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All-trans retinoic acid up-regulates Prostaglandin-E Synthase expression in human macrophages

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ABSTRACT

All-trans retinoic acid (ATRA) is a potent retinoid, which has been used successfully in different clinical settings as a potential drug to treat COPD and emphysema. In the present study, we analyzed genes modulated by ATRA by performing mRNA expression array analysis on alveolar macrophages after treatment with ATRA. Here we observed a 375-fold up-regulation of Prostaglandin-E Synthase (microsomal PGES-1, NM_004878 PTGES) which mediates the conversion of prostaglandin H_2 (PGH_2) to Prostaglandin E_2 (PGE_2). We furthermore studied the expression of PTGES after treatment with ATRA in human monocyte-derived macrophages (MDMs) and bronchoalveolar lavage (BAL) cells.

ATRA up-regulated PTGES mRNA expression in MDMs generated with M-CSF by 2500-fold whereas in M-CSF+IL-13 macrophages the up-regulation was only 20-fold. Similarly, ATRA up-regulated PTGES mRNA expression by factor 1524 in BAL cells. The up-regulation of PTGES mRNA expression by ATRA is both time and dose dependent. IL-13 suppressed the ATRA induced PTGES expression at both mRNA and protein level in MDM and BAL cells. We also observed that LPS acts synergistically with ATRA in MDMs and strongly induces PTGES expression. ATRA had little impact on cyclooxygenase-1 and -2 (COX-1 and -2) expression as compared to PTGES expression under the same experimental conditions. Furthermore, we observed an induction of PGE_2 levels by ATRA in BAL cells.

These data indicate that ATRA is a potent inducer of PTGES expression in human macrophages but not in alternatively activated macrophages and suggest that the eicosanoid pathway is important for ATRA action in macrophages.

Introduction

Vitamin A is an essential vitamin involved in various processes in the body, including embryonic development, vision, cell differentiation, and gene expression (Evans and Kaye 1999). Vitamin A acts via the RAR and RXR nuclear receptors, which after ligand binding can bind to specific motifs in the promoters of various genes leading to either transactivation or repression (Nagpal and Chandraratna 1998). Retinoic acid derivates are already in clinical use for different diseases, such as acute leukemia, for which ATRA is used as a differentiating agent (Petrie et al. 2009) or as a potential drug to treat COPD and emphysema (Mao et al. 2002; Roth et al. 2006). Along these lines ATRA has been demonstrated to promote alveolarisation in elastase-induced emphysema in the rat model (Massaro and Massaro 1997). Furthermore it has been used as liposomal formulation for inhalation in a compassionate treatment of a male patient with severe emphysema, where it could be demonstrated that neutrophil elastase in sputum and desmosine in urine decreased over time (Frankenberger et al. 2009). ATRA has been shown furthermore to suppress many activities of cells of the monocyte lineage (Weber et al. 1995;

Abbreviations: ATRA, all-trans retinoic acid; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; COX-1/-2, cyclooxygenase-1/-2; IL-13, interleukin-13; LPS, lipopolysaccharide; M-CSF, macrophage colony stimulation factor; MDM, monocyte-derived macrophage; MMP-9, matrix metallo-proteinase-9; PBMC, peripheral blood mononuclear cells; PGE_2, Prostaglandin E_2; PTGES, Prostaglandin-E Synthase; RAR, retinoic acid receptor; RXR, retinoic X receptor; TIMP-1, tissue inhibitor of matrix-metallo proteinase-1.

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Zitnik et al. 1994; Gross et al. 1993; Kreutz et al. 1998). In alveolar macrophages, it was shown that ATRA selectively down-regulates MMP-9 and up-regulates TIMP-1 expression on the transcriptional level (Frankenberger et al. 2001). In the present study, where we have studied the effect of Vitamin A in the form of the all-trans retinoic acid (ATRA) derivate, we analyzed genes modulated by ATRA by performing an mRNA expression array on BAL cells after treatment with ATRA. Herein we report on a strong induction of the Prostaglandin-E Synthase (PTGES) gene. PTGES is a key enzyme involved in the eicosanoid pathway, which mediates the conversion of Prostaglandin H_2 (PGH_2) to Prostaglandin E_2 (PGE_2). Based on the induction of PGE_2 in macrophages by ATRA we propose that much of the action of ATRA is via induction of this eicosanoid.

Methods

Patients and bronchoalveolar lavage

BAL samples were obtained from patients (3 patients with sarcoidosis, 1 patient with fibrosis, 2 patients with chronic cough, 1 patient with dyspnea, 1 patient under investigation for interstitial lung disease, 1 patient with NSIP – nonspecific interstitial pneumonia) who underwent the lavage procedure for routine diagnostic purpose and only leftover material was used in the study. After informed consent, lavage was performed during fiberoptic bronchoscopy by instilling 160 ml of 0.9% saline solution in 20 ml aliquots into the lingual or right middle lobe and withdrawing the fluid immediately. Ethics approval was obtained from the local Ethics committee. Total cells were determined, and centrifuge smears were prepared for cytological analysis. Differential cell counts of 400 cells were made by Giemsa-May-Grünwald staining (Diff-Quick, #130832, Dade Behring, Suisse) and percentage of macrophages, lymphocytes, neutrophils and eosinophils were calculated. The percentage of alveolar macrophages in the samples used for the study was above 75%.

Cells and culture conditions

Cells were cultured in RPMI 1640 (Biochrom, #F1415, Berlin, Germany), L-glutamine 2 mM (Gibco, #25030-024), Penicillin 200 U/ml, Streptomycin 200 μg/ml (Gibco, #15140-114), nonessential amino acids 1–2× (Gibco, #11140-35), and OPI-supplement (contains oxalacetic acid, sodium pyruvate, and insulin) 10 ml for 1 L (Sigma, #O-5003). To avoid inadvertent LPS contamination, culture medium was filtered through a Gambro Ultrafilter U 2000 (Martinsried, Germany) to remove LPS. 10% FCS (#S0115, Biochrom, Berlin, Germany) was added after filtration. BAL cells (0.5×10^6) in 1 ml medium were seeded in 24-well Costar low attachment plates (Costar #3473, Bodenheim, Germany) that were pre-treated according to the manufacturer's instructions to prevent cell adhesion and differentiation. To these cultures, free ATRA, liposomal ATRA, both at concentrations of 5×10^{-6} M, or empty liposomes were added, and cells were cultured for 3 days. Cells were harvested and cell counts were assessed using trypan blue exclusion. For RNA isolation, 2×10^4 cells were lysed in 200 μl TRI reagent (#T-9424, Sigma, Taufkirchen, Germany).

Monocyte-derived macrophages

Human $CD14^{++}$ monocytes were isolated from heparinised blood from healthy volunteers using MACS magnetic separation technique (all antibodies from Miltenyi Biotec, Bergisch Gladbach, Germany). PBMC were first isolated by density gradient centrifugation and depleted of CD16 positive cells. For this, a total of 15×10^6 cells were resuspended in 60 μl of PBS containing 25 μl of anti-CD16 microbeads (#130045701). Cells were incubated for 30 min at 4 °C washed and resuspended in 1.5 ml PBS. Cell suspension was then loaded onto a LD column (#120-000-497) that was positioned in a MidiMACS magnet (#130-042-302). Nonlabelled cells were recovered and used for enrichment of $CD14^{++}$ cells. For this anti-CD14 microbeads (#130-050-201) were diluted 1:5 in PBS and added to the cells to a final volume of 100 μl. After incubation for 30 min at 4 °C cells were washed and resuspended in 1.5 ml PBS and loaded onto a LS column (#120-000-475) that is placed in a MidiMACS magnet. The column was washed five times with 2 ml PBS each. $CD14^{++}$ cells were recovered from the column by flushing the column five times with 2 ml PBS using a plunger. The purity of isolation was determined by staining the $CD14^{++}$ cells with FITC-labelled anti-CD14 antibody (My4-FITC, #6603511, Coulter, Krefeld, Germany), PE-labelled anti-CD16 antibody (Leu11c-PE, #332779, Becton-Dickinson, Heidelberg, Germany) and PC5-labelled anti-CD45 antibody (#A07785, Coulter, Krefeld, Germany or the corresponding isotype control and measured by flow cytometry. $CD14^{++}$ monocytes with a purity of 90–95% or above were used for further experiments.

Cells were resuspended in RPMI 1640-supplemented medium (mentioned above) and cultured at a final concentration of 1×10^6 cells/ml in 24-well Costar low attachment plates (Costar #3473, Bodenheim, Germany). The cell suspension was supplemented with M-CSF 100 ng/ml (M-CSF, lot no. Ex3-001, was kindly provided by Genetics Institute, Cambridge Massachusetts, USA) or IL-13 50 ng/ml (generously provided by IDM, Paris, France) or LPS 100 ng/ml (#L-6261, Sigma, Taufkirchen, Germany). The cells were incubated at 37 °C in the presence of 5% CO_2 for 5 days and subjected to further analysis.

Preparation of liposomes

Liposomes were prepared as previously described (Frankenberger et al. 2001). In brief, Di-oleyl-phosphoserine (OOPS) and palmitoyl-oleyl-phosphocholine (POPC) were dissolved at an OOPS/POPC ratio of 0.43 in chloroform. All-trans retinoic acid (Tretinoin, #700121-0005 Fagron, Barsbüttel, Germany) in 96% ethanol was added at a concentration of 5×10^{-4} M. Organic solvent was evaporated with rotation in a round-bottomed glass flask. LPS free PBS (1 ml) was added to the dry lipid layer, and formation of multilamellar liposomes was enhanced by the addition of glass beads to recover the lipid layer. The liposomes were washed five times by centrifugation for 5 min at $21{,}000 \times g$ to remove non-incorporated ATRA. This preparation was then reduced to ≤0.4 μm in diameter by a three-time passage through polycarbonate filters (Nucleopore 110407, Costar, Bodenheim, Germany) in an extruder device (B002, Lipex Biomembranes, Vancouver, Canada). The resultant unilamellar liposomes were sterile filtered through 0.2 μm and stored up to 4 weeks at 4 °C.

Total RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from cells by using TRI reagent (#T-9424, Sigma, Taufkirchen, Germany) according to the manufacturer's instruction. The MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin et al. 2009) were followed as far as applicable. In brief cells were lysed in 200 μl TRI reagent and 15 μg of transfer RNA from brewer's yeast (#109517, Roche Diagnostics, Mannheim, Germany) or 10 μg of linear acrylamide (#AM9520, Ambion, Germany) as carrier were added per sample. After isolation, RNA was reverse transcribed with oligo(dT) as primer. Quantitative PCR was performed using the LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Gene-specific primers for amplification for real-time

monitoring of PCR reactions were designed with the online program http://frodo.wi.mit.edu/primer3/. Rozen and Skaletsky (2000). Amplified products were run out on a 2% agarose gelelectrophoresis and bands were observed at the expected molecular weight (data not shown). The following primers were used:

Gene	Primer (5′ → 3′)	Product length (bp)
Prostaglandin E synthase (microsomal PGES-1)	F–GCGTATCACCATACCCAGCTA R–CACACCTGAGCCAGAGAGAAG	370
Angiopoietin like-4	F–GCAAGATGACCTCAGATGGAG R–GAAGTACTGGCCGTTGAGGTT	446
CD300 molecule like family member b	F–AGCAAGGATGGGAGACCTACA R–GCAGTGACCAAGATGAGCAAG	388
Heparin-binding EGF like growth factor	F–AGTCTTGCCTAGGCGATTTTGT R–CACCTCCAACCTTCTCGGTAG	385
Pro-platelet basic protein (Chemokine (C-X-C motif) ligand 7)	F–GCAGCAACTCACCCTCACTCA R–GGCAGATTTTCCTCCCATCCT	383
Hydroxysteroid (11-beta) dehydrogenase 1	F–TGGCTTATCATCTGGCGAAGA R–GCCAGAGAGGAGACGACAAC	367
COX-1	F–GTTGGAGCGACTGTGAGTAC R–CGCCAGTGAATCCTGTTGTTACTA	290
COX-2	F–GCTTTTTACCTTTGACACCC R–CTGCTCAACACCGGAATTTT	310
α-Enolase	F–GTTAGCAAGAAACTGAACGTCACA R–TGAAGGACTTGTACAGGTCAG	619

cDNA (3 μl) was used for amplification in the SYBR Green format using the LightCycler-FastStart DNA Master SYBR Green I kit (#2239264, Roche, Mannheim, Germany) or using 5× SensiMix Lite kit (#06-QT405-99, peqLab, Erlangen, Germany). The amplification program started with denaturation at 95 °C for 10 min, followed by 40 cycles of amplification: denaturation for 10 s at 95 °C, annealing for 10 s at 56 °C, and final elongation for 25 s at 72 °C. Finally, the amplification products were cooled down to 40 °C for 30 s. For quantitative PCR, the LightCycler system offers the advantage of speed and real-time measurement of fluorescent signals during amplification. The SYBR Green dye binds specifically to the minor groove of double-stranded DNA. Fluorescence intensity is measured after each amplification cycle. During PCR, a doubling of template molecule occurs in each cycle only during the log-linear phase. Melting curves were analyzed to make sure that the amplified product was specific and devoid of primer dimers. As an internal control, the housekeeping gene, α-Enolase was amplified and used for normalization of the data.

Microarray analysis

For gene expression analysis, GeneChip Human Genome U133 Plus 2.0 array (Affymetrix) was used according to manufactures' instructions. BAL cells from five independent donors were cultivated either in the presence of liposomal ATRA or left untreated for 3 days. Total RNA was isolated from liposomal ATRA or untreated BAL cells and pooled at 0.4 μg each to give a final amount of 2 μg. This pooled RNA was used for microarray analysis using the Affymetrix array. The complete microarray data are deposited in the GEO data base with the accession number GSE23704.

Western blot

For Western blot analysis total cell lysates were prepared with RIPA lysis buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0). After 4 days incubation with and without ATRA, BAL cells or MDMs were harvested and pellet was lysed in 4 volumes of RIPA lysis buffer (R-0278, Sigma) in the presence of protease inhibitor mixture containing of 10 μg/ml aprotinin (#A-6279, Sigma), 1 mM PMSF (#P-7626, Sigma), 40 μg/ml leupeptin-propinoyl (#L-3402, Sigma), 20 μg/ml leupeptin-acetate (#L-2023, Sigma), 20 μg/ml antipain (#A-6191, Sigma), 20 μg/ml pepstatin A (#P-4265, Sigma), 400 μM ALLN (#A-6185, Sigma) and 2 mM DTT (#19474, Merck).

Protein concentrations were determined using the Bradford reagent (#500-0006, Biorad). Cell proteins (10 μg) were resolved on a 4–12% Novex bis-tris Gel (#NP0329BOX, Invitrogen) and transferred to Nitrocellulose membrane (#LC2001, Novex, Invitrogen) using the Novel X-Cell II Mini Cell. Membranes were blocked with blocking buffer (TBS supplemented with 5% fat-free milk powder (#1.15363, Merck) and 0.1% Tween 20 (#2323003, Wasserfuhr) and then membranes were incubated with 1:200 dilution of anti-PTGES antibody (mouse anti-human, #10004350, Cayman, USA) over night. As a loading control, membranes were stripped in buffer containing 100 mM 2-Mercaptoethanol, 2%SDS, 62.5 mM Tris–HCl pH 6.7 and incubated with anti-actin antibody (#A-2066, Sigma) for 1 h. As a secondary antibody a peroxidase-conjugated anti-mouse IgG (#A-4416, Sigma) and anti-rabbit IgG (#A0545, Sigma) was used. Bound antibody was detected using the ECL kit (#RPN2106, Amersham) and membranes were then exposed to Hyperfilm™ ECL (#RPN3103, Amersham).

Detection of PGE_2

To determine the PGE_2 in culture supernatants, 1×10^6 BAL cells were incubated for 18–20 h in the presence of ATRA (5×10^{-6} M) alone or in combination of ATRA+IL-13 (50 ng/ml) or left untreated. Cell free supernatants were harvested by centrifuging BAL cultures at 21,000 × *g* at 4 °C for 10 min. PGE_2 was determined using commercially available PGE_2 ELISA kit (#KGE004, R&D Systems, USA) with regular sensitivity assay according to the protocol of the manufacturer. Sensitivity of the assay was 10 ng/ml.

Statistics

For statistical analysis of the data, Student's *t*-test was used. Results were considered significant when $p \leq 0.05$.

Results

Gene expression profiling of ATRA and validation of microarray analysis

In this study, the effect of ATRA on gene expression was analyzed by a microarray-based system using Affymetrix GeneChip Human Genome U133 Plus 2.0 array on a pool of BAL cells obtained from five donors. The cells were treated either with liposomal ATRA or they were left untreated. For array analysis we focused on genes with an expression level of "1000" fluorescence intensity and with a ratio of fluorescence intensity of treated versus control signals of at least 10. Here we observed 4 genes up-regulated 10-fold and 2 genes down-regulated 10-fold in the ATRA treated cells compared to the control cells. Validation of the microarray data using quantitative LightCycler RT-PCR confirmed the expression patterns of four up-regulated genes (PTGES, ANGPTL4, CD300LB, and HBEGF) and two down-regulated genes (PPBP and HSD11B1) were consistent with the data obtained from microarray analysis (Table 1). Only the gene CCL2, which gave a 53-fold induction in the array, was not confirmed showing induction in one but no induction in three additional samples (data not shown).

Effect of ATRA on PTGES mRNA expression in BAL cells

We then selected the most strongly induced PTGES gene for more detailed studies. BAL cells were incubated with 5 μM ATRA or left untreated for 3 days. PTGES mRNA expression was quantitatively analyzed by LightCycler RT-PCR allowing real-time monitoring of cDNA amplification. The expression of PTGES mRNA

Table 1
List of strongly induced or reduced genes in response to L-ATRA in BAL cells.

Gene description	Accession ID	Gene symbol	Fold up-regulated	Fold down-regulated	Fold change in LC-PCR ($n = 3$)
Prostaglandin E synthase	NM_004878	PTGES	373.1		1524
Angiopoietin-like 4	NM_016109	ANGPTL4	127.6		33.71
CD300 molecule-like family member b	BC028091	CD300LB	17.1		12.76
Heparin-binding EGF-like growth factor	NM_001945	HBEGF	10		6.43
Pro-platelet basic protein (chemokine (C-X-C) ligand 7)	R64130	PPBP		14.8	50.1
Hydroxysteroid (11-beta) dehydrogenase 1	NM_005525	HSD11B1		12.4	10.2

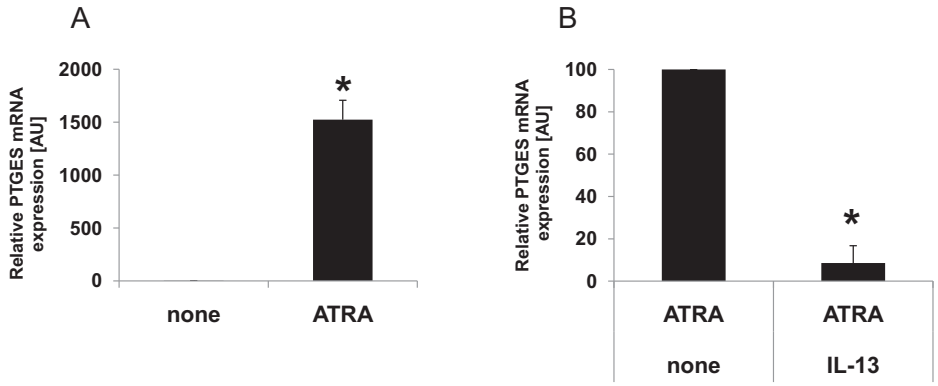

Fig. 1. ATRA induction and IL-13 suppression of PTGES expression in bronchoalveolar lavage cells. (A) ATRA induces PTGES mRNA expression in BAL cells. BAL cells (5×10^5 cells) were treated with ATRA at 5×10^{-6} M or left untreated for 3 days. PTGES mRNA was analyzed by LightCycler RT-PCR. Baseline is untreated cells (none) and was set as 1 ($n = 3$; mean ± SD; $^*p < 0.05$). (B) IL-13 down-regulates PTGES mRNA expression in BAL cells. BAL cells were treated with ATRA at 5×10^{-6} M in the presence of M-CSF (100 ng/ml) or M-CSF (100 ng/ml) + IL-13 (50 ng/ml) for 3 days. PTGES mRNA expression in M-CSF treated cells was set as 100% ($n = 3$; 100% = 50.9, 1370, 1652-fold; mean ± SD; $^*p < 0.05$).

was induced by 1524-fold ± 183 in BAL cells treated with ATRA relative to untreated cells (Fig. 1A).

Next we analyzed the effect of IL-13 on ATRA induced PTGES mRNA expression in alveolar macrophages. BAL cells were treated with 5×10^{-6} M ATRA in the presence of M-CSF or M-CSF+IL-13 for 3 days. IL-13 prevents ATRA induced PTGES mRNA expression from M-CSF alone (100%) by more than factor 10 after treatment with M-CSF+IL-13 (8.9% ± 7.8%, Fig. 1B).

Expression analysis of PTGES mRNA in monocyte-derived macrophages

Since *in vitro* generated monocyte-derived macrophages are readily available cells and therefore can be used for more detailed studies, we next analyzed the effect of ATRA on PTGES mRNA expression in monocyte-derived macrophages. For this, we isolated $CD14^{++}$ monocytes and cultured them for 5 days either in the presence of M-CSF alone or with combined M-CSF+IL-13. ATRA at 5 μM was added at day 5 to these differentiated macrophages and the cells were cultured for further 3 days. RT-PCR analysis demonstrates that ATRA up-regulates PTGES mRNA expression by 2489 ± 558-fold in macrophages cultured in the presence of M-CSF. These data show that for the study of ATRA action the *in vitro* generated MDM can be used as a model for alveolar macrophages. When monocyte-derived macrophages were cultured with M-CSF+IL-13 the up-regulation was only 19 ± 8-fold (Fig. 2). This demonstrates that IL-13 has a suppressive effect on ATRA induced PTGES mRNA expression in monocyte-derived macrophages similar to what has been observed in alveolar macrophages.

Time course and dose–response analysis of PTGES mRNA expression in monocyte-derived macrophages

We next analyzed the time dependence of ATRA induced PTGES mRNA expression in monocyte-derived macrophages. ATRA treatment for 24 h of MDM cultured with M-CSF showed a moderate induction (58-fold ± 28) in PTGES transcript levels, but there was a more pronounced after 48 and 72 h (350-fold ± 190, and 576-fold ± 305, respectively) (Fig. 3A). Hence, maximum induction of PTGES mRNA requires 72 h of culture with ATRA. Dose–response analysis of ATRA in monocyte-derived macrophages cultured with M-CSF showed that the standard dose of 5×10^{-6} M was most effective (576-fold ± 305) in PTGES mRNA induction. The lower doses of 5×10^{-7} M and 5×10^{-8} M also showed significant induction of PTGES mRNA expression (102-fold ± 37, and 44-fold ± 22, respectively) (Fig. 3B).

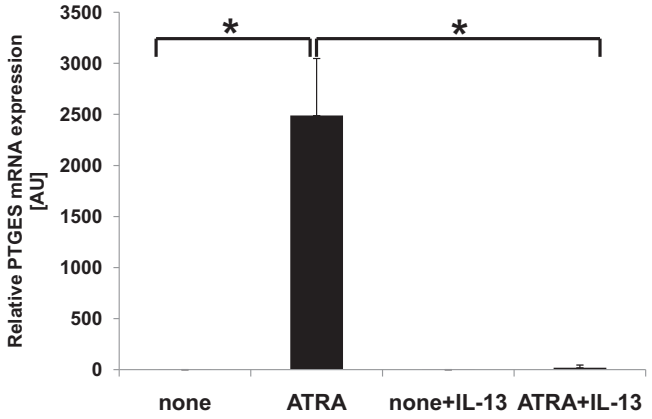

Fig. 2. Expression of PTGES in monocyte-derived macrophages. $CD14^{++}$ monocytes were isolated from peripheral blood and cultured in the presence of M-CSF (100 ng/ml) or M-CSF (100 ng/ml) + IL-13 (50 ng/ml) for 5 days and followed by ATRA at 5×10^{-6} M for 3 days. PTGES mRNA was analyzed by LightCycler RT-PCR. Baseline is set as one in untreated cells ($n = 3$; mean ± SD; $^*p < 0.05$).

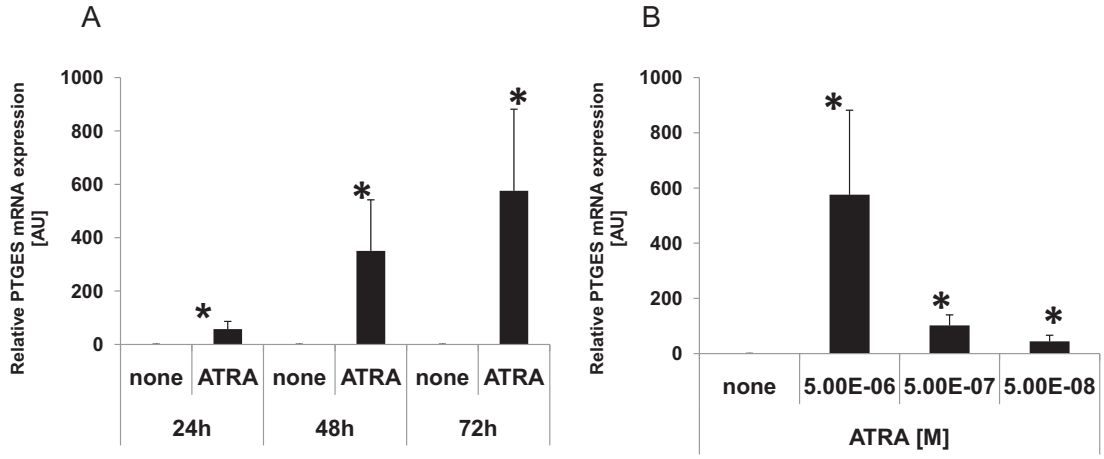

Fig. 3. Time course and dose response analysis of ATRA induced PTGES expression in human monocyte-derived macrophages. (A) Kinetics of PTGES mRNA induction by ATRA in human monocyte-derived macrophages. $CD14^{++}$ monocytes were isolated from peripheral blood and cultured in the presence of M-CSF (100 ng/ml) for 5 days and followed by ATRA at 5×10^{-6} M for 24 h, 48 h and 72 h. PTGES mRNA expression was induced gradually from 24 h to 72 h ($n = 3$; mean ± SD; $^*p < 0.05$). (B) ATRA induces PTGES mRNA in a dose-dependent manner. $CD14^{++}$ monocytes were cultured in the presence of M-CSF (100 ng/ml) for 5 days and followed by ATRA at 5×10^{-6} M, 5×10^{-7} M and 5×10^{-8} M for 3 days. Baseline was set as 1 in untreated cells (none) ($n = 3$; mean ± SD; $^*p < 0.05$).

Time course and dose–response analysis of PTGES mRNA expression in IL-13 treated monocyte-derived macrophages

In contrast to monocyte-derived macrophages cultured with M-CSF, IL-13 treated MDM showed no significant up-regulation of PTGES mRNA expression after treatment with ATRA with a low level of induction of 5-fold ± 4 (24 h), 6-fold ± 4 (48 h) and 7-fold ± 4 (72 h) (Fig. 4A). Dose–response analysis of ATRA in monocyte-derived macrophages cultured with M-CSF+IL-13 also showed no significant up-regulation of PTGES mRNA expression at the standard dose of 5×10^{-6} M (7-fold ± 4) and observed also low induction at 5×10^{-7} M and 5×10^{-8} M (4-fold ± 2 and 3 ± 2, respectively) (Fig. 4B).

COX-1 and COX-2 mRNA expression in BAL cells after treatment with ATRA

Cyclooxygenase (COX)-1/-2 are up-stream modulators of PTGES in the eicosanoid pathway. We therefore analyzed whether ATRA has effect on COX-1 and -2 expressions in BAL cells. LightCycler RT-PCR analysis of COX-1 and -2, in same ATRA-treated BAL samples used for PTGES analysis, showed very low induction by only 3-fold ± 0.6 and 5-fold ± 1.1, respectively (Fig. 5A and B) in comparison to 1524-fold ± 183 PTGES induction (Fig. 1A).

Western blot analysis of PTGES protein in BAL cells

Next, we analyzed the effect of ATRA on PTGES protein expression in BAL cells. After 4 days of incubation, total cell lysates were analyzed for PTGES protein by Western blot analysis. A strongly elevated level of PTGES protein was detected in ATRA treated cells compared with untreated cells, while PTGES protein was down-regulated in cells incubated with ATRA+IL-13 (Fig. 6A). This pattern of response to ATRA is in accordance with the results obtained at the mRNA levels.

Effect of ATRA and LPS exposure on PTGES protein expression in monocyte-derived macrophages

ATRA-induced PTGES expression was confirmed at the protein level in monocyte-derived macrophages. Western blot analysis of total cell lysates of ATRA treated macrophages is shown in Fig. 6B. PTGES protein was detected at very low amounts in cells treated with ATRA only and its expression was enhanced after concomitant

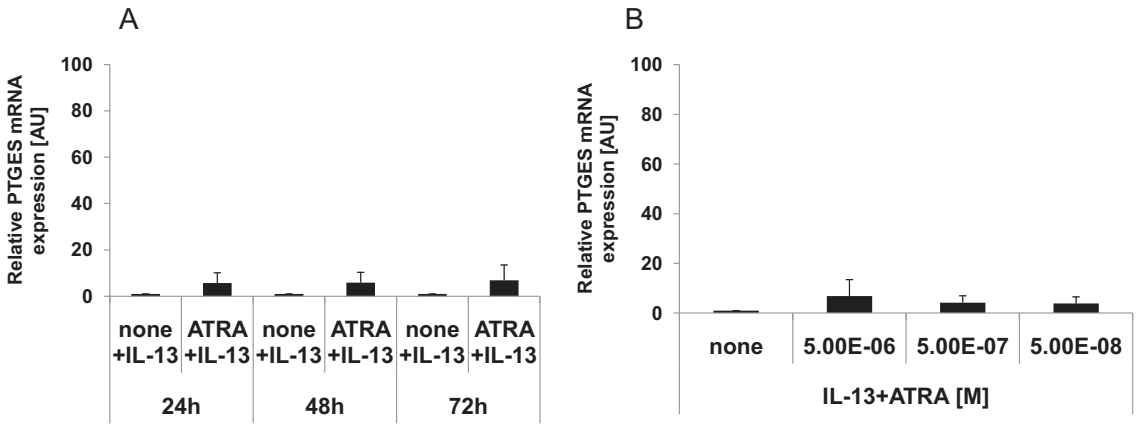

Fig. 4. Time course and dose–response analysis of ATRA induced PTGES expression in IL-13 treated human monocyte-derived macrophages. Kinetics of PTGES mRNA induction by ATRA in IL-13 treated monocyte-derived macrophages. $CD14^{++}$ monocytes were isolated from peripheral blood and cultured in the presence of M-CSF (100 ng/ml) + IL-13 (50 ng/ml) for 5 days and followed by ATRA at 5×10^{-6} M for 24 h, 48 h and 72 h (A) and ATRA at 5×10^{-6} M, 5×10^{-7} M and 5×10^{-8} M for 3 days (B). PTGES mRNA expression was analyzed by LightCycler RT-PCR. Baseline is untreated cells (none) and was set as 1 ($n = 3$; mean ± SD). Differences are not significant.

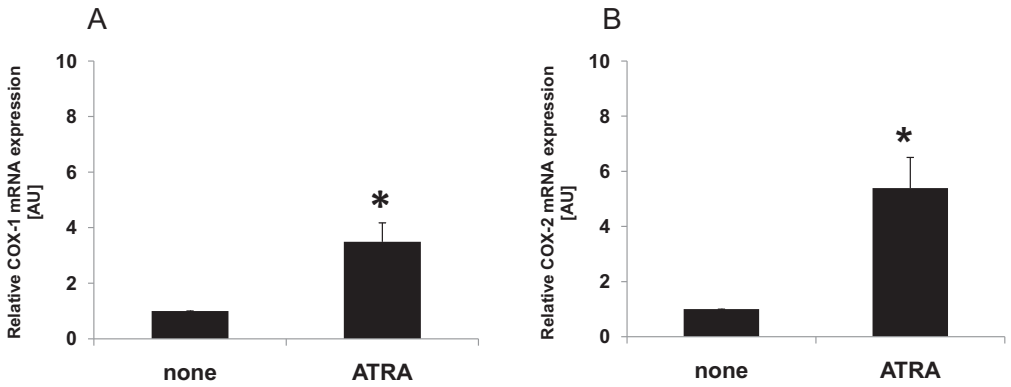

Fig. 5. Effect of ATRA on COX-1 and COX-2 mRNA expression in BAL cells. BAL cells were treated with ATRA at 5×10^{-6} M or left untreated for 3 days. COX-1 (A) and COX-2 (B) mRNA was analyzed by LightCycler RT-PCR. Baseline is untreated cells (none) and was set as 1 ($n = 3$, mean ± SD; $^*p < 0.05$).

exposure to LPS. This indicates the synergistic effect of both stimuli. However, this synergistic effect was suppressed in the presence of IL-13, which is consistent with the results obtained for PTGES mRNA that was measured in the same samples.

Induction of Prostaglandin E_2 by ATRA

PTGES mediates the conversion of prostaglandin H_2 (PGH_2) to Prostaglandin E_2 (PGE_2). To demonstrate the physiological significance of PTGES induction by ATRA, PGE_2 levels were measured in the supernatants of BAL cells. We observed a 14-fold induction (from 70.6 ± 50 pg/ml in controls up to 1026 ± 855 pg/ml after ATRA treatment) of PGE_2 levels in the supernatants of BAL cells incubated with ATRA, whereas, IL-13 suppressed PGE_2 production alone or in combination with ATRA (Fig. 7). These results are in line with the induction of PTGES at the mRNA and protein level and they demonstrate a potent pathway for a therapeutic action of ATRA.

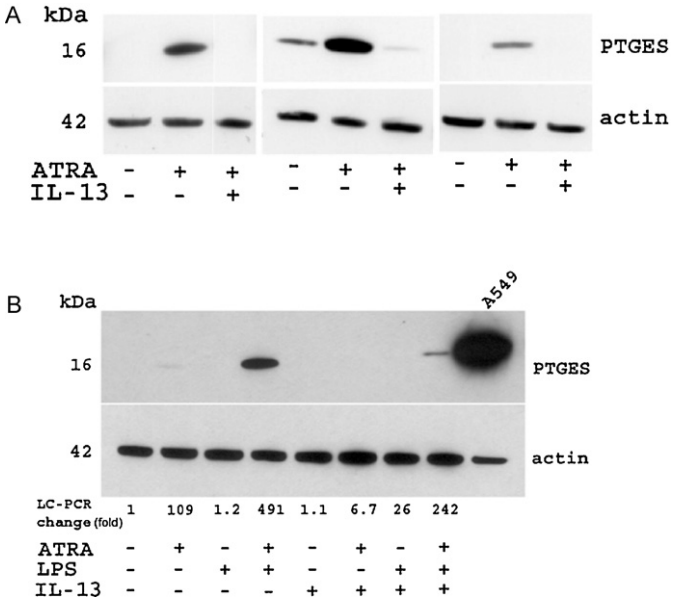

Fig. 6. Western blot analysis of PTGES protein expression. (A) BAL cells. BAL cells (1×10^6 cells/ml) were cultured in the presence of M-CSF (100 ng/ml) and treated with ATRA (5×10^{-6} M) alone or in combination with IL-13 (50 ng/ml) or left untreated for 4 days. Total cell lysates were analyzed by Western blotting. Shown are data from three independent experiments. (B) Monocyte-derived macrophages. $CD14^{++}$ monocytes were isolated from peripheral blood and cultured in the presence of M-CSF (100 ng/ml) for 5 days. After 5 days, cells were left untreated or treated either by ATRA (5×10^{-6} M) or IL-13 (50 ng/ml) or ATRA+IL-13 for 4 days. LPS (100 ng/ml) was added 24 h before harvesting in indicated combinations. Total cell lysates were analyzed by Western blotting. PTGES mRNA expression in same samples was analyzed by LightCycler RT-PCR. Fold change values were indicated below Western blot and baseline is untreated cells (none) and was set as 1. In lane 9 the human alveolar epithelial cell line A549 was used as reference. Shown is one representative of two independent experiments.

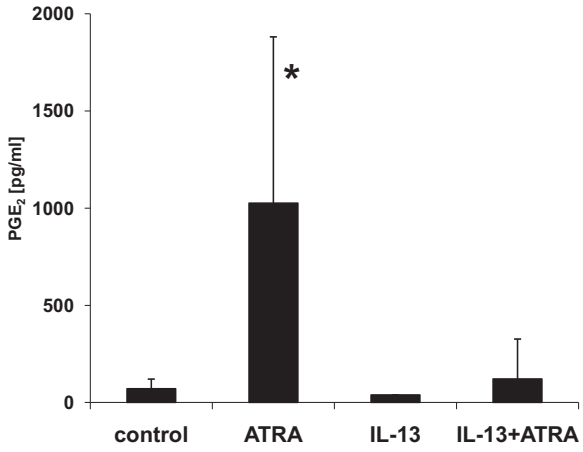

Fig. 7. Induction of Prostaglandin E_2 by ATRA. BAL cells were cultured overnight at a concentration of 1×10^6 cells/1 ml/well on low attachment plates in the presence of M-CSF (100 ng/ml) and either ATRA (5×10^{-6} M), IL-13 (50 ng/ml) or both ATRA+IL-13. After 24 h cell free supernatants were harvested and PGE_2 protein was determined by ELISA. PGE_2 concentrations are given in pg/ml (sensitivity of the assay: 10 ng/ml). $n = 6$; mean ± SD; $^*p = 0.02$ in control versus +ATRA.

Discussion

ATRA, all-trans retinoic acid, is the active form of vitamin A that plays an essential role for the growth and development of cells and tissue (Biesalski and Nohr 2003). There is evidence in the mouse that retinoic acid is required during alveologenesis, because the levels of the retinoid binding proteins, the RA receptors and two RA synthesizing enzymes peak postnatally. Furthermore retinoic acid is required throughout life for the maintenance of lung alveoli because rats that lack dietary retinol lose alveoli and show features of emphysema (Maden and Hind 2004). In another animal model, the elastase-induced emphysema in rat, intraperitoneally administration of ATRA resulted in the rebuilding of alveolar septae (Massaro and Massaro 1997), an effect which to date could not be reproduced in the human system.

Treatment of primary human monocytes with ATRA, as described by Liu et al. (2005), led to the down-regulation of TLR2 as well as its coreceptor CD14, but not TLR1 or TLR4. The ability of a TLR2/1 ligand to trigger monocyte cytokine release was inhibited by pre- and cotreatment with ATRA; however, TLR4 activation was

affected by cotreatment only. ATRA also down-regulated monocyte cytokine induction by *Propionibacterium acnes*. These data indicated that ATRA exerts an anti-inflammatory effect on monocytes via two pathways, one specifically affecting TLR2/1 and CD14 expression and one independent of TLR expression.

It is already known from previously studies that ATRA selectively down-regulates MMP-9 and up-regulates TIMP-1 expression in human alveolar macrophages (Frankenberger et al. 2001; Mao et al. 2003). Since a pilot study with inhalative liposomal ATRA in a compassionate use (Frankenberger et al. 2009) showed only moderate effects on the sputum and plasma levels of MMP-9 and TIMP-1, we performed an Affymetrix expression array using macrophages isolated from bronchoalveolar lavage (BAL) treated without and with liposomal ATRA for 3 days in order to investigate different markers that are modulated by ATRA application and may be suitable for monitoring inhalative ATRA application in future clinical studies.

The selection of genes up- and down-regulated by ATRA (Table 1) was based on this array analysis from BAL cells treated with ATRA versus untreated cells. The expression patterns of four more than ten-fold up-regulated genes (PTGES, ANGPTL4, CD300LB, and HBEGF) and two more than ten-fold down-regulated genes (PPBP and HSD11B1) re-analyzed with LightCycler RT-PCR were consistent with the data obtained from microarray analysis. The highest induction at the mRNA level was found for the enzyme Prostaglandin-E Synthase (PTGES). PTGES is a key enzyme involved in eicosanoid pathway, which mediates the conversion of prostaglandin H_2 (PGH_2) to Prostaglandin E_2 (PGE_2). Prostaglandins are biologically active lipid mediators that are synthesized by the sequential action of phospholipase A2, cyclooxygenase (COX) and Prostaglandin synthase (PTGES) enzymes. The production of prostaglandins begins with the release of arachidonic acid from the membrane phospholipids by phospholipase A2 in response to inflammatory stimuli. Arachidonic acid is then cleaved to PGH_2 by COX-1 and COX-2 and further converted by cell-specific prostaglandin synthases into different prostaglandins, including PGE_2 (Harris et al. 2002). Several forms of PTGES including microsomal PGES-1 (PTGES), microsomal PTGES2 (PTGES2) and cytosolic PTGES (PTGES3) have been characterized and are coupled with cyclooxygenases (COX) (Gillio-Meina et al. 2009). Increased expression of PGE_2 mediated by the ATRA-induced production of PTGES may also be effective against bronchoconstriction in the lower airways as described by Hartney et al. (2006) in a mouse model, similar to Sheller et al. (2000) who described that the bronchodilative effect of PGE_2 is dependent on signaling through the EP_2 receptor. In a human study with control donors and mild atopic asthmatics Gauvreau et al. (1999) demonstrated that inhaled PGE_2 attenuates allergen-induced airway responses, hyperresponsiveness and inflammation, when given immediately before the inhaled allergen.

Array analysis of ATRA cultured monocytes and macrophages were also performed by other groups. By Luesink et al. (2009) it was described in the acute promyelocytic leukemia cell line NB4 that stimulation with ATRA resulted in an upregulation of multiple CC-chemokines and their receptors as detected by Exon GeneChip analysis. Barber et al. (2008) performed a CD antibody microarray using HL60 and NB4 cells. ATRA thereby induced major immunophenotypic changes of several CD markers depending on the cell line. Lee et al. (2002) have compared the gene expression profiles in NB4 and HL-60 cells with and without ATRA treatment using a cDNA microarray containing 10,000 human genes. The cDNA microarray identified 119 genes that were up-regulated and 17 genes that were down-regulated in NB4 cells, while 35 genes were up-regulated and 36 genes were down-regulated in HL60 cells. But there was no induction of PTGES by ATRA treatment reported in both studies.

Since ATRA plays an important role in lung tissue development and maintenance and PGE_2 plays an important role in inflammatory processes, we further investigated the effect of ATRA on the expression of PTGES mRNA level in bronchoalveolar lavage cells treated with ATRA alone versus cells cultured with M-CSF and M-CSF+IL-13 (Fig. 1). Addition of M-CSF does not affect the expression of PTGES in BAL cells (data not shown). We could demonstrate a strong increase of PTGES with ATRA alone and an adverse effect in alternatively activated macrophages with M-CSF+IL-13. This is in accordance with the findings of Mosca et al. (2007) who could induce PTGES mRNA expression only in classical activated monocytes with LPS but not in alternatively activated macrophages with IL-4 or IL-13. Also by Martinez et al. (2006) it has been described that IL-4 alternatively activated macrophages (M2) show differentially expressed genes, including PTGES, compared to M1 macrophages that have been generated by IFNγ and LPS.

To work with a more reproducible cell model we isolated $CD14^{++}$ monocytes from peripheral blood obtained from healthy volunteers and differentiated them for 5 days with either M-CSF alone or M-CSF+IL-13 to monocyte-derived macrophages (MDM) followed by ATRA culture for an additional 3 days. Here we could confirm the up-regulation of PTGES mRNA in the classical MDM and the suppression of this effect in the alternatively activated MDMs (Fig. 2). Data show that MDM behave similarly to BAL cells in response to ATRA.Induction of PTGES with ATRA has been reported also by other groups. Recently, Kim et al. (2008) described that retinoic acid in the presence of LPS or thrombin enhances Prostaglandin E_2 production through increased expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 in rat brain microglia. It was shown that RA has a dual effect on cyclooxygenase-2 (COX-2) expression in inflammatory activated microglia, the resident brain macrophages. Treatment of microglia with LPS or thrombin induced COX-2 expression. ATRA enhanced PGE_2 production but had little effect on 15-deoxy-PGJ_2. Moreover, ATRA selectively up-regulated the expression of a PGE_2 synthase, mPGES-1, but had little effect on the PGD_2 synthase, H-PGDS. The results collectively suggest that ATRA modulates microglial responses to inflammatory stimulators, particularly at the late phase, via enhancement of COX-2 expression and PGE_2 production. While these studies demonstrate an induction of PGE_2 by ATRA the mechanism involves COX-2 and not PTGES.

Time course analysis performed with classical monocyte-derived macrophages treated with ATRA revealed a gradually increase of PTGES mRNA tested over 72 h (Fig. 3A). However, maximum induction of PTGES mRNA required 72 h of culture with ATRA. We therefore have chosen 3 days pre-culture with ATRA for all further experiments and did for practical reasons not study longer periods of time. The most efficient dose of ATRA was 5 μM at day 3 as determined in the dose-dependent analysis shown in Fig. 3B. These dose and time points were in line with data obtained in earlier experiments (Frankenberger et al. 2001).

Contrary to monocyte-derived macrophages cultured with M-CSF alone, we could not detect a significant up-regulation of PTGES mRNA in IL-13 plus ATRA treated monocyte-derived macrophages (Fig. 4A). Dose–response analysis of ATRA in monocyte-derived macrophages cultured with M-CSF+IL-13 also showed no significant up-regulation of PTGES mRNA expression (Fig. 4B). PGE_2 is produced by the PGE_2 synthase (PTGES) from PGH_2, which is the product of the upstream enzymes COX 1/2 in the eicosanoid pathway. We therefore determined the effect of ATRA on the expression of these two up-stream transcripts. We could demonstrate only weak induction of COX-1 (Fig. 5A) and COX-2 (Fig. 5B) mRNA after 3 days culture with ATRA in BAL cells, assuming that the up-regulation of PTGES by ATRA is not mediated via an increased cyclooxygenase expression. Next, we asked whether the effect of ATRA on PTGES mRNA is also reflected on the protein level. Expression of PTGES

protein was measured in BAL cells after 4 days of incubation to obtain maximal concentration of PTGES protein. We detected elevated levels of PTGES protein in ATRA treated cells compared with untreated cells. Consistently with transcript data, PTGES protein was down-regulated in cells incubated with ATRA+IL-13 (Fig. 6A).

In order to investigate the effect of ATRA on monocyte-derived macrophages we cultured 5 days old macrophages for an additional 3 days with combinations of ATRA, IL-13 and LPS. Since ATRA treatment resulted in only a weak induction of PTGES protein, we stimulated the cells with an additional small amount of LPS, that alone had no effect, but in combination with ATRA strongly induced PTGES protein (Fig. 6B). The synergistic effect of ATRA plus LPS could be suppressed in the presence of IL-13 which is in line with the results obtained by RT-PCR of the same donor. However, the amount of PTGES protein detectable in monocyte-derived macrophages was low, when compared with the PTGES protein detectable in the A549 cell line, which was used as a reference cell line (Thoren and Jakobsson 2000). Comparing alveolar macrophages and MDM we detected a similarly high induction of PTGES mRNA in both cell types.

Since PTGES mediates the conversion of prostaglandin H_2 (PGH_2) to Prostaglandin E_2 (PGE_2) we measured the end product PGE_2 in cell culture supernatants of BAL cells harvested after 3 days culture with ATRA/IL-13. Results are consistent with PTGES expression data (Fig. 7). We observed induction of PGE_2 levels in the supernatants of BAL cells incubated with ATRA, whereas, IL-13 suppressed PGE_2 production alone or in combination with ATRA. Since ATRA showed very low induction of COX-1 and -2 expression, it is apparent that PTGES induction is responsible for the increase of PGE_2 in these cells.

In conclusion, our data indicate that ATRA is a potent inducer of PTGES expression in human classical macrophages but not in alternatively activated macrophages. The extent of up-regulation of PTGES mRNA expression by ATRA is both time and dose dependent and resulted also in production of PGE_2. The data suggest that the eicosanoid pathway is important for ATRA action in macrophages. Since PGE_2 exhibits anti-inflammatory effects as the inhibition of inflammatory chemokines CCL3 and CCL4, as reported by Jing et al. (2003), the administration of ATRA may be of benefit in the treatment of inflammatory lung diseases. Furthermore PTGES up-regulation may function as a marker in clinical monitoring of ATRA application and has to be evaluated in further clinical settings.

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