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The IL-4/13-induced production of M2 chemokines by human lung macrophages is enhanced by adenosine and PGE_2

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ABSTRACT

Background and purpose: Lung macrophages (LMs) are critically involved in respiratory diseases. The primary objective of the present study was to determine whether or not an adenosine analog (NECA) and prostaglandin E_2 (PGE_2) affected the interleukin (IL)-4- and IL-13-induced release of M2a chemokines (CCL13, CCL17, CCL18, and CCL22) by human LMs.

Experimental Approach: Primary macrophages isolated from resected human lungs were incubated with NECA, PGE_2, roflumilast, or vehicle and stimulated with IL-4 or IL-13 for 24 h. The levels of chemokines and PGE_2 in the culture supernatants were measured using ELISAs and enzyme immunoassays.

Key results: Exposure to IL-4 (10 ng/mL) and IL-13 (50 ng/mL) was associated with greater M2a chemokine production but not PGE_2 production. PGE_2 (10 ng/mL) and NECA (10^{-6} M) induced the production of M2a chemokines to a lesser extent but significantly enhanced the IL-4/IL-13-induced production of these chemokines. At either a clinically relevant concentration (10^{-9} M) or at a concentration (10^{-7} M) that fully inhibited phosphodiesterase 4 (PDE4) activity, roflumilast did not increase the production of M2a chemokines and did not modulate their IL-13-induced production, regardless of the presence or absence of PGE_2.

Conclusions: NECA and PGE_2 enhanced the IL-4/IL-13-induced production of M2a chemokines. The inhibition of PDE4 by roflumilast did not alter the production of these chemokines. These results contrast totally with the previously reported inhibitory effects of NECA, PGE_2, and PDE4 inhibitors on the lipopolysaccharide-induced release of tumor necrosis factor alpha and M1 chemokines in human LMs.

1. Introduction

Lung macrophages (LMs) are key sentinel cells for the host's primary defense and are involved in the pathophysiology of pulmonary inflammatory diseases (such as asthma and chronic obstructive pulmonary disease (COPD)) and viral respiratory diseases (such as respiratory syncytial virus-induced bronchiolitis and COVID-19) [1–7]. Human LMs orchestrate lung inflammation and can be committed to a continuum of functional phenotypes, depending on various environmental cues [8]. The phenotypes at the ends of the spectrum are often referred to as classically activated M1 macrophages and alternatively activated M2 macrophages [9–11]. The commitment to an M1 phenotype is induced by signals generated during Th1-mediated immune responses or by exposure to microbial products, such as lipopolysaccharide (LPS). A M2 state of activation induced by the canonical Th2 interleukins (IL) IL-4 and IL-13 is also called M2a, since it may differ from the M2 states of

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activation induced by other stimulatory factors [12,13]. Human M2a macrophages produce chemokines (such as CCL13, CCL17, CCL18, and CCL22 [14–16]) that differ from the cytokines and chemokines produced by M1 macrophages [14,17,18]. In allergic asthma, an imbalance towards M2 macrophages may be a pathogenic factor [19–21].

Prostaglandin E_2 (PGE_2) can be synthesized by all cell types, and elevated levels are observed during most inflammatory responses [22,23]. PGE_2 promotes inflammatory processes in most organs but has predominantly anti-inflammatory effects in the lung [23–25]. Accordingly, we have previously shown that exogenous PGE_2 can inhibit the production of M1 cytokines by LPS-stimulated human LMs [26]. Although PGE_2 is best documented as having an anti-inflammatory role during type 2 inflammation, this prostaglandin might also have a proinflammatory role or might alter the type 1 vs. type 2 balance; in turn, this would allow or enhance the emergence of a type 2 response [25,27].

Adenosine is an endogenous purine nucleoside that is rapidly released from all cells when ATP is degraded during hypoxia, chronic inflammation, or other stress phenomena [28]. Adenosine signaling can be markedly amplified under these pathological conditions, with a 100-fold increase in the local concentration (i.e. up to 2–30 μM). However, the compound has a short half-life and is metabolized by adenosine deaminase or adenosine kinase [29–31]. Adenosine has a clear role in inflammatory lung diseases like asthma and COPD [32–35]. We have previously shown that 5́–N-ethyl-carboxamidoadenosine (NECA, a metabolically stable, full agonist of the four subtypes of adenosine receptor [31]) can inhibit the production of M1 cytokines by LPS-stimulated human LMs [36]. However, adenosine can also promote alternative activation in the macrophage cell line RAW 264.7, as shown by the up-regulation of IL-4-induced arginase (Arg) activity and tissue inhibitor of metalloprotease 1 (TIMP-1) production [37,38].

Both adenosine and PGE_2 stimulate adenylate cyclase and intracellular cAMP accumulation through the adenosine A_2 receptors and the EP_4 receptor subtypes, respectively. In human LMs, the generation of intracellular cAMP by PGE_2 and NECA is involved in the downregulation of LPS-induced M1 cytokine production [26,36]. Furthermore, the inhibition of phosphodiesterases 4 (PDE4) by roflumilast leads to an increase in intracellular cAMP levels and to the inhibition of LPS-induced production of M1 cytokines by human LMs [26,39].

The putative roles of adenosine and PGE_2 in the regulation of M2a chemokine production by human LMs have not been addressed previously. Hence; the main objectives of the present study were to (i) assess the effects of NECA and PGE_2 on the IL-4/IL-13-stimulated production of chemokines in human LMs, and (ii) establish whether or not roflumilast enhanced the effects of NECA and PGE_2.

2. Methods

2.1. Isolation and culture of primary human LMs

The use of human lung tissues for research was approved by an institutional review board (*Comité de Protection des Personnes Ile de France VIII*, Boulogne-Billancourt, France), and all patients gave their informed consent, prior to surgery. Lung tissues were obtained from 34 patients (23 males and 11 females; mean ± SD age: 62.8 ± 7.8 years; 10 current smokers and 19 ex-smokers; mean pack-years: 41.7 ± 15.2; mean FEV1 = 89.5 ± 14.2 %; mean FEV1/FVC ratio: 0.76 ± 0.09; 9 patients with (non-severe) COPD (FEV1/FVC < 0.7)) undergoing surgical resection for lung carcinoma and who had not received chemotherapy or radiotherapy before surgery. Lung macrophages were isolated from finely minced peripheral tissues far from the tumor [16,26,36]. Briefly, the fluid collected from several washings of the minced lung tissues with sterile culture medium (Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 $\mu g.mL^{-1}$ streptomycin, 100 $U.mL^{-1}$ penicillin and 2 mM glutamine) was centrifuged (300 x g, 10 min), and the cell pellet was resuspended in RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics. Resuspended viable cells were then plated at 10^6 cells mL^{-1} per well in a 24-well plate. After a 90 min incubation at 37 °C (in a humidified 5 % CO_2 atmosphere), non-adherent cells were removed by gentle washing. The adherent cells (≈200 × 10^3 cells per well) were > 95 % pure macrophages, as determined by May–-Grünwald–Giemsa staining and CD68 immunocytochemistry. Cell viability exceeded 90 %, as assessed by trypan blue dye exclusion. Using a CytoFLEX SRT flow cytometer (Beckman Coulter Life Sciences, Villepinte, France), we sought to identifying alveolar (AM) versus interstitial (IM) macrophages with antibodies targeting CD14 (Beckton-Dickinson (BD)-555397), CD45 (Miltenyi (M)-130–110-639), CD43 (Invitrogen (I)-563521), CD206 (M−130−126−637), CD169 (I-63–1699-42), HLA-DR (M−130−111−944), and live/dead cells (I-L3224). Human AMs were defined as CD45+/CD169+/CD206+/CD43+/CD14low and human IMs were defined as CD45+/CD169+/CD206+/CD43-/CD14high. The vast majority (~90 %) of the LMs were AMs, and the mean proportion of IMs after the adherence step was ~ 10 % in four independent samples.

2.2. Incubation of LMs with an adenosine receptor agonist (NECA), PGE_2, IL-4, IL-13, and roflumilast

Stock solutions of 10 mM roflumilast and NECA were prepared in dimethylsulfoxide (DMSO). The PGE_2 stock solution (10 mM) was prepared in ethanol. All subsequent dilutions were prepared daily in supplemented RPMI. The maximal DMSO concentration applied to cells in culture did not exceed 0.1 % and had no effect on either cell viability or cytokine production (data not shown), as reported previously [16,26].

The 24-well plates containing LMs were washed with warm medium, and 1 mL of fresh medium supplemented with 1 % FCS was then added per well. The LMs were preincubated with PGE_2 (10 nM) or NECA (1 μM) in the presence or absence of the PDE4 inhibitor roflumilast (1 or 100 nM), or vehicle for 30 min prior to stimulation with IL-4 (10 $ng.mL^{-1}$) or IL-13 at concentrations of 10 or 50 $ng.mL^{-1}$. Our choice of the IL-4 and IL-13 concentrations and incubation time (24 h) was based on previous experiments on time- and concentration-dependencies [16]. Up to a concentration of 1 μM, roflumilast is a selective PDE4 inhibitor [40]; we tested it at a clinically relevant concentration (1 nM) and a 100-fold higher concentration that fully inhibited PDE4 (100 nM). All wells were run in duplicate for each series of experiments performed with LMs isolated from a resected lung. Following the 24 h incubation with IL-4 or IL-13, cell culture supernatants were collected and stored at −20 °C for later assays of M2a chemokines and PGE_2.

Chemokine and prostaglandin E_2 assays. The production of M2a chemokines was assessed by measuring their concentrations in the LM culture supernatants, using an ELISA (Duoset Development System) according to the manufacturer's instructions (R&D Systems Europe, Lille, France). The supernatants were diluted with RPMI as appropriate, and the optical density was determined at 450 nm with an MRX II microplate reader from Dynex Technologies (Saint-Cloud, France). Cytokine concentrations are expressed in pg per 10^6 LMs. The limit of detection (LOD) was 4 $pg.mL^{-1}$ for the CCL13, CCL18 and CCL22 assays and 16 $pg.mL^{-1}$ for the CCL17 assay. PGE_2 was determined using an enzyme immunoassay kit (LOD: 36 $pg{\cdot}mL^{-1}$), according to the manufacturer's instructions (Cayman Chemical Europe, Tallinn, Estonia).

2.3. Materials

RPMI 1640 medium, penicillin–streptomycin stabilized solution, DMSO, L-glutamine and FCS were purchased from Eurobio Biotechnology (Les Ulis, France). LPS (from *Escherichia coli* serotype 0111:B4, batch 029 K4022), PGE_2, and DMSO were purchased from Sigma Aldrich (St. Louis, MO, USA). Human recombinant IL-13 and IL-4 were obtained from R&D Systems (Lille, France). Roflumilast was synthesized by Nycomed GmbH (Konstanz, Germany) and provided as a generous gift by Dr H. Tenor. NECA was purchased from Tocris Bioscience (Bristol,

UK). All other chemicals were of analytical grade and were obtained from Prolabo (Fontenay sous Bois, France). All cell culture plastics were from CCL (Nemours, France).

Statistical analysis. Data are expressed as the mean ± standard error of the mean (SEM); *n* represents the number of patients from whom LM preparations were obtained. Statistical analyses were performed on log-transformed data using an unpaired or paired Student's *t* test, a one-way, repeated-measures analysis of variance (followed by Sidak's test for multiple comparisons), or a mixed-effects repeated-measures analysis of variance (followed by Tukey's test for multiple comparisons), as appropriate. The threshold for statistical significance was set to $p < 0.05$. All analyses were performed using GraphPad Prism® software (version 8.4.2, GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effects of LPS, IL-4, IL-13, PGE_2, NECA and roflumilast on the production of M2a chemokines and PGE_2 by LMs

Incubation with LPS (10 ng.mL^{-1}) did not alter the production of CCL13, CCL17, and CCL22 but induced a small (2.1-fold), statistically significant elevation in the production of CCL18 (Fig. 1). Incubation with IL-4 (10 ng.mL^{-1}) or IL-13 (50 ng.mL^{-1}) was associated with much greater elevations in the production of CCL13 (19.2- and 20.2-fold, respectively), CCL17 (30.3- and 33.9-fold, respectively) and CCL22 (9.4- and 8.6-fold, respectively) (Fig. 1). The level of CCL18 was slightly higher (2.1- to 2.4-fold) after treatment with LPS, IL-4 or IL-13, which ruled out this chemokine as a true M2a cytokine (Fig. 1). This result differs from a previous report on human monocyte-derived macrophages (MDMs), in which the level of CCL17 (rather than CCL18) was elevated to the same extent by LPS, IL-4, and IL-13 [15]. There were no significant differences in the levels of M2a chemokines after exposure to IL-4 (10 ng.mL^{-1}) or IL-13 (50 ng.mL^{-1}). However, the levels of M2a chemokines were significantly lower (data not shown) after exposure to IL-13 (10 ng.mL^{-1}).

Treatment with LPS was associated with a much higher (141-fold) level of PGE_2 production by LMs, as observed previously [26]. In contrast, treatment with IL-4 and IL-13 weakly influenced the production of this prostaglandin (Table 1). The PGE_2 concentration in the supernatant of LMs exposed to LPS for 24 h was around 10 nM. Exposure to this PGE_2 concentration promoted M2a chemokine production, with a significant greater levels of basal production of CCL13, CCL17, CCL18, and CCL22 (4.8-, 11.3-, 1.6- and 3.2- fold, respectively; Fig. 1).

Furthermore, treatment with NECA was associated with significantly greater levels of CCL13, CCL17, CCL18, and CCL22 (6.2-, 12.8-, 1.8-, and 3.5-fold, respectively; Fig. 1). In contrast, full inhibition of PDE4 by 100 nM roflumilast was not associated with a difference in the basal

Table 1
Prostaglandin E_2 production induced by IL-4, IL-13 and LPS in human LMs.

	Baseline	IL-4 treatment	IL-13 treatment	LPS treatment
PGE_2 (pg/10^6 LM)	37.8 ± 6.9	52.9 ± 8.8 **	42.7 ± 9.6 **	5221.3 ± 1411.1 ##

Lung macrophages were incubated with LPS (10 ng.mL^{-1}), IL-4 (10 ng.mL^{-1}) or IL-13 (50 ng.mL^{-1}). PGE2 was assayed in supernatants collected after 24 h of culture. The results are quoted as the mean ± SEM of 18 to 20 independent experiments. # indicates a significant difference, relative to the baseline condition (##p < 0.01); * indicates a significant difference, relative to the LPS condition (**p < 0.01).

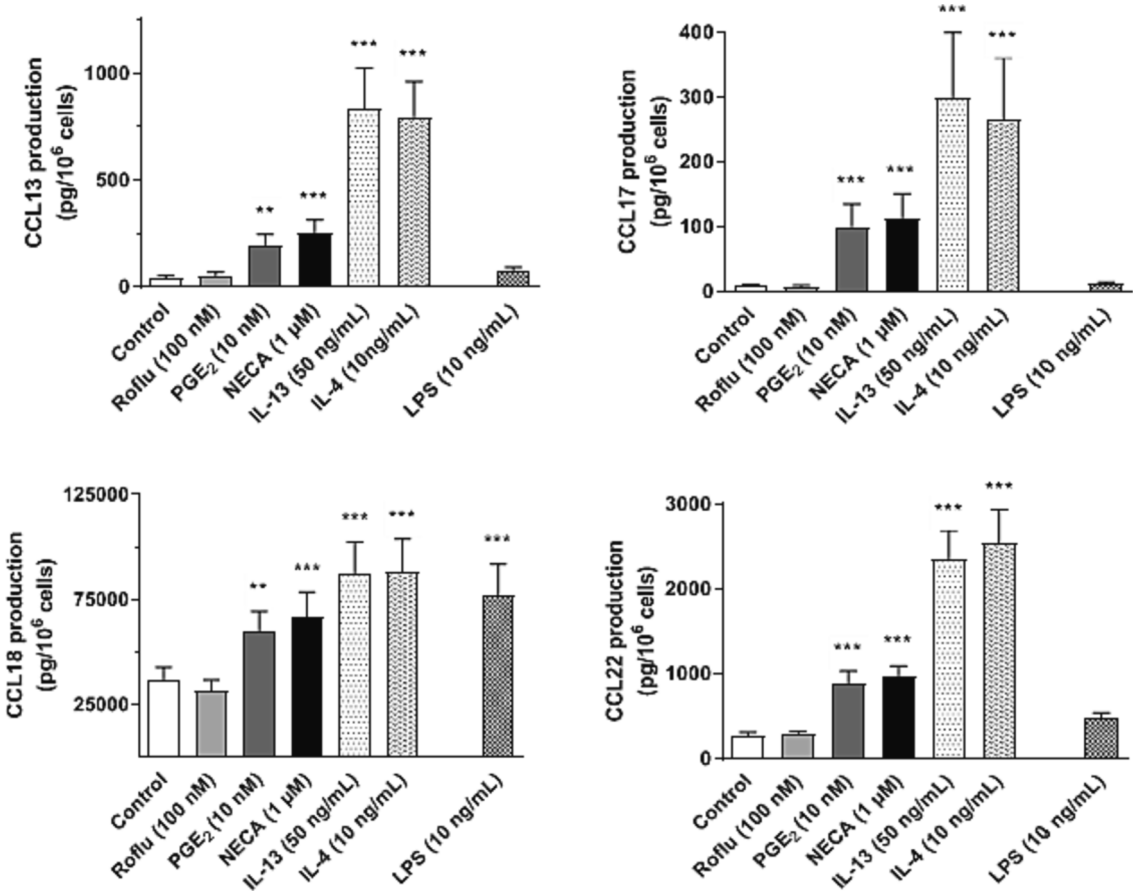

Fig. 1. Effects of IL-13, IL-4, PGE_2, NECA, roflumilast, and LPS on M2a chemokine production.

production of M2a chemokines (Fig. 1).

3.2. Effects of PGE_2, NECA and roflumilast on IL-4/IL-13-induced production of M2a chemokines by LMs

The combination of PGE_2 with IL-4 or IL-13 significantly enhanced the production of the four chemokines, relative to IL-4 or IL-13 alone (Fig. 2). The difference in the production of CCL13, CCL17, CCL18, and CCL22 was respectively 1.4-, 2.4-, 1.3-, and 1.6-fold for IL-4 and 1.7-, 2.6, 1.4- and 1.4-fold for IL-13.

The combination of NECA with IL-4 or IL-13 also significantly enhanced chemokine production, relative to IL-4 or IL-13 alone (Fig. 2). The difference in the production of CCL13, CCL17, CCL18, and CCL22 was respectively 1.6-, 3.2-, 1.3-, and 1.7-fold for IL-4 and 1.6-, 2.7-, 1.3- and 1.6-fold for IL-13. In a limited series of experiments, exposure to the adenyl cyclase activator forskolin (25 μM) was associated with similar relative increases in the IL-13-induced production (1.3- to 1.7-fold) of the M2a chemokines (data not shown).

In contrast, 1 or 100 nM roflumilast did not significantly alter the IL-13-induced production of M2a chemokines (Fig. 2, Table 2). Furthermore, roflumilast did not enhance or inhibit the M2a chemokine production induced by the combination of PGE_2 and IL-13 (Table 2).

Table 2
Effect of roflumilast on the IL-13-induced production of M2a chemokines in culture supernatants of human LMs, according to the presence or absence of PGE_2.

	IL-13	IL-13 + ROF (1 nM)	IL-13 + ROF (100 nM)	IL-13 + PGE_2	IL-13 + PGE_2 + ROF (1 nM)	IL-13 + PGE_2 + ROF (100 nM)
CCL13	826.9 ± 174.8	812.1 ± 171.5	841.7 ± 196.9	1534.6 ± 312.5**	1430.9 ± 333.4	1614.8 ± 380.7
CCL17	290.5 ± 87.5	267.7 ± 64.9	286.2 ± 85.1	1339.9 ± 429.9**	1223.4 ± 401.3	1254.1 ± 439.9
CCL18	130.3 ± 17.7	121.5 ± 16.2	132.5 ± 18.2	174.7 ± 15.7*	169.8 ± 17.3	181.2 ± 13.7
CCL22	4471.2 ± 470.3	4482.9 ± 501.8	4210.3 ± 488.7	7872.3 ± 810.4***	7938.8 ± 976.8	7445.5 ± 853.0

Human LMs were pre-incubated with 1 nM or 100 nM roflumilast (ROF) or vehicle, prior to the addition of IL-13 (50 $ng.mL^{-1}$) and in the presence or absence of PGE_2 (10 nM). The supernatant concentrations of the M2a chemokines were measured using ELISAs after 24 h of culture and are expressed in pg/10^6 LMs (except for CCL18, expressed in ng/10^6 LMs). The data are reported as the mean ± SEM of 6 independent, paired preparations..*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. IL-13 in the absence of PGE_2.

4. Discussion and conclusions

The present study of human LMs is the first to have shown that (i) exposure to NECA and PGE_2 is associated with greater production of CCL13, CCL17, CCL18, and CCL22 and enhancement of the stimulatory effects of IL-4 and IL-13 on the production of these M2a chemokines, and (ii) inhibition of PDE4 did not influence either the basal and the IL-13 induced production of M2a chemokines or the enhancing effect of PGE_2.

We have reported previously on the downregulation of the LPS-induced production of a pattern of M1 cytokines in LMs by NECA and PGE_2, and we have discussed the involvement of the intracellular cAMP level in this phenomenon [26,36]. A cAMP-dependent pathway has been also implicated in the regulation of M2 chemokine production under various experimental conditions. The production of CCL22 by murine spleen cells (including macrophages/dendritic cells) is reportedly promoted by cAMP-elevating agents such as PGE_2, dibutyryl cAMP, and 3-isobutyl-1-methylxanthine [41]. The neuropeptides vasoactive intestinal peptide (VIP) and the structurally related pituitary adenylate cyclase-activating polypeptide (PACAP) reportedly promote CCL22 production by murine bone marrow-derived dendritic cells via the cAMP/PKA pathway [42]. Furthermore, PGI_2 analogues (iloprost and treprostinil), forskolin, and a β_2-adrenoceptor agonist (formoterol) enhanced the LPS-induced production of CCL22 in human primary blood monocytes and in the human monocyte cell line THP-1 [43,44]. Forskolin has been shown to increase the basal expression of CCL13 and CCL18 by human MDMs [45].

However, we reported previously that β_2-adrenoceptor agonists (including formoterol) were devoid of any effect on LPS-induced

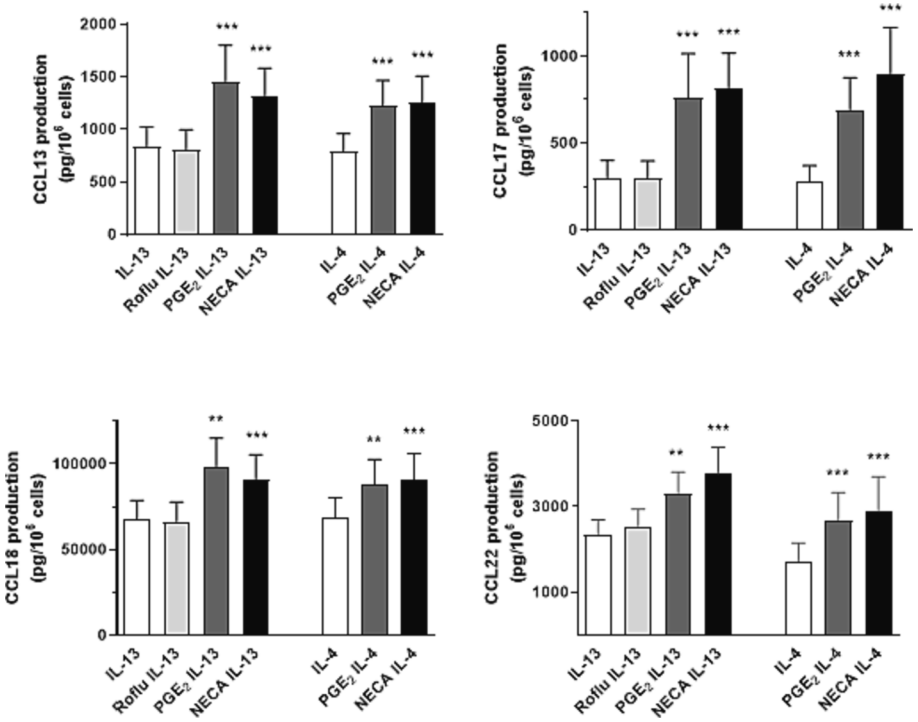

Fig. 2. Effects of PGE_2, NECA, and roflumilast on IL-13 and IL-4-induced M2a chemokine production.

cytokine release by LMs [46]; this contrasts with the agonists' known inhibitory effects on human monocytes and MDMs [46]. Furthermore, both forskolin and dibutyryl-cAMP suppressed CCL17 and CCL22 production by interferon gamma and tumor necrosis factor (TNF)-α -stimulated HaCaT keratinocytes [47]; this contrasted with their enhancing effects on M2 chemokine production in the aforementioned cellular preparations of murine macrophages and human monocytes. Taken as a whole, these results suggest that the regulatory role of the adenylyl cyclase-cAMP system depends on the inflammatory stimulus, the cell type, and the cell's state of differentiation. Lastly, and above all, murine macrophages and dendritic cells and human monocytes, MDMs and macrophage-derived cell lines are all surrogate cell models that do not adequately recapitulate the biology of human primary tissue macrophages [7,48–51]. It was therefore important to assess the effects of cAMP-elevating agents on the production of M2a chemokines by human LMs. In the present study, a PGE_2 concentration close to that induced *in vitro* by LPS and a NECA concentration equivalent to the adenosine concentration induced by hypoxia (taking into account NECA's 10-fold higher affinity for the adenosine receptors, relative to adenosine) promoted the basal production of M2a chemokines although to a lesser extent than IL-4 or IL-13. In sharp contrast, we have previously shown that PGE_2 and NECA did not alter the basal production of M1 chemokines [26,36].

4.1. Regulation of the IL-4/IL-13-induced production of M2a chemokines by PGE_2 and NECA

PGE_2 and NECA also enhanced the IL-4- and IL-13-induced production of M2a chemokines by human LMs. We found that the effect was somewhat smaller and less consistent for CCL18. In murine splenic macrophages and dendritic cells, PGE_2 reportedly enhanced IL-4-induced CCL22 production [41]. Apart from their effect of the production of M2a chemokines, adenosine and NECA act synergistically with IL-4 and IL-13 to induce the production of two M2 markers (Arg and TIMP-1) by the RAW 264.7 murine macrophage cell line [37]. The stimulatory effects of adenosine and NECA on alternative macrophage activation were mediated chiefly by A_2B receptors [37,52], which contrasts with the predominant role of A_2A receptors in downregulating LPS-induced macrophage M1 activation [36,52]. In murine bone marrow-derived macrophages and RAW 264.7 macrophages, two cAMP-elevating agents (db-cAMP or 8-Br-cAMP) and IL-4 were associated with greater expression of Arg-1, and the combination of IL-4 and the cAMP elevating agents exerted a synergistic effect on Arg-1 expression and Arg activity [53–55]. The elevated cAMP concentration resulted in the activation and greater nuclear localization of the transcription factor C/EBPβ and the subsequent increase in the expression of Arg-1 (55).

Taken as a whole, these results contrast sharply with the previously reported inhibitory effects of NECA and PGE_2 on the LPS-induced release of TNF-α and M1 chemokines in human LMs [26,36].

4.2. Effect of PDE4 inhibition on the production of M2a chemokines

A number of previous studies (including some of our own) in human MDMs and LMs have found that the presence of PGE_2 (i.e. an increase in cAMP formation) was required for the reduction in the LPS-induced production of TNF-α and some M1 chemokines by PDE4 inhibitors. These findings suggested that the inhibition of PDE4 only amplified the cAMP response (by inhibiting cAMP degradation) when cAMP synthesis was simultaneously activated [26,56]. In the present study, roflumilast alone did not enhance the basal production or IL-13-stimulated production of the M2a chemokines. This finding was probably due to the very weak production of PGE_2 by unstimulated LMs and IL-13-stimulated LMs. Hence, roflumilast could not have had an enhancing effect on IL-13-induced M2a chemokine production.

In RAW 264.7 macrophages, the PDE4 inhibitor rolipram had no effect alone but enhanced Arg activity when combined with IL-4 [54]. Unlike the 16-fold increase in Arg activity induced by IL-4 in RAW 264.7 cells, Arg activity in human alveolar macrophages (AMs) from healthy volunteers was not increased by IL-4 alone [54]; this finding again emphasized that murine models of macrophages do not recapitulate the biology of human LMs. Furthermore, the use of a combination of forskolin and IBMX (a nonspecific PDE inhibitor) to produce a maximal increase in intracellular cAMP levels was associated with a weak (less than 2-fold elevation) in Arg activity [54] - suggesting that in AMs, cAMP regulates this M2 marker to a modest extent only.

To further assess the effect of roflumilast on the production of M2a chemokines, we performed experiments with PGE_2. At a clinically relevant concentration and at a concentration that fully inhibited PDE4, roflumilast did not enhance the effect of a combination of IL-13 and PGE_2 on the production of the M2a chemokines. Our use of the selective PDE4 inhibitor roflumilast (rather than a non-specific PDE inhibitor like IBMX) is unlikely to explain the absence of an enhancing effect on the production of M2a chemokines in response to PGE_2 and IL-13. Indeed, we have shown previously that the PDE isoenzyme pattern in human LMs [26] is similar to that seen in human MDMs [56] and AMs [57]. These macrophages show PDE4 activity along with PDE3 and PDE1 activities. In experiments with MDMs, a PDE3-selective inhibitor showed little or no potentiation of chemokine expression when combined with forskolin or PGE_2 (suggesting that PDE3 is not greatly involved in controlling chemokine expression), whereas a PDE4-selective inhibitor increased the PGE_2- and the forskolin-induced production of chemokines. Therefore, PDE4 appears to be the main regulator of the cAMP-dependent pathways that increase chemokine expression in human MDMs [45]. Although human LMs have various distinctive features, 10 nM PGE_2 might have caused an optimal, cAMP-dependent enhancement of the IL-13-induced production of M2a chemokines (as suggested by the similar increases observed with NECA and forskolin); this would have left no room for further enhancement by another modulator of the adenylyl cyclase-cAMP system.

Roflumilast has been approved by the regulatory authorities as a once-daily oral tablet for the maintenance treatment of severe COPD. In asthma, roflumilast has been shown to (i) inhibit allergen- and exercise-induced bronchoconstriction [58,59], (ii) confer clinical benefits on patients with mild-to-moderate asthma [60,61] and (iii) be efficacious as an add-on treatment in patients with moderate-to-severe asthma inadequately controlled by combination therapy with an inhaled corticosteroid and a long-acting β2-agonist [62]. In patients with asthma, CCL13, CCL17 and CCL22 are expressed in AMs and in the airways and are upregulated in uncontrolled, severe allergic asthma or after a bronchial allergen challenge [63–66]. In about a quarter of patients with exacerbations of asthma or COPD, blood and sputum levels of type 2 mediators (including IL-13, CCL13, and CCL17) are elevated [67,68]. It is noteworthy that both CCL17 and CCL22 are actively involved in the recruitment of Th2 cells in lung and cutaneous allergic diseases that maintain the Th2 process [69]. Furthermore, CCL13 is known to be involved in the migration of eosinophils, monocytes, T cells, and immature dendritic cells, and can cause eosinophil degranulation and basophil histamine release [66]. CCL18 is highly expressed in human lung tissues, and levels are elevated in asthma and COPD [70–72]. This M2 chemokine has broad chemotactic activity towards immune and inflammatory cell types and is associated with pro-fibrotic activities [70,73]. The absence of an effect of roflumilast on the production of M2a chemokines suggests that PDE4 inhibition does not amplify the harmful effects of M2a chemokines in asthma and COPD. Therefore, the beneficial clinical effect of roflumilast and other PDE4 inhibitors in asthma and COPD are probably related to (i) anti-inflammatory effects on other cell types [39,74–76], (ii) the enhancement of β_2-agonist-induced relaxation of human isolated bronchial preparations, and (iii) β_2-agonist-induced anti-inflammatory effects on isolated human bronchial preparations and human lung parenchyma [77–79].

One of the strengths of our study is the used of human LMs. However, our LM preparations are obtained from patients undergoing surgical

resection for lung carcinoma; about 10 % of the LMs were IMs, the functions of which remain largely unknown. These patients were current smokers or ex-heavy smokers, and some were suffering from (non-severe) COPD. In a previous study of AMs obtained by bronchoalveolar lavage, the results of transcriptional profiling with M1- and M2-related gene probe sets suggested that smoking and the development of COPD were associated with the progressive deactivation of M1-gene expression program and the progressive induction of a particular M2-related gene expression program [80]. Nevertheless, it has been reported that the expression levels of three M2 markers used in the present study (CCL17, CCL18, and CCL22) were similar in AMs from smokers, COPD patients, and healthy non-smoking subjects [80]. However, we cannot rule out an alteration of the cAMP-regulatory pathway involved in production of M2 chemokines by smoking and/or COPD.

5. Conclusion

In experiments with human LMs, exposure to NECA and PGE_2 was associated with greater basal and IL-4/IL-13-induced production of M2a chemokines. Roflumilast was devoid of effects, whether alone or combined with NECA, PGE_2, and IL-13.

CRediT authorship contribution statement

Marion Brollo: Formal analysis, Methodology, Resources, Validation, Writing - review & editing. **Hélène Salvator:** Conceptualization, Writing – review & editing. **Stanislas Grassin-Delyle:** Conceptualization, Writing – review & editing. **Mathieu Glorion:** Resources, Writing – review & editing. **Delphyne Descamps:** Conceptualization, Writing-review & editing. **Amparo Buenestado:** Formal analysis, Methodology, Resources. **Emmanuel Naline:** Methodology, Resources. **Hermann Tenor:** Resources, Writing – review & editing. **Angelica Tiotiu:** Conceptualization, Writing - review & Editing. **Philippe Devillier:** Conceptualisation, Formal analysis, Validation, Writing - review & editing.

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.

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