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Polarized Th1 and Th2 cells are less responsive to negative feedback by receptors coupled to the AC/cAMP system compared to freshly isolated T cells

1,2Irene H. Heijink, 2Edo Vellenga, 3Peter Borger, 4Dirkje S. Postma, 1Jan G.R.de Monchy & *1Henk F. Kauffman

1Department of Allergology, University Hospital Groningen, Groningen, The Netherlands; 2Department of Hematology, University Hospital Groningen, Groningen, The Netherlands; 3Institute of Respiratory Medicine, University of Sydney, Australia and 4Department of Pulmonology, University Hospital Groningen, Groningen, The Netherlands

The adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP) system is known to negatively regulate transcriptional activity of T cells, thereby possibly modulating T-cell-mediated responses at the sites of inflammation. Effects of cAMP have been widely studied in freshly isolated T cells and T-cell clones; yet, effects in differentiated Th1 and Th2 cells are largely unknown.

To obtain differentiated T helper cells, we activated naive T cells for 1 week in the presence of IL-12 plus α-IL-4 to generate Th1-type cells and in the presence of IL-4 plus α-IL-12 to generate Th2-type cells.

We demonstrate that, in contrast to freshly isolated T cells, the production of Th1 (IFN-γ) and Th2 (IL-4, IL-5) cytokines in polarized T helper cells is not strictly controlled by the activation of AC/cAMP-linked β2-adrenergic and prostaglandin (PG)E2 receptors.

In Th2 cells, PGE2 could still activate the G protein-coupled AC/cAMP system and subsequently induce CREB phosphorylation, whereas PGE2 was unable to activate the cAMP-dependent pathway in Th1 cells. In both Th1 and Th2 cells, the induction of CREB phosphorylation by β2-agonist fenoterol was impaired.

The loss of control over cytokine production by cAMP elevating agents in differentiated Th1 and Th2 subsets may have important implications for the regulation of Th1- and Th2-mediated diseases, in particular those associated with the ongoing immune responses.

Keywords: Th1; Th2; cytokines; β2-adrenergic; PGE2; cAMP; CREB

Abbreviations: AC, adenylyl cyclase; β2AR, β2-adrenergic receptor; βARK, β-adrenergic receptor kinase; β2mG, β2-microglobulin; cAMP, cyclic adenosine monophosphate; CRE, cAMP responsive element; CREB, cAMP-responsive element binding protein; db, dibutyryl; GRK, G protein-coupled receptor kinase; IBMX, 1-methyl-3-isobutylxantine; JNK, c-Jun N-terminal kinase; MAP, mitogen activated protein; PDE, phosphodiesterase; PGE2, prostaglandin E2; PI3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; Th, T helper

Introduction

The cyclic adenosine monophosphat (cAMP)-dependent pathway is an important negative feedback system in the regulation of inflammatory activity. Expression of cytokine production in T lymphocytes is regulated by prostaglandin (PG)E2 and β2-agonists, which activate receptors coupled to the cAMP-dependent pathway. Upon receptor binding, the associated G protein, protein triggers adenylyl cyclase (AC) activity, resulting in the formation of intracellular cAMP. Cytokine genes containing a cAMP-responsive element (CRE) in their promoter, like the IFN-γ gene, can be regulated by the protein kinase A (PKA)-mediated phosphorylation of cAMP-responsive element binding protein (CREB) (Nigg et al., 1985; Gonzalez & Montminy, 1989; Masquelier & Sassone-Corsi, 1992). Not all cytokine genes that are controlled by the cAMP-dependent pathway contain a CRE in their promoter. For instance, no binding site for CREB has been found in the IL-5 promoter region. Expression of cytokine genes lacking this binding site can be affected indirectly by cAMP, most likely by the modulation of the signal transduction pathways, including the mitogen activated protein (MAP) kinase pathways (Wu et al., 1993; Tamir et al., 1996; Harada et al., 1999).

In T cells, cAMP elevating substances are known to dose-dependently control the production of both Th1 and Th2 cytokines (Betz & Fox, 1991; Snijdewint et al., 1993; Hilkens et al., 1995; Borger et al., 1996, 1998, 1999), although the ultimate effect on Th2 cytokines appears to be dependent on the activation state and costimulatory signals (Hilkens et al., 1995; Borger et al., 1996, 1998). cAMP has been described to
inhibit Th2 cytokine production in freshly isolated T cells activated with CD3 plus CD28 antibodies (Borger et al., 1996), whereas it slightly enhances the production of IL-5 in the presence of potent PKC activators or high concentrations of IL-2 (Borger et al., 1996, 1998) and strongly upregulates IL-5 in Th2 clones (Snijdewint et al., 1993; Lee et al., 1994). Since T-cell clones may be dysregulated because of many cell divisions, this effect in Th2 clones may not be representative for in vivo conditions. Furthermore, the existence of strictly separated Th1 and Th2 subsets in the human immune system has been under discussion. However, polarized Th1 and Th2 cells may exist at the sites of tissue inflammation; several immune responses are associated with the presence of Th1 and Th2 subsets, including Th1-mediated autoimmune diseases and Th2-mediated allergies (Abbas et al., 1996; O’Garra, 1998). At the initiation of an immune response, autocrine IL-2 induces the proliferation of naive helper cells. Subsequently, the specific cytokine environment determines the final outcome of the T helper subtype (Abbas et al., 1996; O’Garra et al., 1998). From in vitro studies, it has become clear that naive CD4+ cells differentiate into polarized Th1 and Th2 during activation in the appropriate environment, for example, in the presence of IL-12 (Hsieh et al., 1993; Seder et al., 1993) or IL-4 (Seder et al., 1992), respectively. In addition, when T cells are only activated for a short period or in the absence of specific cytokine environment, nonpolarized intermediates or Th0 cells develop, which are able to produce various patterns of cytokines. Recent studies have demonstrated that entering the cell cycle is essential to induce epigenetic remodeling, that is, alterations in chromatin structure, which is crucial for differentiation towards a Th1 or Th2 phenotype and the efficient production of the associated cytokines (Argawal & Rao, 1998; Bird et al., 1998). The effects of cAMP have not been examined thoroughly in polarized Th1 and Th2 subsets, although this may provide insight into the regulation of Th1- and Th2-associated immune responses. We investigated the regulation of cytokine production by cAMP in Th1 and Th2 cells and demonstrate that control over cytokine production by cAMP elevating agents operating via AC-coupled receptors is partially lost in Th2 subsets and completely lost in Th1 subsets.

**Experimental procedures**

**Isolation of T cells**

Peripheral blood cells were obtained from healthy volunteer platelet donors. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll - Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density-gradient centrifugation. T cells were isolated by rosetting with 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells (SRBC). The SRBC were lysed with 155 mmol/l NH₄Cl, 10 mmol/l KHCO₃ and 0.1 mmol/l EDTA. After isolation, T cells were incubated overnight at 37°C in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 5% fetal calf serum (FCS; HyClone, Logan, UT, U.S.A.), supplemented with 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin.

**Th1/Th2 polarization**

T lymphocytes were isolated from peripheral blood of healthy volunteer platelet donors as described above. The T cells were differentiated into Th1 and Th2 subsets as described before (Roozendaal et al., 2001). In short, naive helper T lymphocytes were sorted after staining with z-CD45R0-FITC (UHCL-1) and z-CD4 CyQ (B-F5) (Immune Quality Products (IQP), Groningen, The Netherlands using a MoFlo™ Flow cytometer (Cytomation, Fort Collins, CO, U.S.A.) calibrated using Flow-Check™ Fluorospheres (Beckman Coulter, Paris, France). Purity was above 98% by reanalysis. Cells were cultured in RPMI 1640 medium containing 10% FCS, in the presence of PHA, IL-2, irradiated allogenic PBMC (neutral conditions) and either IL-12 (2 ng ml⁻¹, R&D systems, ITK diagnostics, Uithoorn, The Netherlands) plus z-IL-4 (200 ng ml⁻¹, Becton Dickinson, Erebodegem-Aalst, Belgium) to generate polarized Th1 cells, or IL-4 (200 U ml⁻¹, Becton Dickinson) and z-IL-12 (2 μg ml⁻¹, R&D systems) to generate polarized Th2 cells. To confirm that Th1 and Th2 phenotypes were obtained after 7 days, the cells were analyzed for intracellular cytokines as described before (Jung et al., 1993). In short, the polarized T helper cells were replated in RPMI medium containing 5% FCS and cultured overnight. Next, the cells were stimulated with PMA (10 ng ml⁻¹) and ionomycin (1 μmol/l) for 4 h in the presence of monensin (2 μM, Alexis, Läufelfingen, Switzerland). Cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% saponin/0.1% azide and stained using z-CD4-CyQ (B-F5, IQP, Groningen), z-IL-12/FITC (45-15, IQ P) and z-IL-4-PE (B-T4, CLB, Amsterdam, The Netherlands). Irrelevant specificity antibodies of the same isotype were used for gate setting. Analysis was performed using an Elite™ flow cytometer ( Beckman Coulter). Lymphocyte events were gated on the basis of forward and sideward scatter characteristics. The intracellular cytokine stainings indicated that highly divergent cytokine production patterns were obtained after 1 week of culture. Virtually all cells became CD45R0+ after culturing under polarizing conditions. In the cell population polarized under Th1 conditions, approximately 40% of the CD4+ cells was IFN-γ+/IL-4- and 0.5% was IL-4+/IFN-γ-, whereas in the cell population cultured under Th2 conditions, approximately 10% was IL-4+/IFN-γ- and 0.5% was IL-4+/IFN-γ+. In both cell populations, only a small percentage of the cells was positive for both cytokines.

**Stimulation of the T cells**

After 7 days of culture under polarizing conditions, the cells were rested overnight in RPMI medium containing 5% FCS. For stimulation, polarized T helper cells or freshly isolated T cells (1 · 3 · 10⁶ ml⁻¹) were incubated in RPMI 1640 medium containing 5% FCS with 50 μl ml⁻¹ of z-CD3 and z-CD28 antibodies, as previously described by Borger et al. (1999), in the presence or absence of PGE₂ (Sigma, St Louis, MO, U.S.A.) in a final concentration of 10 μM β₂-agonist fenoterol (Sigma) in a final concentration of 10 μM, cAMP analog dibutyryl (db)-cAMP (Boehringer-Mannheim GmbH, Germany) in a final concentration of 0.5 μM or phosphodiesterase (PDE) inhibitor l-methyl-3-isobutylxantine (IBMX, Alexis, Läufelfingen, Switzerland) in a final concentration of 100 μM.

**Measurement of cytokine protein**

Polarized T cells or freshly isolated T cells (1 · 3 · 10⁶ ml⁻¹) were left unstimulated or stimulated with z-CD3/z-CD28 during 6–8 h. Secreted IL-4, IL-5 and IFN-γ proteins were
measured in cell-free supernatants, using enzyme-linked immunosorbent assay (ELISA) kits for IL-4 and IFN-γ (CLB). The IL-5 ELISA was performed as previously described by Hoekstra et al. (1997).

**Measurement of intracellular cAMP accumulation**

After resting overnight, T lymphocytes (3 x 10⁶ ml⁻¹) were suspended in RPMI 1640 medium. Stimulation of cAMP production was performed as described before (Meurs et al., 1980). In short, the samples were incubated with IBMX (0.5 mM) for 10 min to prevent cAMP degradation. After preincubation, the samples were stimulated for 10 min with PGE₂ (10 μM). Reactions were terminated by adding 2 N HCl - 0.1 M EDTA followed by incubating the samples at 80°C for 10 min. After centrifugation of precipitated protein, the samples were neutralized by CaCO₃ and cAMP was measured using an enzyme immunoassay (Biotrak, Amersham, Buckinghamshire, U.K.) according to the manufacturer’s guidelines. cAMP concentrations are expressed as fmol cAMP/10⁶ T lymphocytes.

**Immunodetection by Western blotting**

Phosphorylation of CREB and expression of G protein-coupled receptor kinase 3 (GRK3/βARK2) were analyzed by Western blotting. T cells (1 - 3 x 10⁶) were cultured overnight in 1 1/2 ml RPMI 1640 medium containing 0.5% FCS. The cells were incubated with PGE₂ (10 μM), fenoterol (10 μM), dbcAMP (0.5 mM), IBMX (100 μM) or sodium fluoride (NaF, Sigma, 10 mM), to directly activate the G protein, in final a concentration of 10 mM for 60 min to study CREB phosphorylation, or left unstimulated to study βARK expression. T cells were harvested and spun down at maximum speed during 30 s. Next, total cell lysates were obtained by resuspension of the pellets in 1 x sample buffer (containing 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 60 mM Tris-Cl pH 6.8 and bromphenol blue) and boiling for 5 min. Samples were loaded on a SDS 10% PAGE gel (acrylamide : bisacrylamide 173 : 1) and transferred to a cellulosenitrate membrane (Schleicher & Schuell, Germany). Immunoblotting was performed by standard procedures and the detection was performed according the manufacturer’s guidelines (ECL, Amersham). Relative protein levels were quantified using the gelscan program Diversity One (Pharmacia, Uppsala, Sweden).

**Reverse transcription (RT) – polymerase chain reaction (PCR)**

Polarized T cells (5 x 10⁶) were rested overnight in RPMI medium containing 5% FCS, harvested and RNA was isolated using the TRIzol method (GIBCOBRL, Burlington, Ontario, Canada). Total cellular RNA was resuspended in diethyl-pyrocarbonate (DEPC; Sigma) treated H₂O. A volume of 1 μg RNA was used for cDNA synthesis. First, the samples were incubated during 10 min at 65°C with a random hexamer (pdN6). After cooling on ice, RT mix containing 5 x RT buffer (GIBCOBRL), 0.1 M DTT, 5 mM of each dNTP and 3 U of Reverse Transcriptase (GIBCOBRL) was added and the samples were incubated at 37°C for 1 h. For the PCR reaction, 10 x PCR buffer (GIBCOBRL), 50 μM of forward and reverse primer, 0.25 μl Taq polymerase, 2 mM dNTP’s and 75 μl MgCl₂ in 25 μl total volume were added. The following specific primer pairs for β₂ microglobuline (β₂μG, housekeeping gene), EP₃ (an AC-coupled subtype of the PGE₂ receptor), EP₃ (a Gₛ-coupled subtype of the PGE₂ receptor) and β₂-adrenergic receptor (β₂AR) were obtained from Bioloeg BV (Malden, The Netherlands):

- β₂μG: 5’CCACGAGAGAATGGAAAGTC3’ sense and 5’GATGCTGCTTACATGTC TCG3’ antisense.
- AR: 5’TTCTTGCTGGACACCTT3’ sense and 5’GGAGGCTCCA AACCTCGACACCA3’ antisense.
- EP₃: 5’CCTCTTGAGAAA GACAGTCTTGTGACCT3’ sense and 5’AAGACACTCTCTGATGC TC3 antisense.
- EP₃: 5’TCTGCGGCGTGGCCGCTACA3’ sense and 5’GACCAACAGCGACAGCACA3’ antisense.

PCR conditions were a denaturation step at 94°C for 5 min followed by 20 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of β₂μG, 35 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 30 s for detection of β₂AR, 25 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of EP₃ and 30 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of EP₃. With these primers, the amplified products were 268, 295, 395 and 300 bp long for β₂μG, β₂AR, EP₃, and EP₃, respectively. After PCR, 10 μl of the reaction mixture was run on a 1.5% agarose gel containing 0.2 μg ethidium bromide in 1 x TAE buffer. A 100 bp ladder (Pharmacia) was used as DNA marker. Relative mRNA levels were quantified using the gelscan program Diversity One (Pharmacia, Uppsala, Sweden).

<table>
<thead>
<tr>
<th>T cell type</th>
<th>Treatment</th>
<th>IFN-γ</th>
<th>IL-4</th>
<th>IL-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly isolated</td>
<td>Basal</td>
<td>3539±3037</td>
<td>15.3±12.8</td>
<td>54.8±47.5</td>
</tr>
<tr>
<td></td>
<td>+ PGE₂ 10 μM</td>
<td>33.6±39.2</td>
<td>5.3±3.8</td>
<td>4.5±3.1</td>
</tr>
<tr>
<td></td>
<td>+ fenoterol 10 μM</td>
<td>679±310</td>
<td>10.1±9.3</td>
<td>26.5±23.9</td>
</tr>
<tr>
<td>Polarized</td>
<td>Basal</td>
<td>455±347</td>
<td>0.4±0.1</td>
<td>14.2±15.8</td>
</tr>
<tr>
<td></td>
<td>+ PGE₂ 10 μM</td>
<td>10213±7803</td>
<td>84.4±88.8</td>
<td>506±316</td>
</tr>
<tr>
<td></td>
<td>+ fenoterol 10 μM</td>
<td>10018±4050</td>
<td>58.3±37.8</td>
<td>400±241</td>
</tr>
<tr>
<td></td>
<td>+ PGE₂ 10 μM</td>
<td>10200±4020</td>
<td>79.4±42.5</td>
<td>499±385</td>
</tr>
</tbody>
</table>

Freshly isolated T cells or polarized Th cells were left unstimulated or stimulated by β₂μG, β₂AR, EP₃ and EP₃, respectively. After PCR, 10 μl of the reaction mixture was run on a 1.5% agarose gel containing 0.2 μg ethidium bromide in 1 x TAE buffer. A 100 bp ladder (Pharmacia) was used as DNA marker. Relative mRNA levels were quantified using the gelscan program Diversity One (Pharmacia, Uppsala, Sweden).
Statistical analysis

For the protein measurements, statistical analysis was performed using a nonparametric test for paired observations (Wilcoxon-signed ranks test). Statistical significance of the secretion data was set at P<0.05.

Results

Control over cytokine production by cAMP elevating agents in polarized helper T cells is reduced compared to freshly isolated T cells

There was a wide range in the levels of cytokines secreted by the different blood donors. Absolute values (mean±s.e.m.) of cytokine secretion are given in Table 1. Since similar effects were exerted by cAMP elevating agents in low and high cytokine producers, cytokine protein levels are expressed as a percentage of the cytokine secretion upon stimulation in the absence of the cAMP elevating agents. As demonstrated in Figure 1a, secretion of IFN-γ was strongly inhibited by 10μM PGE2 (from 100 to 4±3%, P<0.001, n=8) and to a smaller extent by 10μM of short-acting β2-agonist fenoterol (from 100 to 20±12%, P<0.001, n=8) in freshly isolated, α-CD3/α-CD28 stimulated T cells. The secretion of the Th2 cytokines IL-4 and IL-5 was also under firm control of 10μM PGE2 in these cells (inhibition from 100 to 32±9 and 15±10%, respectively, P<0.001). Fenoterol of 10μM moderately, but significantly, reduced IL-4 and IL-5 secretion (from 100 to 71±17 and 64±20%, respectively, P<0.03, Figure 1a). Dose-dependent effects of fenoterol and PGE2 on cytokine production in stimulated freshly isolated T cells are depicted in Figure 1b (n=4). The secretion of IFN-γ appeared to be more sensitive to fenoterol and PGE2 compared to the secretion of IL-5, whereas IFN-γ production was still significantly inhibited by 10nm fenoterol and PGE2 (P<0.01). IL-5 was only significantly inhibited when fenoterol and PGE2 were used in higher concentrations (from 100nm to 10μM).

Interestingly, cytokine production appeared to be differently regulated by cAMP elevating agents in polarized T helper cells. In contrast to the strong inhibitory effects on IFN-γ protein secretion in freshly isolated T cells (Figure 1a), there was a complete absence of control over IFN-γ secretion by PGE2 and fenoterol (10μM) in polarized Th1 cells (Figure 1b). In polarized Th2 cells, the control of IL-4 and IL-5 secretion was also reduced compared to freshly isolated T cells. No inhibitory effect was found upon stimulation of the β2-adrenoceptor with fenoterol (10μM). In contrast, the secretion of IL-4 and IL-5 protein was still modestly, but significantly, inhibited by 10μM PGE2 (from 100 to 62±19 and 74±10%, P<0.05 and P<0.01, respectively). The loss of control in Th2 and Th1 subsets may be because of differential regulation of the cytokine gene promoters or by impaired activation of the cAMP-dependent pathway.

CREB phosphorylation induced by different cAMP elevating agents

To investigate whether the cAMP-dependent pathway can still be efficiently activated in polarized Th1 cells and Th2 cells, we studied the phosphorylation of downstream effector CREB. In
freshly isolated T cells, both PGE2 and fenoterol (10 μM) clearly induced CREB phosphorylation (Figure 2), the effect of PGE2 being most pronounced. In contrast, in Th1 cells, no phosphorylation of CREB could be observed upon stimulation with PGE2 and fenoterol. These results suggest that the defective regulation of cytokine production by fenoterol and PGE2 in Th1 cells is because of impaired activation of the cAMP downstream pathway. In Th2 cells, PGE2 was able to induce a clear increase in CREB phosphorylation, whereas fenoterol only slightly enhanced the levels of phosphorylated CREB. Thus, the ability of PGE2 to activate the cAMP-dependent pathway is impaired in Th1 cells and still intact in Th2 cells, whereas β2-adrenergic activation of this pathway appears to be disturbed in both Th1 and Th2 cells. To study if this β2-adrenergic hyporesponsiveness in polarized cells is a consequence of the proliferation and activation induced by culturing under polarizing conditions, we studied the effect of neutral (Th0) conditions, that is, PHA, IL-2 and irradiated APC. In T cells cultured under these conditions for 7 days, fenoterol was not able to induce phosphorylation of CREB either. In contrast, PGE2 could still enhance CREB phosphorylation. These data suggest that β2-adrenergic hyporesponsiveness may develop by polyclonal activation of T cells during polarization.

**Impaired formation of intracellular cAMP by PGE2 in Th1 cells**

To study in more detail the defects of PGE2 and β2-receptor function, we measured the capacity of these agents to enhance the accumulation of intracellular cAMP in freshly isolated and polarized T cells. To prevent degradation of cAMP during the assay, cells were incubated with PDE inhibitor IBMX. Addition of IBMX strongly enhanced accumulation of intracellular cAMP in both polarized and freshly isolated T cells: from 0.32±0.06 to 2.09±0.58 pmol cAMP in Th1 cells, from 0.18±0.07 to 1.83±1.0 pmol cAMP in Th2 cells and from 0.22±0.09 to 1.93±0.58 pmol cAMP in freshly isolated T cells (Figure 3a). In both Th1 and Th2 cells, fenoterol was unable to increase intracellular cAMP levels (data not shown). Addition of PGE2 (10 μM) induced a significant increase in cAMP production in both freshly isolated T cells and polarized Th2 cells (fold increase 263±71 and 208±49%, P<0.05 and P<0.01, respectively), but not in Th1 cells (110±41%, Figure 3b). Thus, these data show a difference in PGE2 functionality in Th1 cells versus Th2 and freshly isolated T cells. Together, our findings suggest that the defective regulation of cytokine production by cAMP elevating in polarized T helper is caused by the inability of PGE2 receptor and/or β2-AR stimulation to increase intracellular cAMP levels.

As previously described, one of the mechanisms responsible for the impaired capacity to increase cAMP accumulation may be the upregulation of cAMP-specific PDE’s (Seybold et al., 1998), which are responsible for the degradation of cAMP. However, basal levels of cAMP were not reduced in polarized T helper cells compared to freshly isolated T cells, indirectly indicating that basal activity of PDE’s is not enhanced. Additionally, the responsiveness of cytokine production to PDE inhibitors was not enhanced in polarized T cells compared to freshly isolated T cells (data not shown). Thus,
PDE’s are unlikely to play a role in the reduced responsiveness to cAMP elevating substances.

**CREB phosphorylation is induced and cytokine production is strongly inhibited by db-cAMP, IBMX and direct stimulation of the Gs protein in polarized Th1 and Th2 cells**

Next, it was of interest to study whether cAMP is still able to activate its downstream pathway and to negatively regulate cytokine production in polarized T helper cells. As demonstrated in Figure 4a, strong induction of CREB phosphorylation was observed when db-cAMP was added to Th1 cells. These results clearly demonstrate that cAMP-induced signaling is not impaired in these cells. Moreover, addition of IBMX increased both cAMP accumulation (see the results described above) and CREB phosphorylation in Th1 cells, indicating that intracellular cAMP generation is not disturbed. Finally, direct activation of the AC-coupled Gs protein by NaF resulted in strong induction of CREB phosphorylation. This indicates that the defective induction of CREB phosphorylation by PGE2 and fenoterol is most likely caused at receptor level. Similar results with db-cAMP, IBMX and NaF were observed in Th2 cells (Figure 4a) and freshly isolated T cells (data not shown).

In addition, we examined whether IBMX or db-cAMP could also inhibit IFN-γ and IL-5 protein secretion in Th1 and Th2 cells, respectively. Indeed, enhancement of intracellular levels of cAMP by the addition of IBMX reduced x-CD3/x-CD28 stimulated IL-5 and IFN-γ protein secretion by approximately 85%. Similar results were obtained with cAMP analog db-cAMP (Figure 4b). These data demonstrate that cytokine production can still be efficiently regulated by the cAMP-dependent pathway in polarized T helper cells, supporting the findings that the reduced responsiveness to PGE2 and/or β2-agonist fenoterol in polarized Th cells is because of impaired activation of the AC system and not differential regulation of cytokine production.

**Enhanced expression of βARK (GRK3) in polarized T helper cells**

We were interested in the possible mechanisms involved in the desensitization of the PGE2 and/or fenoterol effects in polarized Th1 and Th2 cells. Desensitization of the β2-AR can be induced by either downregulation of mRNA and protein levels of the receptor (Rademaker et al., 1990) or by phosphorylation of the receptor, which results in functional uncoupling from the Gs protein and internalization of the receptor. First, the expression level of mRNA was determined using RT–PCR. We observed that, similar to freshly isolated T cells, β2AR mRNA was clearly expressed in both Th1 and Th2 subsets (Figure 5a). Thus, downregulation of β2AR mRNA is not likely the cause of desensitization of the β2-adrenergic system in Th1 and Th2 cells. GRK’s, as well as PKA and protein kinase C (PKC) are able to phosphorylate the β2-AR (Meurs et al., 1987; Hausdorff et al., 1990). Enhanced expression and activation of βARK are known to induce β2-AR desensitization because of enhanced and more rapid phosphorylation of the receptor (Lohse et al., 1992; McGraw & Liggett, 1997; Penn et al., 1998). It has been described that treatment of T cells with polyclonal activators (PHA, x-CD3 and IL-2) for 3–7 days results in enhanced βARK (GRK3) and GRK6 mRNA and protein levels as well as increased activity of both kinases (Loudon et al., 1996). Therefore, we analyzed the levels of βARK2/GRK3 in polarized Th cells and freshly isolated T cells by immunoblotting. As demonstrated in Figure 5b, the expression of βARK was dramatically enhanced in polarized Th1 cells compared to freshly isolated T cells. The expression in Th2 cells was lower, but still strongly enhanced compared to freshly isolated T cells. In addition, we studied the expression of βARK in T cells cultured under neutral conditions, which also showed defective induction of CREB phosphorylation by fenoterol. In these cells, levels of βARK were strongly enhanced compared to freshly isolated T cells as well. Thus, polyclonal activation of T
cells during polarization might induce an increase in βARK expression, which in turn might be involved in the loss of β2-adrenergic control.

**Altered levels of PGE2 receptor subtypes in polarized T helper cells**

In addition to β2-adrenergic unresponsiveness, polarized Th1 were unresponsive to PGE2. Although, the mechanism of β2-AR desensitization has been widely described, little information is available about desensitization and internalization of the PGE2 receptor. It is known that PGE2 can exert differential effects through activation of different subtypes of the receptor. At least four different subtypes are known, that is, the EP1, EP2, EP3 and EP4 subtypes. It has been described that EP4 is susceptible to agonist-promoted internalization, whereas the EP3 receptor is resistant. EP2 and EP4 are known to be coupled to the Gs protein and induce AC activation. On the other hand, activation of EP1 induces Ca2+ accumulation, while the EP3 subtype preferentially couples to Gi, thereby inhibiting cAMP generation (An et al., 1993; Funk et al., 1993; Yang et al., 1994; Breyer & Breyer, 2001; Castleberry et al., 2001). To study if differential expression of the PGE2 receptor could be involved in the altered PGE2 signaling in polarized T helper cells, we measured the mRNA expression of the Gs-coupled EP2 subtype and the Gi-coupled EP3 subtype of the PGE2 receptor using RT–PCR. As demonstrated in Figure 6, the expression of EP2 was reduced in both Th1 and Th2 cells compared to freshly isolated T cells. This may be involved in the reduced responsiveness of cytokine production to PGE2 that was observed in both Th1 and Th2 cells compared to freshly isolated T cells; yet, this does not explain the difference of PGE2 reactivity in Th1 and Th2 cells. As described above, PGE2 was able to activate the AC/cAMP-dependent pathway in Th2 cells, while this was not the case in Th1 cells. In contrast to the Gs-coupled EP2 subtype, the expression of the Gi-coupled EP3 subtype appeared to be about a two-fold higher in Th1 cells compared to freshly isolated T cells as well as Th2 cells (Figure 6). Since activation of the Gi protein is known to result in activation of the AC/cAMP system, while activation of the Gs protein is known to inhibit this system, the altered ratio in EP2 and EP3 expression in Th1 cells might be an explanation for the net zero effect of PGE2 on cAMP production in Th1 cells (see Figure 3b). Thus, differential expression of subtypes of the PGE2 receptor might be involved in the unresponsiveness of Th1 cells to PGE2.

**Discussion**

Specialized Th1 and Th2 subsets direct immune responses at sites of inflammation by the production of a defined pattern of cytokines. An important regulator of proinflammatory activity is the cAMP-dependent pathway. At sites of tissue inflamma-
tion, T cells have ample opportunity to encounter cells that produce cAMP elevating agents, for example, PGE2. The regulatory effects of cAMP have been widely studied in freshly isolated T cells and in T-cell clones, but not in specialized T helper subset cells, which can be obtained by in vitro differentiation under Th1 or Th2 polarizing conditions. In the present report, we demonstrate that cytokine secretion in polarized T helper cells is not strictly controlled by cAMP elevating agents. Although the x-CD3/x-CD28 stimulation method cannot necessarily be extrapolated to the activation of T cells by antigen, our results may have important implications for the regulation of Th1- and Th2-mediated immune responses. Our findings are in contrast to the results in freshly isolated T cells, where the production of the Th1-like cytokine IFN-γ was almost fully blocked by cAMP elevating substances and significant inhibition of Th2-like cytokine (IL-4, IL-5) production was observed. The inhibitory effect on IFN-γ secretion appeared to be completely abolished in polarized Th1 cells, while the inhibitory effect on IL-4 and IL-5 in polarized Th2 cells was reduced compared to freshly isolated T cells. Thus, whereas in freshly isolated T cells, Th1 cytokine production was observed. The inhibitory effect on IFN-γ secretion appeared to be completely abolished in polarized Th1 cells, while the inhibitory effect on IL-4 and IL-5 in polarized Th2 cells was reduced compared to freshly isolated T cells. Thus, whereas in freshly isolated T cells, Th1 cytokine production was more susceptible to cAMP inhibition than Th2 cytokine production, polarized Th2 cells appear to be more sensitive to PGE2 than polarized Th1 cells. PGE2 was able to induce an increase in cAMP production and CREB phosphorylation in Th2 cells, while PGE2 was unable to enhance cAMP production and subsequently activate downstream signals in Th1 cells. This may have implications for the Th1/Th2 balance at sites of tissue inflammation, where specialized Th1 or Th2 subsets may be found. Our data indicate that the loss of PGE2 control over IFN-γ production in polarized Th1 cells is most likely because of a defect at receptor level and is not caused by differential regulation of cytokine production. This is supported by the finding that preincubation with db-cAMP, IBMX or NaF significantly enhances CREB phosphorylation and strongly inhibits IFN-γ production, indicating that IFN-γ production is still under the control of the cAMP-dependent pathway. Defective induction of CREB phosphorylation might indeed lead to a loss of control over IFN-γ production, since it has been shown that CREB inhibits Jun-mediated activation of the IFN-γ promoter by competitive binding (Zhang et al., 1998). In addition to the regulation of cytokine production, the cAMP-dependent pathway has been described to induce apoptosis in T lymphocytes (Gu et al., 2000). Thus, our results might also have implications for the survival of Th1 and Th2 subsets in inflamed tissue, where T cells have opportunity to encounter PGE2 secreting cells.

In contrast to the differential effects of PGE2 in Th1 and Th2 subsets, we found reduced responsiveness to the β2-agonist fenoterol in both Th1 and Th2 cells. This seems to be because of a defect at receptor level, since the ability of fenoterol to activate the cAMP-dependent pathway was impaired in polarized T cells. If the in vitro polarized T cells are indeed representative for specialized Th1 and Th2 subsets in vivo, our results suggest that the use of β2-mimetics, for instance in asthma, may not efficiently inhibit proinflammatory T-cell activity. Several mechanisms may be responsible for the desensitization of the β2-AR. First, prolonged agonist binding can result in downregulation of the total cellular levels of the receptor (both mRNA and protein) and contribute to desensitization of the receptors (Rademaker et al., 1990). However, no indications for a prolonged repression of receptor gene transcription, resulting in decreased receptor expression, were observed in polarized Th cells; mRNA for the β2-AR was clearly expressed in polarized Th1 cells and Th2 cells. Thus, a functional uncoupling of the β2-AR and PGE2 receptor from the G protein seems to be more likely. This can be caused by phosphorylation of the receptor by GRKs, PKA or PKC (Meurs et al., 1987; Hausdorff et al., 1990). Phosphorylation of the active form of the receptor by βARK (also GRK3) promotes binding of β-arrestin to the receptor, resulting in uncoupling from the G protein and finally internalization of the receptor (Gu et al., 2000). Enhanced expression and activation of βARK may induce β2-adrenergic hyporesponsiveness (Lohse et al., 1992). It has been demonstrated that overexpression of βARK leads to hyporesponsiveness of the β2-AR because of enhanced and more rapid agonist-induced phosphorylation of the receptor (McGraw & Liggett, 1997; Penn et al., 1998). Indeed, we found that βARK levels in polarized Th1 and Th2 cells were strongly enhanced compared to freshly isolated T cells. In addition, high levels of βARK were observed in T cells polarized under neutral (Th0) conditions, where reduced responsiveness to β2-agonist fenoterol was also found. These data suggest that polyclonal activation of T cells during polarization induces upregulation of βARK, thereby reducing the responsiveness to β2-agonists. In this way, T cells may become less sensitive to circulating β2-agonists (e.g. epinephrine) during the induction of cell cycle progression at the initiation of immune responses.

So far, little information is available on the regulation of the PGE2 receptor. It has been demonstrated that the EP4 subtype of the PGE2 receptor is susceptible to agonist-promoted internalization, whereas the EP3 subtype receptor is resistant (Penn et al., 2001). EP3 desensitization can occur by a similar mechanism as β2-adrenergic desensitization, involving βARK and β-arrestin. Therefore, a role for enhanced βARK expression in PGE2 unresponsiveness in Th1 cells cannot be excluded, although it is not clear why Th2 cells, expressing high levels of βARK, were still responsive to PGE2. We show that another possible mechanism involved in reduced responsiveness to PGE2 might be the altered balance in EP3/EP1 mRNA expression. EP1 mRNA expression was higher in both Th1 and Th2 subsets compared to freshly isolated T cells, whereas EP3 mRNA expression was only enhanced in Th1 cells. Thus, the reduced expression of EP1 could explain why Th2 cells are less responsive to PGE2 than freshly isolated T cells, while the additional upregulation of mRNA for the Gi-coupled EP3 receptor in Th1 cells may be responsible for the complete unresponsiveness to PGE2 in these cells. It is known that activation of the Gi protein by PGE2 and β2-agonists does not result in cAMP formation, but instead causes activation of the PI3-kinase-dependent antiapoptotic pathways (Zhu et al., 2001). Our data suggest that coupling of the PGE2 receptors to the Gi protein is enhanced in polarized Th1 cells compared to freshly isolated T cells. This might result in a net zero effect on cAMP production and activation of downstream pathways, since activation of Gi counteracts the effects induced by activation of the G protein. It still remains unclear as to what causes the Th1 specific upregulation of EP3 mRNA. Incubation of T cells with the Th1-directing cytokine IL-12 alone for prolonged periods did not result in reduced ability of PGE2 to activate the cAMP-dependent pathway (data not shown). Possibly, entering the cell cycle and epigenetic remodeling during Th1 polarization are required to induce upregulation of
EP2 expression. Another G-coupled receptor that can trigger different intracellular events through different receptor subtypes is the histamine receptor. It has been demonstrated that histamine reduces cytokine (IL-4, IL-13) production and elevates cAMP production in differentiated murine Th2 cells, which preferentially expressed the histamine receptor type 2 (HR2) (Jutel et al., 2001). In contrast, Th1 cells preferentially expressed HR1, leading to an increase in calcium influx instead of an increase in intracellular cAMP levels upon activation and in addition an upregulation of IFN-γ production (Jutel et al., 2001). Thus, reduced linking of G protein-coupled receptors to the AC system seems to be a general phenomenon in polarized Th1 cells. Together, our findings suggest that resting T cells in peripheral blood are under strict control of G protein-coupled receptors, while this control is lost when T cells become activated at the sites of tissue inflammation and differentiate into effector T cells. The loss of control may allow appropriate activation and increase the survival of effector Th cells. Th1 cells appear to be less responsive to G protein-coupled receptors than Th2 cells, favoring activation of the Th1 subtype.

In summary, our study demonstrates that polarized T helper cells are less responsive to PGE2 and the β2-agonist fenoterol than circulating T cells. This lack of negative feedback control may have implications for ongoing inflammatory processes, since cytokine production in effector Th2 cells may not be efficiently suppressed and the activity of Th1 cells may not be suppressed at all by a β2-agonist or PGE2 secreted by surrounding tissue cells.

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