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Synergistic effect of phosphodiesterase 4 inhibitor and serum on migration of endotoxin-stimulated macrophages

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Abstract

Macrophage migration is an essential step in host defense against infection and wound healing. Elevation of cAMP by inhibiting phosphodiesterase 4 (PDE4), enzymes that specifically degrade cAMP, is known to suppress various inflammatory responses in activated macrophages, but the role of PDE4 in macrophage migration is poorly understood. Here we show that the migration of Raw 264.7 macrophages stimulated with LPS was markedly and dose-dependently induced by the PDE4 inhibitor rolipram as assessed by scratch wound healing assay. Additionally, this response required the involvement of serum in the culture medium as serum starvation abrogated the effect. Further analysis revealed that rolipram and serum exhibited synergistic effect on the migration, and the influence of serum was independent of PDE4 mRNA expression in LPS-stimulated macrophages. Moreover, the enhanced migration by rolipram was mediated by activating cAMP/exchange proteins directly activated by cAMP (Epac) signaling, presumably via interaction with LPS/TLR4 signaling with the participation of unknown serum components. These results suggest that PDE4 inhibitors, together with serum components, may serve as positive regulators of macrophage recruitment for more efficient pathogen clearance and wound repair.

Keywords

Phosphodiesterase 4, serum, macrophage migration, cAMP/Epac signaling, inflammation

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Introduction

Macrophages play key roles in innate immune responses and wound repairing. To undertake these functions, they must respond rapidly to wound or infection signals and migrate efficiently to sites of inflammation. While macrophage migration is critical to the success of pathogen clearance and wound healing, inadequate regulation of this step may lead to progression of a number of diseases, such as atherosclerosis, rheumatoid arthritis, and cancer.[1] Therefore, a better understanding of the mechanisms underlying the control of macrophage migration is crucial for the development of therapies to treat these diseases.

Macrophages can be activated by numerous stimuli, including the Gram-negative bacterial component LPS. Through activation of TLR4, LPS potently stimulates the production of various pro-inflammatory cytokines and mediators, which in turn facilitate the recruitment of neutrophils and monocytes, leading to local inflammation. Additionally, LPS stimulation has also been shown to induce migration of macrophages, enabling the cells to clear infection and restore tissue homeostasis.[2–4] This LPS-mediated cell migration involves both chemokinetic (random cell motility) and chemotactic

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(directional cell motility) activity, in which the lipid mediators prostaglandin E_2 (PGE_2) and PGD_2 and the chemokine MCP-1, all secreted by LPS-stimulated macrophages, are considered major regulators.[2]

The second messenger cAMP is known to regulate diverse cellular processes. In immune cells, elevation of cAMP generally leads to negative modulatory effects on inflammatory responses, such as generation of pro-inflammatory mediators, receptor-mediated phagocytosis, and microbicidal ability.[5] For instance, the endogenous cAMP-elevating agent PGE_2, cAMP agonist 8-bromo-cAMP, and inhibitors of phosphodiesterase 4 (PDE4), a family of cAMP-hydrolyzing enzymes, have been shown to suppress the production of many pro-inflammatory mediators, such as TNF-α, IL-12, CCL3, and leukotriene B4, while up-regulating the anti-inflammatory cytokines IL-1Ra and IL-10 in LPS-stimulated macrophages.[5–8] Several studies also point to a role of cAMP signaling in cell migration, although conflicting effects are reported. Poole and co-workers have revealed that by increasing cAMP, PGE_2 induces platelet-derived growth factor (PDGF)-stimulated migration in murine IC21 macrophages while inhibiting PDGF-stimulated fibroblast migration.[9] This induction of macrophage migration by PGE2/cAMP signaling is in line with the finding that a late phase LPS-induced migration in Raw 264.7 macrophages is mediated mainly by increasing secretion of macrophage PGE_2 and subsequent activation of the cognate receptor EP4/cAMP signaling.[2] Conversely, the cAMP-elevating agent sphingosine 1-phosphate (S1P) was found to inhibit C5a-induced migration in bone marrow-derived macrophages.[10] It is likely that the effects of cAMP vary depending on the stimuli and macrophage type under investigation.

The PDE4 isozymes are expressed in almost all immune and inflammatory cells, implicating their importance in regulation of intracellular cAMP level and thereby, immune responses in these cells.[11] By increasing cAMP, presumably in discrete compartments rather than global massive change in a cell,[12,13] PDE4 inhibitors have been shown to suppress many inflammatory responses in most inflammatory cells.[6] In fact, the PDE4 inhibitors roflumilast and apremilast are used as anti-inflammatory drugs for the treatment of chronic obstructive pulmonary disease and psoriasis and psoriatic arthritis, respectively.[14,15] Moreover, evidence also indicates that inhibition of PDE4 generally suppresses migration in both immune and non-immune cells. For instance, PDE4 inhibitors have been shown to inhibit epithelial cell migration *in vivo* during dextran sodium sulfate-induced colonic damage,[16] IL-1β- or nerve growth factor-induced fibroblast migration,[17,18] and vascular endothelial growth factor-induced endothelial cell migration.[19,20] The PDE4 inhibitor rolipram has also been documented to suppress the formation of integrin-dependent actin adhesion microspikes and impair the rate of random cell migration in rat embryo fibroblasts.[21] Additionally, eosinophil chemotaxis triggered by eotaxin, platelet-activating factor, or leukotriene B_4,[22,23] and T lymphocyte trans-endothelial migration induced by the chemokine CXCL12,[24] are all demonstrated to be inhibited by rolipram. These findings clearly indicate an involvement of PDE4 in cell migration.

Cell motility is a main characteristic of macrophages and is essential for fulfilling most of their immune/inflammatory effects. Although PDE4 inhibitors have been shown to suppress migration of various types of cells, the knowledge on the PDE4 regulation of macrophage migration is still lacking. Thus, in this study we used murine Raw 264.7 macrophages to test whether PDE4 plays a role in their migration. To our surprise, inhibition of PDE4 greatly induced the migration of LPS-stimulated macrophages when the cells were cultured in the medium containing 10% serum. Further analysis showed that this induction was the result of a synergistic effect of PDE4 inhibitor and serum, since rolipram or serum alone produced negligible or minimal effect on migration. Moreover, this migration induction is mediated by activation of cAMP/exchange proteins directly activated by cAMP (Epac) signaling but not cAMP/PKA pathway.

Materials and methods

Reagents

Escherichia coli LPS (O55:B5), 8-bromoadenosine 3′,5′-cyclic monophosphate (8-bromo-cAMP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Giemsa stain, rolipram, and roflumilast were purchased from Sigma-Aldrich (St Louis, MO, USA), murine monocyte chemoattractant protein-1 (MCP-1) is from PeproTech (Rocky Hill, NJ, USA), and the Epac inhibitor ESI-09 and PKA inhibitor Rp-8-CPT-cAMPS were from BioLog Life Science Institute (Bremen, Germany). The DMEM medium base, FBS, and horse serum were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Cell line

Raw 264.7, a murine leukemia macrophage, cell line was obtained from Bioresource Collection and Research Center (Hsinchu City, Taiwan). The cells were maintained in DMEM medium supplemented with 100 u/ml penicillin, 100 μg/ml streptomycin, and 10% FBS at 37°C in 5% CO_2. For scratch wound healing assay, cells were plated at 2.5×10^5 or

1.2×10^5 cells/well in 24- or 48-well plate, respectively, grown overnight, and then the wound was created followed by LPS and drug treatment. For quantitative PCR analysis of PDE4 mRNA expression, the cells were plated at 2×10^6 cells/well in 6-well plate, cultured to 80–90% confluency, and then treated with 0.1 μg/ml LPS for 3 and 12 h in serum-free or 10% FBS-containing medium.

Scratch wound healing assay

Raw 264.7 cells were grown until they had reached about 80% confluence. Then a wound was created in the cell monolayer by scratching a vertical line at the center of each well using a sterile 200-μl pipette tip. Cells were washed twice with PBS to remove debris, followed by incubation with 0.1 μg/ml LPS in the absence or presence of PDE4 inhibitors or cAMP analogs in serum-free or serum-containing medium. The wound area was photographed at time 0 and designated incubation times under a light microscope (magnification 40×, Coolpix 4500, Nikon, Tokyo, Japan). Pictures of each well were taken exactly at the same position before and after the treatment to verify the healing process. Cells migrated off the scratch edges toward the wound area were counted using ImageJ 1.47v software. All experiments were performed at least in duplicate wells and repeated more than five times.

Cell viability assay

Raw 264.7 cells were plated in 96-well plate, cultured overnight, followed by incubation for 24 h under the conditions corresponding to those conducted in the wound healing assay, and then cell viability was determined by adding MTT solution (at the final concentration of 0.5 mg/ml). Following 3 h incubation at 37°C in 5% CO_2, the medium was removed and 200 μl DMSO added to dissolve the insoluble formazan formed in the cells. Then the absorbance at 570 nm was measured using a microplate reader (VersaMax, Molecular Devices, San Jose, CA, USA). Cell viability was expressed as percent survival of untreated cells.

Transwell migration assay

To determine whether PDE4 inhibition affects LPS- and the chemokine MCP-1-mediated chemotactic migration, a modified Boyden chamber assay was carried out in 24-well plates with 8-μm pore-size polycarbonate membrane (Corning, NY, USA) in DMEM medium supplemented with 2% FBS. Following pretreatment of Raw 264.7 cells with 10 μM rolipram or vehicle for 20 min, 5×10^5 cells in 200 μl of the medium were seeded in the upper chamber, and increasing concentrations of LPS or 20 ng/ml recombinant murine MCP-1 in 600 μl medium were added in the lower chamber. After 4 h incubation at 37°C, the cells remained on the upper side of the membrane were wiped off with a cotton swab and the migrating cells on the underside of the membrane were fixed with 10% formalin in PBS for 10 min. The cells then were stained with 5% Giemsa stain for 40 min followed by washing with water. The cells were counted in four randomly selected fields by light microscopy at 200× magnification.

RNA isolation, cDNA synthesis, and quantitative PCR

Total RNA was extracted from Raw 264.7 cells with the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. First strand cDNA was synthesized from 1 μg of total RNA in the presence of random primer using M-MLV reverse transcriptase according to the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was carried out with the SensiFAST SYBR Hi-ROX kit (Bioline, London, UK) using Applied Biosystems Prism 7900 Real-time PCR Detection System (Applied Biosystems, CA, USA). The reaction was performed in a 10 μl reaction mixture with preliminary denature for 10 min at 95°C, followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Oligonucleotide primer sequences were as follows: PDE4A, 5′-CTTCTGCGA GACCTGCTCCA-3′ and 5′-GAGTTCCCGGTT CAGCATCC-3′; PDE4B, 5′-GCCACTGGATGAG AGGAGCA-3′ and 5′-CCTTTTCCGGTCCCT CAGAA-3′; PDE4C, 5′-CTCTGCCCACAGACT GGAAT-3′ and 5′-ACAGAGTCCGACTGCA TGTG-3′; PDE4D, 5′-ACCGCCAGTGGACGGACC GGA-3′ and 5′-CATGCCACGCTCCCGCTCTCGG-3′; MIF, 5′-GCCAGAGGGGTTTCTGTCG-3′ and 5′-GTTCGTGCCGCTAAAAGTCA-3′; GAPDH, 5′-GGAGCGAGACCCCACTAACA-3′ and 5′-ACA TACTCAGCACCGGCCTC-3′. All primers used were synthesized by Tri-I Biotech (New Taipei City, Taiwan). Target gene expression was calculated by the comparative ΔΔ cycle threshold (Ct) method for relative quantification after normalization to the housekeeping gene *GAPDH* expression.

Statistical analysis

All data are presented as mean ± SEM of at least three independent experiments. Comparisons of two treatment groups were performed by unpaired Student's t-test. Most data also were analyzed using Mann-Whitney/Wilcoxon rank sum test (for sample size ≥ 4)

to confirm the statistical significance. Values were considered statistically significant when $P < 0.05$.

Results

PDE4 inhibitor enhances cell migration in LPS-stimulated Raw 264.7 macrophages

To investigate whether PDE4 regulates macrophage migration, scratch wound healing assay was performed in Raw 264.7 macrophages incubated with LPS in the presence or absence of the PDE4 inhibitor rolipram. The initial microscopic examination revealed that LPS treatment for 24 h led to an increasing number of cells migrating off the scratch edges toward the center of the wound area as compared with the basal migration of untreated cells. Surprisingly, the migration activity was markedly enhanced when the cells were co-treated with rolipram, while rolipram alone had no significant effect (Figure 1a). Counting migration cells using ImageJ software revealed that there was a time-dependent increase in migration activity up to 24 h for all test conditions (Figure 1b). When compared with the untreated cells, LPS mildly, but significantly, increased cell migration during the 4-24 h incubation period ($P < 0.05$), a result consistent with other reports

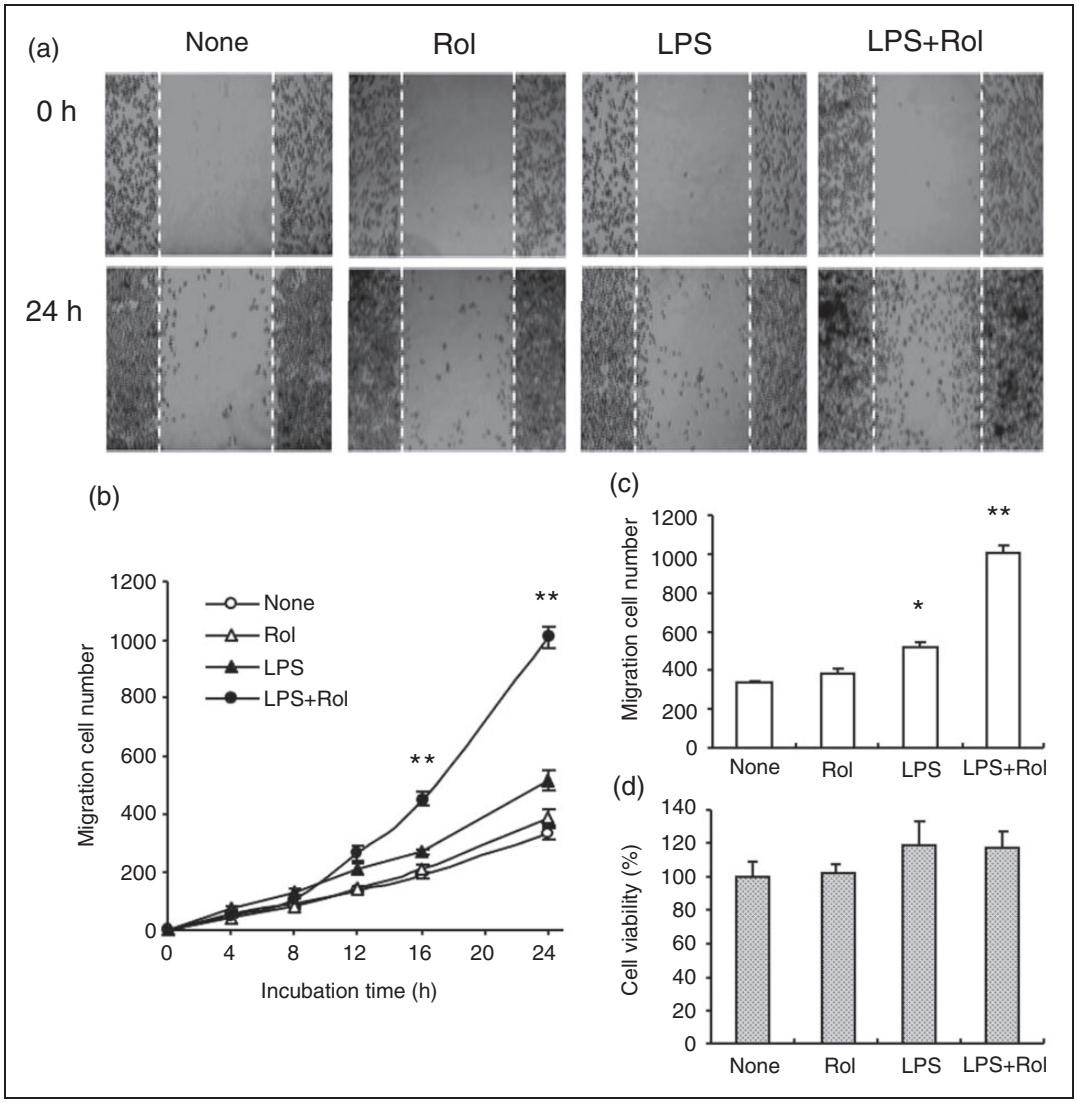


Figure 1. PDE4 inhibitor enhances migration of LPS-stimulated macrophages. Raw 264.7 macrophages were pre-treated with 10 μM rolipram (Rol) or vehicle for 20 min followed by stimulation with or without 0.1 μg/ml LPS for 24 h. Cell migration was measured by scratch wound healing assay. (a) Images taken at 0 and 24 h are shown (40× magnification). Representative of at least five experiments. (b) Cells migrated into the wound area at the indicated times were counted using ImageJ software. (c) Migration cell numbers counted at 24 h in (b) are presented as bar graphs. (d) The cell viability at 24 h was determined by MTT assay. Data are the mean ± SEM (b, $n = 6$–9; c, $n = 9$; d, $n = 6$). *$P < 0.001$, compared with untreated cell; **$P < 0.001$, compared with the cells treated with LPS alone.

using a modified Boyden chamber assay.[2–4] The LPS-stimulated migration was escalated by rolipram after 12 h incubation ($P < 0.001$), showing approximately 1.7- and 2.0-fold increase at 16 and 24 h, respectively (Figure 1b and c). This enhanced chemokinetic migration was not caused by an increase in cell viability as no significant difference in cell survival was detected among the four test conditions (Figure 1d).

To further confirm the rolipram effect on macrophage migration, Raw 264.7 cells were incubated with LPS in the presence of increasing concentrations of rolipram for 24 h. Figure 2a shows that rolipram enhances the cell migration in a concentration-dependent manner. A similar dose response also was obtained when the cells were treated with the cAMP agonist 8-bromo-cAMP (Figure 2b). Moreover, the clinically used PDE4 inhibitor roflumilast was found to recapitulate the migration effect of rolipram (Figure 2c). These results suggest that Raw 264.7 cell migration enhanced by PDE4 inhibitors is mediated by activating cAMP signaling.

To further determine whether PDE4 inhibition also increases directional migration in macrophages in response to LPS as well as to the chemotactic mediator MCP-1, a modified Boyden chamber migration assay was performed. As shown in Figure 3a, migration of Raw 264.7 cells through the transwell membrane was increased in a LPS-dose-dependent manner, and the migration activity at 10 and 100 ng/ml LPS were significantly enhanced when the cells were pre-treated with rolipram for 20 min ($P < 0.01$ and 0.005, respectively). However, rolipram alone had no significant effect on the migration (Figure 3b). As expected, the cell motility was increased when the chemokine MCP-1

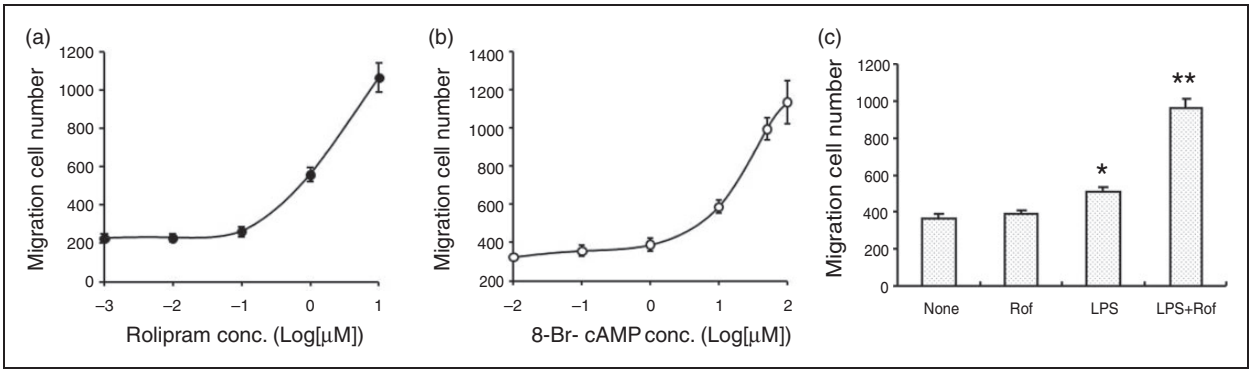


Figure 2. cAMP-elevating agents dose-dependently enhance cell migration in LPS-stimulated macrophages. Raw 264.7 cells were pre-treated with increasing concentrations of rolipram (a) or 8-brome-cAMP (b) for 20 min, followed by stimulation with 0.1 μg/ml LPS for 24 h. (c) Cells were treated with 1 μM roflumilast (Rof) or vehicle for 20 min before stimulation with or without 0.1 μg/ml LPS for 24 h. Cell migration was monitored and measured as described in Figure 1. Data are the mean ± SEM (a, $n = 6$–8; b, $n = 4$–5; c, $n = 3$–4). *$P < 0.05$, compared with untreated cell; **$P < 0.001$, compared with the cells treated with LPS alone.

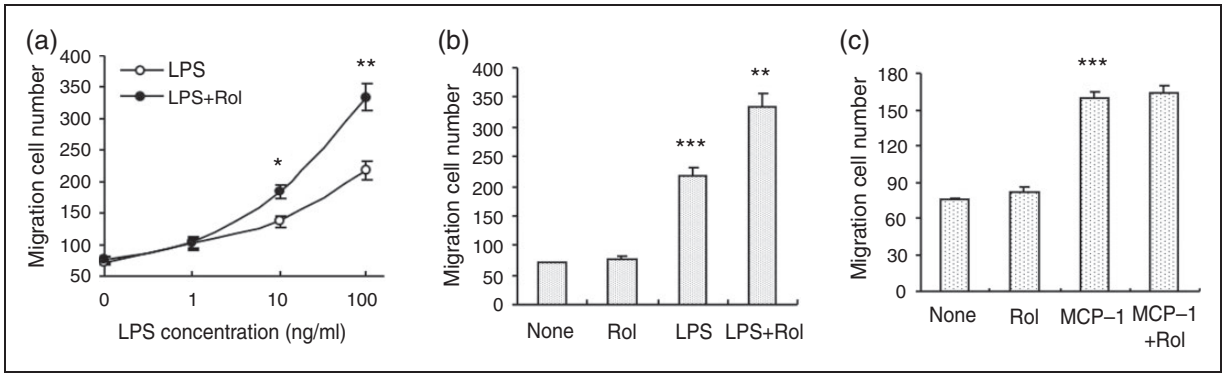


Figure 3. PDE4 inhibitor enhances directional migration of macrophages in response to LPS but not to MCP-1. *In vitro* chemotactic migration of Raw 264.7 cells was measured by a modified Boyden transwell chamber assay. The cells were pre-treated with 10 μM rolipram (Rol) or vehicle for 20 min and then placed in the upper chamber of a transwell. The lower chamber contained either increasing concentrations of LPS (a), 0.1 μg/ml LPS (b), or 20 ng/ml MCP-1 (c). After 4 h incubation, migration was quantified as described under "Materials and methods." Data are the mean ± SEM ($n = 6$). *$P < 0.01$, **$P < 0.005$, compared with cells treated with LPS alone; ***$P < 0.001$, compared with untreated cells.

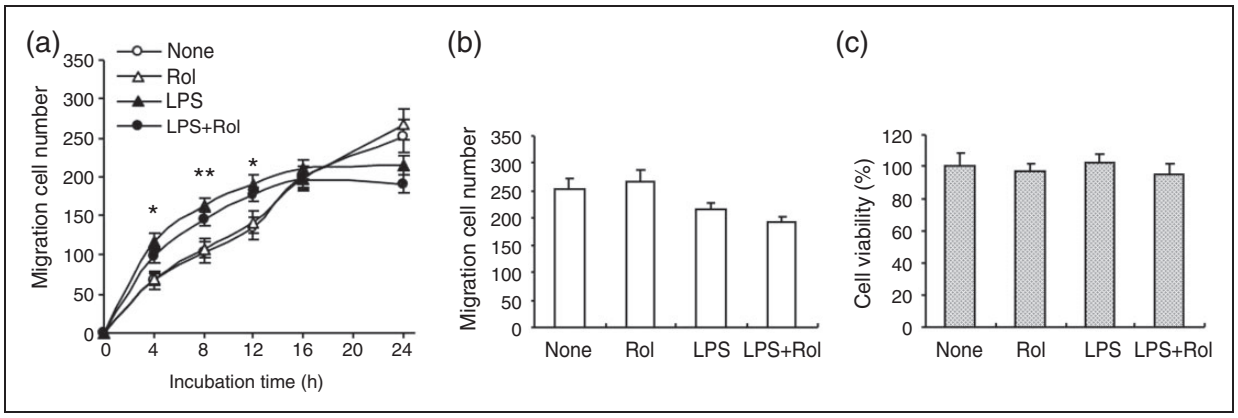


Figure 4. Rolipram enhancement of cell migration in LPS-stimulated macrophages is abrogated in serum-free condition. Raw 264.7 cells incubated in the serum-free medium were treated with 10 μM rolipram or vehicle for 20 min, followed by stimulation with or without 0.1 μg/ml LPS for 24 h. (a) Cells migrated into the wound area at the indicated times were counted using ImageJ software. (b) Migration cell numbers counted at 24 h in (A) are presented as bar graphs. (c) The cell viability at 24 h was determined by MTT assay. Data are the mean ± SEM (a and b, $n = 10$; c, $n = 6$). *$P < 0.01$, **$P < 0.005$, cells treated with LPS alone compared with untreated cells.

was added to the lower chamber of the transwell, yet the migration activity was unaffected by rolipram pretreatment (Figure 3c). Taken together, these data indicate that inhibition of PDE4 selectively enhances LPS-mediated chemotactic migration in macrophages.

The rolipram-enhanced migration in LPS-stimulated macrophages requires the involvement of serum

Several studies have shown that serum promotes cell migration in various cell types, such as epithelial cells, fibroblasts, and cancer cells.[25–27] Given the fact that our data presented in Figures 1 and 2 were obtained from the cells incubated in 10% FBS-containing medium, the literature reports prompted us to examine whether serum also contributes to the rolipram enhancement of macrophage migration. To this purpose, Raw 264.7 cells were treated with LPS with or without rolipram in the serum-free medium for 24 h. As shown in Figure 4a, a time-dependent increase in cell migration was obtained under all four test conditions albeit the migration reached a plateau after 16 h in the cells treated with LPS alone or LPS plus rolipram. Compared with Figure 1b and c, there were less migration cells detected under serum starved conditions (Figure 4a and b). Additionally, it was obvious that rolipram had no effect on cell migration since cell treatment with rolipram alone or LPS plus rolipram displayed no significant difference in migration from their control groups (Figure 4a and b). This lack of increase in cell migration was not caused by a decrease in cell viability as no significant difference in cell survival was detected among the four conditions (Figure 4c). These data indicate that serum is required for the observed rolipram effect in macrophage migration under LPS stimulation.

Rolipram and serum exhibited synergistic effect on migration of LPS-stimulated macrophages

To further investigate whether the enhanced macrophage migration by PDE4 inhibitors is serum concentration dependent, Raw 264.7 cells were incubated in the medium containing 0, 2, or 10% FBS and the cell migration was evaluated in the absence or presence of LPS or rolipram. The results showed that the migration of untreated cells (Figure 5a) and the cells treated with rolipram alone (Figure 5b) or LPS alone (Figure 5c) was significantly increased at 24 h in 2% FBS medium ($P < 0.01$), while the increase was either reduced (Figure 5a and b) or only marginally increased (Figure 5c) in 10% FBS medium. However, a serum dose-dependent increase in migration was detected in cells treated with LPS plus rolipram (Figure 5d), suggesting that serum constituents specifically up-regulate the rolipram effect on macrophage migration under LPS stimulation. Moreover, we also observed that cells treated with LPS alone without an influence of serum exhibited a trend of decrease in cell migration (0% FBS in Figure 5c), while rolipram alone showed a negligible or minimal increase in migration (0% FBS in Figure 5B) as compared with the cell migration in untreated cells (0% FBS in Figure 5a). Since 10% serum alone (Figure 5a) and rolipram alone without serum (Figure 5b) produced limited effect on cell migration, the substantial increase in migration cell number under the condition of LPS plus rolipram in 10% serum (Figure 5d) indicates a synergistic effect of rolipram and serum on the migration of

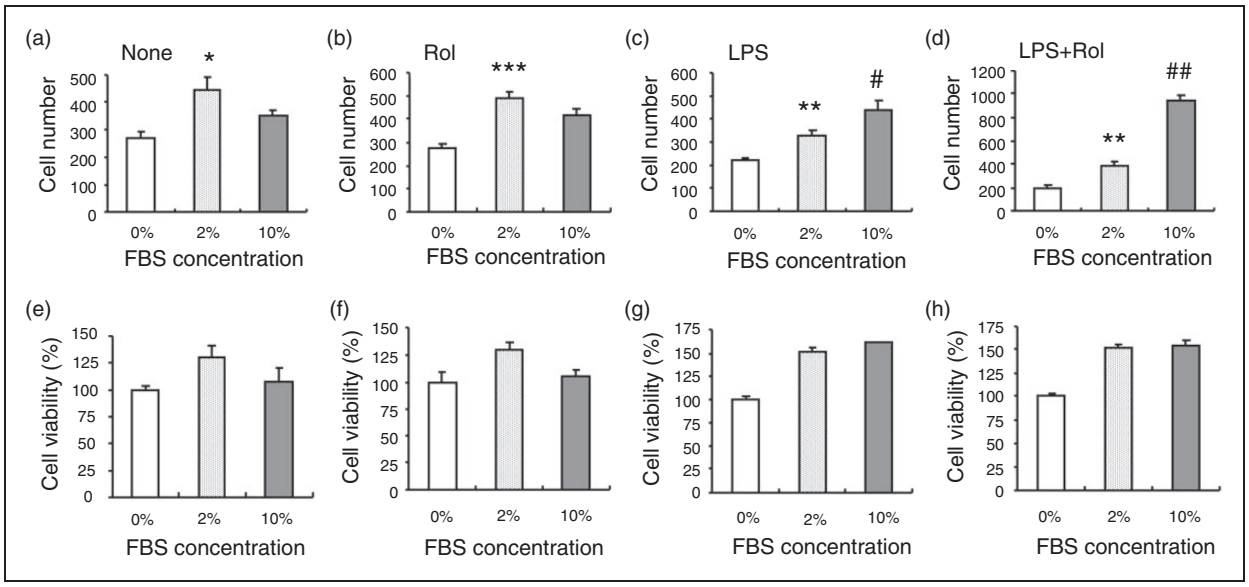


Figure 5. Rolipram-enhanced cell migration in LPS-stimulated macrophages is serum concentration dependent. Raw 264.7 cells incubated in the medium containing 0, 2, or 10% FBS were treated with 10 μM rolipram or vehicle for 20 min, followed by stimulation with or without 0.1 μg/ml LPS for 24 h. (a–d) Migration cell numbers were measured using ImageJ software. (e–h) The cell viability at 24 h was determined by MTT assay. Data are the mean ± SEM (a–d, $n = 7$–10; e–h, $n = 4$–6). $^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.001$, compared with cells incubated in 0% serum; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, compared with cells incubated in 2% serum.

LPS-stimulated cells. Further MTT assay revealed that this synergistic induction in migration was not caused by an increase in cell viability as comparable cell survival was detected under the conditions of 2% FBS and 10% FBS medium (Figure 5e to h).

To assess whether sera from other animal sources also produce the same migration effect as FBS, mouse and horse sera were tested. The results showed that similar pattern and levels of migration induced by LPS and rolipram were detected at 24 h in 10% mouse serum (migration cell numbers: none, 399 ± 32; Rol, 438 ± 40; LPS, 599 ± 44; LPS + Rol, 1091 ± 86; $n = 4$) as well as in 10% horse serum (migration cell numbers: none, 287 ± 22; Rol, 315 ± 31; LPS, 530 ± 80; LPS + Rol, 1020 ± 132; $n = 5$). These data indicate the presence of common factor(s) in the sera that is (are) necessary for the rolipram enhancement of macrophage migration.

Serum does not alter PDE4 expression in LPS-stimulated macrophages

To test whether serum influences PDE4 expression and thereby contributes to the observed migration effect in LPS-stimulated macrophages, Raw 264.7 cells were incubated with LPS in the presence or absence of rolipram in 0% and 10% FBS-containing medium for 3 and 12 h. The mRNA levels of the four PDE4 isoforms (PDE4A∼D) were measured by quantitative PCR. As expected, PDE4C expression was undetectable or at low levels (data not shown).[6] As depicted in Figure 6, PDE4D was the predominant isoform expressed in the control cells cultured in both media at both incubation times. Following stimulation with LPS alone, the expression profiles of the three PDE4 isoforms under serum-free and 10%-serum conditions were comparable, with PDE4B expression being upregulated the greatest at 3 h incubation (approximately 31- and 22-fold increase, respectively) and then declined at 12 h (approximately 7- and 8-fold increase, respectively), and no induction detected with PDE4A and PDE4D expression (Figure 6). These results indicate that serum has no major effect on PDE4 mRNA expression in LPS-stimulated macrophages.

The PDE4 expression profiles in Raw 264.7 cells treated with rolipram alone and LPS plus rolipram were also evaluated. Previous studies by us and others have revealed that in addition to LPS, cAMP also induces PDE4B expression in monocytes and macrophages, and this expression is synergistically upregulated by cAMP and LPS.[28,29] Indeed, as shown in Figure 6, by elevating cAMP, rolipram elicited a marked increase in PDE4B expression when the cells were co-treated with LPS. Additionally, the expression profiles of PDE4 isoforms were comparable under serum-free and 10%-serum conditions at both incubation times. However, it is worthy of note that the profound increase in the PDE4B expression under the condition of LPS plus rolipram would have no functional impact, simply because the presence of 10 μM

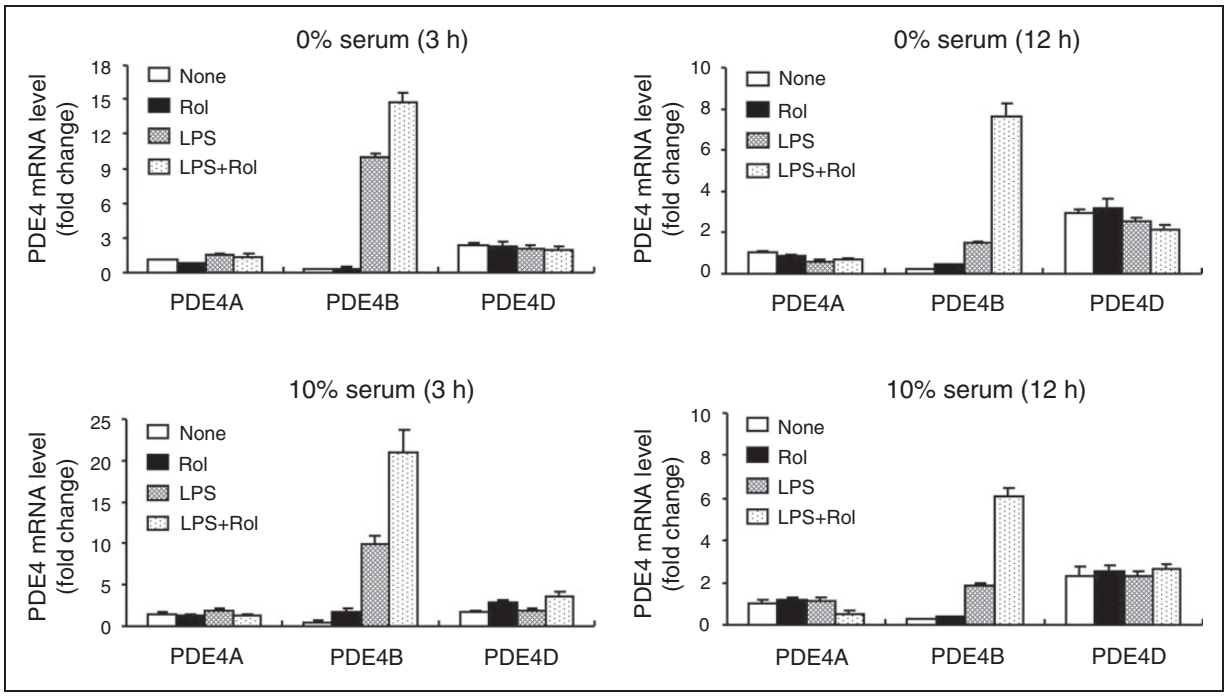


Figure 6. Serum does not alter PDE4 isoform expression in LPS-stimulated macrophages. Raw 264.7 cells incubated in the medium containing 0% or 10% FBS were treated with 10 μM rolipram or vehicle for 20 min, followed by stimulation with or without 0.1 μg/ml LPS for 3 and 12 h. The mRNA levels of PDE4A, PDE4B, and PDE4D in the cells were measured by real-time PCR and normalized to GAPDH mRNA levels. Data are presented as fold change compared with the PDE4A mRNA level at 3 h in the control cells cultured in the serum-free medium. Data are the mean ± SEM ($n = 5$–6).

rolipram would inhibit almost all PDE4 enzymatic activity and therefore is regarded as no functional PDE4 in the cell. Taken together, these data clearly indicate that the serum effect on the macrophage migration is independent of PDE4 expression in these cells.

Rolipram and serum enhanced migration in LPS-stimulated macrophages is mediated by activating cAMP-Epac signaling

To further assess which cAMP effector pathway mediates the migration effect of rolipram and serum, Raw 264.7 cells cultured in the 10% FBS-containing medium were treated with LPS in the absence or presence of the PKA inhibitor Rp-8-CPT-cAMPS or the Epac inhibitor ESI-09 for 24 h. Figure 7a shows that the rolipram-enhanced cell migration was dose-dependently inhibited by ESI-09, and a full inhibition was obtained at 10 μM ESI-09. Contrarily, PKA inhibitor had minimal or no impact on the cell migration (Figure 7b). These results indicate that the rolipram enhancement of macrophage migration is mediated by activation of cAMP/Epac but not cAMP/PKA signal pathway.

Discussion

The main goal of this study was to investigate whether PDE4 modulates macrophage migration under LPS stimulation. Using murine Raw 264.7 macrophages cultured in 10% serum-containing medium, we show that LPS mildly, but significantly, induces macrophage migration as assessed by scratch wound healing assay and this effect is greatly enhanced by the PDE4 inhibitors rolipram and roflumilast. We further demonstrate that this migration induction by PDE4 inhibitor requires the involvement of serum in the medium, whereas the PDE4 inhibitor or serum alone has negligible or minimal effect. Moreover, this enhanced migration is mediated by activation of cAMP-Epac signaling.

In addition to the scratch wound healing assay to assess chemokinetic migration, we also performed a transwell migration assay to evaluate directional motility in macrophages. Our data show that LPS significantly induces directional motility of macrophages (Figure 3), a result consistent with other reports.[2–4] Additionally, this LPS-induced migration is further enhanced by rolipram, indicating that inhibition of PDE4 promotes both chemokinetic and chemotactic activity of LPS-mediated migration in macrophages. Conversely, the directional migration mediated by the

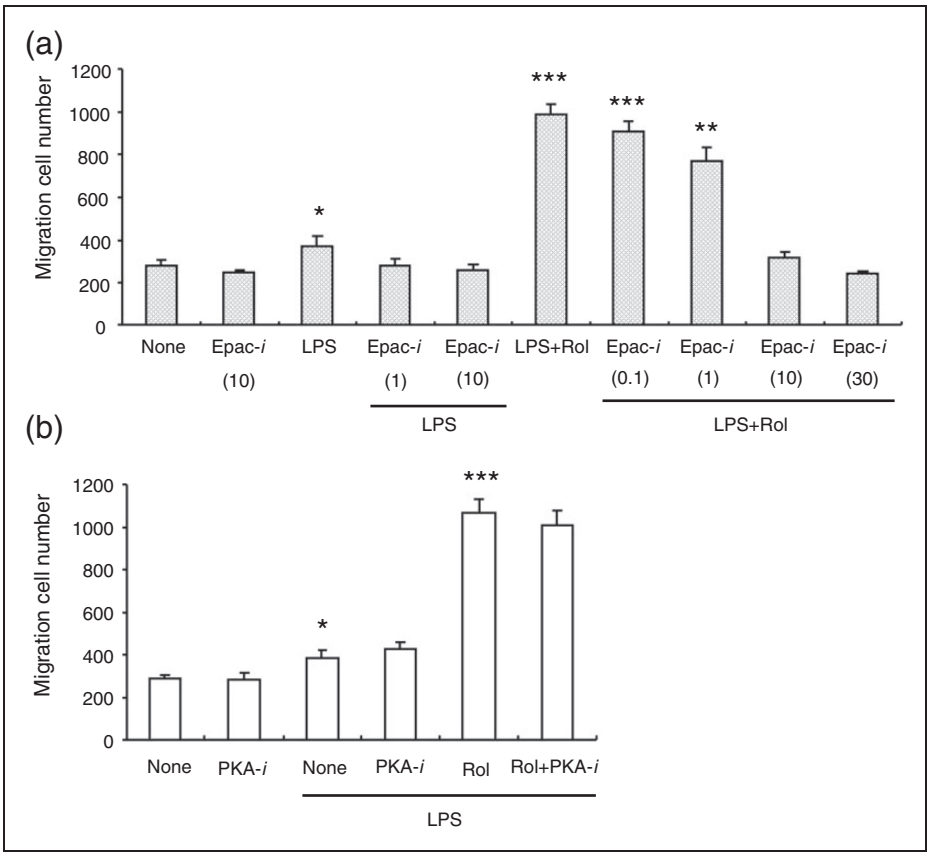


Figure 7. Rolipram and serum induced migration in LPS-stimulated macrophages is mediated by activation of Epac. Raw 264.7 cells were pre-treated with 10 μM rolipram, increasing concentrations of the Epac inhibitor ESI-09 in the presence or absence of rolipram (a) or the PKA inhibitor Rp-8-CPT-cAMPS (500 μM) or a combination of rolipram and Rp-8-CPT-cAMPS (b) for 20 min prior to LPS stimulation for 24 h. Cell migration was assessed as described in Figure 1. Data are the mean ± SEM (a, $n = 4$–8; b, $n = 3$–6). *$P < 0.05$, compared with untreated cell; **$P < 0.01$, ***$P < 0.001$, compared with the cells treated with LPS alone.

chemokine MCP-1 is not affected by rolipram, suggesting that PDE4 inhibitor, hence cAMP signaling, selectively regulates LPS-mediated chemotaxis in macrophages.

FBS is a well-known stimulant for cell migration.[30] It contains a wide variety of growth factors, such as PDGF, fibroblast growth factor, TGF, and insulin-like growth factor (IGF). These factors promote not only the fibroblast and cancer cell motility, but also macrophage migration.[31–33] By supplementing FBS in the incubation medium, we observed significant increases in macrophage migration under the basal (no drug treatment) condition (Figure 5a), in which the increase is more pronounced in 2% than in 10% FBS medium (2%, $P < 0.01$; 10%, $P < 0.05$, compared with the 0% control). This finding is consistent with the previous report on fibroblasts, where the migration speed of fibroblasts measured in the presence of increasing concentrations of FBS reaches the highest at 2% FBS and then trends toward the baseline migration when FBS concentration increases up to 20%.[30] Compared with the untreated cells (Figure 5a), the cells treated with rolipram alone displayed similar pattern and levels of increase in migration at both 2% and 10% FBS concentrations (Figure 5b), indicating rolipram itself has limited impact on macrophage migration. The serum effect on the basal migration probably is instigated by a combined effect of several serum factors. A study by Furundzija et al. has shown that the serum factor IGF-1 is chemotactic to macrophages and its effect on migration induction involves activation of integrins and focal adhesion formation via *inside-out* 3-kinase/PKC/p38-dependent signaling.[33] Additionally, Krettek et al. found that the homodimer of PDGF B chain (PDGF-BB) also stimulates the migration of macrophages, with an increase primarily in random motility (chemokinesis).[31] However, TGF-β1 was shown to stimulate macrophage migration only in the early phase of the treatment because an inhibitory effect was detected during the late phase.[32]

In LPS-stimulated macrophages, we observed a mild but consistent decrease in migration cell number under serum-free and 2% serum conditions (Figure 5c) as compared with the untreated cells (Figure 5a).

However, these cells displayed a marginal but significant increase in migration when the serum concentration increased from 2% to 10% ($P < 0.05$, Figure 5c), showing a positive impact of serum on migration in LPS-treated cells. Additionally, the migration of LPS-stimulated macrophages was greatly induced by 10% serum when the cells were co-treated with rolipram (ca. 2.5-fold increase compared with the cells treated with 2% serum; Figure 5d), suggesting PDE4 negatively regulates macrophage migration when the cells are under a combined stimulation of LPS and serum component(s). From these results, we propose that with the participation of unknown serum factor(s), LPS/TLR4-elicited signaling might crosstalk to the cAMP signaling activated by PDE4 inhibitors, from which cell migration is escalated.

In addition to the growth factors, LPS-binding protein (LBP), a protein found in abundance in serum, is also likely to be a critical factor for the rolipram-enhanced migration in LPS-stimulated Raw 264.7 cells. It is well established that by initiating pro-inflammatory responses during infection and inflammation, LPS is recognized by LBP and then transferred to CD14 receptor and subsequently to the MD2/TLR4 complex on inflammatory cell surface.[34] Evidence indicates that in the presence of serum, cells that possess surface CD14 display an increased sensitivity to LPS by 100 to 1000 folds, and this increased sensitivity is mediated by LBP.[35,36] Additionally, unlike Kupffer cells and most intestinal macrophages that lack or express low level of CD14 receptor, Raw 264.7 cells, a peritoneal macrophage cell line, are abundant in surface CD14.[37,38] On the basis of these findings, we postulate that LBP, and thereby the formation of LPS/LBP complex, is essential for the binding of LPS to CD14 and then to MD2 in Raw 264.7 cells. The binding of LPS and MD2 initiates the formation of MD2/TLR4 complex which then activates the MyD88 (myeloid differentiation factor)-dependent and -independent pathways, leading to the induction of cell migration (LPS-induced migration). When PDE4 inhibitor is present, the elicited cAMP/Epac signaling interacts with the LPS/TLR4 signaling resulting in further increase in cell migration (PDE4 inhibitor-enhanced migration). This proposed essential role of LBP in macrophage migration explains why the migration activity induced in LPS and LPS+Rol treated cells is abrogated when serum is absent (Figures 4b and 5c and d). Nevertheless, further experiments are necessary to verify whether LBP is essential for activating downstream LPS/TLR4 signaling and cell migration regulation.

Macrophage migration inhibitory factor (MIF), a cytokine released by inflammatory cells, is known to inhibit monocyte/macrophage migration.[39] A study by Calandra et al. has shown that low dose LPS (<1 μg/ml) stimulates MIF release in Raw 264.7 cells.[40] To determine whether the rolipram-enhanced migration is associated with a decrease in MIF expression, we also quantified MIF mRNA levels in Raw 264.7 cells and found there was a significant increase ($P < 0.05$) in MIF mRNA expression in the cells treated with LPS for 3 h, but the mRNA level was not affected by co-treatment with rolipram (data not shown).

Figure 2 shows that the cAMP-elevating agents rolipram and 8-bromo-cAMP dose-dependently increase migration in LPS-stimulated macrophages. This cAMP effect is consistent with the reports of other groups, where macrophage migration is induced by PGE_2 through activation of EP2 and/or EP4 receptors to promote cAMP production.[2,9,41] These results also are in line with the finding that roflumilast treatment of COPD patients induces, rather than suppresses, phagocytic activity of peripheral blood cells, which results in an improvement of lung function.[42] On the contrary, increasing cAMP has also been shown to inhibit macrophage migration. Moon et al. found that elevation of cAMP by long-term (24 h) treatment of Raw 264.7 cells with TGF-β1 activates sequentially Epac, Rap1, and ARAP3 (Rap-dependent RhoGAP), resulting in suppression of RhoA activity and then macrophage migration.[43] A study by Michaud et al. also showed that activation of S1P receptor 2 in macrophages induces cAMP production while inhibits Akt phosphorylation, which leads to a decrease in C5a-induced chemotaxis.[10] The reason for the opposite effects of cAMP on migration is unknown, but could possibly be due to different stimulation conditions and types of macrophage being used.

Cyclic cAMP regulation of immune cell functions generally is mediated by activating its effector protein PKA or Epac.[5] Here we demonstrate that Epac, but not PKA, is involved in the rolipram/serum-enhanced migration in LPS-stimulated macrophages. This result coincides with the finding that the synthetic Epac activator promotes migration of smooth muscle cells in rat ductus arteriosus.[44] Conversely, cAMP-Epac signaling has been shown to inhibit migration of various cell types, such as human prostate carcinoma cells,[45] PDGF-treated human vascular smooth muscle cells,[46] and human pancreatic ductal adenocarcinoma cells.[47] The reason for the contradictory results is unknown, possibly due to cell type and species differences.

LPS-elicited macrophage migration can be explained by several mechanisms. LPS is known to activate macrophages via TLR4,[48] from which the production of immunomodulatory factors is evoked. The production of MCP-1, a chemokine known to regulate the migration and infiltration of monocytes/macrophages, is induced by LPS,[49] via activation of the

LPS/TLR4/MyD88/NF-κB signaling pathway.[48] However, the MCP-1induced migration is not further enhanced by rolipram, as indicated in Figure 3c. A study by Tajima et al. has demonstrated that LPS stimulation of macrophage triggers secretion of PGD2 and PGE2, two lipid inflammatory mediators, through which the receptor signaling of CRTH2 and EP4, respectively, are activated to promote LPS-mediated migration.[2] Additionally, LPS has been shown to stimulate reactive oxygen species (ROS) production in Raw 264.7 cells through activation of the ERK1/2 signal pathway, which in turn promotes MMP-9 expression and cell migration.[3] A following study by Yang et al. has shown that TLR4/NF-κB signaling is required for LPS-induced MMP9 expression and cell migration in Raw 264.7 cells.[4] Further studies are necessary to elucidate which pathway is modulated by PDE4/cAMP signaling.

In summary, our data demonstrate that with the involvement of serum constituents, the migration of LPS-stimulated macrophages is markedly enhanced by PDE4 inhibition. The enhancement of migration is mediated by activation of cAMP/Epac signaling, presumably via interaction with LPS/TLR4 signaling with the participation of unknown serum factors. While it is not clear as yet whether the anti-inflammatory effects of PDE4 inhibitors have input to macrophage migration, here the observed rolipram effect on migration may imply a more efficient clearance of infection and thereby wound repair by this family of cAMP-elevating agents.

Declaration of conflicting interests

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References

1. Pollard JW. Trophic macrophages in development and disease. *Nat Rev Immunol* 2009; 9: 259–270.
2. Tajima T, Murata T, Aritake K, et al. Lipopolysaccharide induces macrophage migration via prostaglandin D(2) and prostaglandin E(2). *J Pharmacol Exp Ther* 2008; 326: 493–501.
3. Kim SY, Lee JG, Cho WS, et al. Role of NADPH oxidase-2 in lipopolysaccharide-induced matrix metalloproteinase expression and cell migration. *Immunol Cell Biol* 2010; 88: 197–204.
4. Yang YH, Li DL, Bi XY, et al. Acetylcholine Inhibits LPS-Induced MMP-9 Production and Cell Migration via the alpha7 nAChR-JAK2/STAT3 Pathway in RAW264.7 Cells. *Cell Physiol Biochem* 2015; 36: 2025–2038.
5. Serezani CH, Ballinger MN, Aronoff DM, et al. Cyclic AMP: master regulator of innate immune cell function. *Am J Respir Cell Mol Biol* 2008; 39: 127–132.
6. Jin SL, Ding SL and Lin SC. Phosphodiesterase 4 and its inhibitors in inflammatory diseases. *Chang Gung Med J* 2012; 35: 197–210.
7. Lai CR, Lo HC, Chen YL, et al. Phosphodiesterase 4B is essential for lipopolysaccharide-induced CC chemokine ligand 3 production in mouse macrophages. *J Med Sci* 2015; 35: 111–121.
8. Yang JX, Hsieh KC, Chen YL, et al. Phosphodiesterase 4B negatively regulates endotoxin-activated interleukin-1 receptor antagonist responses in macrophages. *Sci Rep* 2017; 7: 46165.
9. Poole NM, Mamidanna G, Smith RA, et al. Prostaglandin E(2) in tick saliva regulates macrophage cell migration and cytokine profile. *Parasit Vectors* 2013; 6: 261.
10. Michaud J, Im DS and Hla T. Inhibitory role of sphingosine 1-phosphate receptor 2 in macrophage recruitment during inflammation. *J Immunol* 2010; 184: 1475–1483.
11. Torphy TJ. Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. *Am J Respir Crit Care Med* 1998; 157: 351–370.
12. Houslay MD. Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 2010; 35: 91–100.
13. Jin SL, Lan L, Zoudilova M, et al. Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. *J Immunol* 2005; 175: 1523–1531.
14. Garnock-Jones KP. Roflumilast: a review in COPD. *Drugs* 2015; 75: 1645–1656.
15. Deeks ED. Apremilast: a review in psoriasis and psoriatic arthritis. *Drugs* 2015; 75: 1393–1403.
16. Zimmerman NP, Kumar SN, Turner JR, et al. Cyclic AMP dysregulates intestinal epithelial cell restitution through PKA and RhoA. *Inflamm Bowel Dis* 2012; 18: 1081–1091.
17. Kohyama T, Liu X, Wen FQ, et al. Cytokines modulate cilomilast response in lung fibroblasts. *Clin Immunol* 2004; 111: 297–302.
18. Schafer PH, Truzzi F, Parton A, et al. Phosphodiesterase 4 in inflammatory diseases: effects of apremilast in psoriatic blood and in dermal myofibroblasts through the PDE4/CD271 complex. *Cell Signal* 2016; 28: 753–763.
19. Netherton SJ and Maurice DH. Vascular endothelial cell cyclic nucleotide phosphodiesterases and regulated cell migration: implications in angiogenesis. *Mol Pharmacol* 2005; 67: 263–272.
20. Abusnina A, Keravis T, Zhou Q, et al. Tumour growth inhibition and anti-angiogenic effects using curcumin

correspond to combined PDE2 and PDE4 inhibition. *Thromb Haemost* 2015; 113: 319–328.
21. Fleming YM, Frame MC and Houslay MD. PDE4-regulated cAMP degradation controls the assembly of integrin-dependent actin adhesion structures and REF52 cell migration. *J Cell Sci* 2004; 117: 2377–2388.
22. Alves AC, Pires AL, Cruz HN, et al. Selective inhibition of phosphodiesterase type IV suppresses the chemotactic responsiveness of rat eosinophils in vitro. *Eur J Pharmacol* 1996; 312: 89–96.
23. Santamaria LF, Palacios JM and Beleta J. Inhibition of eotaxin-mediated human eosinophil activation and migration by the selective cyclic nucleotide phosphodiesterase type 4 inhibitor rolipram. *Br J Pharmacol* 1997; 121: 1150–1154.
24. Layseca-Espinosa E, Baranda L, Alvarado-Sanchez B, et al. Rolipram inhibits polarization and migration of human T lymphocytes. *J Invest Dermatol* 2003; 121: 81–87.
25. Brink HE, Miller GJ, Beredjiklian PK, et al. Serum-dependent effects on adult and fetal tendon fibroblast migration and collagen expression. *Wound Repair Regen* 2006; 14: 179–186.
26. Banerjee S, Li G, Li Y, et al. RNase L is a negative regulator of cell migration. *Oncotarget* 2015; 6: 44360–44372.
27. Etxebarria J, Sanz-Lazaro S, Hernaez-Moya R, et al. Serum from plasma rich in growth factors regenerates rabbit corneas by promoting cell proliferation, migration, differentiation, adhesion and limbal stemness. *Acta Ophthalmol* 2017; 95: e693–e705.
28. Ma D, Wu P, Egan RW, et al. Phosphodiesterase 4B gene transcription is activated by lipopolysaccharide and inhibited by interleukin-10 in human monocytes. *Mol Pharmacol* 1999; 55: 50–57.
29. Jin SL and Conti M. Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-alpha responses. *Proc Natl Acad Sci USA* 2002; 99: 7628–7633.
30. Sandulache VC, Parekh A, Li-Korotky H, et al. Prostaglandin E2 inhibition of keloid fibroblast migration, contraction, and transforming growth factor (TGF)-beta1-induced collagen synthesis. *Wound Repair Regen* 2007; 15: 122–133.
31. Krettek A, Ostergren-Lunden G, Fager G, et al. Expression of PDGF receptors and ligand-induced migration of partially differentiated human monocyte-derived macrophages. Influence of IFN-gamma and TGF-beta. *Atherosclerosis* 2001; 156: 267–275.
32. Kim JS, Kim JG, Moon MY, et al. Transforming growth factor-beta1 regulates macrophage migration via RhoA. *Blood* 2006; 108: 1821–1829.
33. Furundzija V, Fritzsche J, Kaufmann J, et al. IGF-1 increases macrophage motility via PKC/p38-dependent alphavbeta3-integrin inside-out signaling. *Biochem Biophys Res Commun* 2010; 394: 786–791.
34. Ohto U, Fukase K, Miyake K, et al. Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. *Science* 2007; 316: 1632–1634.
35. Schumann RR, Leong SR, Flaggs GW, et al. Structure and function of lipopolysaccharide binding protein. *Science* 1990; 249: 1429–1431.
36. Wright SD, Ramos RA, Tobias PS, et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990; 249: 1431–1433.
37. Lichtman SN, Wang J and Lemasters JJ. LPS receptor CD14 participates in release of TNF-alpha in RAW 264.7 and peritoneal cells but not in kupffer cells. *Am J Physiol* 1998; 275: G39–G46.
38. Smith PD, Smythies LE, Shen R, et al. Intestinal macrophages and response to microbial encroachment. *Mucosal Immunol* 2011; 4: 31–42.
39. Weiser WY, Temple PA, Witek-Giannotti JS, et al. Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. *Proc Natl Acad Sci USA* 1989; 86: 7522–7526.
40. Calandra T, Bernhagen J, Mitchell RA, et al. The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J Exp Med* 1994; 179: 1895–1902.
41. Cantarow WD, Cheung HT and Sundharadas G. Effects of prostaglandins on the spreading, adhesion and migration of mouse peritoneal macrophages. *Prostaglandins* 1978; 16: 39–46.
42. Porpodis K, Domvri K, Zarogoulidis P, et al. Roflumilast, a phosphodiesterase-4 inhibitor, induces phagocytic activity in Greek COPD patients. *Int J Chron Obstruct Pulmon Dis* 2015; 10: 1123–1128.
43. Moon MY, Kim HJ, Kim JG, et al. Small GTPase Rap1 regulates cell migration through regulation of small GTPase RhoA activity in response to transforming growth factor-beta1. *J Cell Physiol* 2013; 228: 2119–2126.
44. Yokoyama U, Minamisawa S, Quan H, et al. Prostaglandin E2-activated Epac promotes neointimal formation of the rat ductus arteriosus by a process distinct from that of cAMP-dependent protein kinase A. *J Biol Chem* 2008; 283: 28702–28709.
45. Grandoch M, Rose A, ter Braak M, et al. Epac inhibits migration and proliferation of human prostate carcinoma cells. *Br J Cancer* 2009; 101: 2038–2042.
46. McKean JS, Murray F, Gibson G, et al. The cAMP-producing agonist beraprost inhibits human vascular smooth muscle cell migration via exchange protein directly activated by cAMP. *Cardiovasc Res* 2015; 107: 546–555.
47. Burdyga A, Conant A, Haynes L, et al. cAMP inhibits migration, ruffling and paxillin accumulation in focal adhesions of pancreatic ductal adenocarcinoma cells: effects of PKA and EPAC. *Biochim Biophys Acta* 2013; 1833: 2664–2672.
48. Akira S, Takeda K and Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001; 2: 675–680.
49. Kopydlowski KM, Salkowski CA, Cody MJ, et al. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J Immunol* 1999; 163: 1537–1544.