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ORIGINAL ARTICLE

Apremilast effectively inhibits TNFα-induced vascular inflammation in human endothelial cells

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Abstract

Background Patients with chronic inflammatory diseases (e.g. psoriasis and rheumatoid arthritis) are at increased risk for the development of atherosclerosis and cardiovascular diseases (CVD). Previous studies have suggested that phosphodiesterase 4 (PDE4) inhibitors possess anti-inflammatory properties.

Objectives Here we examined the effect of the PDE4 inhibitor apremilast, a well-established anti-psoriatic drug, on pro-inflammatory responses in TNFα-activated endothelial cells.

Methods Human umbilical vein endothelial cells (HUVEC) were treated with tumour necrosis factor-α (TNFα) in the presence or absence of apremilast. Expression levels of pro-inflammatory cytokines, chemokines and adhesion molecules were assessed by ELISA, western blot and RT-PCR. Effects of apremilast on adhesion and transendothelial migration (TEM) of THP-1 monocytic cells were analysed in transwell assays.

Results Apremilast suppressed TNFα-induced expression and secretion of important endothelial and monocytic pro-inflammatory factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF), C-X-C motif chemokine ligand 10 (CXCL10), chemokine (C-C motif) ligand 2 (CCL2), vascular cell adhesion molecule 1 (VCAM-1), E-selectin and matrix metalloproteinase-9 (MMP9). Functionally, apremilast reduced adhesion of THP-1 cells to activated HUVECs and TEM in response to TNFα. Mechanistically, apremilast suppressed activation of nuclear factor κB (NFκB) and mitogen-activated protein kinases (MAPK) signalling in activated HUVECs. Furthermore, inhibition of p38, C-Jun-N-terminale Kinase (JNK) and NFκB in activated HUVECs decreased expression of GM-CSF, VCAM-1 and E-selectin. Additionally, apremilast decreased IL-17A-induced secretion of IL-6 and CCL2.

Conclusions We demonstrate that apremilast has distinct anti-inflammatory effects in activated HUVECs, indicating that apremilast could have the therapeutic potential to prevent higher risk for CVD in patients with chronic inflammatory diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Introduction

Epidemiologic studies demonstrate that patients with chronic inflammatory diseases (e.g. psoriasis and rheumatoid arthritis) are at increased risk for development of atherosclerosis and cardiovascular diseases (CVD).[1] Early atherogenesis is characterized by recruitment of monocytes and lymphocytes into endothelium. The process of monocytic and lymphocytic adhesion to inflammatory activated endothelial cells is regulated by a broad range of pro-inflammatory cytokines and chemokines such as GM-CSF, CCL2, CXCL10, CCL5 and several endothelial adhesion molecules including VCAM-1 and E-selectin.[2]

3′,5′-cyclic nucleotide phosphodiesterases (PDEs) are highly conserved enzymes which catalyse the conversion of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) into 5′-AMP and 5′-GMP by which mammalian cells regulate intracellular levels of cAMP and cGMP. cAMP is a crucial second messenger which negatively regulates the synthesis and release of pro-inflammatory

mediators and cytokines.[3] Increased PDE activity is associated with low intracellular cAMP-levels and subsequent initiation and progression of several inflammatory disorders, such as psoriasis, chronic obstructive pulmonary disease (COPD) and inflammatory bowel diseases.[4] Eleven human PDEs have been identified so far and only PDE4, PDE7 and PDE8 are cAMP-specific. Each PDE family has multiple isoforms that differ in structure and size. PDEs are expressed in almost all tissues such as brain, heart, adipose tissue, kidney, lung and various cell types including T-lymphocytes, monocytes, keratinocytes, endothelial cells and smooth muscle cells.[5] Within this group, aberrant expression and activity of PDE4 was found in peripheral blood mononuclear cells (PBMC) of psoriasis and COPD patients.[6,7]

Several synthetic PDE4 inhibitors (PDE4i) have been identified so far that elevate intracellular levels of cAMP, which results in decreased de novo synthesis of pro-inflammatory cytokines such as TNFα, IL-17 and IL-23, while inducing anti-inflammatory mediators such as IL-10 and IL-1 receptor antagonist. Anti-inflammatory effects of PDE4i are mainly mediated via modulation of Epac1/2-, protein kinase A (PKA)- and NFκB-dependent signalling in various immune cells such as T-lymphocytes, monocytes and dendritic cells.[8] Furthermore, PDE4i have been shown to reduce obesity-related inflammation by augmenting lipolysis, enhancing formation of brown adipose tissue and reducing infiltration of macrophages in white adipose tissue.[9–11] Taken together, cAMP elevation by PDE4 inhibition is closely associated with suppression of pro-inflammatory responses. So far, two compounds exhibiting activity against PDE4 – apremilast and roflumilast – have been approved in the United States and Europe for the treatment of psoriasis, active psoriasis arthritis, M. Behcet (Apremilast) and COPD (Roflumilast).[8] First evidence in an oxidized low-density lipoprotein (ox-LDL)-challenged human endothelial cell model has demonstrated anti-atherogenic activities of apremilast.[12] However, the underlying molecular mechanisms of apremilast-mediated inhibition of pro-inflammatory responses in human endothelial cells are not fully understood. Here, we used TNFα-activated primary human endothelial cells as in vitro model for inflamed endothelium to analyse the underlying molecular mechanisms of anti-inflammatory properties by apremilast.

Material and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs; PromoCell, Heidelberg, Germany) were cultured until the sixth passage in endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 5% fetal calf serum (FCS) and gentamicin/amphotericin B. THP-1 cells (ATCC, Manassas, VA, USA) were maintained in RPMI-1640 medium (Gibco-Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2 mM L-glutamine, 50 μM β-mercaptoethanol, 10% FCS and penicillin/streptomycin solution. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Chemicals

Detailed list of the chemicals used for the experiments are provided in the supplemental data (Appendix S1, Supporting Information).

Cell proliferation assay and cytotoxicity assay

The effects of apremilast and roflumilast on cell proliferation and cytotoxicity were measured by quantifying BrdU via a cell proliferation immunoassay from Roche Diagnostics (Mannheim, Germany) and using a lactate dehydrogenase-based cytotoxicity detection kit from Roche. The detailed cell assay processes were performed as described previously.[13]

Western blot analysis

Protein extracts were prepared as described previously.[14] Following SDS-PAGE and electroblotting, membranes were incubated with primary antibodies (Table S1, Supporting Information). Western blot analysis was performed as described earlier.[13] Densitometry was used to quantify band intensities using ImageJ software v1.52a (National institutes of Health, Bethesda, MD, USA). Optical densities of the bands were corrected for loading differences based on corresponding control bands.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of GM-CSF, CCL2, CXCL8, CXCL10, IL-6 and MMP-9 in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA; BioLegend, San Diego, CA, USA).

RNA extraction and reverse transcription–PCR

Reverse transcription–PCR (RT-PCR) analyses of GM-CSF were performed on total RNA (500 ng) extracted from subconfluent cell cultures. Detailed protocols and primer sequences are provided in the supplemental data (Table S2 and Appendix S1, Supporting Information).

Cell adhesion assay

To analyse the adherence of THP-1 on activated HUVEC monolayers, we used the CytoSelect Leukocyte-Endothelium Adhesion Assay kit (Cell Biolabs inc., San Diego, CA, USA) as described by the manufacturer. Briefly, 3×10^4 HUVECs were seeded into each well of gelatin-coated 96-well plates in 200 μL of endothelial cell growth medium supplemented with 5% FCS and grown for 24 h. Confluent HUVECs were treated with TNFα (20 ng/mL) in the presence or absence of apremilast (20, 40 μM) and DMSO as solvent control for 10 h. In the meantime, THP-1 cells were labelled with fluorescent LeukoTracker for 60 min at 37°C and then added to the endothelial cell monolayer for 60 min. After washing with washing buffer, remaining adherent THP-1 cells were lysed and fluorescence measured with a fluorometer

(Tecan Infinite M200, Tecan, Männedorf, Switzerland) at excitation and emission wavelengths of 480 and 520 nm, respectively.

Transendothelial migration assay

To determine THP-1 TEM across activated endothelial cells, we used a 24-well transwell system (Transwell® polycarbonate membrane cell culture inserts with 8 μm pore sizes, Corning, Corning, NY, USA). Briefly, 8×10^4 HUVECs were seeded to the upper chamber of gelatin-coated transwell inserts in 500 μL of endothelial cell growth medium supplemented with 5% FCS and grown for 24 h. After 48 h confluent HUVECs were treated with TNFα (20 ng/mL) in the presence or absence of apremilast (20, 40 μM) and DMSO as a control for 24 h. After washing the HUVEC monolayers with PBS, THP-1 cells were resuspended in serum-free RPMI-1640 and labelled with 10 μM BCEFC-AM (Invitrogen, Carlsbad, CA, USA), then added to the upper chamber of the transwell inserts ($1x10^5$/well). RPMI-1640 containing 10% FCS (as a chemotactic factor) was placed in the bottom chamber. After 4-h migration at 37°C in a humidified atmosphere containing 5% CO_2, transmigrated THP-1 cells from the bottom chamber were collected and fluorescence measured with a fluorometer (Tecan Infinite M200, Tecan, Männedorf, Switzerland) at excitation and emission wavelengths of 480 and 520 nm, respectively.

NFκB transcription factor activity assay

To analyse the DNA binding activity of NFκB (p50/p65) in HUVECs, we used the Trans-AM NFκB ELISA kit (ActiveMotif, Carlsbad, CA, USA). HUVECs were treated with TNFα (20 ng/mL) in the presence or absence of apremilast (20, 40 μM) and solvent control (=DMSO) for 1 h. Nuclear extracts were prepared as described previously.[15] Protein-DNA complexes were detected according to the manufacturer's protocol.

Immunolocalization of p65

Immunofluorescence staining was performed as described previously.[13] Detailed protocols and used antibodies are provided in the supplemental data (Appendix S1, Supporting Information).

Statistical analysis

Statistical analyses were done with GraphPad PRISM 5 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SE from ≥3 independent experiments. Significance testing was performed by Student's *t*-test and *P*-values of <0.05 were considered to be significant.

Results

Effects of PDE4i on cell proliferation and cytotoxicity in HUVECs

HUVECs were treated with apremilast and roflumilast or solvent only (0.2% DMSO) at indicated concentrations for 24 h. Apremilast and roflumilast did not induce cytotoxicity or affect cell proliferation of HUVECs at all tested concentrations, as determined by lactate dehydrogenase and BrdU assays (Fig. 1a, b), demonstrating that PDE4i in the dose range of 2.5–40 μM had no effects on cellular viability of HUVECs.

PDE4i effectively suppresses protein expression of important pro-inflammatory cytokines in TNFα-activated HUVECs

The impact of PDE4 inhibition was addressed by analysing the effects of apremilast and roflumilast on the protein expression of GM-CSF, CXCL8, CXCL10 and CCL2 of TNFα-activated HUVECs. We treated HUVECs with TNFα (20 ng/mL) in the presence or absence of apremilast (40 μM) and roflumilast (10 μM) and DMSO as solvent control for 24 h. Apremilast (40 μM) downregulated the expression of GM-CSF, CCL2 and CXCL10, whereas roflumilast (10 μM) downregulated the expression of GM-CSF and CCL2. Apremilast did not influence the expression of CXCL8 and roflumilast did not affect the expression of CXCL8 and CXCL10. Apremilast, however, showed more pronounced effects in inhibition of GM-CSF and CXCL10 protein expression (Fig. 2a,b). The inhibition of GM-CSF production by apremilast was dose- and time dependent and confirmed at protein and mRNA level (Fig. 2c-f). In addition, treatment with roflumilast inhibited GM-CSF release in a dose-dependent manner in activated HUVECs, suggesting that suppression of pro-inflammatory cytokines occurs in part through a signalling pathway dependent on PDE4 inhibition (Fig. 2g). Finally, we investigated the effects of apremilast and roflumilast on interleukin-17A (IL-17A)-induced endothelial inflammation. IL-17A is a major pro-inflammatory cytokine that initiates and maintains inflammation in psoriasis and vascular diseases.[16,17] Interestingly, we demonstrated that apremilast (10–40 μM) reduced IL-6 and CCL2 secretion in an dose-dependent manner, whereas roflumilast (2.5–10 μM) failed to inhibit the secretion of these pro-atherogenic factors in IL-17A-induced HUVECs (Fig. S1, Supporting Information). Taken together, our results suggest that apremilast inhibits IL-17A-induced secretion of pro-inflammatory molecules in part through a PDE4-independent pathway.

Apremilast suppresses MAPK-signalling pathways in TNFα-treated HUVECs

Several studies revealed the crucial role of MAPK signalling pathways in TNFα-induced expression of various pro-inflammatory cytokines and mediators.[18] Therefore, we analysed the phosphorylation status of JNK, p42/44 and p38 in HUVEC over time (0–60 min) in response to TNFα (20 ng/mL) or TNFα plus apremilast. Apremilast significantly inhibited the protein level of phosphorylated JNK and p38 in TNFα-induced HUVECs, while inhibition of phosphorylation of p42/44 was not affected. JNK phosphorylation was inhibited already at 5 min, inhibition, while p38 phosphorylation was blocked after 30 min

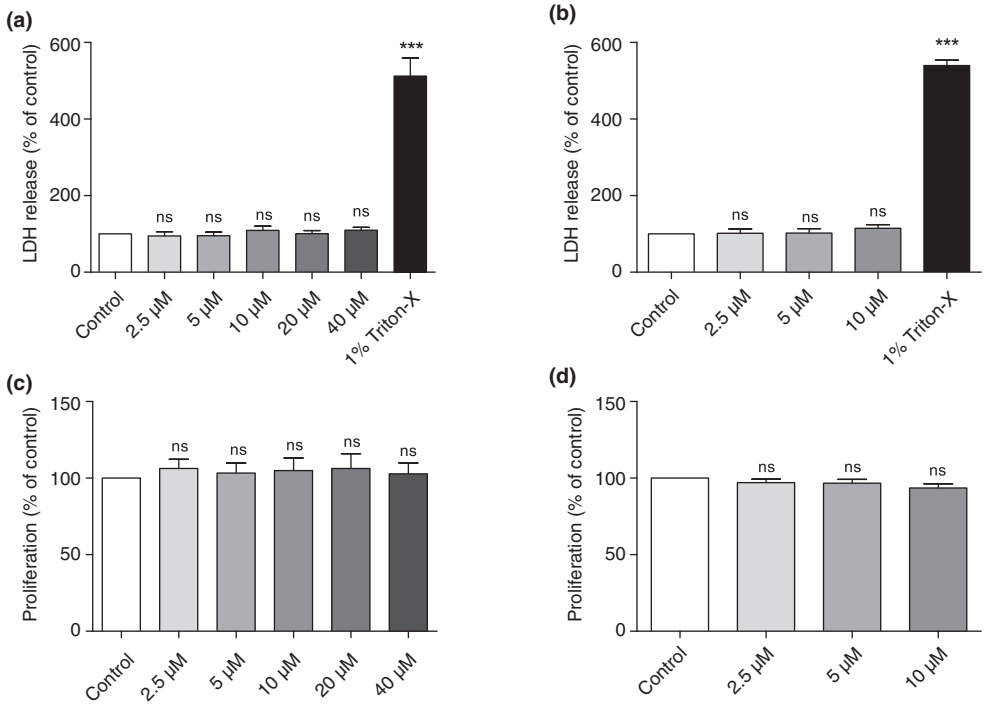

Figure 1 Effects of PDE4i on the HUVEC cytotoxicity and proliferation. (a and b) Quantification of cytotoxicity. Cells were incubated with increasing concentrations of apremilast and roflumilast for 24 h as indicated. Cytotoxicity was quantified by using the LDH assay. Average absorbance values (mean ± SE) from triplicate determinations per experimental condition were calculated; data are expressed as cytotoxicity in percentage (%). ***$P < 0.001$ vs. ctrl. (c and d) Cells were exposed to increasing concentrations of apremilast and roflumilast for 24 h as indicated. Cell proliferation was measured using the BrdU assay. Average absorbance values (mean ± SE) from 3 wells per experimental condition are displayed; data are expressed as cell proliferation in percentage (%) with regard to solvent controls (=100%; 0.2% DMSO). Results were confirmed in five independent sets of experiments. ***$P < 0.001$ vs. ctrl. ns, not significant.

treatment with apremilast (Fig. 3a,b). To dissect signalling elements in more detail we performed assays in the presence or absence of different MAPK inhibitors (SB203580, SP600125 and U0126) or a NFκB inhibitor (resveratrol). Consistent with our previous data, we found that blocking of JNK- and p38-signalling significantly inhibited the induction of GM-CSF, while inhibition of p42/44-signaling did not affect the expression of GM-CSF. Additionally, treatment with the NFκB inhibitor resveratrol blocked the expression of GM-CSF in TNFα-activated HUVECs (Fig. 3c).

Apremilast effectively blocks endothelial-monocytic cell interactions

Recruitment and accumulation of monocytes and transendothelial migration is an important step in various vascular diseases such as atherosclerosis.[19] Therefore, we examined the effect of apremilast on monocyte-endothelial adhesion and TEM. Apremilast suppressed dose-dependently the TNFα-induced adhesion of monocytic THP-1 cells to HUVECs (Fig. 4a). In addition, apremilast effectively blocked TEM of THP-1 cells in a concentration-dependent manner (Fig. 4b).

Since endothelial adhesion molecules such as VCAM-1, PECAM-1 and ICAM-1 are involved in the induction of endothelial injury with subsequent activation of early pro-inflammatory steps,[20] we next analysed the impact of apremilast on endothelial adhesion molecules. TNFα alone significantly increased the HUVEC protein expression of VCAM-1, ICAM-1, E-selectin, while expression of JAM-A and PECAM-1 was not affected. Apremilast treatment resulted in a marked downregulation of TNFα-induced E-selectin and VCAM-1 expression in a dose-dependent manner (Fig. 4c,d).

We next examined the potential role of MAPK and NFκB for the effects of apremilast on VCAM-1 and E-selectin protein expression in activated HUVECs using selective inhibitors. Inhibition of p38 (SB203580) and NFκB (resveratrol) significantly downregulated TNFα-induced VCAM-1 protein expression in HUVECs, whereas TNFα-induced E-selectin expression was blocked by p38 (SB203580) and JNK (SP600125) inhibition. Interestingly, a superinduction of VCAM-1 after treatment with SP600125 and E-selectin after treatment with U0126 was observed (Fig. 4e,f). In summary, apremilast treatment blocked early stages of vascular inflammation and the expression of

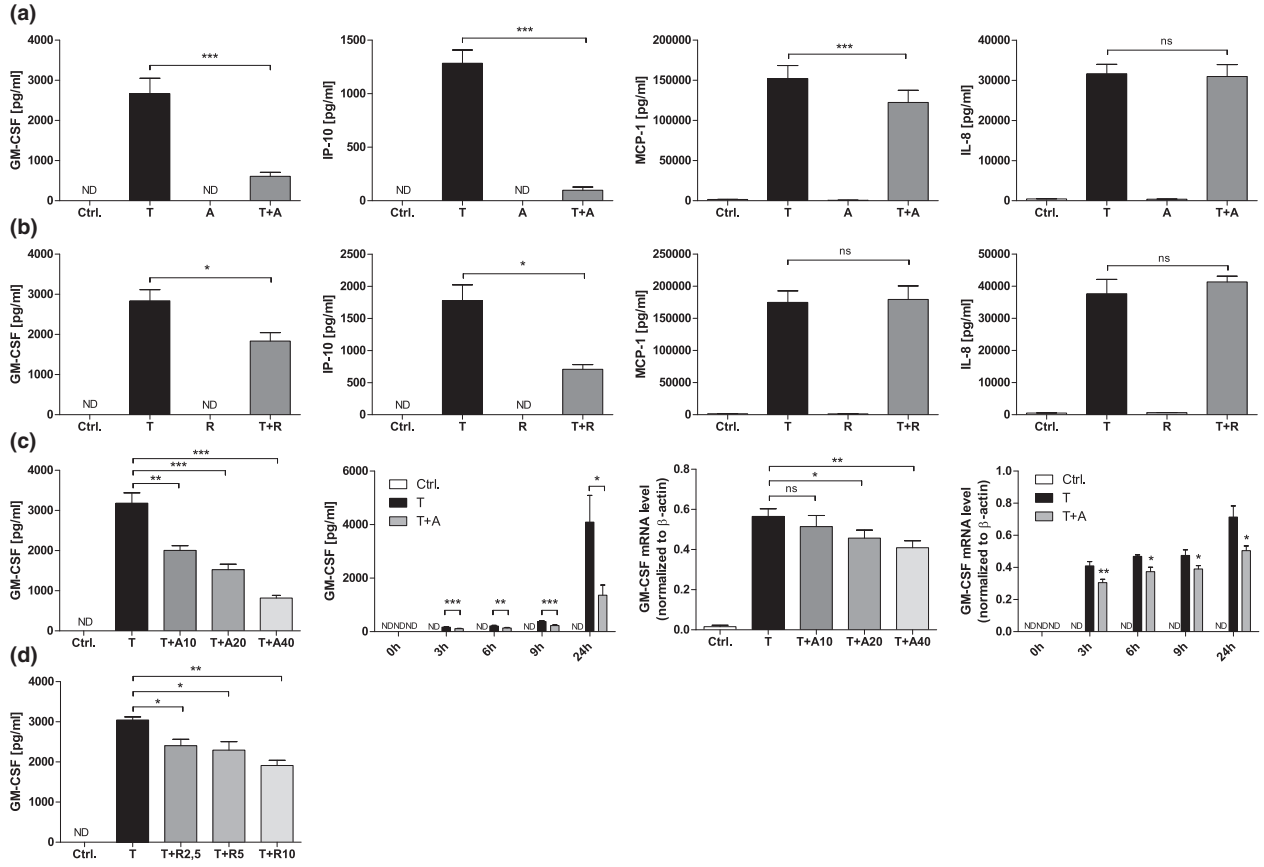

Figure 2 PDE4i inhibits various pro-inflammatory cytokines/chemokines in activated HUVECs. (a and b) We assayed the protein content in culture supernatants by ELISA (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. The HUVECs were treated with TNFα (20 ng/mL) in the presence or absence of apremilast (40 μM) or roflumilast (10 μM) and DMSO as solvent control for 24 h. (c) Cells were incubated with TNFα (20 ng/mL) or apremilast (10, 20 and 40 μM) + TNFα and DMSO as a control for 24 h. (d) Endothelial cells were mock-treated (solvent only) or treated with TNFα (20 ng/mL) and apremilast (40 μM) + TNFα for the indicated times. The mean values from five triplicate experiments are presented as the mean ± SEM. Semiquantitative RT-PCR analyses for GM-CSF and β-actin as loading control were performed on total RNA extracted from subconfluent HUVECs. (e) HUVECs were treated as indicated for 24 h and (f) indicated times. Mean values from six independent experiments are depicted as the mean ± SEM. (g) Cells were incubated with TNFα (20 ng/mL) or roflumilast (2.5, 5 and 10 μM) + TNFα and DMSO as a control for 24 h. We analysed the data using the Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. TNFα. A, Apremilast; ND, not detectable; ns, not significant; R, Roflumilast; T, TNFα.

important endothelial adhesion molecules VCAM-1 and E-selectin in activated HUVECs. Inhibition studies suggest that JNK- and NFκB-dependent pathways are involved in the regulation of VCAM-1, while the expression of E-selectin is regulated by JNK- and p38-signalling pathways, suggesting that apremilast differentially regulate the expression of both molecules.

Apremilast inhibits the activation of NFκB-signalling pathways in activated endothelial cells and the expression of MMP9 in activated monocytic cells

Previous studies demonstrated that PDE4i inhibits the expression of pro-inflammatory mediators in part through the modulation of NFκB-dependent pathways.[8] In line with this, we observed reduced activation of p65 and additionally demonstrated reduced nuclear p65 and p-p65 translocation. Furthermore, apremilast reduced the degradation of the NFκB inhibitor IκBα in activated HUVECs (Fig. 5a-c). DNA binding activity assay demonstrated that apremilast suppressed TNFα-induced p65-mediated NFκB DNA-binding in a concentration-dependent manner in activated HUVECs. This suggests that apremilast inhibits the activation of endothelial NFκB-signalling pathways induced by TNFα (Fig. 5d).

Since increased monocytic expression of MMP9 plays an important role in the inflammatory cell recruitment into the vessel wall,[21,22] we finally examined the effect of apremilast on MMP9 protein expression in activated THP-1 cells. Our data

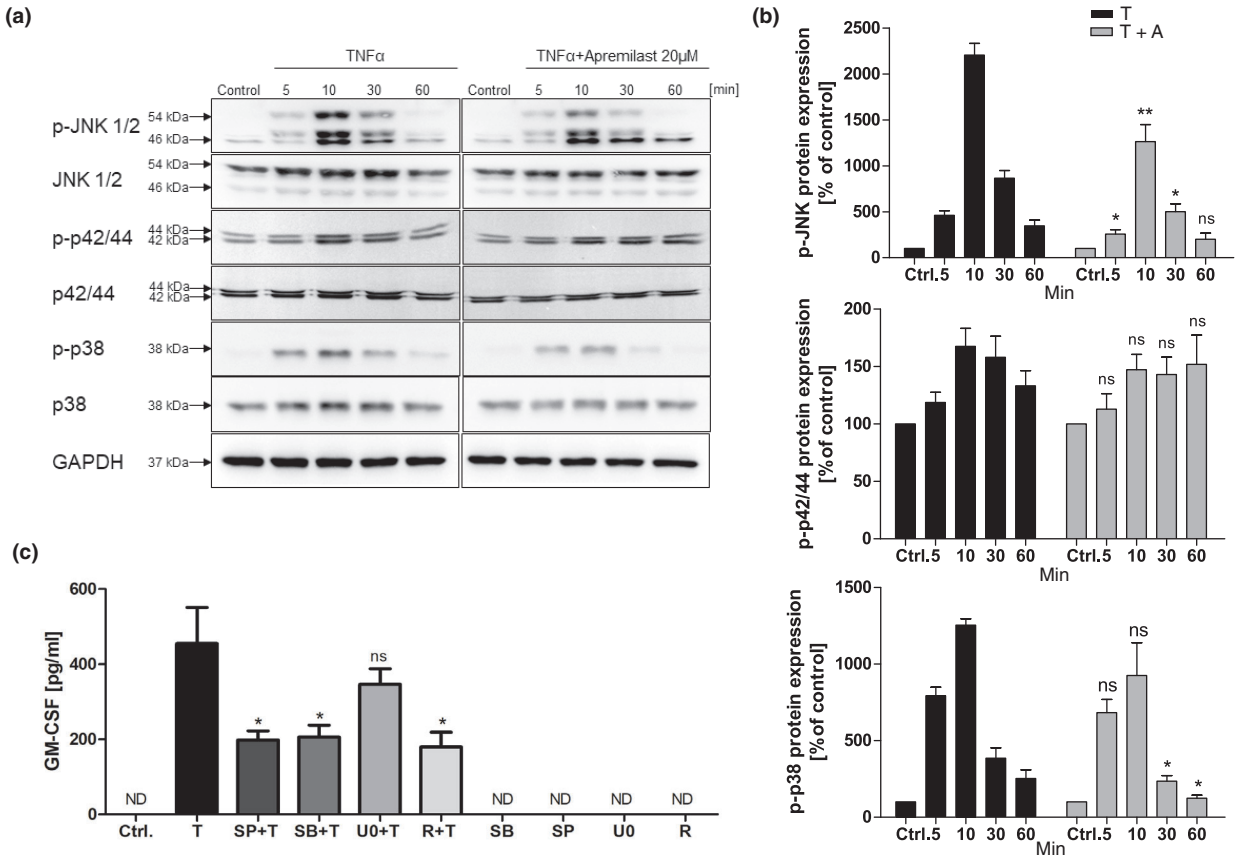

Figure 3 Analysis of the effects of apremilast on MAPK phosphorylation in TNFα-treated HUVECs and the influence on GM-CSF expression. (a) MAPK expression and phosphorylation: Representative western blot analysis of HUVECs treated with vehicle (solvent only), TNFα (20 ng/mL) or apremilast (20 μM) + TNFα for the indicated times. (b) Densitometry analysis: The results were normalized to the expression of the non-posphorylated controls. The relative expression of the posphorylated protein is presented in % of ctrl. Data are expressed as the mean ± SEM, $n = 5$. (c) Signalling pathway blockade: GM-CSF ELISA of HUVECs treated with TNFα (20 ng/mL), MAPK inhibitors or NFκB inhibitor (SB=SB203580, SP=SP600125, U0=U0126 and R=resveratrol) + TNFα. We present the mean values from five triplicate experiments as the mean ± SEM. We analysed the data using the Student's *t*-test. $*P < 0.05$, $**P < 0.01$ vs. TNFα. ND, not detectable; ns, not significant.

demonstrate that apremilast significantly blocked the MMP9 protein expression in THP-1 monocytes in a concentration-dependent manner (Fig. 6).

Discussion

Recent research has shown that chronic inflammatory diseases such as psoriasis, psoriasis arthritis, rheumatoid arthritis and diabetes are associated with vascular inflammation, atherosclerosis and subsequently increased cardiovascular morbidity and mortality.[1] It is known that endothelial inflammation is a crucial early event in the development of atherosclerosis. Vascular injury is mediated by various inflammatory stimuli, such as TNF-α, LPS and ox-LDL, which lead to endothelial cell activation and the upregulation of adhesion molecules, inflammatory cytokines and chemokines (e.g. E-selectin, VCAM-1 and GM-CSF).[2] It is assumed that therapies targeting the inflammatory process shared in psoriasis and atherosclerosis can lead to a reduction of cardiovascular risk in psoriatic patients.[23] In the present study, we analysed the effects of the PDE4i apremilast, a potent anti-psoriatic drug, on TNFα-induced inflammation in HUVECs. Apremilast is a PDE4-selective inhibitor which blocks the synthesis of several inflammatory cytokines and chemokines, such as TNF-α, IL-17, IL-12/IL-23p40 and CXCL8 in multiple cell types (e.g. T cells and macrophages) by elevating intracellular cAMP-levels.[24-26] Interestingly, recent studies demonstrated that apremilast suppresses doxorubicin- and carfilzomib-induced cardiac inflammation *in vivo* by reducing cardiac metabolic stress, inhibition of caspase 3-dependant apoptosis and

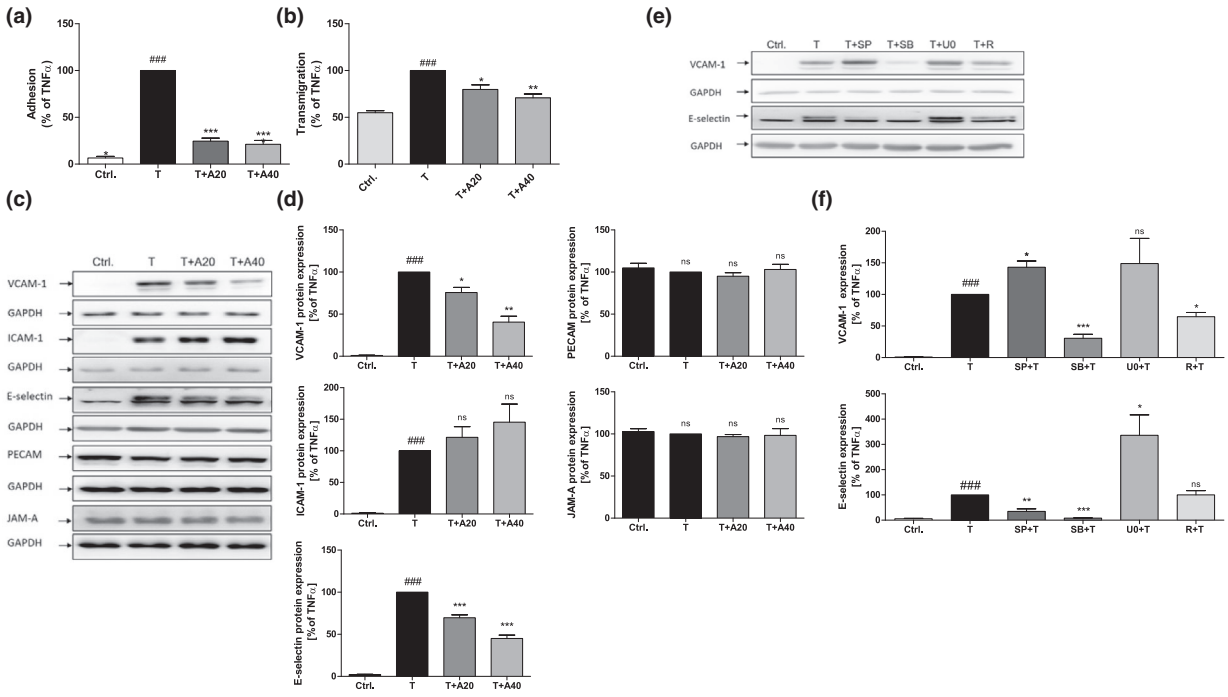

Figure 4 Apremilast inhibits THP-1 monocyte adhesion and migration. (a) HUVECs were treated with TNFα (20 ng/mL) in the presence or absence of apremilast (20, 40 μM) and DMSO as solvent control for 10 h. The percentage of HUVEC-adhering THP-1 cells labelled by LeukoTracker™ was determined. Data are expressed as the mean ± SEM, $n = 5$. (b) TEM of THP-1 monocytes were assessed after stimulation with TNFα (20 ng/mL) in the presence or absence of apremilast (20, 40 μM) and DMSO as solvent control. We present the mean values from five triplicate experiments as the mean ± SEM, $n = 6$. (c and d) Effects of apremilast on protein expression of endothelial adhesion molecules in TNFα-induced HUVECs. HUVECs were treated with vehicle (solvent only), TNFα (20 ng/mL) or apremilast at the indicated concentrations + TNFα for 24 h. Protein expression of adhesion molecules was detected by western blot analysis and quantified by densitometric analysis. Data are expressed as the mean ± SEM, $n = 5$. (e and f) MAPK and NFκB inhibitors block TNFα-induced expression of E-selectin and VCAM-1 in HUVECs. Representative western blot analysis of HUVECs that were incubated with TNFα (20 ng/mL), MAPK inhibitors or NFκB inhibitor (SB=SB203580, SP=SP600125, U0=U0126 and R=resveratrol) + TNFα. VCAM-1 and E-selectin protein was detected by western blot analysis and quantified by densitometric analysis. Data are depicted as the mean ± SEM, $n = 6$. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. ctrl. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. TNFα. A, Apremilast; ns, not significant; T, TNFα.

suppressing the expression of NfκB, JNK and p42/44.[27,28] Concordant to these biological data, Mazzilli and colleagues presented an open-label trial in a cohort of psoriatic and psoriatic arthritis patients treated with apremilast 30 mg twice daily. They observed that apremilast positively regulated metabolic biomarkers of inflammation (e.g. low-density lipoprotein) in diabetic and non-diabetic psoriatic patients.[29] These results suggest the growing importance and the possible applicability of PDE4i for anti-inflammatory therapies. Our data provide evidence that intermediate-dose apremilast (20–40 μM) has distinct anti-inflammatory properties, without any cytotoxic effects in part due to the suppression of GM-CSF, CCL2, CXCL10, VCAM-1 and E-selectin in TNFα-induced HUVECs through the differentiated inhibition of NFκB- and MAPK-signalling pathways, reduced adhesion of monocytic THP-1 cells to activated HUVECs and monocytic TEM, suggesting that suppression of pro-inflammatory cytokines occurs in part through a signalling pathway dependent on PDE4 inhibition. To date, little is known about the effects of apremilast on primary human endothelial cells. There is only one study by Wang *et al.* investigating the role of low-dose apremilast (0.5–1 μM) on ox-LDL-challenged human endothelial cell model.[12] They found that apremilast inhibited the expression of lectin-like oxidized-low-density-lipoprotein receptor-1 (LOX-1), TNFα, IL-6, CXCL8, VCAM-1, CCL2 and reduced adhesion of monocytic U937 cells to activated human aortic endothelial cells (HAECs). The effects were mediated through the rescue of Krüppel like factor 6 (KLF6) expression, which was reduced in response to ox-LDL via increased phosphorylation of JNK. Our findings not only confirm in part these observations but also broaden these results regarding the chemokines GM-CSF, CXCL10, the endothelial adhesion molecules E-selectin, VCAM-1 and monocytic

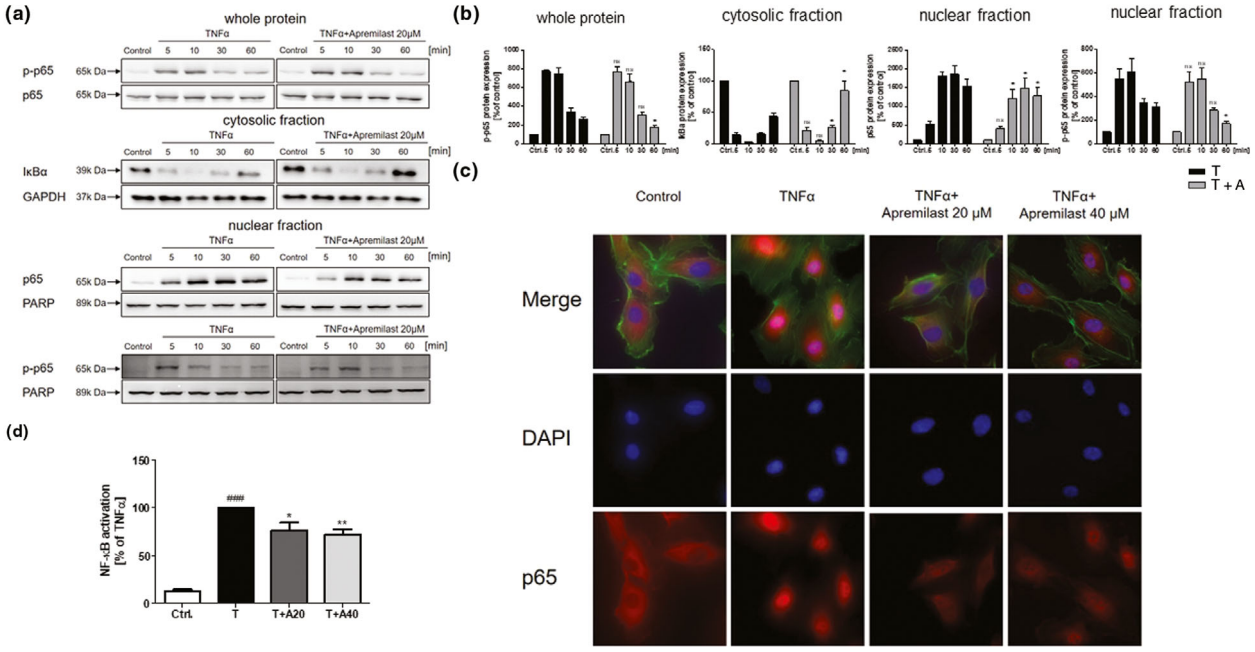

Figure 5 Apremilast blocks NFκB signalling pathways in TNFα-induced HUVECs. (a and b) Nuclear translocation of p65, phospho-p65 and IκBα degradation. Cells were treated with TNFα (20 ng/mL) or apremilast (20 μM) + TNFα for different periods of time (5–60 min). Cytosolic and nuclear protein fractions were prepared, and the protein level of IκBα in the cytoslic, p65 and phospho-p65 in the nuclear protein fraction was detected by western blot analysis and quantified by densitometric analysis. Data are expressed as the mean ± SEM, $n = 5$. (c) Representative immunofluorescent analysis of p65 in HUVECs that were treated with vehicle, TNFα (20 ng/mL) or apremilast (20, 40 μM) + TNFα for 3 h. Comparable results were obtained from four independent experiments. (d) Analysis DNA binding activity of NFκB in HUVECs treated with vehicle (solvent only) TNFα (20 ng/mL) or apremilast (20, 40 μM) + TNFα for 1 h. We present the mean values from five triplicate experiments as the mean ± SEM. We analysed the data using the Student's *t*-test. $^{###}P < 0.001$ vs. ctrl. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. TNFα. A, Apremilast; ns, not significant; T, TNFα.

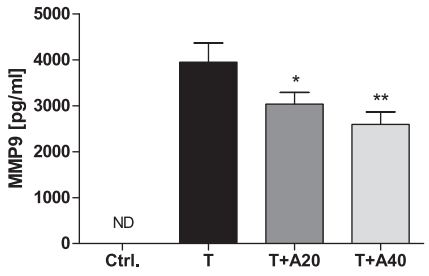

Figure 6 Effects of apremilast on MMP-9 protein expression in activated THP-1 monocytes. MMP-9 protein content was measured in culture supernatants by MMP-9 ELISA according to the manufacturer's instructions. The THP-1 cells were treated with TNFα (20 ng/mL) in the presence or absence of apremilast (20, 40 μM) and DMSO as solvent control for 24 h. Mean values are depicted from five triplicate experiments as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. TNFα. A, Apremilast; ND, not detectable; T, TNFα.

expression of MMP9 and TEM. The NFκB and MAPK pathways have a pivotal role in the regulation of inflammatory cytokines, chemokines and adhesion molecules in TNFα-activated endothelial cells.[18] However, the causal effect between apremilast-induced inhibition of chemokines, endothelial adhesion molecules and MAPK- and NFκB-signalling has not been addressed so far. We now demonstrate that apremilast reduced the TNF-α-induced phosphorylation of JNK, p38 and activation of NFκB-signalling, whereas phosphorylation of p42/44 was unaffected. Our results indicate that GM-CSF expression is regulated through the JNK-, p38- and NFκB-dependent pathways. We observed that p38 and NFκB inhibition downregulated TNFα-induced VCAM-1 protein expression in the HUVECs, whereas induced E-selectin expression was blocked by the treatment with specific inhibitors of JNK and p38. Interestingly, a recent study showed that high-dose treatment with apremilast (300 μM) inhibited the expression of several pro-inflammatory cytokines (e.g. CXCL8) in PGE2-activated human epidermal keratinocytes without any cytotoxic effects and independent of cAMP accumulation. Furthermore, it has been reported

that NFκB signalling was not affected by different PDE4i (apremilast, roflumilast and crisaborole) and that only apremilast abrogated MEK-dependent pathways.[30] Taken together, our data provide for the first time conclusive evidence that apremilast-related inhibition of GM-CSF, VCAM-1 and E-selectin is differentially regulated by MAPK and NFκB signalling pathways in activated HUVECs. Additionally, we provide strong evidence that apremilast inhibits the secretion of pro-atherogenic IL-6 and CCL2 in IL-17A-activated HUVECs, whereas roflumilast failed to suppress these factors, suggesting that inhibition of these cytokines/chemokines occurs in part through a PDE4-independent mechanism. Therefore, these effects might be cytokine- and/or cell type-specific, stimulus-dependent and of dose-dependent character.

Limitations

Some limitations of our study should be noted. Firstly, an *in vitro* approach was used for this study to analyse the potential anti-inflammatory properties of apremilast in vascular diseases. Further studies in animal models and human-derived monocytes from treated and untreated psoriasis patients are necessary to investigate and define the effects of apremilast so that we may observe whether these findings hold true *in vivo*. Secondly, the effects of NFκB and MAPK inhibition on functional relevant monocytic adhesion on activated endothelial cells and TEM should be examined. It is unclear whether these signalling pathways are involved in the effects of apremilast observed herein. Further studies will help to elucidate the exact mechanisms which may help to understand the cardiovascular protective and anti-inflammatory effects of apremilast.

Conclusion

In summary, our study provides conclusive evidence that apremilast mediates distinct anti-inflammatory activities in activated primary human endothelial cells by suppressing the important pro-inflammatory adhesion molecules, cytokines and chemokines GM-CSF, CXCL10, CCL2, VCAM-1 and E-selectin through differentiated inhibition of NFκB and MAPK signalling pathways, reduced adhesion of monocytic THP-1 cells to activated HUVECs, reduced monocytic TEM and diminished expression of MMP9 in activated monocytic cells. Additionally, apremilast reduced secretion of pro-inflammatory molecules IL-6 and CCL2 in IL-17A-activated endothelial cells most likely in part through a PDE4-independent signalling pathway. These newly identified targets for apremilast in primary human endothelial cells may be of relevance for future clinical studies and may provide a potential rationale for the use of apremilast in the treatment of various chronic inflammatory diseases associated with increased risk for CVD.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Apremilast but not roflumilast inhibits IL-6 and CCL2 secretion in IL-17A-induced HUVECs
Table S1. Antibodies and technical details
Table S2. List of the primer sequences used for semiquantitative RT-PCR
Appendix S1. Material and methods