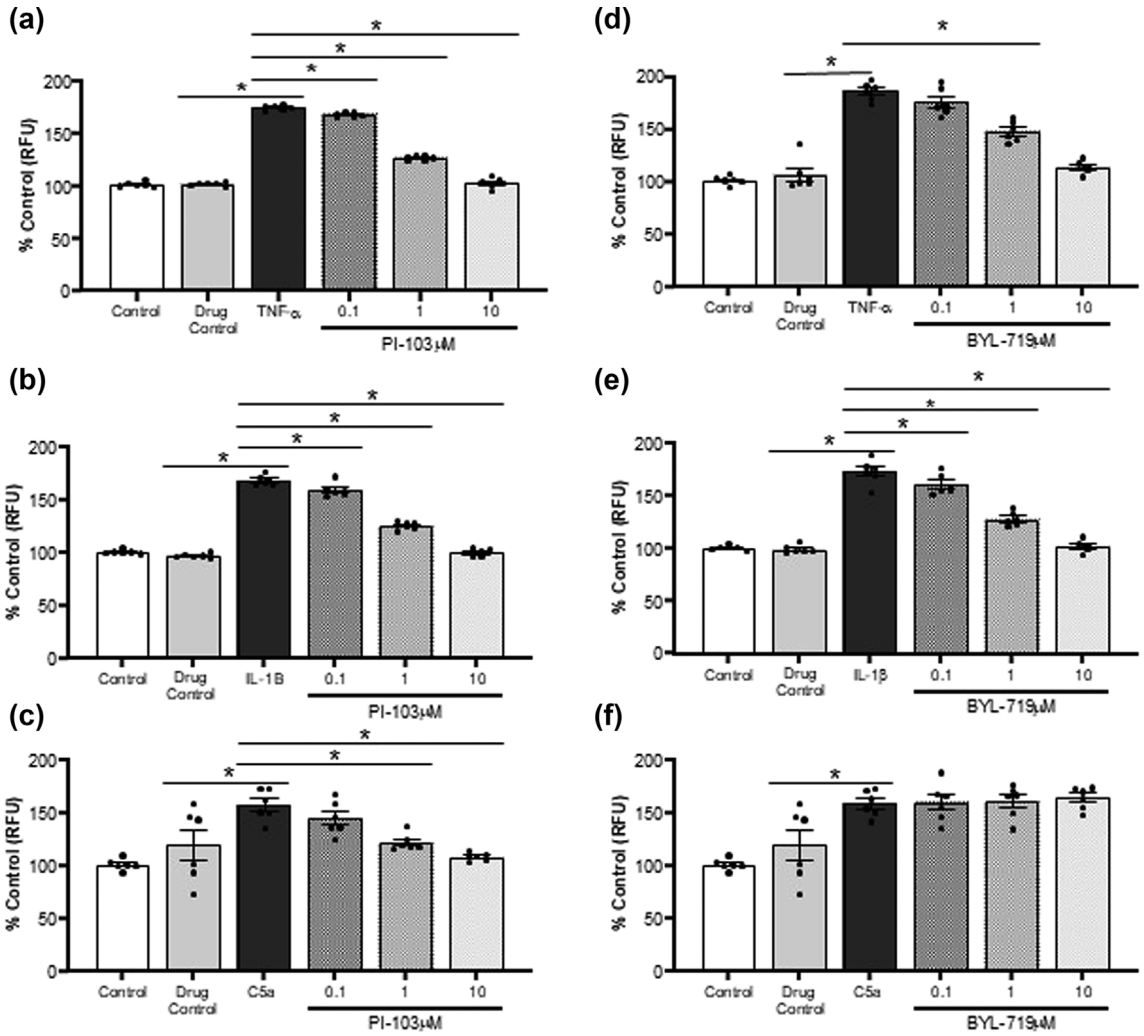

FIGURE 5 Permeability of endothelial cells induced by inflammatory mediators and effects of PI3K inhibitors *in vitro*. Human dermal microvascular endothelial cells (HMVEC-Ds) permeability quantitatively measured by initially treating with TNF-α (0.6 pmol·ml^{-1}), IL-1β (0.6 pmol·ml^{-1}) and C5a (5 pmol·ml^{-1}) for 18 h, followed by 1 h inhibitor treatment at three concentrations: 1, 3 and 10 μM of either PI-103 (a–c) or BYL-719 (d–f) PI3K inhibitor treatment respectively. Data are mean ± SEM one-way ANOVA plus Tukey's test; n = 6 independent experiments in duplicates; *$P < 0.05$, between control and either TNF-α/ IL-1β or between TNF-α/IL-1β and treatment.

during inflammation *in vivo* (Wedmore & Williams, 1981). It is noted that the study of 2D cultures does not always correlate with *in vivo* observations. Therefore, we also carried out *in vivo* experiments to better understand events.

TNFα and IL-1β are well-known cytokine mediators of neutrophil accumulation in inflamed tissues *in vivo* (Petrofsky & Bermudez, 1999). Murine dorsal skin was chosen to investigate the effect of PI3K inhibitors on responses to cytokines *in vivo*. We chose two timepoints for our study:- an acute response over 30 min and a more sustained 4-h response (as Zarban et al., 2023). The neuropeptides were used as acute neutrophil-independent controls as SP is an established mediator of increased microvascular permeability and α-CGRP is a potent microvascular dilator (Brain & Williams, 1985; Grant et al., 2004); they together act synergistically to promote acute plasma extravasation over 30 min. This was utilized here as a valuable control due to their PI3K-independent mechanism. It is noted that the PI3K-inhibitors did not inhibit this oedema formation, leading to us suggesting that these inhibitors are not acting to reduce peripheral microvascular blood flow. More recently acute neutrophil-dependent oedema, over 30 min, has been reported to be mediated via TNFα (Finsterbusch et al., 2014). However, our results support the alternative theory that TNFα and IL-1β require a longer 4 h time period compatible with the up-regulation and expression of endothelial adhesion molecules that are required for neutrophil attachment and extravasation, which then mediate oedema formation (Leirisalo-Repo, 1994b; Zarban et al., 2023). Thus, the longer 4-h period was required for cytokine responses to be clearly observed in this *in vivo* scenario. Notably, it is unlikely that p110α inhibition itself affects endothelial adhesion molecule expression, because p110α depletion did not alter leukocyte attachment to ECs or **ICAM-1** expression *in vitro*, nor did it affect expression of endothelial cell–cell adhesion proteins (Cain et al., 2010). If time had allowed, it would have been interesting to

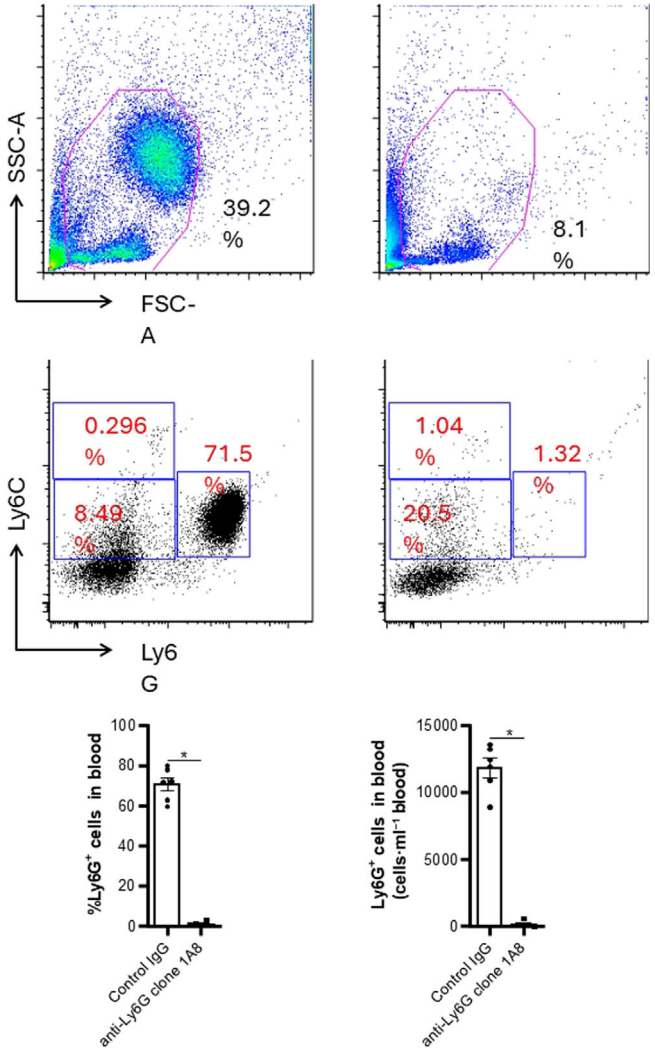

FIGURE 6 Flow cytometry analysis following depletion of circulating neutrophils *in vivo*. Mice were injected with either IgG control or anti-Ly6G antibody (0.17 μmol·kg^{-1}, i.p.). After 24 h, cardiac puncture was performed to obtain blood for flow cytometry analysis. Representative scatter plot of leukocyte populations in blood of mice treated with anti-IgG antibody (a) and anti-Ly6G antibody (b). Depletion of LyG+ cells (neutrophils) are shown in c and d. Graphs showing the relative and absolute number of neutrophils in blood (e and f). Data are mean ± SEM with *t* test; n = 6 independent experiments in duplicate; *$P < 0.05$ between control IgG (nonneutrophil-depleted mice) and anti-Ly6G clone 1A8 antibody (neutrophil depleted mice).

observe the effect of a TNFα antibody (e.g. **infliximab** or **adalimumab**) on the TNFα-induced inflammation. However, based on previous experiments in mice, an inhibition of oedema and neutrophil accumulation would have been observed (Zarpelon et al., 2013). By comparison, the complement fragment C5a, whilst known as an anaphylatoxin and a neutrophil chemotactic agent (Webster et al., 1980), is an acute mediator of neutrophil-dependent oedema formation (Wedmore & Williams, 1981) that can act in a sustained manner.

A question arises as to why mouse skin requires neutrophils to activate ECs in order for them to increase microvascular permeability and become leaky, whereas the cultured cells increase microvascular permeability in the absence of neutrophils. ECs are normally quiescent *in vivo* when associated with the basement membrane/extracellular matrix. One possibility is that ECs, which are grown on a rigid substrate with growth supplements, become primed similar to the effects of neutrophils. Additionally, C5a is known to act directly on ECs to mediate increased permeability (Killackey et al., 1986). A low level of C5a receptors have been detected on ECs and C5a has been previously reported to induce stress fibres in ECs *in vitro* (Schraufstatter et al., 2002); correlating with increased permeability as observed here. Whilst neutrophil-dependent increased microvascular permeability has been established, the precise mechanisms by which neutrophils act remain under debate. Neutrophils can either act directly to release factors that increase vascular permeability or initiate this priming function *in vivo*. These include release of various mediators including heparin-binding protein (Gautam et al., 2001; Soehnlein & Lindbom, 2009).

The results provide clear evidence that the oedema and neutrophil accumulation induced by the pro-inflammatory cytokines was inhibited by the PI3K inhibitors and this is in keeping with the observation that PI3K inhibitors of all subtypes inhibit the general carrageenan-induced oedema and neutrophil accumulation in the mouse paw (Pritchard et al., 2016), although these authors did not investigate specific mediators. We show for the first time that C5a-induced EC permeability is mediated via one of the kinases inhibited by PI-103 but that p110α is unlikely to be involved. Together, these *in vitro* and *in vivo* studies imply that p110α does not mediate C5a responses. Interestingly, neutrophil recruitment induced by C5a in mice is primarily via p110γ (Pinho et al., 2007). Thus, it is possible that EC permeability stimulated by C5a is also mediated by p110γ, which is one of the kinases inhibited by PI-103 (Knight et al., 2006). It has been established that TNFα, IL-1β and C5a induce neutrophil extracellular traps (NET) formation via PI3K-dependent processes (de Carvalho et al., 2023) and C5a also induces neutrophil extracellular traps formation (Chen et al., 2022; Yousefi et al., 2009). Thus, a specific effect at the level of neutrophil extracellular traps formation is unlikely. This is further supported here by the result that, in a neutrophil-independent setting, when neutrophils were depleted, C5a effects were abolished, as no oedema formation in response to C5a was observed.

The finding that C5a-mediated neutrophil accumulation and oedema formation is not affected by the selective p110α inhibitor BYL-719 is important as it indicates that C5a activity may remain as a defensive component, if a selective p110α inhibitor is used to inhibit excessive and damaging cytokine-induced inflammation. There is evidence that C5a can be protective in a range of situations; for example, its deficiency can result in tissue damage and death (von Köckritz-Blickwede et al., 2010). Perhaps most importantly, C5a receptor deficiency has been shown to have no effect on cardiac remodelling in murine heart failure models (de Haan et al., 2017). Therefore, its remaining activity may not influence one of the most common end points associated with vascular dysfunction and hypertension. This together with the present findings leads to the concept that the C5a signalling pathway will remain operational when p110α signalling is

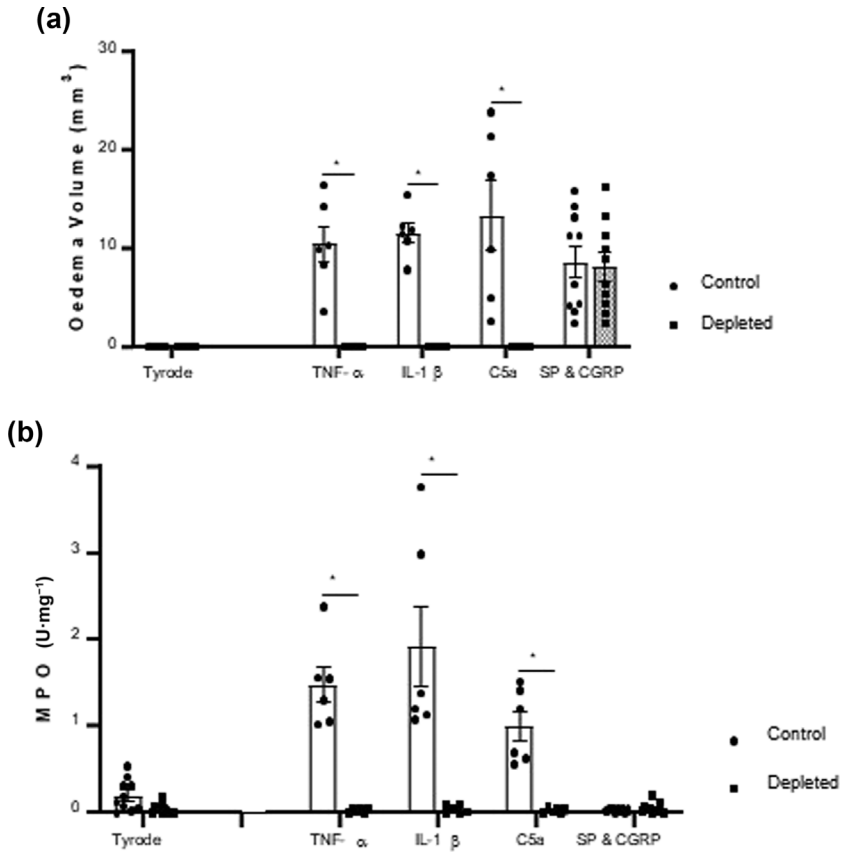

FIGURE 7 Characterization of oedema-inducing mediators and neutrophil accumulation *in vivo* after anti-Ly6G treatment. Mice were treated with either IgG control (anti-isotype control anti-trinitrophenol) or anti-Ly6G clone 1A8 antibody (0.17 μmol·kg^{-1}, i.p., for 24 h). Then with Evan's blue dye (i.v., oedema marker), followed by Tyrode (control), TNF-a (0.6 pmol), IL-1β (0.6 pmol) and C5a (30 pmol) per 50 μl, i.d. for 4 h. SP (300 pmol) and α-CGRP (20 pmol) per 50 μl were given at 3.5 h, for the last 30 min. The experiment was terminated at 4 h. Results for oedema (a) and MPO (b) are shown. Data are mean ± SEM two-way ANOVA plus Sidak's test; n = 6 independent experiments with duplicate sites; *$P < 0.05$ between control and depleted mice.

inhibited, at a time when detrimental oedema forming, and neutrophil accumulation induced by TNFα and IL-1β would occur. This may be critical, as adverse p110α blockade is associated with cardiac side effects risk (Ezeani & Prabhu, 2022). Overall, our results support a model where different PI3K subtypes mediate responses to different stimuli (Bekhite et al., 2011; Fruman et al., 2017; Guillermet-Guibert et al., 2008) and hence the use of a panel of small molecule inhibitors with distinct PI3K subtype selectivity could be important for effective and targeted therapeutic intervention.

Of particular relevance to potential use in therapy, there were no major adverse effects of long-term administration of BYL-719 in middle-aged mice, although there was evidence of insulin resistance (Hedges et al., 2021). Additionally, BYL-719 (alpelisib) was effective in mice for the treatment of Epidermolysis Bullosa Acquisita, indicating the beneficial effect in a disease model (Zillikens et al., 2021). Surprisingly, based on the results described here, selective p110α inhibitors have been associated with peripheral oedema formation in 20% of cases in the SOLAR-1 clinical trial. Additionally, rarely periorbital oedema formation, which is separate to a more usually observed adverse effect of a rash, has been observed in a few cases (Dao et al., 2022). In the SOLAR-1 clinical trial, 20% of subjects experienced peripheral oedema, but in that study, none suffered periorbital oedema formation. The oedema formation is considered to be due to mTOR, which signals downstream of PI3Ks and is linked to platelet-derived growth factor signalling (Esmaeli et al., 2002). The findings here, over the 4-h experimental period, would not be expected to be linked to growth factor signalling.

We conclude that we provide novel evidence that whilst TNFα, IL-1β and C5a induced increased permeability in endothelial cultures *in vitro*; inflammatory oedema formation induced by the same stimuli *in vivo* is neutrophil dependent. This highlights the need to ensure studies carried out in culture are supported by *in vivo* studies. The reversal of TNFα and IL-1β-induced junctional disruption *in vitro* by the selective p110α inhibitor BYL-719 extends knowledge to support a major role of p110α in mediating microvascular permeability in *in vitro* studies. This finding is supported by our *in vivo* studies that provide clear evidence that whilst the less selective PI-103 inhibitor blocks both oedema and neutrophil infiltration induced by TNFα, IL-1β and C5a, the p110α-selective inhibitor BYL-719 blocks neutrophil accumulation and oedema *in vivo* in response to TNFα and IL-1β only. This previously unknown selective effect of BYL-719 on TNFα and IL-1β responses indicates a therapeutic potential to treat vascular

inflammatory diseases where the cytokines play an important proinflammatory role. However, the limited influence of BYL-719 on C5a-related pathways (and SP + α-CGRP) provides a new insight that implies that this innate component of the immune response against infection may continue to protect against injury and infection, in patients treated with a p110α (PI3Kα)-selective inhibitor.

AUTHOR CONTRIBUTIONS

Hiba Chaudhry, Anne J. Ridley and Susan D. Brain designed the study. Hiba Chaudhry, Ali A. Zarban, Silvia C. Trevelin, Camilla Cerutti, Xenia Kodji and Fulye Argunhan performed the study. Silvia C. Trevelin and Ajay M. Shah provided design and strategic support for the flow cytometry analysis. Xiaoqing Xiong performed data analysis. Susan D. Brain, Hiba Chaudhry, Xiaoqing Xiong and Anne J. Ridley drafted the manuscript. All authors contributed to the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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