

Prostaglandin E₂ differentially modulates IL-5 gene expression in activated human T lymphocytes depending on the costimulatory signal

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Background: Protein kinase A (PKA) activation is documented to be inhibitory for T helper cell (T_{H1})-like cytokines (IL-2, IFN- γ), whereas T_{H2}-like cytokines (IL-4, IL-5) are not affected or upregulated. We have recently shown that IL-4 gene expression can be inhibited by PKA activation but depends on the mode of T-cell activation. For IL-5 gene expression, we hypothesized that the mode of T-cell activation also determines the ultimate effect of simultaneous PKA activation.

Objectives: The objective of this study was the examination of IL-5 gene expression in healthy T cells activated with various mitogenic stimuli after simultaneous activation of PKA by dibutyryl-cAMP or prostaglandin E₂ (PGE₂).

Methods: IL-5 mRNA was measured by semiquantitative reverse transcription-polymerase chain reaction or Northern analysis. IL-5 protein was measured by ELISA. Transcriptional mechanisms involved in IL-5 gene expression were determined by nuclear run-on experiments and electrophoretic mobility shift assays. Posttranscriptional mechanisms were determined by actinomycin D chase studies.

Results: Anti-CD2-, anti-CD3-, and anti-CD3 plus anti-CD28-induced IL-5 mRNA were completely inhibited by dibutyryl cyclic AMP (10⁻³ mol/L) and PGE₂ (10⁻⁵ mol/L), whereas concanavalin A-induced IL-5 mRNA was partially reduced. The effect of PGE₂ was accomplished at the transcriptional level, probably as the result of inhibition of DNA binding of nuclear factor- κ B. Anti-CD3 plus anti-CD28-induced IL-5 protein (504 $\pm$ 56 pg/ml) was significantly reduced by PGE₂ (122 $\pm$ 42 pg/ml, p < 0.001). Addition of cytokines that use the IL-2 receptor γ_c chain (IL-2, IL-7, IL-15) abrogated the PGE₂-induced inhibition of IL-5 protein. In contrast, concanavalin A plus PMA-induced IL-5 protein (75 $\pm$ 8 pg/ml) was significantly upregulated by the simultaneous addition of PGE₂ (128 $\pm$ 17 pg/ml, p < 0.03). **Conclusions:** PKA activation differentially modulates IL-5 gene expression and depends on the mode of T-cell activation. (J Allergy Clin Immunol 1998;101:231-40.)

Key words: T cells, IL-5, prostaglandin E₂, protein kinase A, transcription factors

IL-5, mainly produced by activated T lymphocytes, is a highly specific cytokine affecting the proliferation, differentiation, and activation of eosinophils.¹⁻³ Atopic disorders (e.g., allergic asthma and allergic dermatitis) are accompanied by enhanced IL-5 production and consequently eosinophilia, which is reflected by elevated numbers of eosinophils in peripheral blood and accumulation of eosinophils in airway wall tissue.⁴⁻⁶ Although these biologic responses evoked by IL-5 have been well documented, little is known about the control of IL-5 gene expression in human T lymphocytes because IL-5 gene regulation has predominantly been studied in the murine system.⁷⁻⁹ In the murine thymoma cell line EL-4, it has been demonstrated that IL-5 gene expression is induced by the phorbol ester phorbol myristate acetate (PMA), which could synergistically be upregulated by dibutyryl cyclic AMP (db-cAMP), a direct activator of protein kinase A (PKA).^{7,8}

In human peripheral blood T lymphocytes, IL-5 gene expression could be induced by PMA in combination with the calcium ionophore A23187¹⁰ or by PMA in combination with CD3 or CD28 monoclonal antibodies (mAbs).¹¹ Moreover, it has been demonstrated in activated human T_{H2} clones that prostaglandin E₂, which increases intracellular cAMP levels through receptor-mediated activation of adenylyl cyclase, upregulates IL-5 protein secretion.¹² These data suggest that the PKA-dependent signaling pathway is of importance in the regulation of the expression of the T_{H2}-like cytokine IL-5. Because no consensus sequence of the cAMP-responsive element-binding protein is present in the promoter of the IL-5 gene, intracellular cAMP elevation may affect transcription factors such as nuclear factor- κ B (NF- κ B), activator protein 1 (AP-1), CD28-responsive complex (CD28RC), or nuclear factor of activated T cells (NFAT). Potential binding sites for these transcription factors are observed in the murine and/or human IL-5 promoter.^{8, 13-17}

Recently it was shown that the ultimate effect of PKA activation on cytokine gene expression critically depends on coactivation signals, as was demonstrated for T_{H1}-like and T_{H2}-like cytokines.^{18, 19} In this study we hypothe-

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Abbreviations used

AP-1:	Activator protein 1
CD28RC:	CD28-responsive complex
Con A:	Concanavalin A
db-cAMP:	Dibutyl cyclic AMP
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
mAbs:	Monoclonal antibody
NFAT:	Nuclear factor of activated T cells
NF- κ B:	Nuclear factor kappa B
PBS:	Phosphate-buffered saline
PGE ₂ :	Prostaglandin E ₂
PKA:	Protein kinase A
PMA:	Phorbol myristate acetate
RT-PCR:	Reverse transcription-polymerase chain reaction
SDS:	Sodium dodecylsulfate
T _{H1} , T _{H2} , etc.:	T helper cells

esized that the ultimate effect of PKA activation on the expression of IL-5 is also dependent on the mode of T-cell activation. Therefore freshly isolated human T lymphocytes were stimulated with several mitogenic activators in the presence of db-cAMP or prostaglandin E₂ (PGE₂). The results demonstrate a downregulation of IL-5 mRNA in anti-CD2-, anti-CD3-, concanavalin A (Con A)-, and anti-CD3 plus anti-CD28-stimulated T lymphocytes in response to PKA activation. The PGE₂-induced downregulation of IL-5 gene expression observed in anti-CD3 plus anti-CD28-stimulated or Con A-stimulated T lymphocytes resulted from a reduced transcription rate of the IL-5 gene and coincided with a reduced DNA binding of NF- κ B. In contrast, IL-5 gene expression in T lymphocytes stimulated with a combination of Con A plus PMA was not suppressed by db-cAMP and was upregulated significantly by PGE₂. The IL-5 mRNA data were also reflected in the IL-5 protein secretion data. Taken together, our data provide evidence that the ultimate effect of cAMP elevation on IL-5 gene expression is highly dependent on costimulatory signals.

METHODS**Preparation of cells**

Peripheral blood cells were obtained from healthy volunteer platelet donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density-gradient centrifugation. T lymphocytes were isolated by 2-aminoethylisothiuronium bromide-treated sheep red blood cell rosetting. The sheep red blood cells were lysed with 155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L EDTA; the remaining cell preparations contained more than 98% T lymphocytes as assessed by flow cytometric analysis after staining with an anti-CD2 mAb (Becton Dickinson, Mountain View, Calif.) and less than 1% CD14-positive cells (Becton Dickinson). After isolation, T lymphocytes were cultured overnight at 37° C in RPMI 1640 media (Flow, Rockville, Md.) containing 4% fetal bovine serum (Hyclone, Logan, Utah)

supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mmol/L l-glutamine, and 6 ng/ml colistine.

Stimulation

Cells (5×10^6 /ml) were incubated for 6 hours in RPMI 1640 medium with 25 μ g/ml Con A (Calbiochem, La Jolla, Calif.), anti-CD3 and anti-CD28 antibodies in a 5% hybridoma supernatant solution (gift from Dr. Bart Jan Kroesen, Department of Immunology, University of Groningen, The Netherlands), or with 25 μ g/ml Con A plus 50 ng/mL PMA (Sigma, St. Louis, Mo.) in the presence and absence of dibutyl cyclic-AMP (Boehringer, Mannheim, Germany) or prostaglandin E₂ (Sigma). Antibodies against CD2 (T11₂ and T11₃; gift from Dr. E. Reinherz, Dana Faber Cancer Center; Boston, Mass.) were used at a final dilution of 1:250. To determine the stability of mRNA, new RNA synthesis was inhibited by Actinomycin D; (Boehringer), used at a concentration of 10 μ g/ml. The exogenously added cytokines rhIL-2 (Eurocetus, Amsterdam, The Netherlands), rhIL-7 (Genzyme, Cambridge, Mass.), and rhIL-15 (Dr. T. Troutt, Immunex, Seattle, Wash.) were used at an optimal concentration of 50 U/ml, 10 ng/ml, and 20 ng/ml, respectively.

Extraction of mRNA and Northern analysis

Total cellular RNA was isolated by the guanidinium isothiocyanate/cesium chloride method²⁰ or by the single-step method as described by Chomczynski and Sacchi.²¹ Total RNA (20 μ g) was electrophoresed in 2.2 mol/L formaldehyde, 1.1% agarose gels, and blotted onto nylon membranes (Qiabrane; Qiagen, Chatsworth, Calif.).²² cDNA probes were labeled with [α -³²P] dCTP (3,000 Ci/mol; Amersham) by the random hexamer priming method.²³ The following cDNA probes were used: (1) the 2.3 kb *Pst*I insert of human IL-5 cDNA purified from the pMT2-T plasmid (gift from Dr. P. Temple, Genetics Institute, Cambridge, Mass.); (2) the 0.45 kb *Eco*RI/*Ava*II insert of human granulocyte-macrophage colony-stimulating factor (GM-CSF) cDNA (gift from Dr. S. Gillis, Immunex, Seattle, Wash.); and (3) the *Eco*RI linearized pBR322 plasmid containing a 7.8 kb human 28S cDNA insert.

Hybridization was performed at 65° C for 18 hours in 0.5 mol/L Na₂HPO₄ (pH 7.2), 1 mmol/L EDTA, and 7% sodium dodecyl sulphate (SDS). Membranes were washed once in $2 \times$ sodium saline citrate (SSC), 0.1% SDS; once in $1 \times$ SSC, 0.1% SDS; and finally in $0.3 \times$ SSC, 0.1% SDS for 20 minutes at 65° C. The membranes were exposed to Kodak X-Omat XAR films (Eastman Kodak, Rochester, N.Y.) at -80° C with the use of an intensifying screen. Quantification of mRNA levels was performed by densitometry with a Gel Scan laser densitometer (Pharmacia LKB, Uppsala, Sweden).

Reverse transcription and polymerase chain reaction

Reverse transcription. Total cellular RNA isolated from 5×10^6 T lymphocytes was resuspended in 15 L diethyl-pyrocyanate-treated (Sigma) H₂O and incubated at 68° C for 10 minutes. After cooling on ice, the samples were spun briefly in a microcentrifuge. Next, 10 L of $10 \times$ RT mix (135 mmol/L Tris-HCl, pH 8.3, 204 mmol/L KCl, 27 mmol/L MgCl₂, 0.24 mg/ml bovine serum albumin, 5.4 mmol/L of each dNTP, 14.3% glycerol) and 3 units of reverse transcriptase (Promega, Madison, Wis.) was added to the samples. The samples were spun briefly and incubated at 37° C for 60 minutes.

Polymerase chain reaction. The following specific primer pairs for β -2-microglobulin (β 2 μ G), IL-5, and GM-CSF were

obtained from Eurogentec (Seraing, Belgium). $\beta 2\mu$ G: 5'CCA GCAGAGAATGGAAAGTC3' (sense) and 5'GATGCTGCT TACATGTCTCG3' (antisense); IL-5: 5'CTTGGCACTGCT TTCTACTC3' (sense) and 5'GCAGGTAGTCTAGGAATT GG3' (antisense). GM-CSF: 5'AGCATGTGAATGCCAT CCAG3'(sense) and 5'ATAGTCTGGGTTGCACAGGA3' (antisense).

One microliter of 100 μ mol/L sense and antisense of $\beta 2\mu$ G was added to each RT-reaction sample, and diethyl-pyrocabonate-treated H_2O was added to a final volume of 100 μ L. Next, the samples were heated for 3 minutes at 94° C, spun briefly in a microcentrifuge, and placed on ice. *Taq* DNA polymerase 0.5 μ L of 5 U/ μ L (Promega) was added to each sample. The tubes were placed in a thermal cycler (Perkin-Elmer, Foster City, Calif.). PCR was performed for 20 cycles for $\beta 2\mu$ G and 30 cycles for IL-5: 1 minute of denaturation at 94° C, 0.5 minute of annealing at 55° C, and 0.5 minute of extension at 72° C. With these primers, fragments spanning 259 bp, 290 bp, and 268 bp were amplified for IL-5, GM-CSF, and $\beta 2\mu$ G, respectively. After 20 PCR cycles with primers for $\beta 2\mu$ G, 10 μ L of the reaction mixture was run on a 2% agarose gel containing 0.2 μ g/ml ethidium bromide in 1 $\times$ TAE buffer. To be sure that equal amounts of cDNA were used in each PCR reaction, bands were quantified with respect to $\beta 2\mu$ G. Equal amounts of RNA then were amplified with primers for IL-5, GM-CSF, and $\beta 2\mu$ G. A 100 bp ladder (Pharmacia) was used as DNA marker. Quantification of the PCR products was performed with the gelscan program Diversity One (Pharmacia).

Radioactive PCR was performed as described above, except for a last amplification cycle in the presence of (α^{32} P)-dCTP (3000 Ci/mol; Amersham). The labeled PCR products were run on a 2% agarose gel and Southern blotted onto nylon membranes (Qiabran, Qiagen) and quantified by a PhosphorImaging system and the ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

Nuclear run-on transcription assay

T lymphocytes (10^8) were pelleted at 500 g for 5 minutes, washed twice with ice-cold phosphate-buffered saline (PBS), and suspended in 4 ml of lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 3 mmol/L $MgCl_2$, 10 mmol/L NaCl, 0.5% NP-40). After gentle vortexing, the suspension was incubated on ice for 5 minutes. Nuclei were pelleted at 500 g for 5 minutes and the lysis described above was repeated. The nuclei were resuspended in 100 μ L glycerol-buffer (50 mmol/L Tris-HCl, pH 8.0, 40% glycerol, 5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA) and incubated at 26° C for 20 minutes with 80 μ L transcription-buffer (12.5 mmol/L Tris-HCl, pH 8.0, 6.0 mmol/L $MgCl_2$, 125 mmol/L KCl, 2 mmol/L dithiothreitol; and 1 mmol/L each of ATP, CTP, and GTP) and 12.5 μ L (α^{32} P)UTP (3,000 Ci/mol; Amersham). Transcription was terminated by the addition of 40 U DNase I, 150 U RNasin, 40 μ g yeast tRNA, and 200 μ L stop-buffer (10 mmol/L Tris-HCl, pH 7.4, 0.5 mol/L NaCl, 50 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$), and the solution was incubated at 37° C for 20 minutes. After proteinase K (750 U/ml) digestion in 1% SDS, nuclear RNA was isolated by phenol-chloroform extraction and 2 $\times$ ethanol precipitated in 2.5 mol/L ammonium acetate. RNA was further purified by Sephadex G-50 column separation (Boehringer).

Five micrograms of the following single-stranded DNAs were immobilized on Hybond N⁺ membranes: (1) *Eco*RI linearized pGEM (negative control); (2) *Eco*RI linearized pMT2-T plasmid containing a 2.3 kb human IL-5 cDNA fragment; (3) *Eco*RI linearized plasmid containing a 0.45 kb human GM-CSF cDNA

fragment; and (4) *Eco*RI linearized pBR322 plasmid containing a 7.8 kb human 28S cDNA fragment. Hybridization with equal amounts of labeled RNA was performed as described above. Washing was performed as described above, with one extra wash step with 2 $\times$ SSC/1 μ g/ml RNase A (Boehringer). Transcription rates were normalized with respect to the 28S signal.

Electrophoretic mobility shift assay

T lymphocytes (120×10^6) were stimulated for 3 hours and nuclear extracts were prepared by a modification of Park et al.²⁴ In short, cells were harvested by centrifugation at 500 g for 5 minutes, washed once with PBS, and resuspended to 12.5×10^8 cells/ml in buffer A (10 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 0.1 mol/L EDTA, 0.1 mmol/L EGTA, and 1 mmol/L DTT)²⁴ supplemented with protease inhibitors (5 μ g/ml leupeptin (Sigma), 5 μ g aprotinin (Sigma), 1 mmol/L phenylmethylsulfonyl fluoride (Sigma). After a 10-minute incubation on ice, cells were lysed by addition of Nonidet P-40 (NP-40; Boehringer Mannheim) to a final concentration of 0.05% and incubated for another 7 minutes on ice. T-cell lysates were centrifuged at 500 g for 5 minutes at 4° C. The nuclear pellets were resuspended in 45 μ L buffer C, a high salt buffer (20 mmol/L HEPES [pH 7.9], 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT)²⁵ supplemented with protease inhibitors as described above. The suspension was incubated on ice for 30 minutes with regular shaking to extract the nuclear proteins and finally spun down in a microcentrifuge for 5 minutes. The supernatants containing the extracted nuclear proteins were divided into small aliquots and stored at -80° C. The protein concentration of the nuclear extracts was determined by the Bradford assay.²⁶

Double-stranded synthetic oligonucleotide probes containing the NF- κ B AP-1 and the NFAT binding sequences used in the gel retardation assay were as follows: NF- κ B: 5'-AACAAA GAGGGATTTCACCTACAT-3'; AP-1: 5'-AAATTCCAAAG AGTCATCAGA3'; and NFAT: 5'GGAGGAAAACTGTTT CATAcagaAGGCGT3').

Fifty nanograms of high-performance liquid chromatography-purified single-stranded oligonucleotide was labeled with T4-polynucleotide kinase and (γ^{32} P) ATP (3,000 Ci/mmol, Amersham), separated from nonincorporated radiolabel by Sephadex G50 chromatography, ethanol precipitated, dried, and dissolved in 20 μ L of 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, and 1 mmol/L DTT, containing a fourfold excess of the opposite strand. Annealing of the two strands was performed by heating the mixture for 2 minutes at 90° C and slow cooling to room temperature.

Twelve micrograms of nuclear extract and 0.1 ng double-stranded labeled oligonucleotide were incubated in 20 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 0.06 mmol/L EDTA, 0.6 mmol/L DTT, 2 mmol/L spermidine, 10% glycerol, supplemented with 2 μ g poly (dI-dC). The binding reaction was performed at 26° C for 20 minutes. The samples were loaded on prerun (30 minutes, 100 V) 4% polyacrylamide gels and run for 1.5 hours at 130 V in 0.5 $\times$ TBE at room temperature. Gels were dried and quantification of the binding signals was performed with a Phosphor Imaging system and ImageQuant software (Molecular Dynamics).

Measurement of IL-5 protein

T lymphocytes (3×10^6 /ml) were stimulated with Con A, anti-CD3/anti-CD28, and Con A plus PMA in the presence or

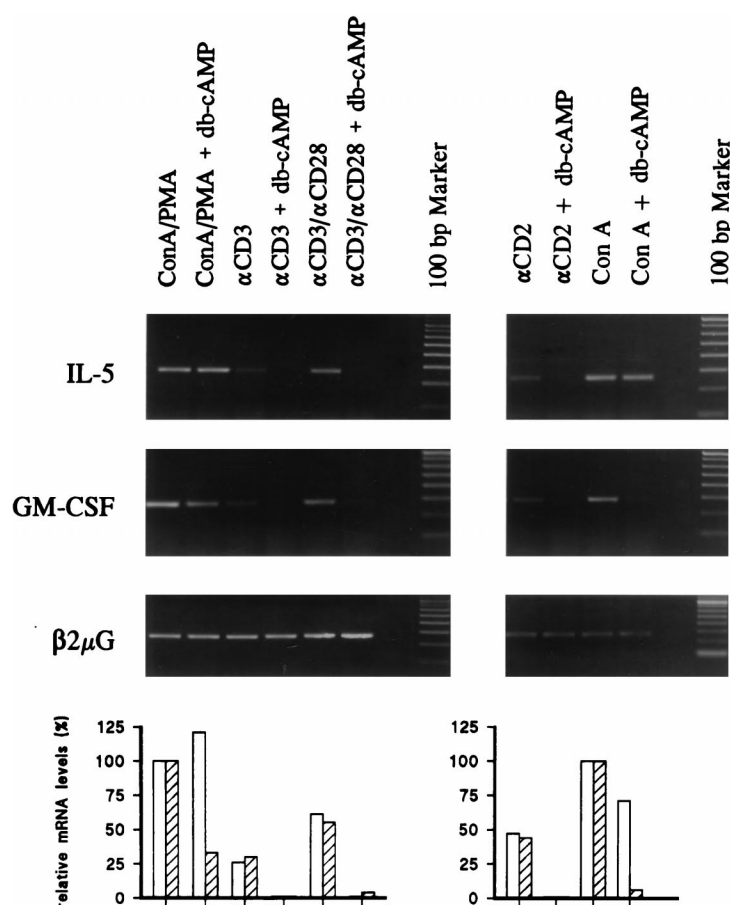


FIG. 1. Ethidium bromide-stained agarose gels demonstrating amplified IL-5, GM-CSF, and $\beta 2\mu$ G products with the expected size. RT-PCR analysis of total cellular RNA extracted from T lymphocytes activated for 5 hours by different T-cell activators. In left and right panels, $\beta 2\mu$ G mRNA signal shows comparable amounts of amplified product in each lane. *Left panel*, RT-PCR-amplified products of RNA isolated from T lymphocytes stimulated with Con A plus PMA, anti-CD3, and anti-CD3 plus anti-CD28 in the absence and presence of 10^{-3} mol/L db-cAMP. *Right panel*, RT-PCR-amplified products of RNA isolated from T lymphocytes stimulated with anti-CD2 and Con A in the absence and presence of 10^{-3} mol/L db-cAMP. Corresponding bar diagrams present relative IL-5 (open bars) and GM-CSF (hatched bars) mRNA levels determined after normalization with respect to the $\beta 2\mu$ G mRNA levels. Experiment shown is representative for three independent experiments.

absence of PGE_2 and/or IL-2, IL-7, and IL-15 for 24 hours. Viability of cells was controlled by trypan blue exclusion. Secreted IL-5 protein levels were quantified in cell-free supernatants by sandwich ELISA and was performed as described by Hoekstra et al.²⁷ The following anti-mouse/human IL-5 mAbs were used: purified rat anti-human IL-5 mAb IgG1 (Pharmingen, San Diego, Calif.) as coating mAbs and biotinylated rat anti-human IL-5 mAbs IgG2a (Pharmingen) as detection mAbs.

Statistical analysis

For the protein measurements, statistical analysis was performed with the Student's *t* test for paired observations. Statistical significance of the secretion data was set at a *p* value less than 0.05.

RESULTS

db-cAMP and PGE_2 downregulate IL-5 mRNA expression depending on costimulatory signals

To study whether the activation pathway is of importance in the ultimate effect of the cAMP-dependent

signaling pathway on IL-5 mRNA expression, T lymphocytes were activated during 5 hours with Con A, anti-CD2, anti-CD3, and anti-CD3 plus anti-CD28 in the absence and presence of db-cAMP (10^{-3} mol/L). As shown in Fig. 1, db-cAMP completely inhibited the IL-5 mRNA expression in anti-CD2-, anti-CD3-, and anti-CD3 plus anti-CD28-stimulated T lymphocytes, whereas IL-5 mRNA accumulation was partially repressed in Con A-activated cells. In contrast, IL-5 mRNA expression was not inhibited by db-cAMP (10^{-3} mol/L) in T lymphocytes costimulated with Con A plus PMA. The observed pattern was specific for IL-5, because GM-CSF mRNA was downregulated by db-cAMP as described before.²⁸

To study the physiologic relevance of the observed inhibitory effect of db-cAMP on IL-5 mRNA expression, anti-CD3 plus anti-CD28-stimulated T lymphocytes were coincubated with different concentrations of PGE_2 ,

an immunomodulator that elevates intracellular cAMP levels by receptor-mediated activation of adenyl cyclase.^{29,30} Fig. 2 demonstrates that anti-CD3 plus anti-CD28-induced IL-5 mRNA was completely inhibited by 10^{-6} mol/L PGE₂, whereas 10^{-8} mol/L PGE₂ was still able to repress IL-5 mRNA expression by approximately 50%.

As shown in Fig. 3, PGE₂ (10^{-5} mol/L) was able to enhance IL-5 mRNA accumulation in Con A plus PMA-activated T lymphocytes without affecting the kinetics of IL-5 mRNA accumulation. The upregulatory effect of PGE₂ was specific for IL-5 gene expression because rehybridization of the membrane with a GM-CSF cDNA probe demonstrated a partial inhibition induced by PGE₂ on Con A plus PMA-induced GM-CSF mRNA expression as described before.²⁸

PGE₂ downregulates IL-5 mRNA expression by transcriptional mechanisms

To analyze whether the downregulatory effect of PGE₂ on IL-5 mRNA expression is accomplished at transcriptional level, nuclear run-on experiments were performed with T lymphocytes stimulated for 3 hours. As shown in Fig. 4, IL-5 gene transcription was not detectable in unstimulated T lymphocytes. IL-5 gene transcription was induced by incubation of the T lymphocytes with a combination of anti-CD3 plus anti-CD28 and by Con A. Costimulation with PGE₂ (10^{-5} mol/L) resulted in a complete abrogation of IL-5 gene transcription in anti-CD3 plus anti-CD28-stimulated T lymphocytes and a slightly diminished IL-5 gene transcription in Con A-activated cells. In accordance with previous observations that GM-CSF gene transcription is inhibited by db-cAMP,²⁸ an inhibitory effect of PGE₂ on GM-CSF transcription was also observed in both anti-CD3 plus anti-CD28-stimulated cells and Con A-stimulated cells.

To investigate whether PGE₂ modulates IL-5 mRNA stability, T lymphocytes were stimulated with a combination of anti-CD3 plus anti-CD28 in the absence and presence of 10^{-7} mol/L PGE₂. To abrogate mRNA synthesis, actinomycin D was added to the cultures after 6 hours of stimulation, and total cellular RNA was extracted after 0, 30, 60, and 90 minutes. As shown in Fig. 5, anti-CD3 plus anti-CD28 induced-IL-5 mRNA decayed with a half-life of approximately 35 minutes. Although PGE₂ diminished the IL-5 mRNA expression, the stability of IL-5 mRNA transcripts was not affected.

PGE₂ inhibits NF- κ B DNA-binding, leaving AP-1 and NFAT binding unaffected

Because PGE₂ downregulated IL-5 gene transcription, we studied DNA binding capacities of the transcription factors NF- κ B, AP-1, and NFAT. Potential binding sites of these transcription factors have been observed in the promoter and/or enhancer of the IL-5 gene.^{8,13,14} T lymphocytes were stimulated with medium (control) or during 3 hours with anti-CD3 plus anti-

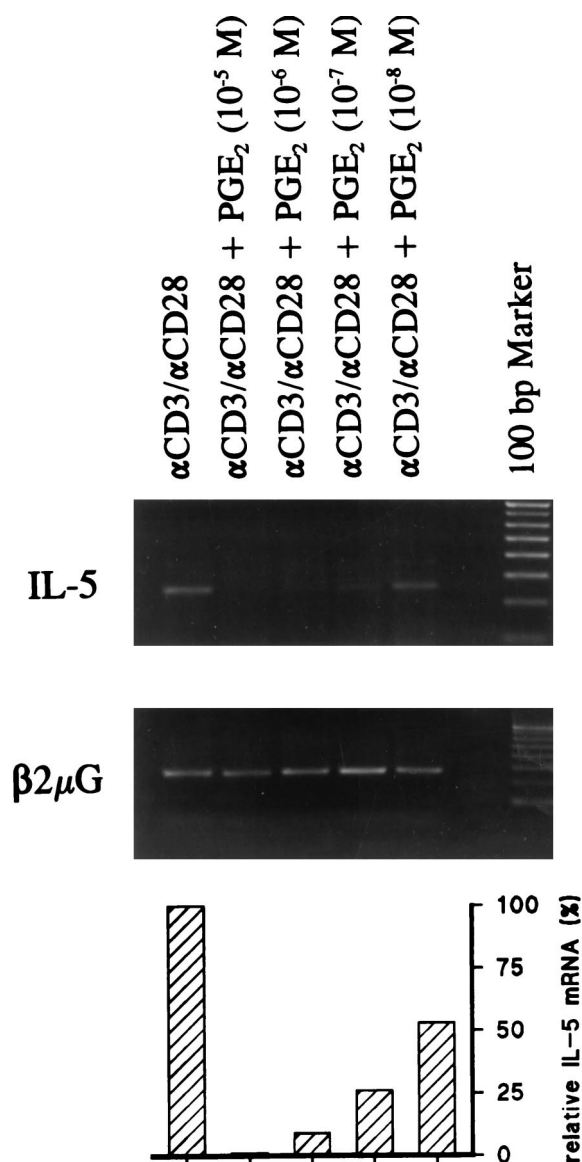


FIG. 2. Dose-response curve of the effect of PGE₂ on IL-5 mRNA accumulation. RT-PCR analysis of total cellular RNA extracted from T lymphocytes activated for 5 hours with antibodies against CD3 and CD28 (α CD3/ α CD28) in the presence of different concentrations of PGE₂ (as indicated at the top of the figure). Bar diagram represents relative IL-5 mRNA levels after normalization with respect to the β 2 μ G mRNA levels. Experiment shown is representative for three independent experiments.

CD28 in the presence and absence of PGE₂ (10^{-5} mol/L). Nuclear extracts were analyzed for binding activity of NF- κ B, AP-1, and NFAT. Stimulation of T lymphocytes with anti-CD3 plus anti-CD28 was shown to induce NF- κ B, AP-1, and NFAT binding. Costimulation with PGE₂ totally inhibited the anti-CD3 plus anti-CD28-induced DNA binding of NF- κ B, whereas AP-1 and NFAT were not affected (Fig. 6). The specificity of the binding activities was demonstrated by a 100-fold molar excess of unlabeled oligonucleotide to nuclear

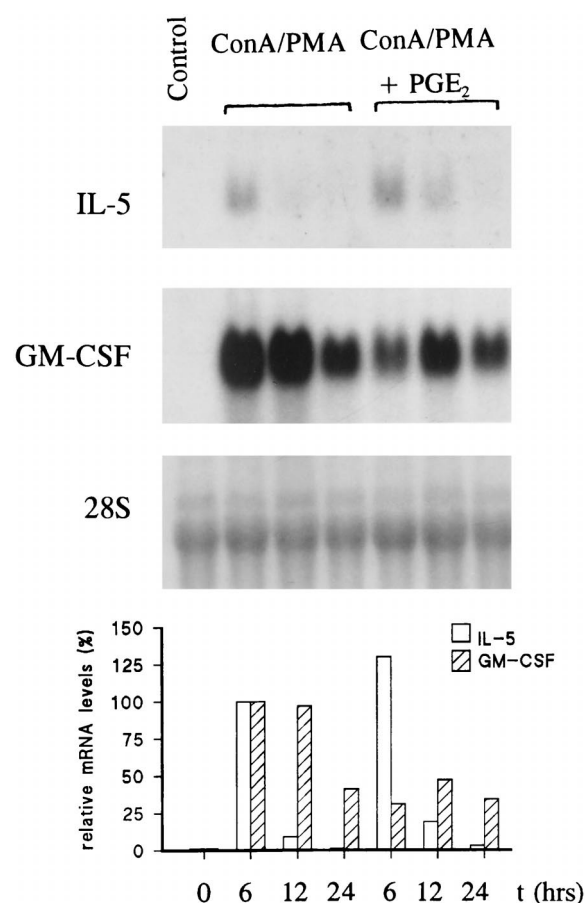


FIG. 3. Kinetics of IL-5 and GM-CSF mRNA accumulation in Con A plus PMA-stimulated T lymphocytes in the absence and presence of PGE₂ (10^{-5} mol/L). Northern analysis of total cellular RNA extracted at various time points indicated at bottom. Bar diagram represents relative IL-5 (open bars) and GM-CSF (hatched bars) mRNA levels after normalization to the respective 28S signals. Experiment shown is representative for three independent experiments.

extracts of T lymphocytes stimulated with anti-CD3 plus anti-CD28 (data not shown).

PGE₂ modulates IL-5 protein secretion depending on costimulatory signals

T lymphocytes obtained from four different donors were stimulated during 24 hours with anti-CD3 plus anti-CD28 or with Con A in the absence and presence of PGE₂ (10^{-5} mol/L). Unstimulated T lymphocytes did not secrete detectable levels of IL-5 protein (data not shown). As shown in Fig. 7, IL-5 protein was secreted after stimulation with anti-CD3 plus anti-CD28 (504 ± 56 pg/ml; $x \pm$ SEM), Con A (44 ± 5 pg/ml; $x \pm$ SEM), and Con A plus PMA (75 ± 8 pg/ml; $x \pm$ SEM). In the presence of PGE₂ the secreted amount of IL-5 protein was significantly diminished in anti-CD3 plus anti-CD28-stimulated T lymphocytes (122 ± 42 pg/ml; $x \pm$ SEM, $p < 0.001$), whereas Con A-induced IL-5 protein was not affected by PGE₂ (43 ± 11 pg/ml; $x \pm$ SEM). In

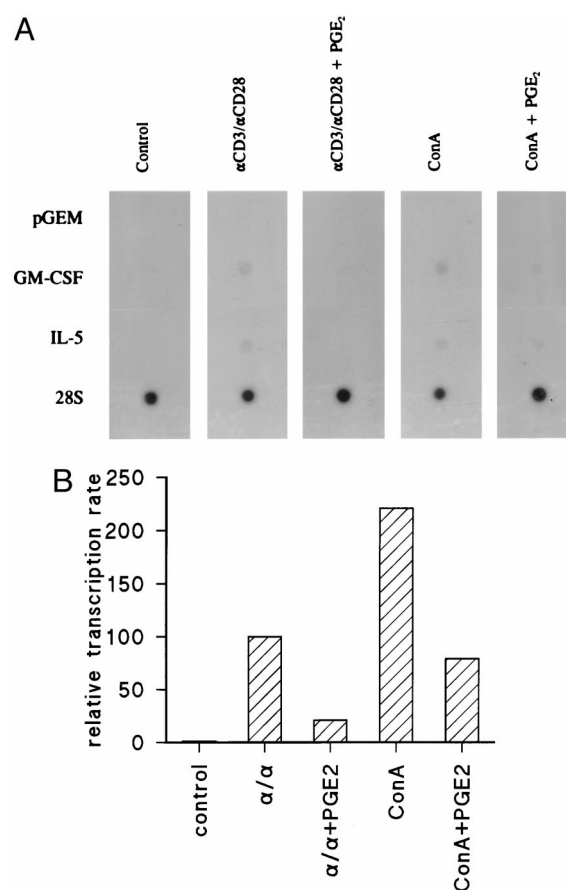


FIG. 4. Nuclear run-on. **A**, Effect of PGE₂ (10^{-5} mol/L) on the transcription rate of the IL-5 and GM-CSF genes in T lymphocytes stimulated during 3 hours with medium alone (control), antibodies against CD3 plus CD28 (α CD3/ α CD28), or Con A in the absence and presence of PGE₂. Nuclei were isolated and incubated during 20 minutes in the presence of α^{32} P-dCTP. Total nuclear RNA was isolated and hybridized to immobilized linearized plasmids containing cDNA probes for IL-5, GM-CSF, and 28S. The linearized plasmid pGEM represents a negative control. **B**, Bar diagram indicates relative IL-5 transcription rates after normalization to the respective 28S signals. Experiment shown is representative for three independent experiments.

accordance with the mRNA data, Con A plus PMA-induced IL-5 protein was significantly upregulated by PGE₂ (128 ± 17 pg/ml; $x \pm$ SEM; $p < 0.03$).

Because the PKA-mediated modulation of IL-5 gene expression has been shown to depend on IL-2,¹⁹ we questioned whether exogenously added cytokines that make use of the IL-2 receptor γ_c chain can modulate the inhibitory effect of PGE₂ in anti-CD3 plus anti-CD28-stimulated T lymphocytes. As depicted in Fig. 8, IL-2, IL-7, and IL-15 are able to completely abrogate the PGE₂-induced inhibition of the IL-5 protein secretion ($p < 0.03$, $n = 4$).

DISCUSSION

The activation of PKA by cAMP in conjunction with protein kinase C activation by phorbol esters is generally

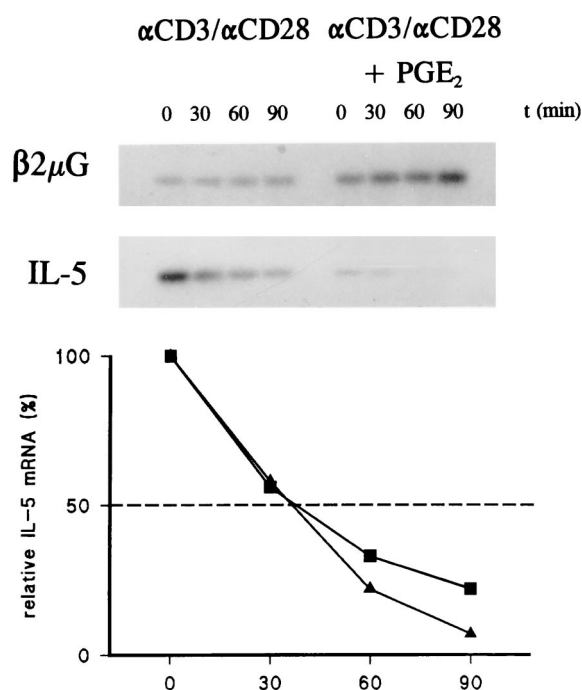


FIG. 5. Effect of PGE₂ (10⁻⁷ mol/L) on the stability of IL-5 mRNA transcripts. T lymphocytes were stimulated for 5 hours with antibody against CD3 and CD28 (αCD3/αCD28) in the absence (*left panel*) and presence (*right panel*) of PGE₂. IL-5 mRNA levels were determined by the radioactive RT-PCR method at various time points (indicated in minutes) after addition of Actinomycin D. IL-5 mRNA half-lives were determined after normalization with respect to the β2μG mRNA levels. Experiment shown is representative for three independent experiments.

regarded to initiate a signaling cascade leading to an enhanced expression of IL-5.^{7,8,31} Our results demonstrate that PKA activation by the synthetic analogue db-cAMP provides a strong negative regulating signal for IL-5 gene expression in freshly isolated human T lymphocytes activated with anti-CD2, anti-CD3, or a combination of anti-CD3 plus anti-CD28. The relevance of this observation for physiologic conditions was demonstrated by PGE₂, which is able to generate intracellular cAMP *in vivo*. PGE₂ at 10⁻⁸ mol/L, a concentration found in epithelial lining fluid in human beings, was still able to reduce IL-5 mRNA accumulation by approximately 50% in anti-CD3 plus anti-CD28-stimulated T lymphocytes. In contrast, IL-5 mRNA accumulation in Con A plus PMA-activated T lymphocytes was upregulated by PGE₂ (10⁻⁵ mol/L). Thus the cAMP-dependent signaling pathway may exert both a negative and a positive regulating effect on IL-5 gene expression dependent on the costimulatory signal.

We have previously shown that a comparable regulation exists for the expression of the IL-4 gene. IL-4 mRNA accumulation was dose-dependently inhibited by PGE₂ in T lymphocytes activated with anti-CD3 plus anti-CD28 or Con A, whereas IL-4 mRNA accumulation was not affected by PGE₂ in A23187 plus PMA-acti-

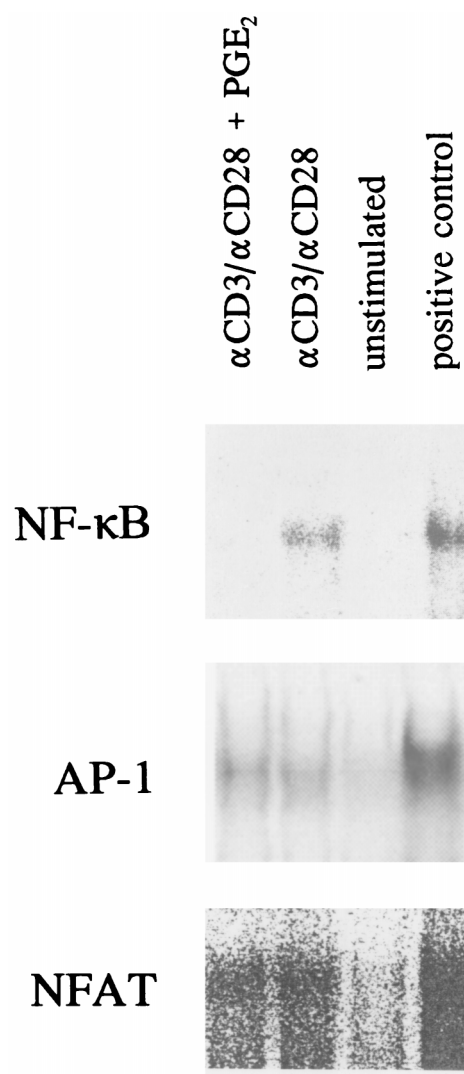


FIG. 6. DNA binding activities of NF-κB, AP-1, and NFAT. Nuclear extracts were prepared from unstimulated T lymphocytes and T lymphocytes stimulated for 3 hours with anti-CD3 plus anti-CD28 in the absence and presence of PGE₂ (10⁻⁵ mol/L).

vated T lymphocytes.¹⁸ In T_{H0} clones, it has been demonstrated that the effect of PGE₂ on IL-4 and IL-5 protein secretion also depended on costimulatory signals. In the presence of high concentrations of IL-2, secretion of IL-4 and IL-5 protein was enhanced by PGE₂. In contrast, when IL-2 concentrations were low, IL-4 and IL-5 protein secretions were inhibited by PGE₂.¹⁹ In our study we extended this observation by demonstrating that exogenously added cytokines, such as IL-2, IL-7, and IL-15—which use the IL-2 receptor γ_C chain—prevented the inhibitory effect of PGE₂. A similar mechanism (or mechanisms) might be responsible for the discrepancy observed with PGE₂ at transcriptional level and protein level in Con A-activated T lymphocytes. Although PGE₂ partially reduced Con A-induced IL-5 gene transcription, no differences were

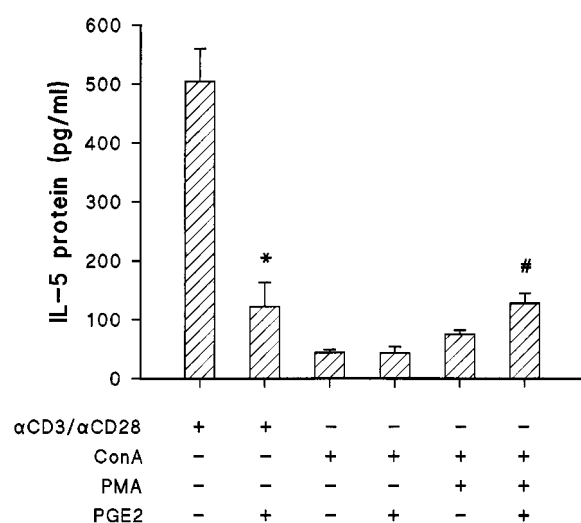


FIG. 7. Effect of PGE₂ (10⁻⁵ mol/L) on the secretion of IL-5 protein. T lymphocytes were cultured at a density of 3 × 10⁶ cells/ml. Supernatants were analyzed for IL-5 protein after 24 hours of stimulation with antibodies against CD3 and CD28 (αCD3/αCD28), Con A, and Con A plus PMA in the absence and presence of PGE₂. Mean values of secreted IL-5 protein are mentioned in the text. Bars indicate mean ± SEM. **p* < 0.001, *n* = 4; #*p* < 0.03, *n* = 4.

observed with respect to secreted IL-5 protein levels. Therefore it is conceivable that in conjunction with IL-5, secretion of cytokines that use the IL-2 receptor γ_C chain may initiate a positive loop, which ultimately leads to detectable levels of IL-5 protein after 24 hours of stimulation. The extended accumulation of IL-5 mRNA in Con A plus PMA in the presence of PGE₂ compared with Con A plus PMA (Fig. 3) may also be the result of cytokines released into the medium.

The observed PGE₂-induced upregulation of IL-5 mRNA in T lymphocytes costimulated in the presence of PMA may suggest a complex cross-talk between several downstream signaling events. In this respect it is worthwhile to mention that cAMP preferentially inhibits the *c-jun* N-terminal kinase pathway, leaving the extracellular signal-regulated kinase pathway unaffected.³² Addition of PMA and subsequent activation of the extracellular signal-regulated kinase cascade may therefore be resistant to cAMP. PGE₂ may exert its upregulatory effect on IL-5 gene expression by intervention and/or activation of these signaling pathways.

Nuclear run-on assays and mRNA stability studies demonstrated that predominantly transcriptional mechanisms are involved in the PGE₂-induced downregulation of IL-5 mRNA accumulation, because PGE₂ did not affect IL-5 mRNA stability but downregulated IL-5 gene transcription. In the transcriptional regulation of IL-5 gene expression, transcription factors such as OCT-1, AP-1, NFAT, NF-κB, and CD28RC might be involved, because potential consensus sequences of these transcription factors have been observed in the IL-5 promoter.^{8, 13-17} Because no cyclic AMP-responsive binding protein site is located in the IL-5 promoter, the observed

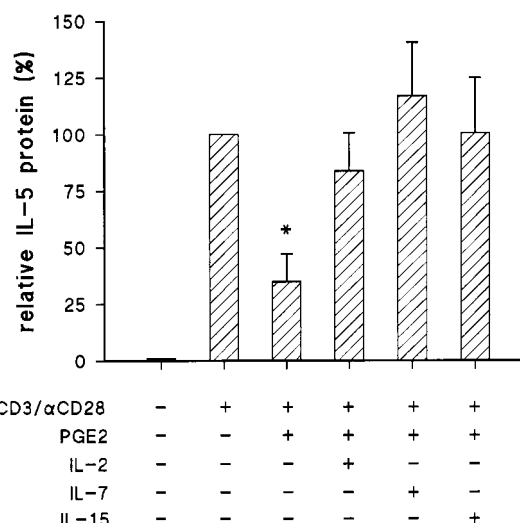


FIG. 8. Effect of cytokines that use the IL-2 receptor γ_C chain on the inhibitory effect of PGE₂ (10⁻⁵ mol/L) with respect to relative IL-5 protein secretion. T lymphocytes (3 × 10⁶ cells/ml) were stimulated with antibodies against CD3 and CD28 (αCD3/αCD28, 100%) in the absence and presence of PGE₂ and IL-2 (50 U/ml), IL-7 (10 ng/ml), and IL-15 (20 ng/ml). Bars represent mean ± SEM. **p* < 0.03, *n* = 4.

inhibitory effect of PGE₂ on IL-5 gene expression might be an indirect one. Anti-CD3 plus anti-CD28-stimulated T lymphocytes showed an enhanced binding of AP-1 and NFAT, yet the binding capacities of AP-1 and NFAT were not affected by PGE₂ (10⁻⁵ mol/L), suggesting that these transcription factors are not involved in PGE₂-induced downregulation. These results are similar to a study with T lymphocytes stimulated with anti-CD3, in which PGE₂ did not affect the binding capacity of AP-1.³³ In contrast, the NF-κB binding capacity was totally inhibited by PGE₂. In accordance with our data is the observation that cAMP antagonized the p21^{ras}-directed signaling pathway,^{34, 35} which leads to the activation of NF-κB. Therefore, NF-κB might fulfill an important role in the regulation of IL-5 gene expression in anti-CD3 plus anti-CD28-stimulated T lymphocytes.

The striking difference between our results and the results described in mouse EL4 cells, in which cAMP elevations lead to an enhanced IL-5 expression,^{7, 8, 31} may have its origin in the fact that EL4 cells are different in their signaling capacity. It has been demonstrated that PMA is sufficient to induce a range of cytokines in these cells, including IL-3, IL-4, IL-5, and GM-CSF.⁷ In our freshly isolated T lymphocytes, none of these cytokines could be induced by PMA alone (unpublished results), which suggests a different signaling mechanism in the EL4 cell line compared with human T lymphocytes, which may be reflected in the ultimate effect of cAMP on IL-5 gene expression. Moreover, it has been demonstrated that T_H clones are very heterogeneous in the sensitivity to PGE₂ in IL-3 and GM-CSF production.³⁶ These observations suggest that the responsiveness to PGE₂ is dependent on the property of the cells studied,

which is particularly reflected in the opposite effects of PGE₂ on cytokine production in extremely polarized T_{H1} and T_{H2} cells.

Our observation that PGE₂ acts as a negative regulator on IL-5 gene expression might be of great significance for understanding of the pathophysiology of asthma. Asthma is characterized by an early and a late bronchoconstrictive reaction after airway allergen challenge. It has been shown that PGE₂ has a protective role in the asthmatic airway, because low doses of inhaled PGE₂ almost completely inhibited both the early and late asthmatic reactions.³⁷ The late asthmatic reaction is accompanied by an influx and activation of eosinophils into the lung. This is most likely the result of chemoattractive and activating properties of IL-5, which action might be suppressed by PGE₂. Moreover, our study suggests that the inhibitory effect of PGE₂ is already observed at relevant physiologic concentrations (10⁻⁸ mol/L), because PGE concentrations in the human airway epithelium lining fluid have been shown to vary between 10⁻⁷ to 10⁻⁸ mol/L.³⁸ Whether these concentrations are reduced after allergen exposure in the airways of asthmatic subjects remains to be established.

Taking these data together, this study demonstrates that activation of the PKA-dependent signaling pathway downregulates IL-5 gene expression subject to the co-stimulatory signaling pathway. These findings may have important implications in the control of ongoing inflammatory processes as found in the airways of asthmatic subjects, especially since airway epithelium and smooth muscle cells are potent producers of PGE₂.^{39, 40} Release of PGE₂ by these cells may be of great importance for the regulation of levels of IL-5 in lung tissue and thereby the control of mucosal eosinophilic inflammation, as is the case in asthmatic subjects.

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