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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

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1 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.

2 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.

3 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 β_2-adrenergic and prostaglandin (PG)E_2 receptors.

4 In Th2 cells, PGE_2 could still activate the G_s protein-coupled AC/cAMP system and subsequently induce CREB phosphorylation, whereas PGE_2 was unable to activate the cAMP-dependent pathway in Th1 cells. In both Th1 and Th2 cells, the induction of CREB phosphorylation by β_2-agonist fenoterol was impaired.

5 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.

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Abbreviations: AC, adenylyl cyclase; β_2AR, β_2-adrenergic receptor; βARK, β-adrenergic receptor kinase; $\beta_2\mu$G, β_2-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; PGE_2, prostaglandin E_2; PI3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; Th, T helper

Introduction

The cyclic adenosine monophosphate (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)E_2 and β_2-agonists, which activate receptors coupled to the cAMP-dependent pathway. Upon receptor binding, the associated G_s 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; Masquilier & 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

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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-aminoethylisothioronium bromide (AET)-treated sheep red blood cells (SRBC). The SRBC were lysed with $155\,\mathrm{mmol\,l^{-1}}$ NH_4Cl, $10\,\mathrm{mmol\,l^{-1}}$ $KHCO_3$ and $0.1\,\mathrm{mmol\,l^{-1}}$ 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 $100\,\mathrm{U\,ml^{-1}}$ penicillin and $100\,\mu\mathrm{g\,ml^{-1}}$ 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 α-CD45RO-FITC (UHCL-1) and α-CD4 CyQ (B-F5) (Immune Quality Products (IQP), Groningen, The Netherlands using a MoFlow™ 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 ($2\,\mathrm{ng\,ml^{-1}}$, R&D systems, ITK diagnostics, Uithoorn, The Netherlands) plus α-IL-4 ($200\,\mathrm{ng\,ml^{-1}}$, Becton Dickinson, Erebodegem-Aalst, Belgium) to generate polarized Th1 cells, or IL-4 ($200\,\mathrm{U\,ml^{-1}}$, Becton Dickinson) and α-IL-12 ($2\,\mu\mathrm{g\,ml^{-1}}$, 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 ($10\,\mathrm{ng\,ml^{-1}}$) and ionomycin ($1\,\mu\mathrm{g\,ml^{-1}}$) for 4 h in the presence of monensin ($2\,\mu$M, Alexis, Läufelfingen, Switzerland). Cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% saponin/0.1% azide and stained using α-CD4-CyQ (B-F5, IQP, Groningen), α -IFN-γ-FITC (45-15, IQ P) and α-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 $CD45RO^+$ 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 ($1-3\times10^6\,\mathrm{ml^{-1}}$) were incubated in RPMI 1640 medium containing 5% FCS with $50\,\mu\mathrm{l\,ml^{-1}}$ of α-CD3 and α-CD28 antibodies, as previously described by Borger *et al*. (1999), in the presence or absence of PGE_2 (Sigma, St Louis, MO, U.S.A.) in a final concentration of 10 (μM β_2-agonist fenoterol (Sigma) in a final concentration of $10\,\mu$M, cAMP analog dibutyryl (db)-cAMP (Boehringer-Mannheim GmbH, Germany) in a final concentration of $0.5\,\mu$M or phosphodiesterase (PDE) inhibitor l-methyl-3-isobutylxantine (IBMX, Alexis, Läufelfingen, Switzerland) in a final concentration of $100\,\mu$M.

Measurement of cytokine protein

Polarized T cells or freshly isolated T cells ($1-3\times10^6\,\mathrm{ml^{-1}}$) were left unstimulated or stimulated with α-CD3/α-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 (3×10^6 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 PGE_2 (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 $CaCO_3$ 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/10^6 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 ($1-3 \times 10^6$) were cultured overnight in 1 1/2 ml RPMI 1640 medium containing 0.5% FCS. The cells were incubated with PGE_2 (10 μM), fenoterol (10 μM), db-cAMP (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 × sample buffer (containing 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 60 mM Tris-Cl pH 6.8 and bromophenol 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 (5×10^6) 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 H_2O. 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 × 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 × PCR buffer (GIBCOBRL), 50 μM of forward and reverse primer, 0.25 μl *Taq* polymerase, 2 mM dNTP's and 75 μl $MgCl_2$ in 25 μl total volume were added. The following specific primer pairs for β_2 microglobuline ($\beta_2\mu$G, housekeeping gene), EP_2 (an AC-coupled subtype of the PGE_2 receptor), EP3 (a G_i-coupled subtype of the PGE_2 receptor) and β_2-adrenergic receptor (β_2AR) were obtained from Biolegio BV (Malden, The Netherlands):

$\beta_2\mu$G: 5′CCAGCAGAGAATGGAAAGTC3′ sense and 5′GATGCTGCTTACATGTC TCG3′ antisense. β_2AR: 5′CC TTCTTGCTGGCACCCCAT3′ sense and 5′GGAAGTCCA AAACTCGCACCA3′ antisense. EP_2: 5′CCTCCTGAGAAA GACAGTGCT3′ sense and 5′AAGACACTCTCTGAGTC CT3 antisense. EP_3: 5′TGCTGGGCGTGGGCCGCTACA3′ sense and 5′GACCAACAGACGGACAGCACA3′ 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 $\beta_2\mu$G, 35 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 30 s for detection of β_2AR, 25 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of EP_2 and 30 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of EP_3. With these primers, the amplified products were 268, 295, 395 and 300 bp long for $\beta_2\mu$G, β_2AR, EP_2 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 × 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 1 Absolute amounts of secreted cytokine protein in freshly isolated and polarized T cells

T cell type	*Treatment*	*Cytokine secretion in pg ml^{-1}*		
		IFN-γ	*IL-4*	*IL-5*
Freshly isolated	Basal	ND	ND	ND
	α-CD3/α-CD28	3539±3037	15.3±12.8	54.8±47.5
	+ PGE2 10 μm	33.6±39.2	5.3±3.8	4.5±3.1
	+ fenoterol 10 μm	679±310	10.1±9.3	26.5±23.9
Polarized	Basal	455±347	0.4±0.1	14.2±15.8
	α-CD3/α-CD28	10213±7803	84.4±88.8	506±316
	+ PGE2 10 μm	10018±4050	58.3±37.8	400±241
	+ fenoterol 10 μm	10200±4020	79.4±42.5	499±385

Freshly isolated T cells or polarized Th cells were left unstimulated or stimulated by α-CD3/α-CD28 in the presence and absence of 10 μM PGE_2 or fenoterol for 6–8 h IFN-γ protein was measured in supernatant from freshly isolated T cells and polarized Th1 cells. IL-4 and IL-5 proteins were measured in supernatant from freshly isolated T cells and polarized Th2 cells. Results arc expressed as means±s.e.m., $n=8$. ND: not detectable.

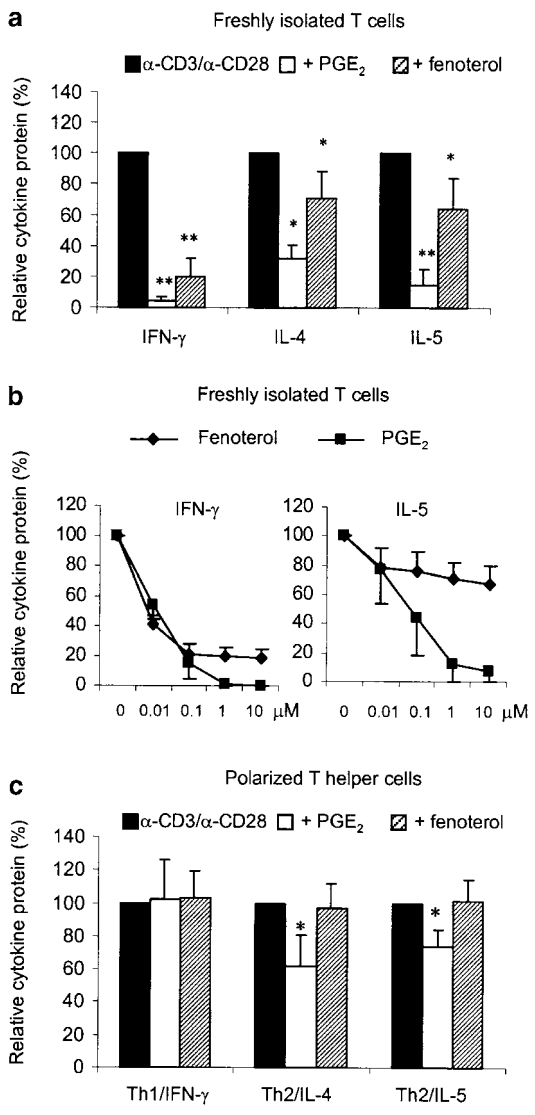

Figure 1 (a) Th1 and Th2-like cytokine production is significantly inhibited by cAMP elevating agents in freshly isolated T cells. T lymphocytes were isolated from healthy donors and rested overnight. PGE_2 and fenoterol were added in a concentration of 10 μM and cells were subsequently stimulated with α-CD3/α-CD28 for 8 h. IFN-γ, IL-4 and IL-5 protein secretion were measured in cell-free supernatants. Protein levels are expressed as percentage ($x \pm$s.e.m., $n=8$) of the secretion after stimulation with α-CD3/α-CD28 in the absence of cAMP elevating agents. $^{*}P<0.05$ and $^{**}P<0.001$ for the cytokine secretion levels after preincubation with cAMP elevating agents compared to the level without these agents. (b) Dose–response curves for PGE_2 and fenoterol on the secretion of IFN-γ and IL-5. Freshly isolated T cells were incubated with different concentrations of PGE_2 or fenoterol and subsequently stimulated with α-CD3/α-CD28 for 8 h. IFN-γ, IL-4 and IL-5 protein secretions were measured in cell-free supernatants. Protein levels are expressed as percentage ($x \pm$s.e.m., $n=4$) of the secretion after stimulation with α-CD3/α-CD28 in absence of cAMP elevating agents. (c) Cytokine production in polarized Th1 and Th2 cells is not under strict control of cAMP elevating agents. Polarized Th1 and Th2 cells were rested overnight in RPMI medium containing 5% FCS and stimulated for 6 h with α-CD3/α-CD28. PGE_2 and fenoterol were added prior to stimulation, in a concentration of 10 μM. IFN-γ, IL-4 and IL-5 proteins were measured in supernatants. Protein levels are expressed as percentage ($x \pm$s.e.m., $n=8$) of the secretion after stimulation with α-CD3/α-CD28 in the absence of cAMP elevating agents. $^{*}P<0.05$ for the cytokine secretion levels after preincubation with cAMP elevating agents compared to the level without these agents.

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 $\pm$ 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 10 μM PGE_2 (from 100 to $4 \pm 3\%$, $P<0.001$, $n=8$) and to a smaller extent by 10 μM of short-acting β_2-agonist fenoterol (from 100 to $20 \pm 12\%$, $P<0.001$, $n=8$) 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 10 μM PGE_2 in these cells (inhibition from 100 to 32 ± 9 and $15 \pm 10\%$, respectively, $P<0.001$). Fenoterol of 10 μM moderately, but significantly, reduced IL-4 and IL-5 secretion (from 100 to 71 ± 17 and $64 \pm 20\%$, respectively, $P<0.03$, Figure 1a). Dose-dependent effects of fenoterol and PGE_2 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 PGE_2 compared to the secretion of IL-5, whereas IFN-γ production was still significantly inhibited by 10 nm fenoterol and PGE_2 ($P<0.01$). IL-5 was only significantly inhibited when fenoterol and PGE_2 were used in higher concentrations (from 100 nm to 10 μM).

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 PGE_2 and fenoterol (10 μM) 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 β_2-adrenoceptor with fenoterol (10 μM). In contrast, the secretion of IL-4 and IL-5 protein was still modestly, but significantly, inhibited by 10 μM PGE_2 (from 100 to 62 ± 19 and $74 \pm 10\%$, $P<0.05$ 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 PGE_2 and fenoterol (10 μM) clearly induced CREB phosphorylation (Figure 2), the effect of PGE_2 being most pronounced. In contrast, in Th1 cells, no phosphorylation of CREB could be observed upon stimulation with PGE_2 and fenoterol. These results suggest that the defective regulation of cytokine production by fenoterol and PGE_2 in Th1 cells is because of impaired activation of the cAMP downstream pathway. In Th2 cells, PGE_2 was able to induce a clear increase in CREB phosphorylation, whereas fenoterol only slightly enhanced the levels of phosphorylated CREB. Thus, the ability of PGE_2 to activate the cAMP-dependent pathway is impaired in Th1 cells and still intact in Th2 cells, whereas β_2-adrenergic activation of this pathway appears to be disturbed in both Th1 and Th2 cells. To study if this β_2-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, PGE_2 could still enhance CREB phosphorylation. These data suggest that β_2-adrenergic hyporesponsiveness may develop by polyclonal activation of T cells during polarization.

Impaired formation of intracellular cAMP by PGE_2 in Th1 cells

To study in more detail the defects of PGE_2 and β_2-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 PGE_2 (10 μM) induced a significant increase in cAMP production in both freshly isolated T cells and polarized Th2 cells (fold increase 263 ± 71 and $208 \pm 49\%$, $P<0.05$ and $P<0.01$, respectively), but not in Th1 cells ($110 \pm 41\%$, Figure 3b). Thus, these data show a difference in PGE_2 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 PGE_2 receptor and/or β_2-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,

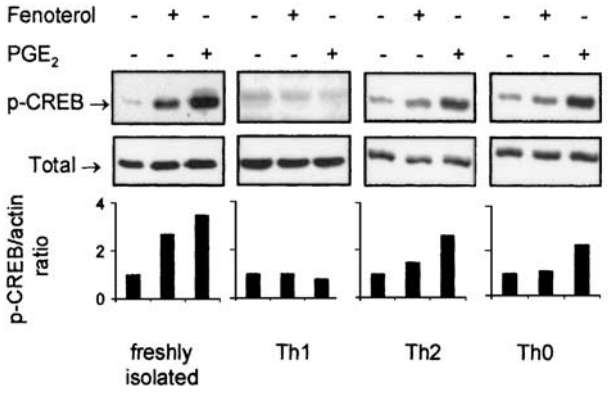

Figure 2 CREB phosphorylation in the presence of fenoterol and PGE_2 in freshly isolated T cells and T cells polarized under Th1, Th2 and Th0 conditions. T cells were stimulated with 10 μM PGE_2 or 10 μM fenoterol for 60 min. Total cell lysates were prepared and phosphorylated CREB was detected by Western blotting. Phospho-CREB is depicted in the upper panel and total protein levels (actin) are shown in the lower panel (marked by arrows). The corresponding diagram shows the relative phospho-CREB values after normalisation for actin. Results shown are representative of three independent experiments.

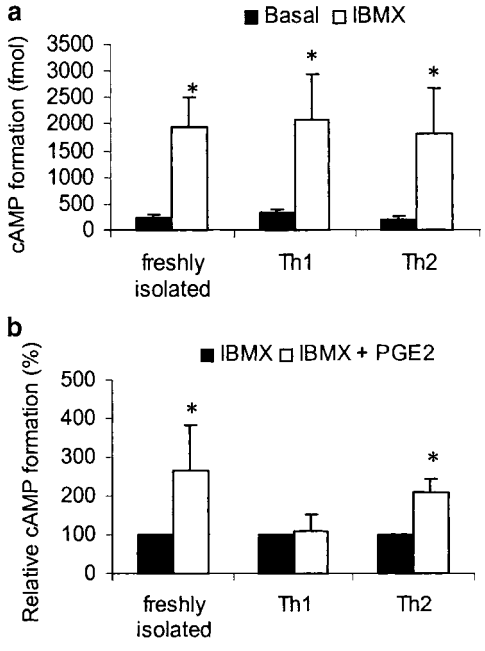

Figure 3 (a) Incubation with IMBX strongly enhances cAMP accumulation in Th1, Th2 and freshly isolated T cells. Basal intracellular cAMP accumulation (fmol) and cAMP accumulation in the presence of IBMX (100 μM) was measured in freshly isolated T cells and polarized Th1 and Th2 cells. Values are presented as the means $\pm$ s.e.m. of four independent experiments. $^*P<0.001$ for the values with IBMX compared to the values without IBMX. (b) PGE_2 enhances cAMP formation in freshly isolated T cells and polarized Th2 cells, but not in polarized Th1 cells. cAMP accumulation was induced by PGE_2 (10 μM) in the presence of IBMX and measured in freshly isolated T cells and Th1 and Th2 cells. cAMP levels are expressed as percentage ($x \pm$ s.e.m., $n=4$) of the formation in absence of PGE_2. $^*P<0.05$ for the values with PGE_2 compared to the values without PGE_2.

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 G_s 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 G_s protein by NaF resulted in strong induction of CREB phosphorylation. This indicates that the defective induction of CREB phosphorylation by PGE_2 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 α-CD3/α-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 PGE_2 and/or β_2-agonist fenoterol in polarized Th cells is because of impaired activation of the AC system and not differential regulation of cytokine production.

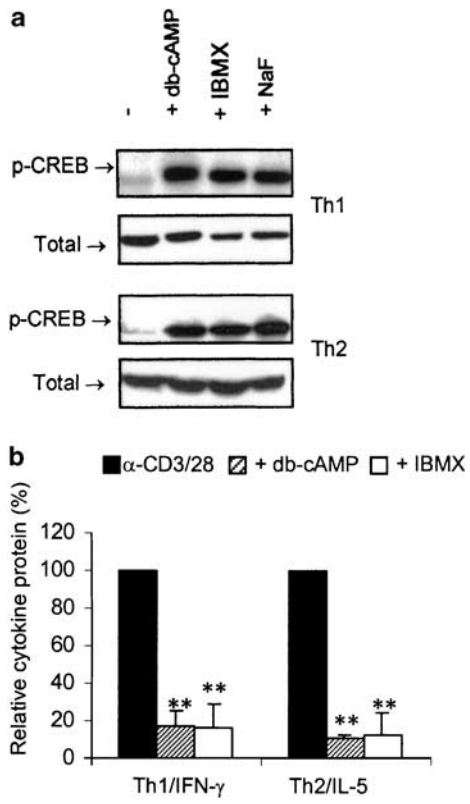

Figure 4 (a) CREB phosphorylation is induced by cAMP elevating agents in polarized Th1 and Th2 cells. T cell subsets were stimulated with db-cAMP, IBMX or NaF for 60 min. Total cell lysates were prepared and phosphorylated CREB was detected by Western blotting. Phospho-CREB is depicted in the upper panel and total protein levels (actin) are shown in the lower panel (marked by arrows). Results shown are representative of three independent experiments. (b) Cytokine production in polarized Th1 and Th2 cells is under strict control of potent activators of the cAMP-dependent pathway. Polarized Th1 and Th2 cells were cultured overnight in RPMI medium containing 5% FCS and stimulated for 6 h with α-CD3/α-CD28. IBMX or db-cAMP was added prior to stimulation, in a concentration of 100 μM, 0.5 and 10 mM respectively. IFN-γ and IL-5 protein secretions were measured in cell-free supernatants. Protein levels are expressed as percentage ($x \pm$ s.e.m., $n = 3$) of the secretion after stimulation with α-CD3/α-CD28 in the absence of cAMP elevating agents. $^*P < 0.05$ for the cytokine secretion level after preincubation with cAMP elevating agents compared to the level without these agents.

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

We were interested in the possible mechanisms involved in the desensitization of the PGE_2 and/or fenoterol effects in polarized Th1 and Th2 cells. Desensitization of the β_2-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 G_s 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, β_2AR mRNA was clearly expressed in both Th1 and Th2 subsets (Figure 5a). Thus, downregulation of β_2AR mRNA is not likely the cause of desensitization of the β_2-adrenergic system in Th1 and Th2 cells. GRK's, as well as PKA and protein kinase C (PKC) are able to phosphorylate the β_2-AR (Meurs *et al*., 1987; Hausdorff *et al*., 1990). Enhanced expression and activation of βARK are known to induce β_2-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, α-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

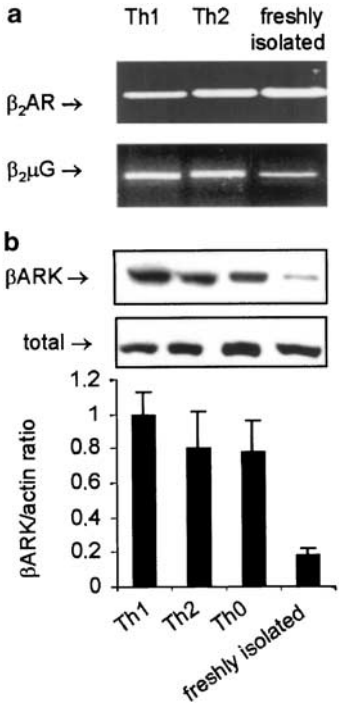

Figure 5 (a) β_2AR mRNA is clearly expressed in Th1 and Th2 cells. RNA was isolated from polarized Th1 and Th2 cells and total cellular RNA was analyzed by RT – PCR. In the upper panel, the β_2AR signal is shown. The $\beta_2\mu$G signal in the lower panel shows that comparable amounts of product were amplified for each condition. Results shown are representative of two independent experiments. (b) Expression of βARK is strongly enhanced in polarized Th1, Th2 and Th0 cells compared to freshly isolated T cells ($n=3$). Expression of βARK in polarized Th1 and Th2 cells was analyzed by Western blotting. βARK is depicted in the upper panel and actin is depicted in the lower panel. A representative blot is shown. The βARK levels in Th1, Th2, Th0 and freshly isolated T cells were normalized for actin and mean relative βARK values $\pm$ s.e.m. of four different experiments are depicted in the corresponding diagram.

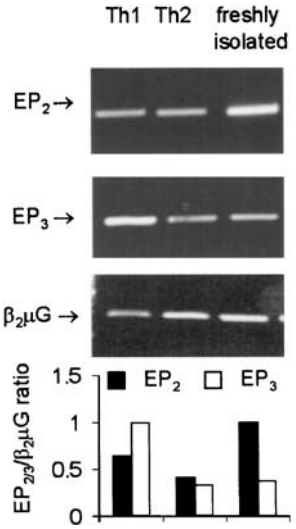

Figure 6 Altered expression of the EP_2 and EP_3 subtypes of the PGE_2 receptor in Th1 and Th2 cells compared to freshly isolated T cells. RNA was isolated from polarized Th1 and Th2 cells and total cellular RNA was analyzed by RT – PCR. In the upper panel, the EP_2 signal shows reduced expression in Th1 and Th2 cells compared to freshly isolated T cells. In the middle panel, the EP_3 signal shows enhanced expression in Th1 cells compared to Th2 cells and freshly isolated T cells. The $\beta_2\mu$G signal is depicted in the lower panel. The corresponding diagram shows the relative EP_2 and EP_3 mRNA values after normalization for $\beta_2\mu$G. Results shown are representative of three independent experiments.

cells during polarization might induce an increase in βARK expression, which in turn might be involved in the loss of β_2-adrenergic control.

Altered levels of PGE_2 receptor subtypes in polarized T helper cells

In addition to β_2-adrenergic unresponsiveness, polarized Th1 were unresponsive to PGE_2. Although, the mechanism of β_2AR desensitization has been widely described, little information is available about desensitization and internalization of the PGE_2 receptor. It is known that PGE_2 can exert differential effects through activation of different subtypes of the receptor. At least four different subtypes are known, that is, the EP_1, EP_2, EP_3 and EP_4 subtypes. It has been described that EP_4 is susceptible to agonist-promoted internalization, whereas the EP_2 receptor is resistant. EP_2 and EP_4 are known to be coupled to the G_s protein and induce AC activation. On the other hand, activation of EP_1 induces Ca^{2+} accumulation, while the EP_3 subtype preferentially couples to G_i, 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 PGE_2 receptor could be involved in the altered PGE_2 signaling in polarized T helper cells, we measured the mRNA expression of the G_s-coupled EP_2 subtype and the G_i-coupled EP_3 subtype of the PGE_2 receptor using RT – PCR. As demonstrated in Figure 6, the expression of EP_2 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 PGE_2 that was observed in both Th1 and Th2 cells compared to freshly isolated T cells; yet, this does not explain the difference of PGE_2 reactivity in Th1 and Th2 cells. As described above, PGE_2 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 G_s-coupled EP_2, subtype, the expression of the G_i-coupled EP_3 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 G_s protein is known to result in activation of the AC/cAMP system, while activation of the G_i protein is known to inhibit this system, the altered ratio in EP_2 and EP_3 expression in Th1 cells might be an explanation for the net zero effect of PGE_2 on cAMP production in Th1 cells (see Figure 3b). Thus, differential expression of subtypes of the PGE_2 receptor might be involved in the unresponsiveness of Th1 cells to PGE_2.

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, PGE_2. 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 α-CD3/α-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 is more susceptible to cAMP inhibition than Th2 cytokine production, polarized Th2 cells appear to be more sensitive to PGE_2 than polarized Th1 cells. PGE_2 was able to induce an increase in cAMP production and CREB phosphorylation in Th2 cells, while PGE_2 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 PGE_2 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 PGE_2 secreting cells.

In contrast to the differential effects of PGE_2 in Th1 and Th2 subsets, we found reduced responsiveness to the β_2-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 β_2-mimetics, for instance in asthma, may not efficiently inhibit proinflammatory T-cell activity. Several mechanisms may be responsible for the desensitization of the β_2-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 β_2AR was clearly expressed in polarized Th1 cells and Th2 cells. Thus, a functional uncoupling of the β_2AR and PGE_2 receptor from the G_s 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_s protein and finally internalization of the receptor (Gu *et al*., 2000). Enhanced expression and activation of βARK may induce β_2-adrenergic hyporesponsiveness (Lohse *et al*., 1992). It has been demonstrated that overexpression of βARK leads to hyporesponsiveness of the β_2-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 β_2-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 β_2-agonists. In this way, T cells may become less sensitive to circulating β_2-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 PGE_2 receptor. It has been demonstrated that the EP_4 subtype of the PGE_2 receptor is susceptible to agonist-promoted internalization, whereas the EP_2 subtype receptor is resistant (Penn *et al*., 2001). EP_4 desensitization can occur by a similar mechanism as β_2-adrenergic desensitization, involving βARK and β-arrestin. Therefore, a role for enhanced βARK expression in PGE_2 unresponsiveness in Th1 cells cannot be excluded, although it is not clear why Th2 cells, expressing high levels of βARK, were still responsive to PGE_2. We show that another possible mechanism involved in reduced responsiveness to PGE_2 might be the altered balance in EP_2/EP_3 mRNA expression. EP_2 mRNA was reduced in both Th1 and Th2 subsets compared to freshly isolated T cells, whereas EP_3 mRNA expression was only enhanced in Th1 cells. Thus, the reduced expression of EP_2 could explain why Th2 cells are less responsive to PGE_2 than freshly isolated T cells, while the additional upregulation of mRNA for the G_i-coupled EP_3 receptor in Th1 cells may be responsible for the complete unresponsiveness to PGE_2 in these cells. It is known that activation of the G_i protein by PGE_2 and β_2-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 PGE_2 receptors to the G_i 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 G_i counteracts the effects induced by activation of the G_s protein. It still remains unclear as to what causes the Th1 specific upregulation of EP_3 mRNA. Incubation of T cells with the Th1-directing cytokine IL-12 alone for prolonged periods did not result in reduced ability of PGE_2 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

EP_3 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_s 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 PGE_2 and the β_2-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 β_2-agonist or PGE_2 secreted by surrounding tissue cells.

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