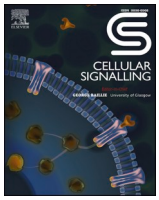
Cellular Signalling 124 (2024) 111444

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig



Non-cyclic nucleotide EPAC1 activators suppress lipopolysaccharide-regulated gene expression, signalling and intracellular communication in differentiated macrophage-like THP-1 cells

Jolanta Wiejak [a,1], Fiona A. Murphy [b,1], Graeme Barker [c], Pasquale Maffia [d,e,f], Stephen J. Yarwood [a,*]

[a] Heriot-Watt University, Institute of Biological Chemistry, Biophysics and Bioengineering, Edinburgh EH14 4AS, United Kingdom
[b] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4 0RE, United Kingdom
[c] Heriot-Watt University, Institute of Chemical Sciences, Edinburgh EH14 4AS, United Kingdom
[d] University of Glasgow, School of Infection & Immunity, College of Medical, Veterinary and Life Sciences, Glasgow G12 8TA, United Kingdom
[e] University of Naples Federico II, Department of Pharmacy, School of Medicine and Surgery, Naples, 80131, Italy
[f] Africa-Europe Cluster of Research Excellence in Non-Communicable Diseases & Multimorbidity, African Research Universities Alliance (ARUA) & The Guild of European Research-intensive Universities, Glasgow G12 8TA, United Kingdom

A B S T R A C T

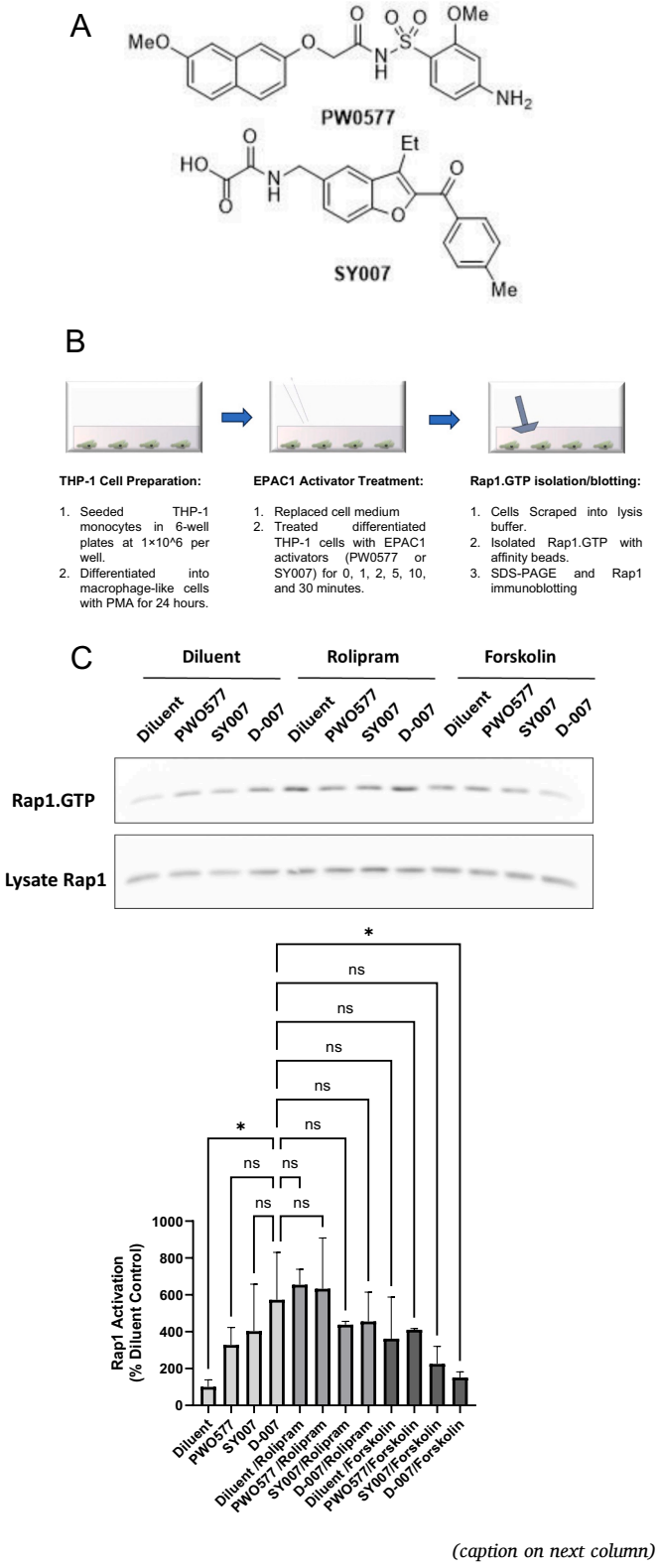
This study explores the anti-inflammatory effects of non-cyclic nucleotide EPAC1 activators, PW0577 and SY007, on lipopolysaccharide (LPS)-induced responses in differentiated THP-1 macrophage-like cells. Both activators were found to selectively activate EPAC1 in THP-1 macrophages, leading to the activation of the key down-stream effector, Rap1. RNA sequencing analysis of LPS-stimulated THP-1 macrophages, revealed that treatment with PW0577 or SY007 significantly modulates gene expression related to fibrosis and inflammation, including the suppression of NLRP3, IL-1β, and caspase 1 protein expression in LPS-stimulated cells. Notably, these effects were independent of p65 NFκB phosphorylation at Serine 536, indicating a distinct mechanism of action. The study further identified a shared influence of both activators on LPS signalling pathways, particularly impacting extracellular matrix (ECM) components and NFκB-regulated genes. Additionally, in a co-culture model involving THP-1 macrophages, vascular smooth muscle cells, and human coronary artery endothelial cells, EPAC1 activators modulated immune-vascular interactions, suggesting a broader role in regulating cellular communication between macrophages and endothelial cells. These findings enhance our understanding of EPAC1's role in inflammation and propose EPAC1 activators as potential therapeutic agents for treating inflammatory and fibrotic conditions through targeted modulation of Rap1 and associated signalling pathways.

1. Introduction

Macrophages are specialized cells involved in the detection, phagocytosis, and destruction of bacteria and other harmful organisms [1]. One of the key signalling pathways that modulate macrophage functions is the Toll-like receptor 4 (TLR4) pathway [2–4]. TLR4 is a transmembrane protein that triggers inflammatory responses through interaction with pathogen-associated molecular patterns (PAMPS), including lipopolysaccharide (LPS) a component of the gram-negative bacteria cell wall and initiates a signalling cascade that results in the production of inflammatory cytokines, chemokines and other mediators of the immune response [2–4]. LPS is recognized by the extracellular domain of TLR4 in combination with the co-receptor, MD-2, which is required for high-affinity binding of LPS, leading to receptor dimerization and recruitment of cytoplasmic adaptor proteins, including myeloid differentiation primary response protein 88 (MYD88) [5]. Classically, the MyD88-dependent pathway leads to the activation of transforming growth factor-beta-activated kinase 1 (TAK1), which phosphorylates the IκB kinase (IKK) complex, leading to IKK degradation and release of cytoplasmic NF-κB dimers, primarily p65 (RelA) and p50 in macrophages [6]. Released NF-κB dimers translocate into the nucleus and trigger transcription of a large number of inflammatory genes [6], including pro-interleukin-1β (pro-IL-1β) and pro-IL-18 [7], and other immune response genes, including pro-caspase 1 and the NLRP3 inflammasome component [8,9]. Upon activation, NLRP3 forms the inflammasome complex with the adaptor molecule ASC, and caspase 1,

* Corresponding author.
E-mail address: s.yarwood@hw.ac.uk (S.J. Yarwood).
[1] These authors contributed equally.

https://doi.org/10.1016/j.cellsig.2024.111444
Received 24 June 2024; Received in revised form 24 September 2024; Accepted 30 September 2024
Available online 3 October 2024



(caption on next column)

Fig. 1. Effects of EPAC1 Activators on Rap1 Activation in THP-1 Macrophages. THP-1 cells were differentiated into macrophages as described in Materials and Methods and then incubated with EPAC1 activators 100 μM PWO577 or SY007 (A). (B) Rap1 activation in THP-1 macrophages were treated with EPAC1 activators and cyclic AMP elevating agents as indicated in the workflow diagram in the *upper panel*. THP-1 macrophages were stimulated with EPAC1 activators (100 μM PWO577, SY007 or D-007) in the presence of diluent or the cyclic AMP elevating agents (Rolipram, 10 μM; Forskolin, 50 μM). Rap1 activation (Rap1.GTP) was assessed *via* an affinity pull-down assay followed by Western blotting. EPAC1 activators increased Rap1.GTP levels compared to the control. However, treatment with Rolipram or Forskolin elevated basal Rap1.GTP levels and prevented further activation by 100 μM EPAC1 activators, indicating a modulation of EPAC1-mediated Rap1 activation by elevated cyclic AMP levels. Significant differences in Rap1 activity at 5 min, relative to D-007-stimualted cells, are shown in the bar graphs in the *lower panel*; *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$ ($n = 3$), 1-way ANOVA. Non-significant changes are also indicated, ns.

leading to cleavage and release of IL-1β and IL-18 [7,8].

The cyclic AMP signalling cascade appears to play an important role in maintaining immune homeostasis and the prevention of excessive inflammation through the control of macrophage differentiation, response to pathogens and injury-related danger molecules [10–13]. There is also evidence that cAMP exerts positive effect on tissue fibrosis, by reducing fibroblast proliferation, decreasing extracellular matrix synthesis, and promoting matrix degradation [14–18], which is the final stage of chronic inflammation, and involves macrophages, besides fibroblasts, as the key cells in its development [19].

Cyclic AMP exerts its actions through direct interaction with cyclic nucleotide-gated ion channels [20], regulatory subunits of protein kinase A (PKA) [21], POPDC proteins [22] or the cyclic nucleotide binding domains of exchange proteins activated by cyclic AMP (EPACs 1 and 2) [21]. The levels of cyclic AMP in the cell are regulated by opposing actions of adenylate cyclase enzymes, which synthesise cyclic AMP from cellular ATP, and cyclic AMP phosphodiesterase enzymes (PDEs), which hydrolyse cAMP to 5'-AMP [23]. There are reports that pharmacological inhibition of the type 4 class of PDEs (PDE4s), knockdown of PDE4 genes in mice or activation of adenylate cyclase by Gs-coupled receptors (GsPCRs) have been widely shown to dramatically inhibit the inflammatory responses of LPS-stimulated macrophages, including cytokine induction, cell adhesion, and FcγR-mediated phagocytosis [24,25]. These observations indicate on essential role of cyclic AMP in the regulation of TLR4 signalling in macrophages.

Many of these immune suppressive actions of cAMP in macrophages appear to occur through the activation of both PKA and/or EPAC1, resulting in the inhibition of pro-inflammatory TLR4 and NFκB signalling [26]. For example, once activated, PKA phosphorylates and activates the cyclic AMP response element-binding protein (CREB; Ser 133), which competes for limiting amounts of the co-activator, CBP, thereby preventing the activation of NF-κB transcription through p65 [27]. Other actions of PKA on the NF-κB pathway have also been reported, including effects on NF-κB dimer formation through phosphorylation of p65 (Ser 276). However, the cyclic AMP-activated PKA appears to act at multiple levels within the NF-κB signalling cascade, resulting in either positive or negative effects on gene expression that appear to be largely cell-type dependent [26].

Similarly, the role of EPAC proteins in the modulation of TLR4 signalling and NF-κB-regulated gene expression remains to be determined. LPS has been shown to increase cyclic AMP levels in Raw 264.7 murine macrophages and activation of EPAC1 with a selective agonist led to NF-κB activation and downstream signalling in the same cells [28,29]. In contrast, EPAC1 activation has been shown to inhibit the induction of the NF-κB gene targets, NLRP3, caspase 1 and IL-1β, in retinal endothelial cells [30,31] and IκBα-degradation and p65 nuclear translocation in airway smooth muscle cells [32], whereas EPAC1 activation appears to be required for nuclear translocation of NF-κB during osteoclast

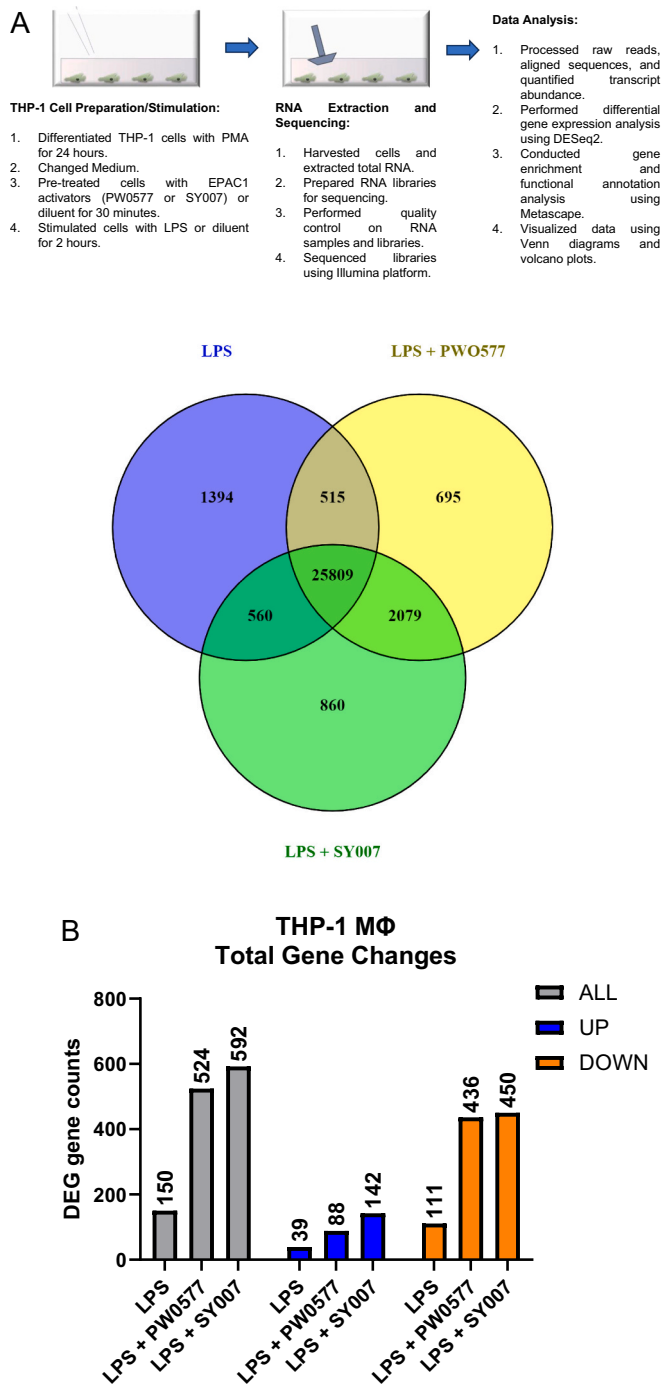


Fig. 2. The Impact of EPAC1 Activators, PWO577 and SY007, on Gene Expression in THP-1 Macrophages Exposed to LPS.
THP-1 macrophages were either left unstimulated or stimulated with LPS for two hours, with or without the addition of EPAC1 activators. Following a 20-h incubation in fresh medium, total RNA was extracted for RNA sequencing analysis. (A) In the *upper panel* is a workflow diagram for this experiment and a Venn diagram, in the *lower panel*, highlights the differential gene expression (DEG) profiles under varying conditions: solely LPS-stimulated, EPAC1 activator-stimulated, and the combined stimulation of LPS with each EPAC1 activator. The diagram provides insights into the specific and common gene regulatory effects of LPS and EPAC1 activators on THP-1 cells. (B) A histogram that quantifies the differentially expressed genes, identifying those up-regulated or down-regulated under each treatment condition, analysed using DESeq2 software with a *p*-value threshold of ≤ 0.05 for significant gene expression changes.

differentiation [33]. In retinal endothelial cells, EPAC1 was shown to act upstream of TLR4 and have a protective action on LPS-stimulated signalling [34].

To begin to address these apparent contradictions and to further understand the anti-inflammatory role of EPAC1 proteins, we have applied high-throughput screening and medicinal chemistry [35–37] to devise chemically distinct, small molecule agonists of EPAC1, SY007 and PWO577 (Fig. 1A), that selectively promote EPAC1 activation over EPAC2 and PKA in cells [35,37]. We have recently shown that both PWO577 and SY007 display limited cytotoxicity in HUVECs, and regulate similar global gene expression patterns, including inhibition of IL-6 induced STAT3 activation and associated downstream signalling, with few off-target effects [38]. We now aim to apply these novel compounds to begin to understand the impact on TLR4-regulated gene expression, signalling and intracellular communication in macrophage-like derived from human monocytic leukaemia THP-1 cell line [39]. We demonstrate a largely inhibitory effect of PWO577 and SY007 on LPS-stimulated gene expression and immune-vascular interplay in these cells.

In this study, we aim to elucidate the anti-inflammatory roles of EPAC1 proteins in macrophages, focusing on the TLR4 pathway, which is known for initiating inflammatory responses through interactions with pathogen-associated molecular patterns, including lipopolysaccharide (LPS) [40]. We investigate the effects of novel, structurally distinct, small molecule non-cyclic nucleotide EPAC1 activators, SY007 and PWO577 [38], on TLR4-regulated gene expression in THP-1 macrophages. This study aims to provide insights into the modulation of inflammatory responses by these EPAC1 activators in THP-1-derived macrophage-like cells, contributing to our understanding of their potential therapeutic applications.

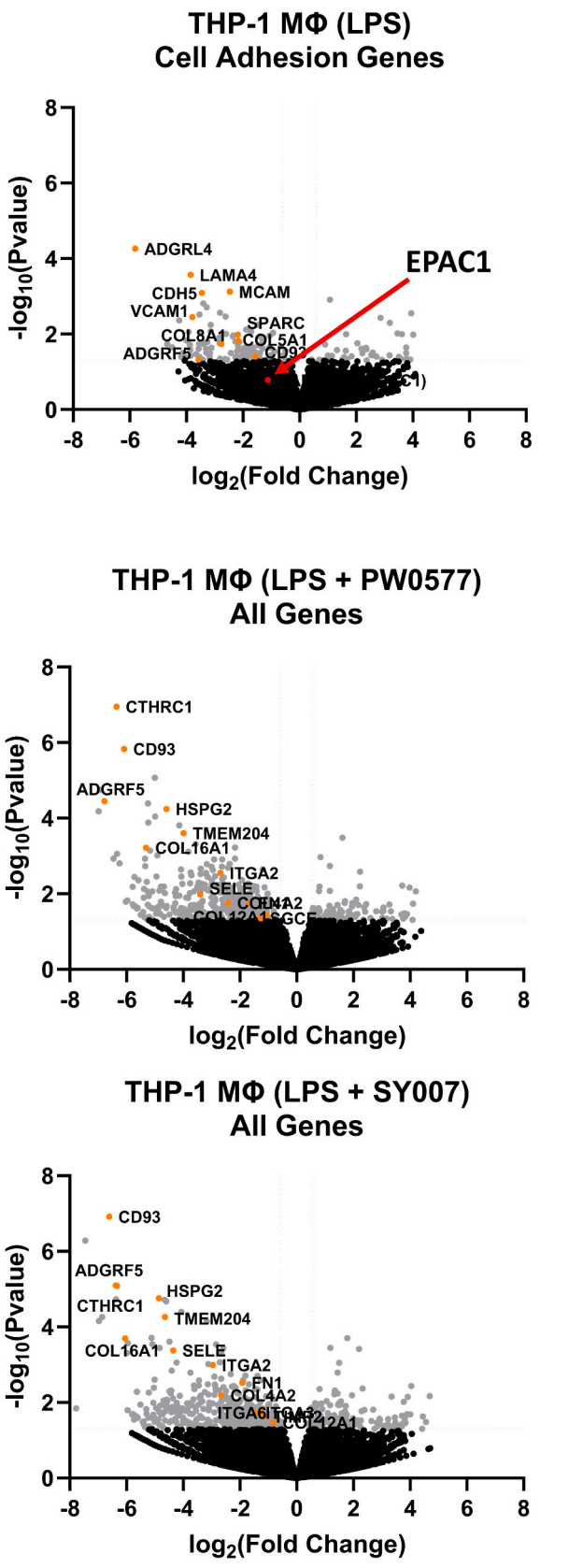
2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were obtained from commercial sources and used without further purification unless otherwise noted. The cyclic nucleotide analogue 8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3′,5′-cyclic monophosphate (D-007) was sourced from Biolog Life Science Institute, Bremen, Germany. Dimethyl sulfoxide (DMSO) for molecular biology, and anti-Rabbit IgG, HRP conjugate, were procured from Sigma-Aldrich, St. Louis, MO, USA. The antibodies against Rap1A/Rap1B (26B4), IL1β, NLR3P, and Caspase 1 were acquired from Cell Signalling Technology, Danvers, MA, USA. Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide from *Escherichia coli* (LPS) were obtained from Merck Life Sciences UK Limited, Glasgow, Scotland. Corning™ Transwell™ Multiple Well Plate with Permeable Polyester Membrane Inserts (0.4 μm pore; 6 well format) were purchased from Fisher Scientific UK Ltd., Loughborough, UK.

2.2. Cell lines and culture media

Cryopreserved human coronary artery endothelial cells (HCAEC; 500,000 cells; Cat# C-12221) and human coronary artery smooth muscle cells (VSMC; 500,000 cells; Cat# C-12511), along with their respective growth media, were purchased from PromoCell GmbH, Heidelberg, Germany. Specifically, endothelial cell growth medium MV2 (Cat# C-39226 for supplement mix and Cat# C22022B for media) and smooth muscle cell medium 2 (Cat# C-39267 for supplement mix and Cat# C22062B for media) were used. THP-1 cells were sourced from the American Type Culture Collection (ATCC; Cat# TIB-202). Heat-inactivated foetal bovine serum (FBS; Cat# 758093) was obtained from Greiner Bio-One, Gloucestershire, UK. Roswell Park Memorial Institute (RPMI) 1640 Medium containing L-Glutamine was procured from ThermoFisher Scientific, Renfrew, Scotland.



(caption on next column)

Fig. 3. Distribution of Differentially Expressed Genes in LPS-stimulated THP-1 Macrophages Cultured in the Presence or Absence of EPAC1 Activators.
This figure displays volcano plots representing the distribution of differentially expressed genes in THP-1 cells treated with LPS for 2 h, in the presence or absence of EPAC1 activators PWO577 and SY007, followed by 20 h culture in fresh medium. The plots compare gene expression changes across different treatment conditions: LPS stimulation, EPAC1 activator stimulation, and their combinations. Significant gene changes ($p \leq 0.05$; $n = 5$) are shown in light grey, with non-significant changes in black. The plots visualize the fold change in gene expression and its statistical significance, highlighting the impact of these treatments on THP-1 cell gene expression.

2.3. Chemical synthesis

Compounds PWO577 and SY007 (Fig. 1A) were synthesised in-house, as previously described [35,37].

2.4. Cell culture and differentiation of THP-1 cells

THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10 % (*v*/v) foetal bovine serum and 1 % (v/v) penicillin-streptomycin. Differentiation into macrophage-like cells was induced by adding phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) to cultures for 24 h. After differentiation, adherent cells were washed and further cultured in PMA-free medium for 24 h to allow for the maturation of macrophage-like cells. All experiments were conducted using fully differentiated THP-1 cells.

2.5. Western blotting

Differentiated THP-1 macrophage-like cells were plated in 6-well plates (1×10^6 cells / well), pre-treated with PWO577 and SY007 (100 μM) for 30 min, and then stimulated with or without LPS (100 ng/ml) for appropriate periods of time (30 min to 24 h). Western blot analysis was performed as previously described [38]. Briefly, samples for SDS-PAGE were prepared by scraping the cells directly into 1 × SDS Sample Loading Buffer and then denaturing for 5 min at 95 °C. Proteins were separated using SDS-PAGE method and transferred to nitrocellulose membranes. After blocking in 5 % (*w*/*v*) non-fat dry milk in 1 × TBS-Tween 20 solution (1 h at RT) membranes were incubated overnight (4 °C) with primary antibodies (anti-NLRP3, NF-κB, phospho-NF-κB Ser536, pro-IL1b and pro-CASP1), followed by incubation with HRP-conjugated anti-rabbit secondary antibody (1 h at RT). After extensive washing in TBS-Tween 20 (3 × 10 min), membranes were incubated for 5 min with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, Paisley, UK). Images were acquired using a Fusion FX7 camera platform (Vilber, Collégien, France) on the chemiluminescence setting. Signal intensities were measured densitometrically, using ImageJ software (National Institutes of Health, Bethesda, USA).

2.6. Rap1 activation assay

Detection of active GTP-bound Rap1 in THP-1 macrophages was performed using isolated RalGDS-RBD, as previously described (Parnell 2017). Briefly, a pGEX-5×-1 vector expressing GST-RalGDS-RBD (788–884) (McPhee 2000), was transformed into *E. coli* One Shot BL21 Star (DE3) cells (ThermoFisher Scientific), following the manufacturer's protocol. Protein expression was induced by addition of IPTG, followed by the affinity purification of the GST-RalGDS-RBD fusion protein using Glutathione Sepharose 4B (GE Healthcare). Protein concentration was measured using a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific).

For Rap1 Activation Assay, THP-1 monocytes were seeded on 6-well plates at a concentration of 1×10^6 per well and differentiated into macrophage-like cells as described above. Then, cells were treated with

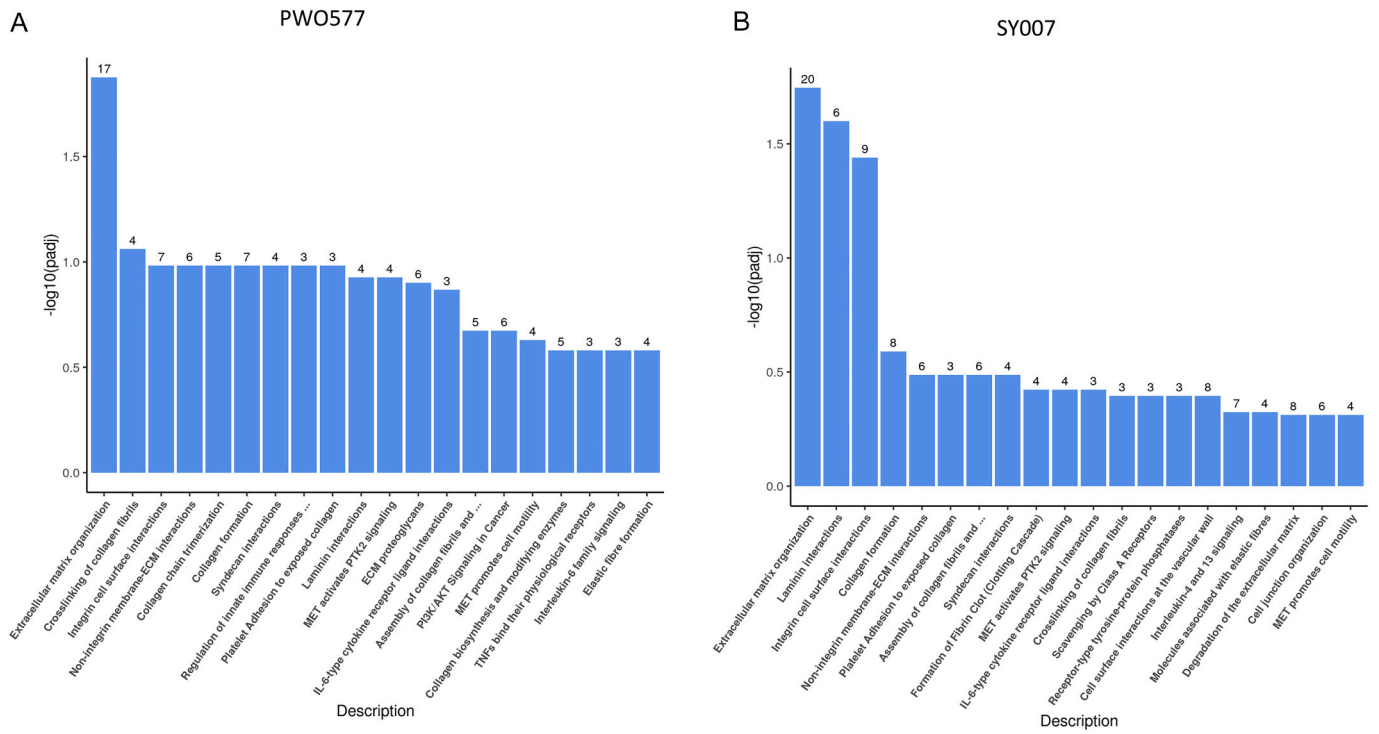


Fig. 4. KEGG Pathway Analysis of Gene Expression Changes in THP-1 Macrophages Treated with LPS in the Presence or Absence of EPAC1 Activators.
Gene expression changes in THP-1 cells, stimulated with LPS and subsequently treated with EPAC1 activators PWO577 (A) and SY007 (B), were analysed for biological function using the KEGG database (https://www.genome.jp/kegg/pathway.html). The histogram displays the top 20 significant KEGG pathways influenced by these treatments. The x-axis represents the specific KEGG pathways, while the y-axis indicates the significance level of pathway enrichment. Gene counts associated with each pathway are noted above each bar, illustrating the impact of EPAC1 activators on gene expression in THP-1 cells stimulated with LPS.

PWO577 or SY007 (100 μM) or DMSO for 1, 2, 5, 10 and 30 min, washed with ice-cold PBS and then scraped into 0.5 ml of ice-cold lysis buffer (55 mM Tris–HCl, pH 7.4132 mM NaCl, 22 mM NaF, 11 mM, $Na_4P_2O_7$, 10 mM $MgCl_2$, 1 % (*v*/v) Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were transferred to Eppendorf tubes, mixed by vortex and kept on ice for 5 min followed by centrifugation at 16,000 xg at 4 °C for 15 min to clear the lysates. The supernatants were transferred to new Eppendorf tubes and used directly for GTPase assay (50 μl of cell lysate was left for analysis of total Rap1 content) or frozen at −80 °C for further use.

To selectively capture active Rap1, cell lysates were incubated with 40 μg of GST-RalGDS-RBD immobilized on Glutathione Sepharose 4B (GE Healthcare, Chicago, IL, USA), for 1 h at 4 °C (with gentle rocking). After incubation, the glutathione resin was separated from the supernatant by centrifugation at 500 xg for 5 min and then the beads were washed three times in 0.4 ml of lysis buffer. The beads were then resuspended in 2 × SDS sample loading buffer (containing 200 mM DTT) and denatured by heating for 5 min at 95 °C. Prepared pull-down and input control samples were then subjected to SDS-PAGE and Western blotting with an anti-Rap1 antibody to detect Rap1.GTP levels.

2.7. Co-culture of cells

Here we used a modification of the protocol originally devised in the Maffia laboratory [41]. Briefly, VSMCs were seeded onto the basal side of an upturned Transwell insert (1.8 × 10^5 cells in 500 μl) and allowed to adhere for 6 h at 37 °C. After adherence, the Transwell inserts were flipped into individual wells of 6-well plates containing 1.5 ml of media and incubated for 72 h at 37 °C until confluent. Concurrently, THP-1 cells were seeded onto 6-well plates (1 × 10^6 cells per well) and differentiated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) for 24 h at 37 °C. Post-differentiation, the media was replaced with fresh RPMI containing 10 % (*v*/v) FBS and the cells were rested for an additional 24 h at 37 °C. *prior* to LPS stimulation, differentiated THP-1 cells were treated with EPAC1 activators PW0577 or SY007 (100 μM) for 30 min to ensure activation of EPAC1 prior to any inflammatory stimulus. This pre-treatment aims to assess the modulatory effects of these activators on the LPS response. Following pre-treatment, THP-1 cells were exposed to lipopolysaccharide (LPS; 100 ng/ml) in 2 ml of media per well for 2 h at 37 °C to induce an inflammatory response. In control conditions, cells were treated with diluent alone. After the LPS stimulation period, the media was removed, and the wells were replenished with fresh media. Transwell inserts containing monolayers of HCAECs (with or without underlying VSMCs) were then transferred to the wells containing LPS-stimulated THP-1 cells pre-treated with PW0577 or SY007. An additional 1 ml of fresh media was added to the apical side of each Transwell. The complete assembly was incubated at 37 °C for 20 h to allow for cellular interactions and signalling under the influence of EPAC1 activators. Cells were then washed with ice-cold sterile phosphate-buffered saline (PBS), and HCAECs were harvested by scrapping with a rubber-tipped plunger from a sterile 1 ml syringe into 0.5 ml of cold PBS. The cell suspensions from duplicate treatment wells were pooled, centrifuged (10,000 xg, 10 min), and resuspended in 350 μl RTL lysis buffer (Qiagen, Manchester, UK) for subsequent RNA extraction and storage at −20 °C.

2.8. RNA isolation and sequencing

Total RNA isolation from THP-1-derived macrophage-like cells and HCAECs, in co-culture with activated THP-1 cells and VSMCs, was performed using the RNeasy Mini Kit (Qiagen, Manchester, UK) following the manufacturer's protocol. RNA concentration was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Paisley, UK). RNA sample quality control, RNA library preparation, library quality control, Illumina library sequencing, data quality control and bioinformatics analysis were then carried out by

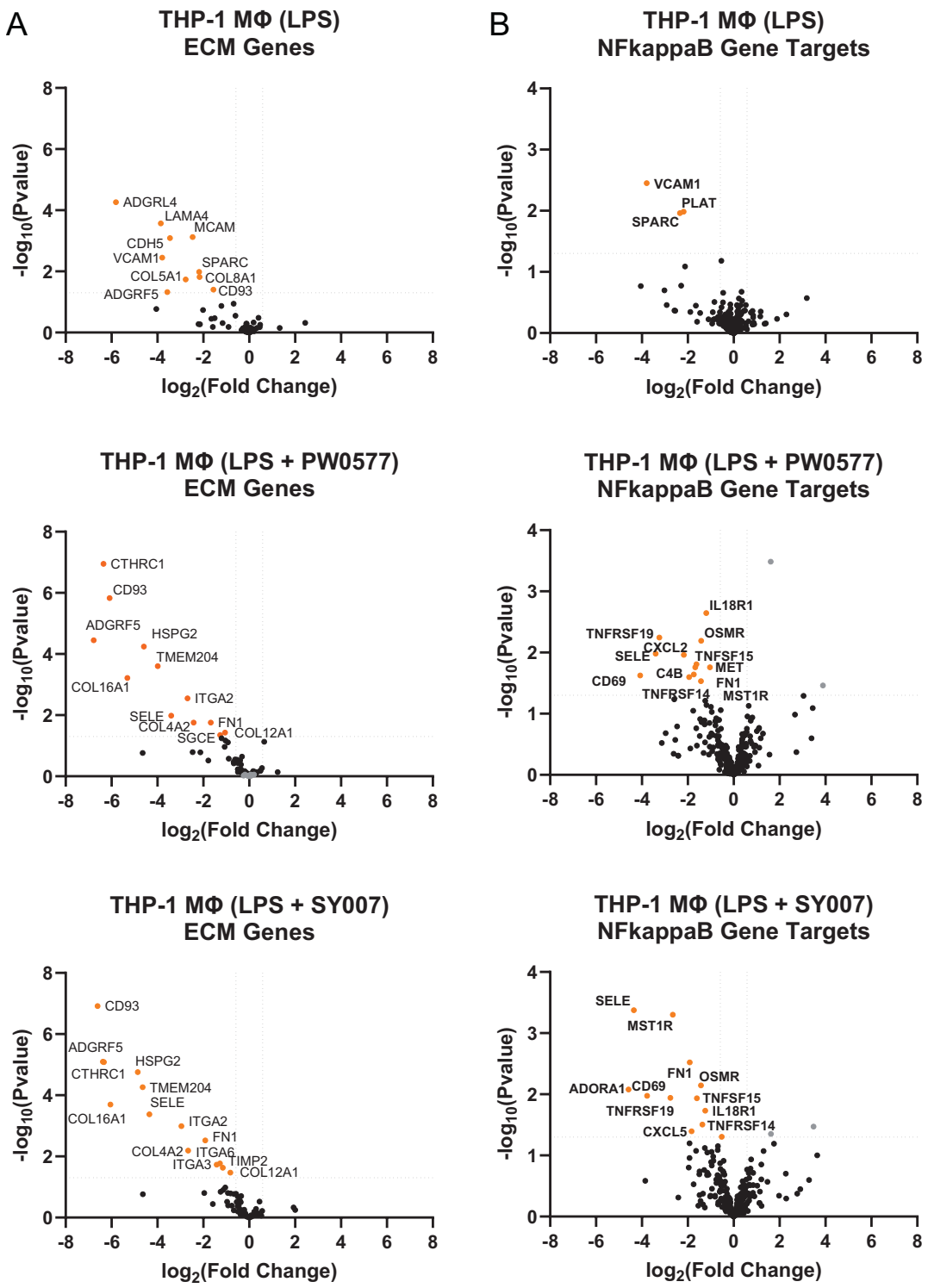


Fig. 5. Volcano Plot Analysis of Extracellular Matrix (ECM)- and NF-κB- Gene Targets in THP-1 Macrophages Following EPAC1 Activators Treatment.
This figure presents volcano plots depicting gene expression changes in THP-1 cells treated with LPS and EPAC1 activators PWO577 and SY007. The focus is on genes involved in ECM (A) and NF-κB gene targets (B), as identified in the study by Sharif et al. (2007). Significant gene expression alterations are represented in light grey and down-regulations in orange, highlighting the specific impact of EPAC1 activators on these pathways in response to inflammatory stimuli.

Novogene UK (Cambridge, UK), as described previously [38]. Bioinformatics analysis included processing of raw reads, alignment, quantification of transcript abundance, and differential gene expression analysis. The DESeq2 package was used for differential gene expression analysis, with genes having an adjusted p-value <0.05 considered differentially expressed. Gene enrichment and functional annotation analyses can be conducted using Metascape, with data sourced from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) Pathway. The profileR package can be employed for statistical enrichment analysis of differentially expressed genes in KEGG pathways. Venn diagrams were produced using the VIB/UGent online package (https://bioinformatics.psb.ugent.be/webtools/Venn/).

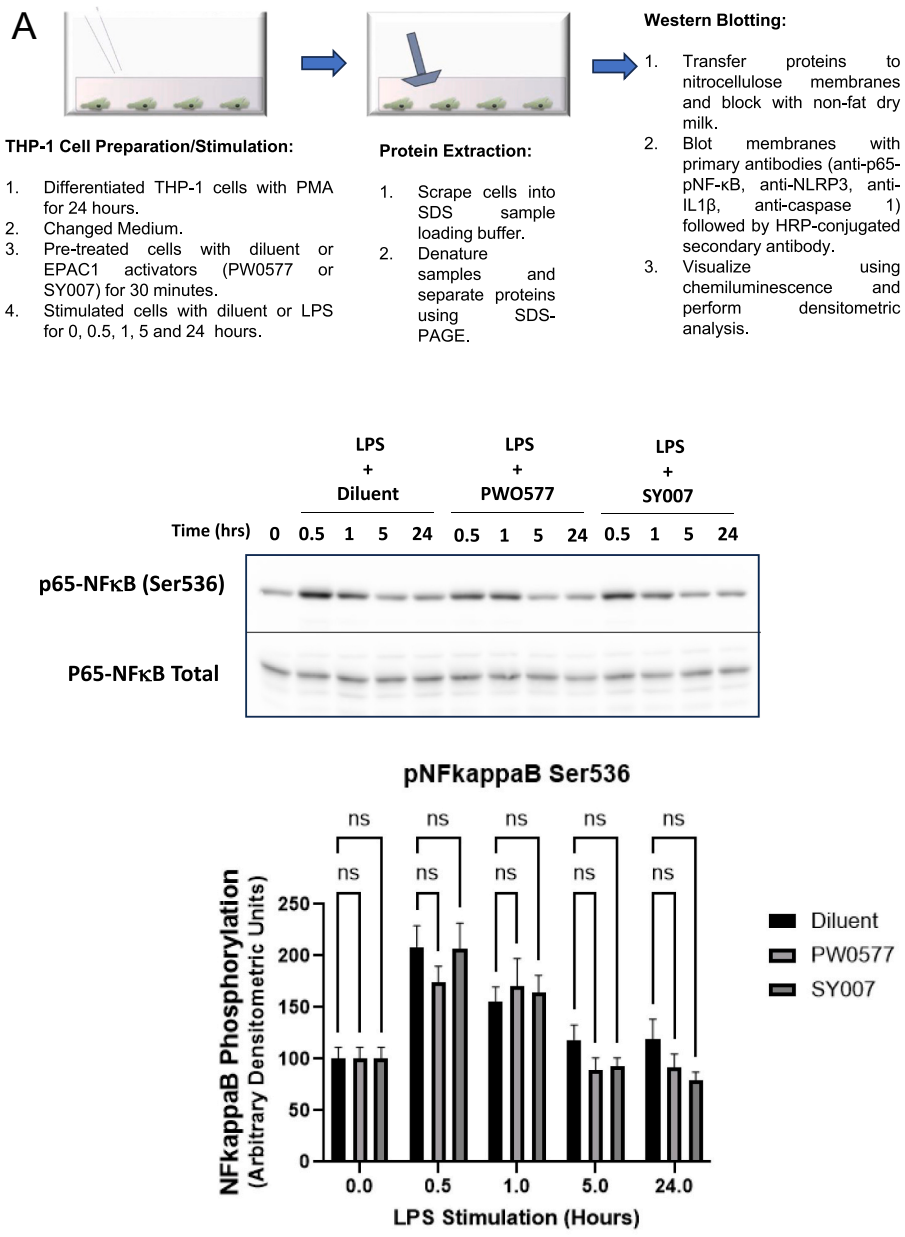


Fig. 6. Western Blot Analysis of NF-κB Gene Targets in THP-1 Macrophages Treated with LPS and EPAC1 Activators.
Western blots were done of cell extracts isolated from THP-1 macrophages treated with LPS, both in the presence and absence of EPAC1 activators, over the indicated time courses, up to 24 h. Antibodies specific to p65-pNF-κB (Ser536), and total p65-pNF-κB (A; $n = 10$), NLRP3 (B; n = 10), IL1β (C; $n = 11$), caspase 1 (D; $n = 9$), were used. Densitometric analysis of the immunoblots is shown in the accompanying graphs for Figs. 6A-6D, with significant changes in protein expression in LPS plus EPAC1 activator stimulated cells, relative to LPS plus diluent stimulated cells at the same time-point, are indicated- * ($p < 0.05$), ** ($p < 0.01$),*** ($p < 0.001$) or **** ($p < 0.0001$), using 2-way ANOVA. A workflow diagram for this experiment is shown in the *upper panel* of Fig. 6A.

2.9. Statistical analysis

Two-way analysis of variance (ANOVA) and Mann-Whitney tests were performed using GraphPad Prism 10 software (GraphPad Software, San Diego, USA).

3. Results

3.1. Activation of EPAC1 by SY007 and PWO577 in THP-1-derived macrophage-like cells -

We first explored the effects of EPAC1 activators PWO577 and SY007 on EPAC1 activation in differentiated THP-1 macrophages. The activation in THP-1 macrophages was assessed using Rap1 activation assays, as previously described [38]. Given that EPAC1 mRNA is expressed in these cells (arrow in Fig. 3), we stimulated THP-1-derived macrophages for 5 min, and then isolated GTP-bound Rap1 using RalGDS-GST fusion proteins, as previously described [38]. Fig. 1B shows Rap1 activation in THP-1 cells treated with EPAC1 activators (PWO577, SY007, D-007) in the presence of cyclic AMP elevating agents (Rolipram at 10 μM or Forskolin at 50 μM). Rap1 activation (Rap1.GTP) was then measured using the affinity pull-down assay followed by Western blotting. Results indicate that all EPAC1 activators increase Rap1.GTP levels, and this activation was further enhanced in the presence of cAMP elevating agents Rolipram and Forskolin, which raised basal Rap1.GTP levels and prevented further activation by the EPAC1 activators. These

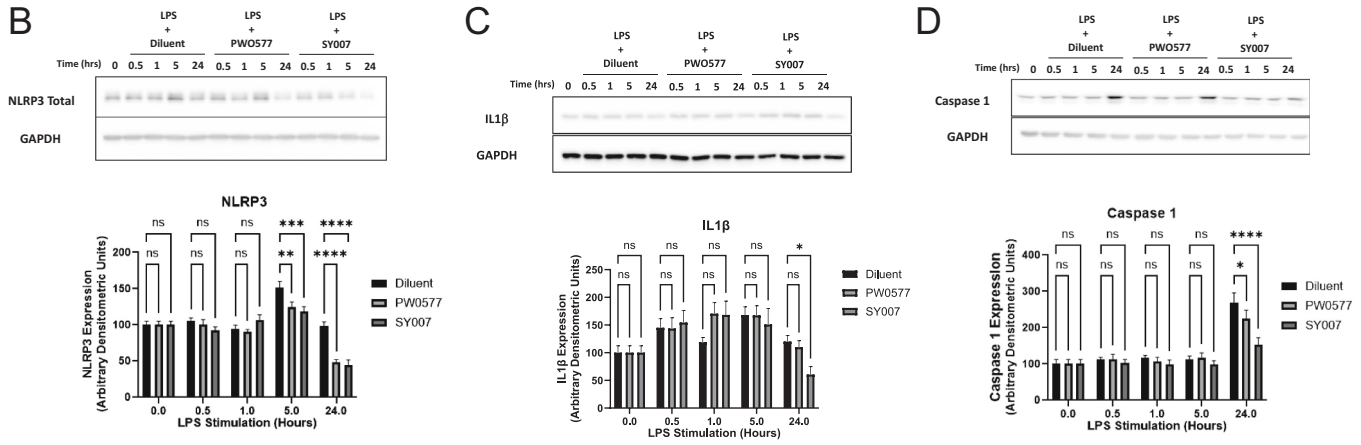


Fig. 6. (*continued*).

findings underscore the role of cyclic AMP in modulating Rap1 activity, the inhibitory effects of EPAC inhibitors on this pathway and the selectivity of PWO577 and SY007 for the cyclic AMP binding pocket of EPAC1 in THP-1 macrophages.

Notably, EPAC1 activators were not as effective as D-007 [42] at activating Rap1 (Fig. 1B). As outlined in our previous study by Wiejak et al. [38], both SY007 and PWO577, and the gold-standard EPAC1 activator, D-007, exhibit similar binding affinities to EPAC1. To further elucidate the relationship between EPAC binding affinity and functional outcomes, we previously examined the effects of SY007 and PWO577 on Rap1 activation [38]. Both agonists were tested in U2OS cells expressing EPAC1, and the activation of Rap1 was assessed. Despite their similar binding affinities to EPAC1, SY007 and PWO577 demonstrated partial agonist effects on Rap1 activation, as compared to the full agonist D-007, when used at 100 μM [38]. This partial agonism likely arises because both SY007 and PWO577 engage Ala322 within EPAC1, which destabilizes the inactive conformation, but do not engage Lys395, which is essential for stabilizing the active conformation, as achieved by D-007 (unpublished observations). This incomplete engagement results in suboptimal activation of EPAC1. Consequently, the levels of stimulated Rap1 activation induced by SY007 and PWO577 were therefore marginally lower than those achieved with D-007 (Fig. 1B), indicating that the extent of EPAC1 activation correlates with the degree of Rap1 activation.

With regards to potential off-target effects of PWO577 and SY007, our previous transcriptomic analysis revealed that both agonists regulate a common set of approximately 11,000 genes in HUVECs, with some differences in gene expression [38]. The differential gene expression observed can primarily be attributed to the partial activation of EPAC1 rather than off-target effects. This conclusion is supported by proteomic analysis, which indicated that the few differential gene changes were not translated into significant changes in protein expression [38]. Thus, while off-target effects cannot be completely ruled out, the data suggest that the observed differences are more likely due to differences in EPAC activation.

In conclusion, any differences observed between the agonists SY007 and PWO577 on Rap1 activation in THP-1 macrophages are likely primarily due to their partial activation of EPAC1 rather than off-target effects. The partial agonism, resulting from incomplete engagement with key residues Ala322 and Lys395 within EPAC1, leads to suboptimal activation of Rap1. This correlation between EPAC binding affinity, partial agonist activity, and the resultant Rap1 activation underscores the nuanced interplay between ligand binding, EPAC1 activation, and downstream signalling outcomes. Our data suggest that the partial agonism might contribute to the failure of pharmacological EPAC1 inhibitors, ESI-09 and CE3F4, to block the activities of these compounds fully (unpublished data). This indicates that PW0577 and SY007 likely induce a unique conformational state of EPAC1 that allows partial Rap1 activation but does not engage the canonical pathways typically inhibited by EPAC1 inhibitors. This is consistent with our earlier findings that pharmacological inhibitors of EPAC1 do not completely inhibit Rap1 activation induced by these compounds, suggesting that they may act through an alternative mechanism.

3.2. *RNAseq analysis of the effects of SY007 and PWO577 on LPS-signalling in THP-1-derived macrophage-like cells*

In terms of downstream signalling outcomes, we next focused on the effects of EPAC1 activators, PWO577 and SY007, on short-term (2 h) LPS-stimulated gene expression in THP-1-derived macrophage-like cells. A 2-h time point for with lipopolysaccharide (LPS) stimulation has previously been determined as being optimal for soluble mediator production in cultures of differentiated THP-1 macrophages [41]. Using RNA sequencing (RNAseq), we analysed the transcriptomic responses, which revealed that 25,809 gene expression changes could be attributable to stimulation of cells with LPS, or a combination of LPS and either PWO577 or SY007 (Fig. 2; Supplementary Data). A Venn diagram of total transcriptional changes indicated that a core of 2079 changes were regulated by both PWO577 or SY007 in LPS-stimulated cells, indicating a shared influence of these compounds on LPS signalling in THP-1-derived macrophages (Fig. 2A). There were also individual effects of either SY007 (860 genes) or PWO577 (695 genes) on LPS-regulated gene expression (Fig. 2A), indicating nuanced actions of each of these activators that do not overlap.

We next examined the statistically significant ($P < 0.05$) differential gene expression (DEG) levels in THP-1-derived macrophages stimulated with LPS in the presence or absence of EPAC1 activators. The specific DEG counts, reflecting the magnitude of gene expression alterations under these conditions, are detailed in Fig. 2B, while the distribution of statistically significant gene changes is shown in grey in the volcano plots in Fig. 3. The analysis revealed that LPS stimulation for 2 h significantly altered gene expression patterns, leading to both upregulation (39 genes) and downregulation (111 genes) of transcripts (Fig. 2B). The presence of EPAC1 activators strikingly influenced these changes (Figs. 2B and 3). In addition to a modest upregulation of gene expression in LPS-stimulated cells (88 genes in the presence of PWO577 and 142 genes in the presence of SY007, compared to 39 for LPS alone) there was a dramatic down regulation of 436 and 450 genes following PWO577 and SY007 treatment of LPS-stimulated cells, respectively, compared to 111 genes for LPS stimulation alone (Fig. 2B). This data underscores the profound impact of EPAC1 activators on the gene expression landscape of THP-1-derived macrophage-like cells in response to LPS stimulation.

3.3. Categorisation of gene expression changes in THP-1 cells

We next conducted KEGG pathway analysis on the existing RNAseq data to categorize gene expression changes in LPS-stimulated EPAC1 macrophage-like THP-1 cells treated with PWO577 (Fig. 4A) or SY007 (Fig. 4B). Our findings revealed significant involvement in genes associated with extracellular matrix (ECM) interaction and specific signalling pathways associated with cytokine signalling, notably the IL-6-signalling pathway, in agreement with our previous studies [38,43]

In support of a role in controlling LPS-induced ECM components, volcano plot analysis demonstrated that both PWO577 and SY007 treatment led to a significant down regulation of genes involved in ECM composition and adhesion, including collagens (Fig. 5A). Moreover, we also noted that both PWO577 and SY007 led to a significant

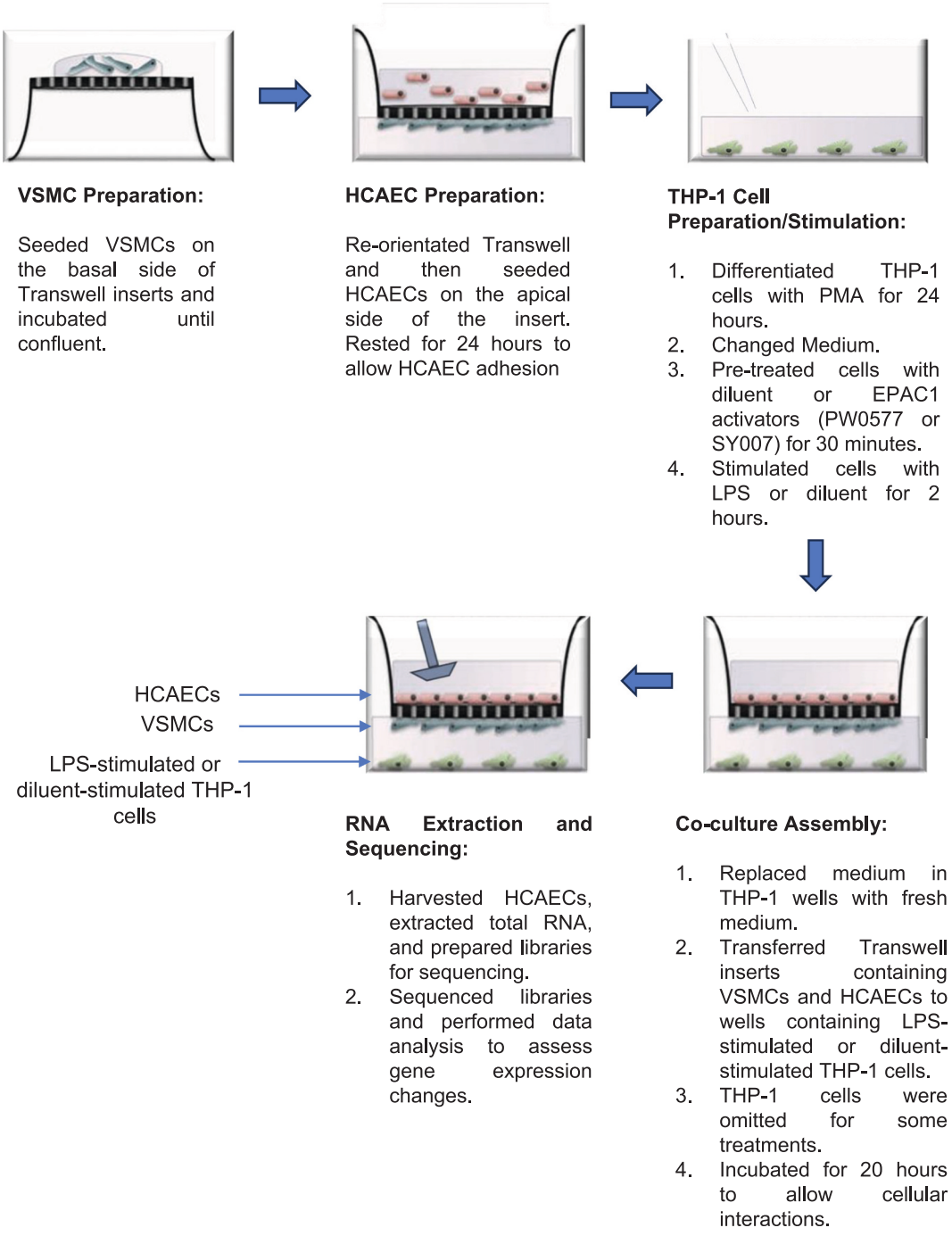


Fig. 7. Differential Gene Expression Analysis in HCAECs co-cultured with VSMC and THP-1 Macrophages.
A workflow diagram for this experiment is shown in the *upper panel* of the figure. In the *lower panel*, volcano plots illustrate the gene expression modulation in HCAEC cells growing in co-culture with VSMCs under varying conditions: (A) Baseline without macrophages, (B) with control macrophages treated with diluent, (C) with LPS-stimulated macrophages, (D) with LPS-stimulated macrophages treated with PWO577, and (E) with LPS-stimulated macrophages treated with SY007. The plots visualize the fold change in gene expression and its statistical significance, highlighting the impact of treatments on HCAEC gene expression, with significant gene changes ($p \leq 0.05$; $-\log10$ *p*-value ≥ 1.3; $n = 5$) are shown in light grey, with non-significant changes in black. Labelled genes highlight notable inflammatory mediators and pathway components, elucidating the impacts of PWO577 and SY007 on LPS-mediated activation. Here, key significant gene expression changes are denoted by blue (up-regulation) and orange (down-regulation) dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

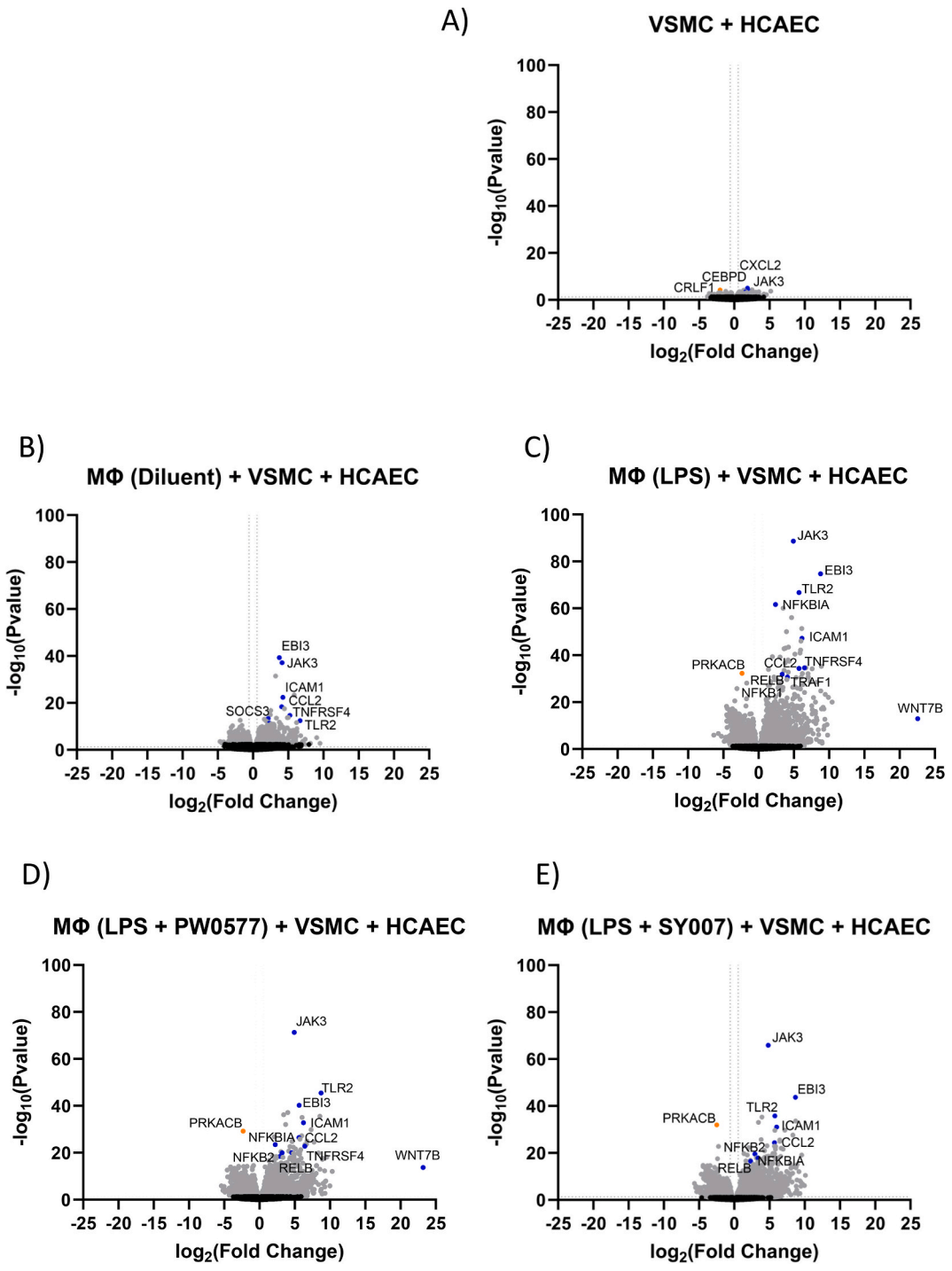


Fig. 7. (*continued*).

downregulation of genes involved in LPS-induced NF-κB signalling, originally identified in THP-1 cells by Sharif et al. [44], including fibronectin (FN1) and *E*-selectin (SELE; Fig. 5B) as well as members of the tumour necrosis factor receptor superfamily (TNFRSF), which are normally involved in the upregulation of components of the IL-6-signalling pathway in THP-1 macrophage-like cells [45] and other cell types [46,47]. Notably, the presence of EPAC1 activators influenced the expression of several genes associated with ECM function and cytokine signalling networks, which highlights their impact on inflammatory and, potentially, fibrotic responses modulation in THP-1-derived macrophage-like cells.

3.4. Western Blot Analysis of NF-κB gene targets in THP-1 Cells

Considering the observed effects of PWO577 and SY007 on genes associated with NF-κB signalling, as illustrated in Fig. 5B, we subsequently conducted Western blot analyses on THP-1 macrophage extracts. These cells were treated with LPS, both in the presence and absence of EPAC1 activators, over the time courses depicted in Fig. 6. Our first objective was to quantify the protein levels of p65-pNF-κB and to assess the phosphorylation status of p65-pNF-κB at Ser536. The phosphorylation at this site is recognized as a crucial mechanism for the downregulation of pro-inflammatory gene expression [48].

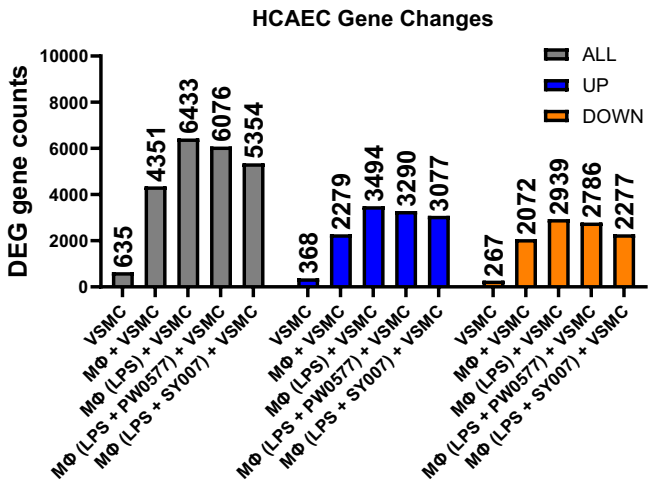


Fig. 8. Quantitative analysis of HCAECs gene expression modulation by PWO577 and SY007 in co-culture with LPS-stimulated THP-1 Macrophages and VSMCs.
The bar graph represents the number of significantly regulated genes in HCAECs across five different conditions. Each bar denotes the count of genes exceeding the established significance threshold of p-value >0.05 and an absolute log2 fold change >1. Grey bars indicated total gene expression changes HCAECs in response to the indicated co-culture conditions. Blue bars indicated the number of up-regulated genes in HCAECs in response to the same conditions, whereas orange bars indicate the number of genes that are down-regulated in HCAECs, in response to the same treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

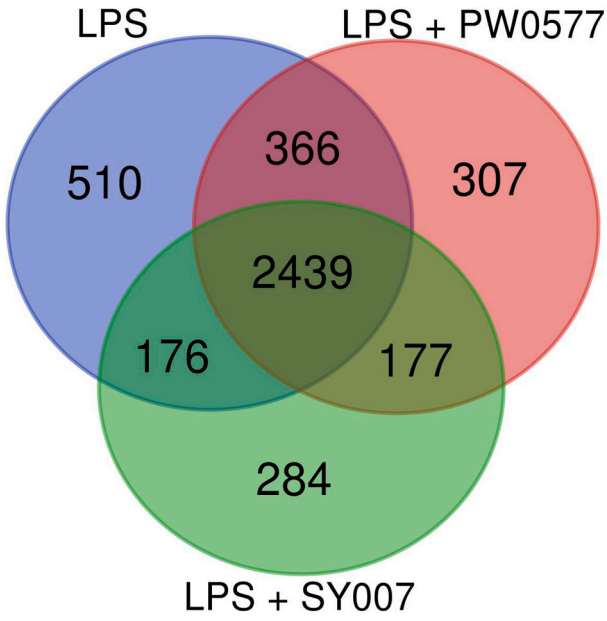


Fig. 9. Venn diagram illustrating the unique and overlapping sets of genes up-regulated in HCAECs in Response to LPS-stimulated THP-1 Macrophages and the impact of PWO577 and SY007.
The blue circle represents genes uniquely up-regulated in HCAECs by co-incubation with LPS-stimulated THP-1 macrophages (510 genes). The red circle shows genes up-regulated uniquely by LPS in combination with PWO577 (307 genes), and the green circle represents those uniquely up-regulated by LPS in combination with SY007 (284 genes). The central overlapping region indicates genes commonly up-regulated by all three conditions (2439 genes), suggesting a core set of inflammation-related genes responsive to LPS stimulation. The numbers in the non-overlapping segments of the LPS + PWO577 and LPS + SY007 circles (366 and 176, respectively) indicate genes whose up-regulation is specific to the interaction of LPS with each compound. The diagram was generated using the online tool available at https://bioinformatics.psb.ugent.be/webtools/Venn/. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To evaluate the potential influence of NF-κB inhibition on downstream gene targets, we also examined the protein expression of key components in the inflammasome signalling pathway, namely NLRP3, IL1β, and caspase 1. These components are known to be upregulated following LPS stimulation and subsequent NF-κB activation in macrophages [49,50] and contribute to inflammatory and fibrotic responses [51].

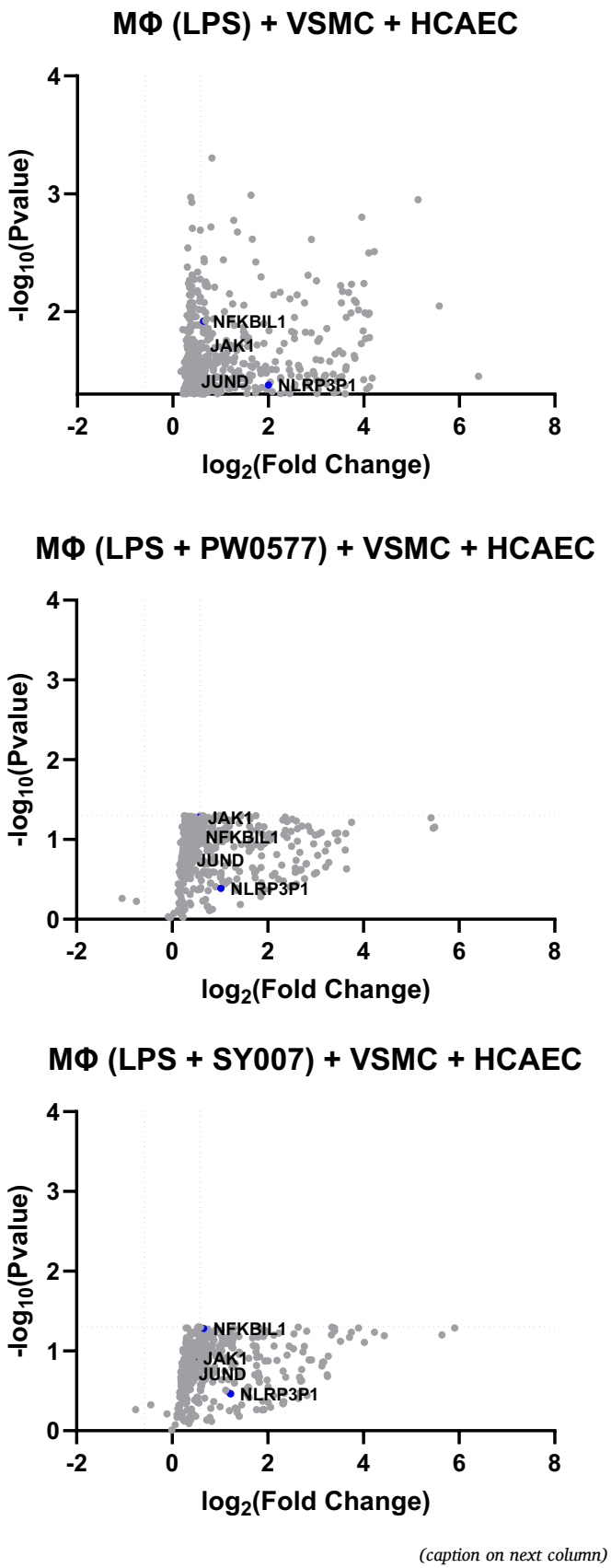
Densitometric analysis of the immunoblots, as presented in Fig. 6A, revealed a notable increase in p65-pNF-κB (Ser536) phosphorylation after 30 min of LPS stimulation (Fig. 6A). This phosphorylation level returned to baseline within 24 h while the total protein levels of p65-pNF-κB remained unchanged. Co-treatment with PWO577 or SY007 did not significantly reduce the duration or magnitude of p65-pNF-κB (Ser536) phosphorylation (Fig. 6A). This finding suggests that the action of EPAC1 activators in THP-1 cells might be independent of the phosphorylation of p65-pNF-κB at Ser536. In contrast, when LPS-stimulated cells were co-treated with PWO577 or SY007, there was a marked inhibition in the protein expression of NLRP3 starting at 5 h and lasted until 24 h (Fig. 6B), and IL1β (Fig. 6C) and caspase 1 (Fig. 6D) after 24 h of LPS stimulation. These results support the hypothesis that EPAC1 activators can suppress the expression of NF-κB-regulated gene targets in THP-1-derived macrophage-like cells. This also contributes to our understanding of the molecular mechanisms by which EPAC1 activators modulate inflammatory and fibrotic responses, particularly highlighting their potential role in influencing NF-κB-mediated pathways.

3.5. *Effects of PWO577 and SY007 on Vascular Immune Interplay*

In order to measure the impact of EPAC1 activators on cell-cell communication, we next investigated the modulatory effects of the EPAC1 activators PWO577 and SY007 on inflammatory responses in a co-culture system consisting of Human Coronary Artery Endothelial Cells (HCAECs), Vascular Smooth Muscle Cells (VSMCs), and LPS-stimulated THP-1 cells that had been differentiated into macrophage-like cells, as described in *Materials and Methods* and originally devised by Noonan et al. [41]. This model is technically simple and allows for the isolation of pure populations of each cell type without the need for cell sorting. The study by Noonan et al. [41] demonstrates that these triple-cell interactions produce unique cellular phenotypes and mediator profiles, highlighting the necessity for multi-cellular *in vitro* systems to better represent *in vivo* conditions in cardiovascular research.

The goal here was to use this 3-cell culture system to understand how EPAC1 activators influence soluble mediator release from activated THP-1 cells as a measure of immune/vascular interplay. The inclusion of VSMCs in the co-culture model aims to replicate the complex cellular interactions within the vascular wall during inflammation more accurately. VSMCs' phenotypic plasticity and their ability to produce a range of inflammatory mediators make them crucial for studying the interplay between immune cells and vascular cells, as demonstrated in our previous publication [52]. By incorporating VSMCs in co-culture experiments, we provide a more comprehensive model of the cellular and molecular mechanisms driving immune/endothelial interplay.

To explore the effects of the EPAC1 activators PWO577 and SY007 on HCAECs co-cultured with VSMCs and LPS-activated THP-1 macrophage-like cells under various conditions (described in Fig. 7), RNAseq experiments were carried out to capture and analyse transcriptional changes in HCAECs. The study began with maintaining HCAECs under standard conditions (co-cultured with VSMCs only) while differentiated THP-1 cells were then exposed to different treatments: a control group with only diluent, an LPS-stimulated group to induce inflammation, and groups pre-treated with PWO577 or SY007



(caption on next column)

Fig. 10. Differential Gene Expression in HCAECs in the Presence of VSMCs and of LPS-stimulated THP-1 Macrophages and Treated with PWO577 or SY007. The series of volcano plots illustrate the comparative gene expression profiles of the 501 genes that were up regulated in HCAECs, following co-culture with differentiated THP-1 cells that had been pre-treated with LPS identified in Fig. 9 (upper plot). The middle plot represents the response of the same 501 genes in HCAECs to co-culture of THP-1 macrophages that had been pre-stimulated with LPS plus PWO577. The bottom plot represents the response of the same 501 genes in HCAECs to co-culture with THP-1 cells pre-stimulated with LPS plus SY007. The upper plot displays a strong inflammatory response to LPS-stimulated THP-1 MΦs with increased expression of key inflammatory genes, such as NFKB1, JAK1, JUND, and NLRP3 Collectively, these plots demonstrate how PWO577 and SY007 may mitigate the effects of LPS-stimulated MΦs in HCAECs by inhibiting immune-vascular interplay and down-regulating inflammatory genes in HCAECs.

prior to LPS exposure. This pre-treatment was intended to assess how EPAC1 activators modulate the inflammatory response elicited by LPS in THP-1 macrophage. HCAECs/VSMCs were placed in Transwell inserts either alone or with THP-1 macrophages below them, allowing cellular communication, without direct contact. Total RNA was extracted from HCAECs after a further 20 h incubation using a standard RNA isolation kit. Libraries prepared from these RNA samples were then sequenced for transcriptome analysis.

The analysis of RNAseq data was visualized using volcano plots to highlight the differential expression of genes across different treatment conditions (Fig. 7). These plots demonstrated that in the absence of macrophage activation, gene expression in HCAECs showed minimal fluctuations, indicating a stable endothelial phenotype without external stimuli. However, prior LPS stimulation of THP-1 macrophages markedly increased inflammatory markers in co-cultured HCAECs, underscoring the potent inflammatory response elicited by LPS stimulation of macrophage-like THP-1 cells. Notably, pre-treatment of THP-1 cells with PWO577 or SY007 before LPS exposure resulted in a significant reduction in the number of genes up regulated in HCAECs that had been co-cultured with the pre-stimulated THP-1 cells. Fig. 8 quantitatively captures the extent of gene expression changes in HCAECs across different conditions. LPS pre-stimulation of co-cultured THP-1 cells increased the number of altered genes, emphasizing its potent inflammatory effect. The addition of PWO577 and SY007 to LPS-stimulated THP-1 cells adjusted these changes in HCAECs, with SY007 showing a more pronounced reduction in both up-regulated and down-regulated genes compared to PWO577.

Venn diagram analysis of this data (Fig. 9) provided an illustration of how the transcriptional landscape of HCAECs was altered under different treatment conditions involving LPS-stimulated THP-1 cells and the EPAC1 activators, PWO577 and SY007. Key findings show that 510 genes were uniquely up regulated in HCAECs following co-culture with LPS-stimulated THP-1 macrophages alone, highlighting the robust activation of the macrophage immune response in HCAECs through classic inflammatory pathways. In contrast, when HCAECs were co-cultured with LPS-stimulated THP-1 cells, in the presence of PWO577, 307 genes were uniquely up regulated, indicating that PWO577 alters the response of THP-1 cells to LPS by modulating different subsets of genes, potentially steering the response of HCAECs, to stimulated THP-1 cells, along a modified pathway. Similarly, treatment of THP-1 cells with LPS and SY007 resulted in a unique up-regulation of 284 genes in co-cultured HCAECs, suggesting that SY007 treatment of THP-1 cells also uniquely influences co-cultured HCAEC gene expression, possibly targeting alternative or additional pathways compared to PWO577.

The analysis also highlighted genes that were up regulated in HCAECs across all treatments, with 2439 genes consistently expressed under each condition, emphasizing a core set of inflammatory genes that are fundamental to the response of HCAECs to macrophage activation by LPS, which remained unaffected by the presence of EPAC1 activator. This suggests that while activator-treatment of THP-1 cells modify the

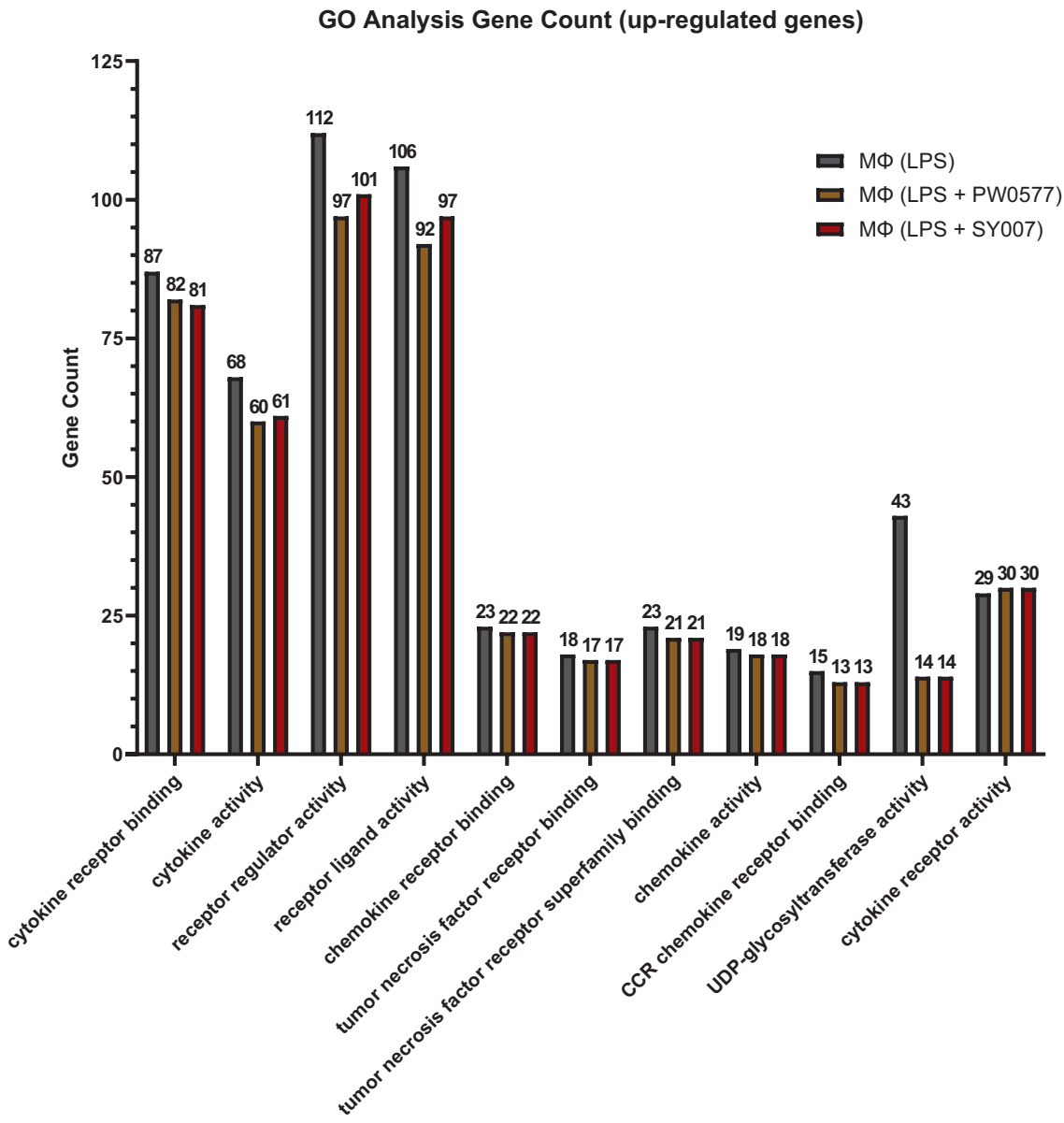


Fig. 11. Comparative Analysis of the Effects of PWO577 and SY007 on Gene Ontology (GO) Terms in LPS-stimulated THP-1 Macrophages Co-cultured with VSMCs. The figure presents an analysis of the 501 genes up-regulated in HCAECs from Fig. 9, which were co-incubated with LPS-stimulated THP-1 macrophages and VSMCS and demonstrates the impact of treatments PWO577 and SY007 on various GO terms, including cytokine receptor binding, cytokine activity, receptor ligand activity, and receptor regulator activity in the context of THP-1 macrophages (MΦ) stimulated with LPS and co-cultured with VSMCs.

response of HCAECs, they do not completely abolish the fundamental LPS-driven inflammatory reaction from THP-1 cells. Furthermore, the diagram identified genes that were co-up-regulated by both LPS and PWO577 (366 genes) and LPS and SY007 (176 genes), indicating that while each compound has its unique targets, they also share common pathways in THP-1 cells that modulate the inflammatory response in co-cultured HCAECs. This shared gene expression could point to common regulatory mechanisms or signalling pathways that are impacted by both treatments.

Fig. 10 illustrates the impact of the EPAC1 activators on the expression of the 510 genes in HCAECs in response to LPS-stimulated THP-1 cells and presents volcano plots that detail how the addition of PWO577 and SY007 modifies the expression profiles of these genes in HCAECs. The data demonstrate that both PWO577 and SY007 reduce the number of genes strongly up-regulated in HCAECs in response to LPS-stimulated macrophages, including JAK1, NFKBIL1, JUND and NLR3P1, suggesting a dampening of the LPS-driven pro-inflammatory response in THP-1 cells, indicating that EPAC1 activators may shift the macrophage response of HCAECs from a classic pro-inflammatory profile to a more balanced or even anti-inflammatory profile. Indeed, Fig. 11 expands on these findings by providing a GO analysis of the genes affected in HCAECs by LPS-stimulated THP-1 macrophages, which highlights significant changes in terms related to cytokine activity and cytokine receptor binding, indicating a suppression of key inflammatory mediators.

4. Discussion

This study investigates the role of EPAC1 activators in modulating TLR4-triggered inflammatory responses in THP-1 macrophages. TLR4, activated by LPS, initiates a cascade leading to cytokine production and immune response. Cyclic AMP signalling, regulated by adenylate cyclase and cyclic AMP phosphodiesterases (PDEs), plays a crucial role in controlling TLR4-regulated inflammation. Adenylate cyclase catalyses the

conversion of ATP to cyclic AMP, while PDEs degrade cyclic AMP, thus modulating its intracellular levels. Cyclic AMP-specific phosphodiesterases (PDE4s), specifically PDE4B, have been shown to regulate TLR4-mediated inflammatory responses in various cell types [25,53,54]. The PDE4 inhibitor, roflumilast, has been shown to effectively reduced the production of inflammatory mediators such as nitric oxide (NO) and tumour necrosis factor-alpha (TNF-α), and inhibited NF-κB pathway activation in response to LPS exposure [54]. In addition, it was found that inhibition of PDE4B enhances the production of interleukin-1 receptor antagonist (IL-1Rα) in response to TLR4 activation, mediated through the cAMP-PKA pathway [53]. Indeed, PDE4B, as a major cyclic AMP metabolizing enzyme in inflammatory cells, plays a critical role in the regulation of inflammation [55]. These studies collectively suggest that the cyclic AMP signalling pathway, particularly the role of PDE4B, is crucial in modulating TLR4-regulated inflammatory responses. They also point towards the potential therapeutic applications of PDE4 inhibitors in treating inflammation-related conditions.

The role of EPAC1 in the control of inflammation, particularly in relation to PDE4s, is a complex interplay of cellular signalling pathways. PDE4s, including PDE4B, regulate inflammation by controlling cyclic AMP levels within cells. When PDE4s are inhibited, intracellular cAMP levels rise, leading to the activation of PKA and EPAC1/2 [56]. Activation of EPAC1 has been found to influence the balance between pro-inflammatory and anti-inflammatory cytokines. For example, investigation into the role of EPAC1 and EPAC2 in airway inflammation and remodelling, in response to cigarette smoke, indicated that while EPAC2 plays a more prominent role in enhancing inflammatory processes, EPAC1 appears to have a capacity to inhibit remodelling processes, showcasing its potential role in anti-inflammatory pathways [57]. Additionally, another study investigating the effects of EPAC activation on cardiac myocytes showed that EPAC1 can modulate cytokine signalling. Specifically, the activation of EPAC1 was observed to inhibit the IL-6-induced phosphorylation of STAT3 and STAT1, and increase the mRNA expression of SOCS3 and SOCS1, which are negative regulators of the Jak-STAT pathway [58]. These findings indicate that EPAC1 can act as an inhibitor in the IL-6-mediated Jak-STAT pathway activation.

The EPAC1 activators, SY007 and PWO577, selectively promote EPAC1 activation over EPAC2 and PKA [35,37,38], showing limited cytotoxicity and altering gene expression patterns, including IL-6 induced STAT3 suppression [38]. Given the role of cyclic AMP and EPAC1 in the suppression of inflammation, we investigated here the impact of PWO577 and SY007 on LPS-stimulated gene expression in THP-1 derived macrophages. RNAseq analysis showed significant gene expression changes, including a shared influence of both activators on LPS signalling. KEGG pathway analysis revealed their involvement in extracellular matrix (ECM) interaction and cytokine signalling pathways, suggesting an influence on the mechanisms underlying fibrosis.

Cyclic AMP plays a pivotal role in the regulation of fibrosis, a condition characterized by excessive accumulation of ECM components, leading to organ dysfunction and failure [14,59]. Some of the anti-fibrotic effects of cyclic AMP appear to be mediated through the activation of EPAC1 [15,17]. These effects encompass the inhibition of fibroblast activation and proliferation, regulation of ECM production and degradation, and modulation of inflammatory responses, all central to the fibrotic process [15,17]. Moreover, inhibition of PDE4B leads to increased cyclic AMP concentrations, enhancing its anti-fibrotic effects [18]. This mechanism is particularly relevant in fibrotic diseases, where PDE4B inhibitors, currently in clinical trials for the treatment of fibrosis, could be used as therapeutic agents [60]. These inhibitors not only activate PKA but also EPAC1, a guanine nucleotide exchange factor directly activated by cAMP [61].

Here we have shed light on the specific actions of EPAC1 activators in inhibiting the expression of ECM components in THP-1 macrophagess (Fig. 5A). This inhibition is crucial in the context of fibrotic diseases, as overproduction of ECM components is a defining feature of fibrosis [62,63]. Furthermore, EPAC1 activators have been shown here to inhibit the induction of inflammasome components involved in fibrosis, such as IL-1β, caspase 1, and NLRP3 (Fig. 6). These components are key players in the inflammatory response and their overactivation can exacerbate inflammatory and fibrotic conditions [64,65]. By inhibiting these components, EPAC1 activators can potentially reduce inflammation and fibrosis, offering a promising therapeutic strategy. The role of EPAC1 and cyclic AMP in modulating TLR4 signalling and NF-κB-regulated gene expression is intricately cell-type dependent, with EPAC1 notably activation NF-κB activation and its downstream signalling in various cell types [28,29] and here, inhibition of NF-κB-mediated gene activation in THP-1 cells (Fig. 6A), although the specifics of this interaction can be complex and context-dependent. For instance, one study discussed the essential role of EPAC1 and EPAC2 for osteoclast differentiation, indicating that the activation of these proteins promotes NF-κB p50/p105 nuclear translocation [33]. Studies like this suggest that the role of EPAC1 in regulating NF-κB is multifaceted and likely depends on the cell type, the physiological or pathological context, and the downstream effectors involved in specific signalling cascades. This research contributes to this field by showcasing the effects of EPAC1 activators on LPS-stimulated gene expression in THP-1 derived macrophages, highlighting their influence on both inflammatory and fibrotic responses, thereby providing new insights into the intricate mechanisms of TLR4 signalling and NF-κB pathway modulation. Further research is needed to fully elucidate the mechanisms by which EPAC1 interacts with the NF-κB pathway and to determine the therapeutic potential of targeting this interaction in various diseases. Such research could contribute significantly to the development of novel treatment strategies for conditions where the dysregulation of NF-κB is a contributing factor.

In addition to analysing the impact of EPAC1 activators on the suppression of LPS signalling in macrophages, we investigated further on how this modulation influences inflammatory signalling in HCAECs in the presence of VSMCs. The results underscore the intricate ways in which co-culture systems can elucidate cellular interactions that are crucial for understanding the pathophysiology of inflammation and the therapeutic potential of targeted interventions like EPAC1 activators. We found that both PWO577 and SY007 significantly dampened LPS-induced cell-cell inflammatory signalling from THP-1 macrophages, as highlighted in the RNAseq analysis of co-cultures HCAECs and subsequent gene expression profiling.

Our study provides critical insights into the effects of non-cyclic nucleotide EPAC1 activators SY007 and PWO577 on LPS-regulated gene expression in THP-1-derived macrophage-like cells. Despite exhibiting similar binding affinities to EPAC1, these two activators showed qualitative differences in the number of genes modulated (860 genes for SY007 and 695 genes for PWO577). This raises intriguing questions about the underlying mechanisms that drive these differences. The differential gene expression observed between SY007 and PWO577 can be primarily attributed to their partial agonistic activity. Both compounds engage the Ala322 residue within EPAC1, which is crucial for destabilizing its inactive conformation. However, neither SY007 nor PWO577 effectively engages Lys395, a key residue required for stabilizing the active conformation of EPAC1 (results not shown). This partial engagement results in suboptimal activation of EPAC1, leading to differential downstream signalling events. The extent of EPAC1 activation by these agonists is likely to influence specific subsets of target genes, contributing to the observed qualitative differences in gene expression. KEGG pathway analysis of the RNA sequencing data revealed that both SY007 and PWO577 significantly impacted genes associated with extracellular matrix (ECM) interaction and cytokine signalling pathways. However, the specific genes within these pathways that are regulated by each agonist differ, indicating selective pathway modulation. The unique influence of each agonist on distinct signalling pathways likely contributes to the qualitative differences in gene expression profiles observed in LPS-stimulated THP-1 cells. The cellular context of THP-1-derived macrophage-like cells further influences the differential gene expression responses to SY007 and PWO577. The state of

differentiation, the presence of other signalling molecules, and the specific cellular environment can all affect how these agonists modulate gene expression. The unique cellular context of THP-1 cells might lead to distinct interactions between EPAC1 activation and other intracellular pathways, thereby driving the observed qualitative differences in gene modulation.

In summary, the qualitative differences in gene modulation by SY007 and PWO577 in LPS-stimulated THP-1 cells can be explained by their partial agonistic activity, differential temporal dynamics of activation, selective pathway modulation, and cell type-specific effects. These findings underscore the complexity of EPAC1 signalling and highlight the importance of considering these factors when interpreting the effects of EPAC1 activators on gene expression. Further research is needed to fully elucidate the intricate mechanisms by which EPAC1 activation leads to specific gene expression outcomes and to explore the potential therapeutic applications of these EPAC1 activators in managing inflammation and fibrosis.

The exploration of EPAC1 activators, particularly in the context of modulating inflammation through macrophage signalling pathways, opens promising avenues for therapeutic interventions in vascular and systemic inflammatory diseases. The anti-inflammatory effects of EPAC1 activators, such as PWO577 and SY007, are mediated by altering key inflammatory pathways within macrophages, which in turn exert protective effects on vascular endothelial cells. This multi-cellular impact highlights the central role of macrophages in orchestrating inflammatory responses within the vascular microenvironment. For example, by influencing macrophage function, EPAC1 activators can shift the inflammatory profile from a pro-inflammatory to a more balanced or even anti-inflammatory state. Research has shown that EPAC1 selectively modulates cyclic AMP (cAMP) within immune cells, affecting various downstream signalling pathways including the mitigation of NF-κB signalling, which is a critical pathway in the transcription of inflammatory genes [26]. Moreover, by dampening the inflammatory response in macrophages, EPAC1 activators indirectly protect endothelial cells from the harmful effects of prolonged inflammatory exposure. This protection could help maintain endothelial integrity and function, critical factors in preventing vascular permeability and plaque formation associated with atherosclerosis [15]. In addition, the anti-inflammatory properties of EPAC1 activators hold potential therapeutic value for a range of diseases characterized by chronic inflammation, such as rheumatoid arthritis, inflammatory bowel disease, and pulmonary diseases like asthma and COPD [32].

To fully harness the therapeutic potential of EPAC1 activators, further studies are needed to understand their long-term effects on vascular health and their safety profile in chronic administration. Longitudinal studies in animal models and eventually in human trials will be crucial to ascertain the efficacy of these activators in chronic disease settings. Additionally, exploring the specific molecular mechanisms through which EPAC1 activators exert their effects will enable the design of more targeted therapies that minimize side effects. Research should also focus on the development of EPAC1 activators that can selectively target specific tissues or cell types, enhancing their efficacy and reducing potential systemic effects. Advanced drug delivery systems, such as nanoparticle-based therapies, could provide precision targeting of these activators to inflamed tissues, thereby maximizing therapeutic outcomes.

CRediT authorship contribution statement

Jolanta Wiejak: Writing – review & editing, Software, Methodology, Investigation. **Fiona A. Murphy:** Methodology, Investigation. **Graeme Barker:** Writing – review & editing, Methodology, Funding acquisition. **Pasquale Maffia:** Writing – review & editing, Funding acquisition, Conceptualization. **Stephen J. Yarwood:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The research here funded by the British Heart Foundation (BHF; grant numbers PG/21/10557 and PG/23/11680 awarded to GB, SY and PM)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2024.111444.

References

[1] T.A. Wynn, A. Chawla, J.W. Pollard, Macrophage biology in development, homeostasis and disease, Nature 496 (7446) (2013) 445–455.
[2] P.N. Moynagh, TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway, Trends Immunol. 26 (9) (2005) 469–476.
[3] C.U. Onyishi, G.E. Desanti, A.L. Wilkinson, S. Lara-Reyna, E.M. Frickel, G. Fejer, O. D. Christophe, C.E. Bryant, S. Mukhopadhyay, S. Gordon, R.C. May, Toll-like receptor 4 and macrophage scavenger receptor 1 crosstalk regulates phagocytosis of a fungal pathogen, Nat. Commun. 14 (1) (2023) 4895.
[4] L.A. O'Neill, D. Golenbock, A.G. Bowie, The history of Toll-like receptors - redefining innate immunity, Nat. Rev. Immunol. 13 (6) (2013) 453–460.
[5] B.S. Park, J.O. Lee, Recognition of lipopolysaccharide pattern by TLR4 complexes, Exp. Mol. Med. 45 (12) (2013) e66.
[6] D. Bhatt, S. Ghosh, Regulation of the NF-κB-mediated transcription of inflammatory genes, Front. Immunol. 5 (2014) 71.
[7] J. Zheng, L. Yao, Y. Zhou, X. Gu, C. Wang, K. Bao, Y. Sun, M. Hong, A novel function of NLRP3 independent of inflammasome as a key transcription factor of IL-33 in epithelial cells of atopic dermatitis, Cell Death Dis. 12 (10) (2021) 871.
[8] F.G. Bauernfeind, G. Horvath, A. Stutz, E.S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B.G. Monks, K.A. Fitzgerald, V. Hornung, E. Latz, Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression, J. Immunol. 183 (2) (2009) 787–791.
[9] Y. Yang, H. Wang, M. Kouadir, H. Song, F. Shi, Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors, Cell Death Dis. 10 (2) (2019) 128.
[10] G.L. Negreiros-Lima, K.M. Lima, I.Z. Moreira, B.L.O. Jardim, J.P. Vago, I. Galvão, L. C.R. Teixeira, V. Pinho, M.M. Teixeira, M.A. Sugimoto, L.P. Sousa, Cyclic AMP regulates key features of macrophages via PKA: recruitment, reprogramming and efferocytosis, Cells 9 (1) (2020) 128.
[11] T. Veremeyko, A.W.Y. Yung, M. Dukhinova, I.S. Kuznetsova, I. Pomytkin, A. Lyundup, T. Strekalova, N.S. Barteneva, E.D. Ponomarev, Cyclic AMP pathway suppress autoimmune neuroinflammation by inhibiting functions of encephalitogenic CD4 T cells and enhancing M2 macrophage polarization at the site of inflammation, Front. Immunol. 9 (2018) 50.
[12] A.L. Hertz, A.T. Bender, K.C. Smith, M. Gilchrist, P.S. Amieux, A. Aderem, J. A. Beavo, Elevated cyclic AMP and PDE4 inhibition induce chemokine expression in human monocyte-derived macrophages, Proc. Natl. Acad. Sci. U. S. A. 106 (51) (2009) 21978–21983.
[13] V.K. Raker, C. Becker, K. Steinbrink, The cAMP pathway as therapeutic target in autoimmune and inflammatory diseases, Front. Immunol. 7 (2016) 123.
[14] M. Delaunay, H. Osman, S. Kaiser, D. Diviani, The role of cyclic AMP signaling in cardiac fibrosis, Cells 9 (1) (2019) 69.
[15] P.A. Insel, F. Murray, U. Yokoyama, S. Romano, H. Yun, L. Brown, A. Snead, D. Lu, N. Aroonsakool, cAMP and Epac in the regulation of tissue fibrosis, Br. J. Pharmacol. 166 (2) (2012) 447–456.
[16] U. Yokoyama, H.H. Patel, N.C. Lai, N. Aroonsakool, D.M. Roth, P.A. Insel, The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals, Proc. Natl. Acad. Sci. 105 (17) (2008) 6386–6391.
[17] X. Cao, Y. Li, J. Shi, H. Tang, The potentially therapeutic role of EPAC in curbing the process of idiopathic pulmonary fibrosis via differential cellular pathways, J. Inflamm. Res. 14 (2021) 611–619.
[18] M. Kolb, B. Crestani, T.M. Maher, Phosphodiesterase 4B inhibition: a potential novel strategy for treating pulmonary fibrosis, Eur. Respir. Rev. 32 (167) (2023) 220206.

[19] L. Lis-López, C. Bauset, M. Seco-Cervera, J. Cosín-Roger, Is the macrophage phenotype determinant for fibrosis development? Biomedicines 9 (12) (2021) 1747.
[20] M. Biel, S. Michalakis, Cyclic nucleotide-gated channels, Handb. Exp. Pharmacol. 191 (2009) 111–136.
[21] P. Sassone-Corsi, The cyclic AMP pathway, Cold Spring Harb. Perspect. Biol. 4 (12) (2012) a011148.
[22] R.F. Schindler, T. Brand, The Popeye domain containing protein family – a novel class of cAMP effectors with important functions in multiple tissues, Prog. Biophys. Mol. Biol. 120 (1–3) (2016) 28–36.
[23] A.T. Bender, J.A. Beavo, Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use, Pharmacol. Rev. 58 (3) (2006) 488–520.
[24] C.H. Serezani, M.N. Ballinger, D.M. Aronoff, M. Peters-Golden, Cyclic AMP: master regulator of innate immune cell function, Am. J. Respir. Cell Mol. Biol. 39 (2) (2008) 127–132.
[25] S.L. Jin, L. Lan, M. Zoudilova, M. Conti, Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages, J. Immunol. 175 (3) (2005) 1523–1531.
[26] S. Gerlo, R. Kooijman, I.M. Beck, K. Kolmus, A. Spooren, G. Haegeman, Cyclic AMP: a selective modulator of NF-κB action, Cell. Mol. Life Sci. 68 (23) (2011) 3823–3841.
[27] G.C. Parry, N. Mackman, Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription, J. Immunol. 159 (11) (1997) 5450–5456.
[28] E.Y. Moon, S. Pyo, Lipopolysaccharide stimulates Epac1-mediated Rap1/NF-kappaB pathway in Raw 264.7 murine macrophages, Immunol. Lett. 110 (2) (2007) 121–125.
[29] E.Y. Moon, J.H. Lee, J.W. Lee, J.H. Song, S. Pyo, ROS/Epac1-mediated Rap1/NF-kappaB activation is required for the expression of BAFF in Raw264.7 murine macrophages, Cell. Signal. 23 (9) (2011) 1479–1488.
[30] Y. Jiang, J.J. Steinle, Epac1 inhibits PKR to reduce NLRP3 inflammasome proteins in retinal endothelial cells, J. Inflamm. Res. 12 (2019) 153–159.
[31] Y. Jiang, L. Liu, E. Curtiss, J.J. Steinle, Epac1 blocks NLRP3 inflammasome to reduce IL-1β in retinal endothelial cells and mouse retinal vasculature, Mediat. Inflamm. 2017 (2017) 2860956.
[32] A. Oldenburger, S.S. Roscioni, E. Jansen, M.H. Menzen, A.J. Halayko, W. Timens, H. Meurs, H. Maarsingh, M. Schmidt, Anti-inflammatory role of the cAMP effectors Epac and PKA: implications in chronic obstructive pulmonary disease, PLoS One 7 (2) (2012) e31574.
[33] A. Mediero, M. Perez-Aso, B.N. Cronstein, Activation of EPAC1/2 is essential for osteoclast formation by modulating NFκB nuclear translocation and actin cytoskeleton rearrangements, FASEB J. 28 (11) (2014) 4901–4913.
[34] L. Liu, Y. Jiang, J. Steinle, Epac1 regulates TLR4 signaling in the diabetic retinal vasculature, Cytokine 144 (2021) 155576.
[35] E.M. Beck, E. Parnell, A. Cowley, A. Porter, J. Gillespie, J. Robinson, L. Robinson, A.D. Pannifer, V. Hamon, P. Jones, A. Morrison, S. McElroy, M. Timmerman, H. Rutjes, P. Mahajan, J. Wiejak, U. Luchowska-Stańska, D. Morgan, G. Barker, H. Rehmann, S.J. Yarwood, Identification of a novel class of benzofuran oxoacetic acid-derived ligands that selectively activate cellular EPAC1, Cells 8 (11) (2019) 1425.
[36] E. Parnell, S.P. McElroy, J. Wiejak, G.L. Baillie, A. Porter, D.R. Adams, H. Rehmann, B.O. Smith, S.J. Yarwood, Identification of a novel, small molecule partial agonist for the cyclic AMP sensor, EPAC1, Sci. Rep. 7 (1) (2017) 1–12.
[37] P. Wang, U. Luchowska-Stańska, B. van Basten, H. Chen, Z. Liu, J. Wiejak, P. Whelan, D. Morgan, E. Lochhead, G. Barker, H. Rehmann, S.J. Yarwood, J. Zhou, Synthesis and biochemical evaluation of noncyclic nucleotide exchange proteins directly activated by cAMP 1 (EPAC1) regulators, J. Med. Chem. 63 (10) (2020) 5159–5184.
[38] J. Wiejak, U. Luchowska, P. Wang, J. Zhou, P. Maffia, D. Morgan, G. Barker, S. Yarwood, Protein interaction, cytotoxic, transcriptomic and proteomic responses to structurally distinct EPAC1 activators in HUVECs, Sci. Rep. 12 (2022) 16505.
[39] S. Tedesco, F. De Majo, J. Kim, A. Trenti, L. Trevisi, G.P. Fadini, C. Bolego, P. W. Zandstra, A. Cignarella, L. Vitiello, Convenience versus biological significance: are PMA-differentiated THP-1 cells a reliable substitute for blood-derived macrophages when studying in vitro polarization? Front. Pharmacol. 9 (2018) 71.
[40] H.J. Kim, H. Kim, J.H. Lee, C. Hwangbo, Toll-like receptor 4 (TLR4): new insight immune and aging, Immun. Ageing 20 (1) (2023) 67.
[41] J. Noonan, G. Grassia, N. MacRitchie, P. Garside, T.J. Guzik, A.C. Bradshaw, P. Maffia, A novel triple-cell two-dimensional model to study immune-vascular interplay in atherosclerosis, Front. Immunol. 10 (2019) 849.
[42] F. Schwede, D. Bertinetti, C.N. Langerijs, M.A. Hadders, H. Wienk, J.H. Ellenbroek, E.J.P. de Koning, J.L. Bos, F.W. Herberg, H.-G. Genieser, R.A.J. Janssen, H. Rehmann, Structure-guided design of selective Epac1 and Epac2 agonists, PLoS Biol. 13 (1) (2015) e1002038.
[43] J. Wiejak, B. van Basten, U. Luchowska-Stańska, G. Hamilton, S.J. Yarwood, The novel exchange protein activated by cyclic AMP 1 (EPAC1) agonist, I942, regulates inflammatory gene expression in human umbilical vascular endothelial cells (HUVECs), Biochimica et Biophysica Acta, Mol. Cell Res. 1866 (2) (2019) 264–276.
[44] O. Sharif, V.N. Bolshakov, S. Raines, P. Newham, N.D. Perkins, Transcriptional profiling of the LPS induced NF-κB response in macrophages, BMC Immunol. 8 (1) (2007) 1.
[45] H.M. Choi, D.H. Oh, J.S. Bang, H.I. Yang, M.C. Yoo, K.S. Kim, Differential effect of IL-1β and TNFα on the production of IL-6, IL-8 and PGE2 in fibroblast-like synoviocytes and THP-1 macrophages, Rheumatol. Int. 30 (8) (2010) 1025–1033.
[46] P. De Cesaris, D. Starace, A. Riccioli, F. Padula, A. Filippini, E. Ziparo, Tumor necrosis factor-alpha induces interleukin-6 production and integrin ligand expression by distinct transduction pathways, J. Biol. Chem. 273 (13) (1998) 7566–7571.
[47] H. Steele, J. Cheng, A. Willicut, G. Dell, J. Breckenridge, E. Culberson, A. Ghastine, V. Tardif, R. Herro, TNF superfamily control of tissue remodeling and fibrosis, Front. Immunol. 14 (2023) 1219907.
[48] J.P. Pradère, C. Hernandez, C. Koppe, R.A. Friedman, T. Luedde, R.F. Schwabe, Negative regulation of NF-κB p65 activity by serine 536 phosphorylation, Sci. Signal. 9 (442) (2016) ra85.
[49] H. Guo, J.B. Callaway, J.P. Ting, Inflammasomes: mechanism of action, role in disease, and therapeutics, Nat. Med. 21 (7) (2015) 677–687.
[50] Y. Qiao, P. Wang, J. Qi, L. Zhang, C. Gao, TLR-induced NF-κB activation regulates NLRP3 expression in murine macrophages, FEBS Lett. 586 (7) (2012) 1022–1026.
[51] W.J. Zhang, S.J. Chen, S.C. Zhou, S.Z. Wu, H. Wang, Inflammasomes and fibrosis, Front. Immunol. 12 (2021) 643149.
[52] J. Wiejak, F.A. Murphy, P. Maffia, S.J. Yarwood, Vascular smooth muscle cells enhance immune/vascular interplay in a 3-cell model of vascular inflammation, Sci. Rep. 13 (1) (2023) 15889.
[53] J.-X. Yang, K.-C. Hsieh, Y.-L. Chen, C.-K. Lee, M. Conti, T.-H. Chuang, C.-P. Wu, S.L. C. Jin, Phosphodiesterase 4B negatively regulates endotoxin-activated interleukin-1 receptor antagonist responses in macrophages, Sci. Rep. 7 (1) (2017) 46165.
[54] D.H. Kang, S. Ahn, J.W. Chae, J.S. Song, Differential effects of two phosphodiesterase 4 inhibitors against lipopolysaccharide-induced neuroinflammation in mice, BMC Neurosci. 24 (1) (2023) 39.
[55] Y. Su, J. Ding, F. Yang, C. He, Y. Xu, X. Zhu, H. Zhou, H. Li, The regulatory role of PDE4B in the progression of inflammatory function study, Front. Pharmacol. 13 (2022) 982130.
[56] M.D. Houslay, Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown, Trends Biochem. Sci. 35 (2) (2010) 91–100.
[57] A. Oldenburger, W. Timens, S. Bos, M. Smit, A.V. Smrcka, A.C. Laurent, J. Cao, M. Hylkema, H. Meurs, H. Maarsingh, F. Lezoualc'h, M. Schmidt, Epac1 and Epac2 are differentially involved in inflammatory and remodeling processes induced by cigarette smoke, FASEB J. 28 (11) (2014) 4617–4628.
[58] H. Jin, T. Fujita, M. Jin, R. Kurotani, Y. Hidaka, W. Cai, K. Suita, R. Prajapati, C. Liang, Y. Ohnuki, Y. Mototani, M. Umemura, U. Yokoyama, M. Sato, S. Okumura, Y. Ishikawa, Epac activation inhibits IL-6-induced cardiac myocyte dysfunction, J. Physiol. Sci. 68 (1) (2018) 77–87.
[59] M. Schmidt, I. Cattani-Cavalieri, F.J. Nuñez, R.S. Ostrom, Phosphodiesterase isoforms and cAMP compartments in the development of new therapies for obstructive pulmonary diseases, Curr. Opin. Pharmacol. 51 (2020) 34–42.
[60] M. Senior, Fighting fibrosis, Nat. Biotechnol. 40 (8) (2022) 1169–1173.
[61] H. Li, J. Zuo, W. Tang, Phosphodiesterase-4 inhibitors for the treatment of inflammatory diseases, Front. Pharmacol. 9 (2018) 1048.
[62] K.E.C. Blokland, S.D. Pouwels, M. Schuliga, D.A. Knight, J.K. Burgess, Regulation of cellular senescence by extracellular matrix during chronic fibrotic diseases, Clin. Sci. (Lond.) 134 (20) (2020) 2681–2706.
[63] V. Sarohi, S. Chakraborty, T. Basak, Exploring the cardiac ECM during fibrosis: a new era with next-gen proteomics, Front. Mol. Biosci. 9 (2022) 1030226.
[64] C.M. Artlett, The mechanism and regulation of the NLRP3 Inflammasome during fibrosis, Biomolecules 12 (5) (2022) 634.
[65] X. Zhang, Z. Wang, Y. Zheng, Q. Yu, M. Zeng, L. Bai, L. Yang, M. Guo, X. Jiang, J. Gan, Inhibitors of the NLRP3 inflammasome pathway as promising therapeutic candidates for inflammatory diseases (review), Int. J. Mol. Med. 51 (4) (2023) 35.