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Regulation of IL-13 synthesis in human lymphocytes: implications for asthma therapy

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1 IL-13 is an important mediator in inflammatory diseases such as asthma. IL-13 is mainly produced by T cells. However, signalling pathways leading to induction of this cytokine are not well-characterized. We analysed the regulation of IL-13 in human peripheral blood mononuclear cells and $CD4^+$ T cells.

2 Cyclosporine (CsA) and FK-506 inhibited IL-13 synthesis, when cells were stimulated by TPA/ionomycin. However, stimulation by α-CD3/α-CD28 led to an enhanced IL-13 synthesis.

3 NF-κB inhibitor N-tosyl-L-lysine chloromethylketone (TLCK) inhibited IL-13 synthesis more effectively after TPA/ionomycin stimulation. After α-CD3/α-CD28 stimulation, only 300 μM TLCK inhibited IL-13 synthesis. Dexamethasone inhibited IL-13 equally effective after α-CD3/α-CD28 and TPA/ionomycin stimulation.

4 p38 MAPK inhibitor SB203580 inhibited IL-13 synthesis only partially. MEK inhibitor U0126 inhibited TPA/ionomycin induced IL-13 synthesis very effectively, whereas α-CD3/α-CD28 stimulated IL-13 induction was resistant to this drug.

5 These results were confirmed in purified $CD4^+$ T cells. In difference to PBMCs α-CD3/α-CD28 stimulated IL-13 synthesis was effectively inhibited by CsA, FK-506 and U0126.

6 Therefore U0126 was tested in an animal model of allergic asthma. We could demonstrate for the first time that inhibition of the MEK–ERK cascade is a therapeutic option for asthma. Intraperitoneal administration of 10 mg kg^{-1} U0126 reduced lung eosinophilia in ovalbumin-challenged Brown Norway rats by 44%.

7 These results demonstrate that different signalling pathways are involved in regulating IL-13 synthesis in primary human T cells. Characterizing highly potent inhibitors of IL-13 synthesis can be exploited to identify new drugs to treat immunological diseases such as asthma.
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Keywords: IL-13; T-cell activation; cyclosporine; FK-506; dexamethasone; TLCK; U0126; SB203580

Abbreviations: BAL, bronchoalveolar lavage fluid; CsA, cyclosporine A; ERK, extracellular signal-regulated kinase; IL, interleukin; JNK, jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; OVA, ovalbumine; PBMC, peripheral blood mononuclear cells; PKC, protein kinase C; TCR, T-cell receptor; TLCK, N-tosyl-L-lysine chloromethylketone; TPA, 12-O-tetradecanoylphorbol-13-acetate; VCAM, vascular cell adhesion molecule

Introduction

IL-13 is a cytokine that is produced in large quantities by appropriately stimulated $CD4^+$ Th2 cells (Brubaker & Montaner, 2001). It has a variety of effects that are relevant to asthma and other Th2-dominated inflammatory disorders, including the ability to induce IgE production, CD23 expression, and endothelial cell VCAM-1 expression (Brombacher, 2000; Corry, 1999). IL-13 and IL-4 have overlapping effector profiles. This overlap is at least partially due to the shared use of receptor components in the multimeric IL-13 and IL-4 receptor complexes (Vita *et al*., 1995). The IL-13 receptor complex is composed of at least three distinct components, including the IL-4α receptor, the low-affinity binding chain IL-13Rα1, and the high-affinity binding chain IL-13Rα2 (Brubaker *et al*., 2001). However, recent *in vivo* studies using different infection and asthma models suggest that IL-13 possesses many important functional activities that are distinct from IL-4. These differences comprise the different ability of these cytokines to drive the differentiation of naive cord blood T cells to a Th2 phenotype, to support the *in vitro* proliferation of activated human or mouse T cells, to regulate prostaglandin biosynthesis, contribute to nematode expulsion, regulate epithelial electrolyte secretion, prolong eosinophil survival, and stimulate eosinophil chemotaxis (Endo *et al*., 1998; Horie *et al*., 1997; Sornasse *et al*., 1996; Urban *et al*., 1998; Zund *et al*., 1996). In addition, IL-13 and IL-4 are produced by different cells and are differentially regulated by mediators such as IFNα (Kaser *et al*., 1998) and transcription factors such as NF-AT (Feske *et al*., 2000).

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Increased production of IL-13 is well documented in extrinsic and intrinsic asthma, atopic dermatitis, allergic rhinitis, and chronic sinusitis (Humbert *et al*., 1997). Asthma is a disease characterized by the infiltration of eosinophils and lymphocytes into airway epithelium and subsequent epithelial damage and tissue remodelling (Busse & Lemanske, 2001). T helper (Th) 2 cells and their cytokine products play a crucial role in this process (Kay, 2001). IL-13 is one of the important Th2-type cytokines which have been implicated in asthma in which they are up-regulated (Huang *et al*., 1995). Recent studies indicate that IL-13 can induce pathologic changes reminiscent of asthma in animals, including infiltration of eosinophils and mononuclear cells, epithelial damage, hyperplasia of goblet cells, and subepithelial fibrosis (Wills-Karp *et al*., 1998). IL-13 probably plays important roles as a mucus-stimulating cytokine as well as in the recruitment of eosinophils. Endothelial cell expression of VCAM-1, an adhesion molecule involved in eosinophil recruitment, has been shown to be induced by IL-13 (Doucet *et al*., 1998). A recent study reported that the inhibition of IL-13 in a mouse model leads to a reduced asthmatic response (Gruenig *et al*., 1998).

IL-13 is mainly produced by T cells. In contrast to IL-2 and IL-4, signalling pathways leading to induction of IL-13 are poorly understood. Furthermore, most studies focusing on T-cell activation use T cell clones or transformed T cell lines, of which the signal transduction pathways may be altered and are largely uncoupled from proliferation (Hughes & Pober, 1996).

Three intracellular pathways have been demonstrated to be important for T-cell activation. Phosphorylation of the T-cell receptor complex allows the association of several 'adaptor' molecules, leading to several signalling cascades (Weiss & Littman, 1994). One of these cascades (reviewed in Crabtree & Clipstone (1994)) involves phosphorylation and activation of PLCγ1, which cleaves phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 1,4,5-trisphosphate. The latter activate PKC and trigger an increase in the concentration of intracellular calcium respectively. The first pathway is initiated by this rapid and sustained increase in $[Ca^{2+}]_i$ resulting in the activation of the calmodulin/calcium-dependent phosphatase calcineurin; this phosphatase regulates IL-2 gene transcription by dephosphorylating the cytoplasmic form of NF-AT and allowing its translocation into the nucleus (Clipstone & Crabtree, 1992; Wesselborg *et al*., 1996).

The second signalling pathway results in the activation of AP-1 (reviewed in Foletta *et al*. (1998)), which binds the IL-2 promoter both directly and as a component of an NF-AT complex (Jain *et al*., 1993). AP-1 is a heterodimer of Fos and Jun family proteins (Foletta *et al*., 1998). It has been established that regulation of c-*fos* gene transcription is mediated by the nuclear factor Elk-1, which is phosphorylated and activated by *ras*-dependent signal cascades involving ERK and JNK (Price *et al*., 1996). On the other hand, c-*jun* is also regulated by JNK for which it appears to be a direct substrate (Derijard *et al*., 1994). ERK1/2, JNK, and another pathway involving the kinase p38, which represent parallel kinase cascades initiated at the *ras* level, are commonly termed MAP kinase pathways (Su & Karin, 1996). In human T-cells, at least two MAP kinases, ERK-1 and ERK-2, are activated through the *ras* pathway in response to occupancy of the TCR (Izquierdo *et al*., 1993). Activation of ERK1/2 is induced by phosphorylation mediated by MEK-1/2 (Cobb & Goldsmith, 1995), the activity of which is itself regulated through phosphorylation by a MAP kinase kinase kinase; the serine/threonine protein kinase Raf-1, which couples p21 *ras* and interacts with MEK-1 to form a ternary signalling complex (Jelinek *et al*., 1994), appears to be this MAP kinase kinase kinase (Leevers *et al*., 1994). Altogether, these results provide a clear and convincing proof demonstration that MEK-1 and ERKs function in conveying stimulatory signals to the IL-2 gene.

Thirdly, activation of NF-κB is important for T-cell activation (Liou *et al*., 1999). This cascade is initiated by PKC. Recently, PKCθ has been identified as the prime PKC isoform in the signalling cascade after TCR activation (Bauer *et al*., 2000; Coudronniere *et al*., 2000; Lin *et al*., 2000). This PKC in turn activates NF-κB *via* small G-proteins *ras* and Raf-1 (Altman *et al*., 2000).

Here we describe the regulation of IL-13 in primary human peripheral leukocytes. By using pharmacological inhibitors, we traced important signalling pathways leading to IL-13 production in primary human cells. Finally, we used a selective MAPK inhibitor to demonstrate the importance of IL-13 production in an animal model of allergic asthma.

Methods

Reagents

Oligonucleotides were synthesized by TIB Molbiol (Berlin, Germany). DMSO, TPA, ionomycin, dexamethasone, TLCK and Histopaque-1077 were from Sigma, (Deisenhofen, Germany). CsA, FK-506 and U0126 were from CALBIOCHEM (San Diego, CA, U.S.A.). SB203580 was from BIOMOL Research Labs., Inc. (Plymouth Meeting, U.S.A.). Purified anti-human CD3 and purified anti-human CD28 were from PharMingen Becton Dickinson Co. (Heidelberg, Germany). RPMI 1640 medium was from Life Technologies (Heidelberg, Germany). $CD4^+$ T Cell Isolation Kit was from Miltenyi Biotec (Bergisch Gladbach, Germany). Unless otherwise indicated, all other chemicals were purchased from the Sigma Chemical Co (Deisenhofen, Germany).

Preparation of PBMC

Buffy coats from healthy human volunteers were obtained from the Erlangen Blood Bank. PBMC were isolated by density gradient centrifugation over Histopaque 1077 (Sigma, Deisenhofen, Germany), washed twice in Hanks buffer (Life Technologies, Heidelberg, Germany) and resuspended in RPMI 1640 medium supplemented with 10% foetal calf serum (Boehringer Mannheim, Penzberg, Germany).

Preparation of $CD4^+$ T cells

PBMC were incubated with hapten-antibody cocktails and MACS anti-hapten microbeads. ($CD4^+$ T cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells were isolated by negative selection on VS+ columns using high gradient magnetic cell separation system MACS

(Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purities of populations were assessed by flow cytometry with over 90% (FACScan, Becton Dickinson, Heidelberg, Germany). Purified $CD4^+$ T cells were resuspended in RPMI 1640 medium supplemented with 10% FCS.

Cell culture

For cytokine production, PBMC were resuspended at 10^6 cells ml^{-1} and incubated in 500 μl aliquots in 24-well tissue culture plates (Falcon Becton Dickinson Labware, Heidelberg, Germany) at 37°C, 5% CO_2. After preincubation with test substances for 30 min, cells were stimulated with soluble α-CD3 mAb (1 μg ml^{-1}), anti-CD3 mAb (1 μg ml^{-1}) plus α-CD28 mAb (0.3 μg ml^{-1}), TPA (25 ng ml^{-1}), ionomycin (1 μM) or TPA (25 ng ml^{-1}) plus ionomycin (1 μM). At the indicated times, cells were sedimented by centrifugation, the supernatants were harvested and kept frozen at −80°C until cytokine protein determination; the cells were lysed by RLT lysis buffer (Qiagen, Hilden, Germany) and frozen at −80°C until RNA isolation.

Enzyme-linked immunosorbent assay

Cytokine measurements in culture supernatants were done by sandwich ELISA using matched antibody pairs (BD Pharmingen, Heidelberg, Germany). ELISA plates (Maxisorb, Nunc) were coated overnight with anti-IL-13 mAb in 0.1 M carbonate buffer, pH 9.5. After being washed, plates were blocked with Assay Diluent (Pharmingen, Heidelberg, Germany) for 1 h and washed again. Appropriately diluted supernatant samples and standards were distributed in duplicates and the plates were incubated for 2 h at room temperature. Plates were washed, incubated for 1 h with working detector (biotinylated anti-IL-13 Ab and Avidin-horseradish peroxidase conjugate). After washing, substrate (TMB and hydrogen peroxide) was added. The reaction was stopped by addition of 1 M H_3PO_4. Plates were read at 450 nm (reference 570 nm) in a microplate reader (Dynatech). The results were expressed as a percentage of the control level of cytokines production by cells stimulated in the presence of the vehicle of the corresponding compound.

Analysis of cytokine mRNA expression by real-time RT–PCR

RNA was prepared from frozen lysates using Rneasy (Qiagen, Hilden, Germany). One-tube RT–PCR was performed using TaqMan EZ RT–PCR Kit from PE Applied Biosystems (Weiterstadt, Germany). Expression of cytokines were determined in relation to beta-actin by real time RT–PCR using TaqMan assay on a ABI Prism 7700. Primers and probes are: β-Actin forward: 5′-CAG CGG AAC CGC TCA TTG CCA ATG G; β-Actin reverse: 5′-TCA CCC ACA CTG TGC CCA TCT ACG A; β-actin probe: 5′-(6FAM)-ATG CCC (TAMRA)T CCC CCA TGC CAT CCT GCG T; IL-13 forward: 5′-GGA GCT GGT CAA CAT CAC CC; IL-13 reverse: 5′-CGT TGA TCA GGG ATT CCA GG; IL-13 probe: 5′-(6FAM)-CCAGAAGGC-(TAMRA)-TCCGCTCTGCAATGGC.

Rat IL-13 was determined using Quantitect SYBR Green RT–PCR Kit from QIAGEN (Hilden, Germany) using the forward primer GTGGCCCTCAGGGAGCTTAT and the reverse primer CTGTCAGGTCCACGCTCCAT. Quantity of mRNA was calculated using the $\Delta\Delta C_T$ method (PE Applied Biosystems User Bulletin #2; ABI PRISM 7700 Sequence Detection System, 1997). For each RT–PCR the threshold cycle (C_T) was determined, being defined as the cycle at which the fluorescence exceeds 10 times the standard deviation of the mean baseline emission for cycles 3 to 10. IL-13 mRNA levels were normalized to the housekeeping gene β-actin according to the following formula: $\Delta C_T = C_T^{\beta\text{-actin}} - C_T^{\text{IL-13}}$. Subsequently, respective IL-13 mRNA levels were calculated using the $\Delta\Delta C_T$ method, i.e., ΔC_T values representing mRNA from cells treated with stimulus in combination with a test compound were set in relation to the ΔC_T value representing mRNA levels from cells treated with stimulus alone according to the following formula: $\Delta\Delta C_T = \Delta C_T$ (drug) $- \Delta C_T$ (vehicle). The relative mRNA level for the respective test compound was calculated as $2^{-\Delta\Delta C_T}$*100% based on the results of control experiments with an efficiency of the PCR reaction of approximately 100%.

Late phase eosinophilia

Male Brown–Norway rats weighing 180–230 g were used. Animals were purchased from Moellegaard Breeding & Research Centre A/S, Skensved, Denmark. The animals were kept under constant environmental conditions (temperature: 22±2°C, humidity: 40–60%, light cycle: 0700–1900). They had free access to standardized food pellets (purchased from Spezialdiäten GmbH, Soest, Westfalen, Germany) and tap water. All animal studies were performed in accordance with the national animal protection rules and permitted by the local governmental authority (Regierungspräsidium Dresden, Germany).

Brown–Norway (BN) rats were actively sensitized by subcutaneous injections of ovalbumin mixed with $Al(OH)_3$ gel and i.p. Bordetella pertussis vaccine on days 1, 14 and 21. On day 28, the animals were used for experiments. Compounds as suspension in tylose, were given intraperitoneally 2 h prior to challenge. Then the rats were exposed to an ovalbumin-containing aerosol in a nose-only inhalation system (TSE GmbH, Bad Homburg, Germany) for 1 h to provoke an influx of inflammatory cells into the airways. Vehicle-treated control animals were sensitized and exposed to saline aerosol. At the time of maximal influx of eosinophilic granulocytes into the airways (48 h later), animals were sacrificed by an urethane overdose and a bronchoalveolar lavage (BAL) was performed by three times 4 ml Hank's balanced solution. The number of eosinophils as well as the total cell number from the pooled BAL samples were counted 48 h post challenge using a haemocytometer (Technicon H1E, Bayer Diagnostics GmbH, Munich, Germany). Each group of animals treated with compounds was compared with vehicle (tylose)-treated saline-challenged and ovalbumin-challenged control groups.

Data analysis

Data are expressed as means±s.e.mean. Significant differences were statistically analysed by the unpaired Student's *t*-test and by ANOVA. IC_{50} values were calculated using the

computer program PRISM 3.0 (GraphPad Software Inc., San Diego, CA, U.S.A.).

Results

Induction of IL-13 in human PBMCs

We compared the stimulation of human PBMCs by different mitogenic agents and antibodies directed against surface receptors all leading to the production of IL-13. For our studies, we developed a real-time RT–PCR method for accurate quantitation of IL-13 mRNA levels (see Methods). Whereas no IL-13 mRNA was detectable in unstimulated cells, each stimulus or combination thereof tested caused an increase of IL-13 mRNA levels in adult blood leukocytes (Figure 1). α-CD3 alone was a poor stimulus, whereas its combination with α-CD28 or ionomycin was much more efficient. α-CD28 alone was insufficient to induce IL-13. Similarly, TPA alone induced hardly detectable levels of IL-13, whereas the combination with ionomycin gave rise to the highest level of IL-13. Combination of two stimuli increased the mRNA levels more than the additive effect of the correspondent single stimuli. IL-13 protein levels in the supernatant correlated with the mRNA levels measured by RT–PCR (Figure 1; $r^2 = 0.94$, $P < 0.0001$).

Time course of IL-13 induction

α-CD3/α-CD28 as a physiologic and TPA/ionomycin as a mitogenic stimulus were chosen to study the time course of IL-13 mRNA induction. As can be seen from Figure 2, TPA/ionomycin induced mRNA levels rapidly peaking at 4 h following a rapid decline to baseline levels. Protein levels follow similar kinetics while the peak is shifted to 8 h. In contrast, IL-13 mRNA after antigen receptor stimulation increased more slowly than with TPA/ionomycin but remained close to peak levels for the whole period. Similarly, IL-13 protein levels started to increase from 24 h onwards and reached much higher levels compared to IL-13 protein induced by TPA/ionomycin.

Effects of inhibitors of the calcium→NF-AT pathway (CsA and FK-506)

Three intracellular pathways have been demonstrated to be important for T-cell activation. One depends on the release of intracellular calcium leading to the subsequent activation of calcineurin and NF-AT. Therefore, we raised the question as to whether IL-13 induction in human leukocytes is sensitive to CsA and FK-506, two specific calcineurin inhibitors. First, we analysed the dose response on IL-13 induced by stimulation with TPA/ionomycin. CsA inhibits mRNA as well as protein induction in the low nanomolar range (Figure 3a). The dose-response curve for mRNA is steeper than for the protein. FK-506 also inhibits IL-13 induction, but this drug is about 30 fold more potent than CsA (Figure 4a). If the cells are stimulated by α-CD3/α-CD28, the cytokines respond quite differently (Figures 3b and 4b). Under these conditions IL-13 is stimulated by CsA and FK-506. This effect was seen for mRNA as well as for the secreted protein. Again, only 30 fold lower concentrations of FK-506 are needed to obtain similar effects compared to CsA.

Effects of NF-κB inhibitors

The activation of NF-κB *via* PKC has also been shown to be important for T cell activation (Trushin *et al*., 1999). We therefore investigated whether the proteasome inhibitor TLCK, which has been shown to inhibit activation of NF-κB by inhibiting the degradation of I-κB, affects IL-13 induction. As shown in Figure 5a, TPA/ionomycin-induced IL-13 is inhibited by TLCK at low micromolar concentrations. In contrast, TLCK inhibited α-CD3/α-CD28 induction of cytokines only at concentrations above 100 μM (Figure 5b).

Steroids have been shown to interfere with NF-κB activation at several stages. Therefore, we compared the

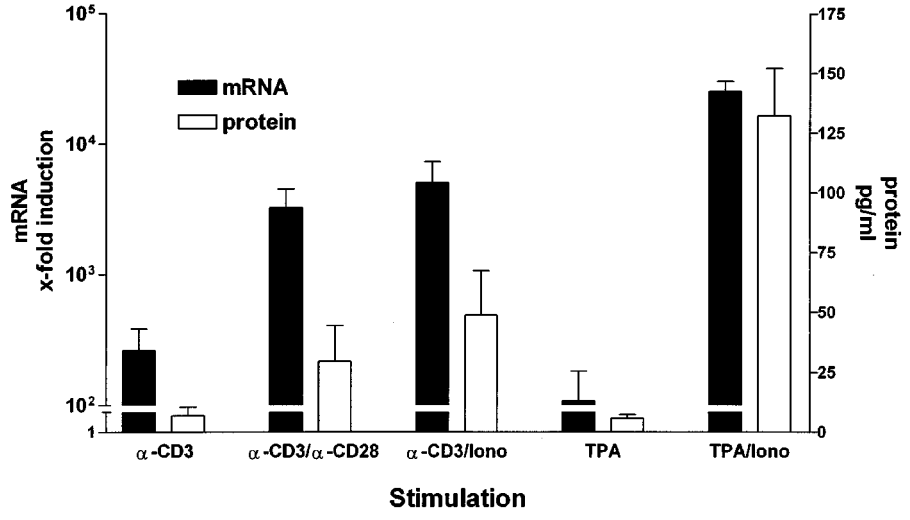

Figure 1 Induction of IL-13 in healthy donors by different stimuli. PBMCs were stimulated for 4 and 24 h. IL-13 mRNA level were determined using real-time RT–PCR after 4 h. Levels were normalized to β-actin and unstimulated cells were set to 1. IL-13 protein was determined in the supernatant by ELISA after 24 h. Each column represents mean±s.e.mean of three different volunteers. Similar results were obtained in three independent experiments.

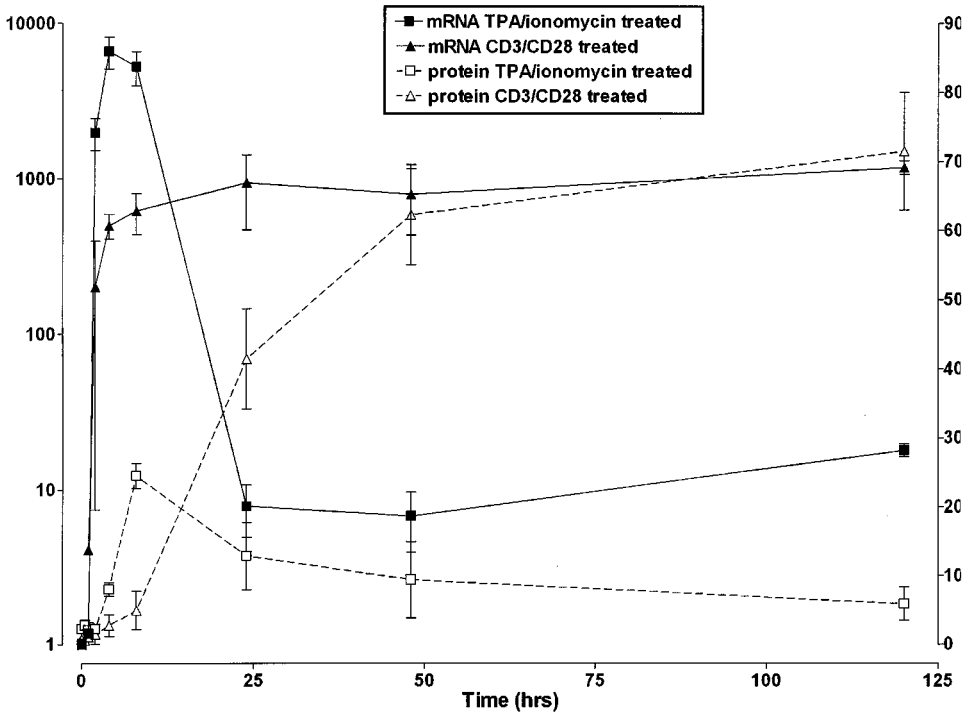

Figure 2 Time course of IL-13 induction. PBMCs were stimulated by TPA/ionomycin and α-CD3/α-CD28 respectively and harvested after different time points. IL-13 mRNA level were determined using real-time RT–PCR. Levels were normalized to β-actin and unstimulated cells were set to 1. IL-13 protein was determined in the supernatant by ELISA. Each point and bar represents mean ± s.e.mean of three different volunteers. Similar results were obtained in three independent experiments.

effect of dexamethasone with that of TLCK (Figure 6). It inhibited IL-13 induced by TPA/ionomycin in low nanomolar concentrations (Figure 6a). Furthermore, dexamethasone inhibited α-CD3/α-CD28 mediated IL-13 induction with similar effectiveness (Figure 6b).

Effects of the MAPK inhibitors

It has been shown that different MAP kinases are involved in signalling events downstream of the TCR. We analysed the effects of two inhibitors of two different MAP kinases. SB 203580 is a specific inhibitor of the p38 MAP kinase pathway. It did not inhibit TPA/ionomycin-induced IL-13 synthesis at concentrations up to 10 μM (Figure 7a). Higher concentrations have been reported to cause unspecific inhibition of other kinases (Lali *et al*., 2000). Only α-CD3/α-CD28 induced IL-13 was inhibited to some extent at 10 μM (Figure 7b). U0126 is a specific inhibitor of the MEK–ERK pathway. It inhibited IL-13 dose dependently after stimulation by TPA/ionomycin (Figure 8a). In contrast, after stimulation with α-CD3/α-CD 28, this compound inhibited IL-13 synthesis only at the highest concentration of 10 μM.

Comparison of IC_{50} with $CD4^+$ T cells

To compare the different compounds, we calculated IC_{50}s from the experiments shown above where possible (Table 1). There was no apparent dose-dependent inhibition by SB 203580 and TLCK to calculate IC_{50} values for these compounds. The IC_{50}s for CsA and FK-506 after TPA/ionomycin stimulation are very similar for mRNA and protein. In sharp contrast, IL-13 is stimulated by these calcineurin inhibitors after α-CD3/α-CD28 stimulation. Dexamethasone is effective for both stimuli with similar affinity. In contrast the MAPK inhibitor, U0126 seems to inhibit the IL-13 protein release more effectively than the mRNA induction for both stimulation conditions. Since PBMC are a heterogeneous cell population, we also determined IC_{50}s for these compounds in purified human $CD4^+$ T cells. Due to the smaller amount of cells available, we focused on the mRNA level. Whereas the inhibition by CsA and FK-506 was similar for TPA/ionomycin, the stimulatory effect for α-CD3/α-CD28 stimulation was abolished in $CD4^+$ T cells. The IC_{50}s are similar to the IC_{50}s for TPA/ionomycin stimulation. Dexamethasone showed similar IC_{50}s for PBMCs and $CD4^+$ T cells at both stimulation conditions. In contrast, for U0126 we observed at least 4 fold lower IC_{50}s in $CD4^+$ T cells after TPA/ionomycin stimulation. Furthermore, α-CD3/α-CD28 stimulated IL-13 synthesis, resistant in PBMCs, was very effectively inhibited by U0126 in $CD4^+$ T cells.

Effect of U0126 in a rat model of asthma: late-phase eosinophilia

The finding that U0126 very effectively inhibits IL-13 in $CD4^+$ T cells prompted us to investigate whether this inhibitor may be active in an animal model of allergic asthma. Brown Norway rats were actively sensitized to ovalbumin and challenged by inhalation of ovalbumin. The inhibition by U0126 compared to CsA of the influx of eosinophils into the lung is shown in Table 2. Intraperitoneally administration of U0126 inhibited dose-dependently the accumulation of eosinophils in the tracheoalveolar lumen. The higher dose of 10 mg kg^{-1} significantly inhibited

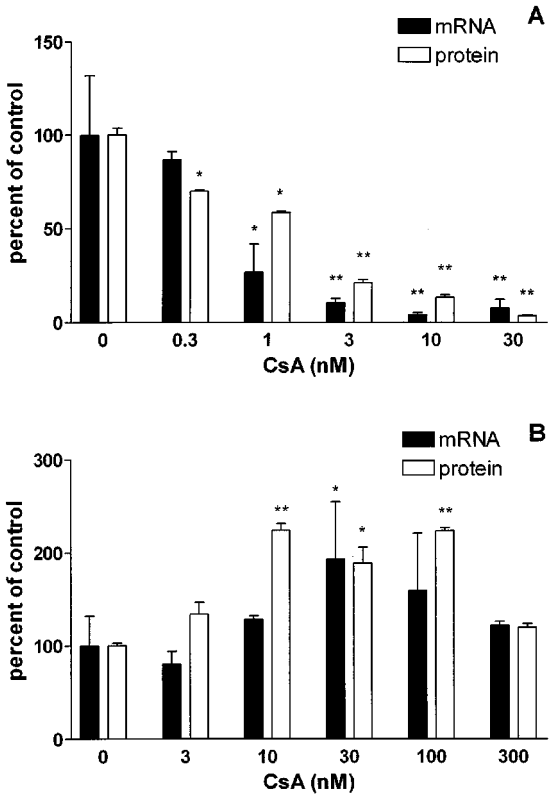

Figure 3 Effect of CsA on IL-13 induction. PBMCs were pre-incubated with different doses of CsA for 30 min. Then cells were stimulated either with TPA/ionomycin (**a**) or with α-CD3/α-CD28 (**b**) for 4 h for mRNA determination and 24 h for protein determination respectively. Cytokine mRNA level were determined using real-time RT–PCR and were normalized to β-actin. Protein was determined in the supernatant by ELISA. Stimulated cells treated with DMSO were used as 100% control. Each column represents mean ± s.e.mean of three replicate measurements of one donor. This experiment is one representative for three different donors. $*P<0.05$, $**P<0.01$ (vs control).

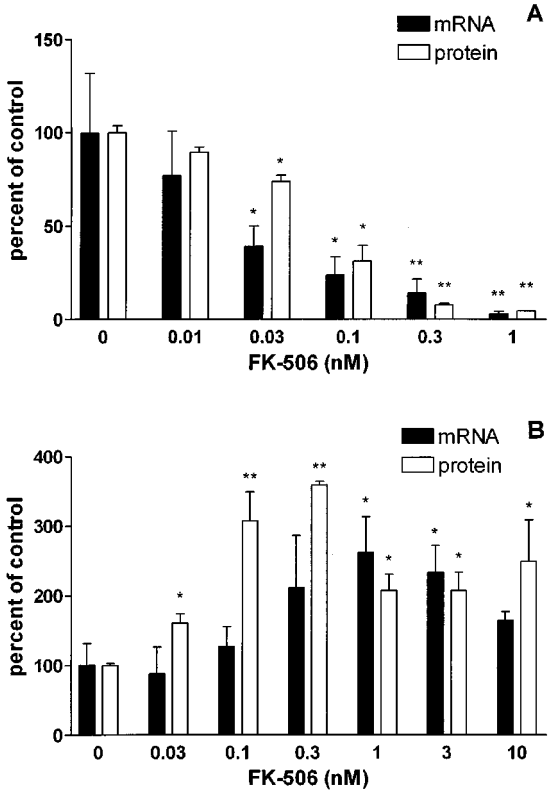

Figure 4 Effect of FK-506 on IL-13 induction. PBMCs were preincubated with different doses of FK-506 for 30 min. Then cells were stimulated either with TPA/ionomycin (**a**) or with α-CD3/α-CD28 (**b**) for 4 h for mRNA determination and 24 h for protein determination respectively. Cytokine mRNA level were determined using real-time RT–PCR and were normalized to β-actin. Protein was determined in the supernatant by ELISA. Stimulated cells treated with DMSO were used as 100% control. Each column represents mean ± s.e.mean of three replicate measurements of one donor. This experiment is one representative for three different donors. $*P<0.05$, $**P<0.01$ (vs control).

eosinophil influx (Table 2). The reference compound CsA also reduced the presence of eosinophils in the BAL. Its IC_{50} was about 3 mg kg^{-1}. Since the reduction of eosinophils may be due to a mechanism different to the inhibition of IL-13, we analysed expression of IL-13 in BAL *in vivo*. As shown in Figure 9, BAL cells from OVA-sensitized rats produced IL-13 in response to OVA. Production of IL-13 in BAL was significantly reduced in mice treated with CsA 30 mg kg^{-1} and U0126 10 mg kg^{-1}.

Discussion

IL-13 is an important mediator of immunological diseases such as asthma. In this study, we have examined the induction and regulation of IL-13 in human PBMCs and $CD4^+$ T cells. Different combinations of mitogenic and surface receptor stimulating antibodies were able to induce IL-13 synthesis. TPA/ionomycin caused most efficient stimulation of IL-13 both at mRNA and protein level. α-CD3 and α-CD28 stimulation increased IL-13 mRNA and protein more slowly than did TPA/ionomycin. We investigated the effects of pharmacological agents inhibiting three different signalling cascades on IL-13 induced by α-CD3/α-CD28 or TPA/ionomycin: (1) the calcineurin inhibiting immunosuppressants CsA and FK-506; (2) NF-κB inhibitors TLCK and dexamethasone; (3) MAPK inhibitors SB 203580 and U0126. Using these drugs, we were able to elucidate signalling pathways leading to IL13 synthesis. After comparing the effects of these drugs on PBMCs with pure human $CD4^+$ T cells, we found that U0126 was at least 10 times more potent in inhibiting IL-13 in $CD4^+$ T cells compared to PBMCs. Finally, we tested this drug in an animal model of asthma in which U0126 was able to reduce the influx of eosinophils into the lungs of sensitized and challenged rats.

The combination of two different stimulating agents increased the IL-13 levels more when compared to the additive effect of the corresponding single stimuli. This indicates a synergistic effect on T cell activation as has been observed for other cytokines such as IL-2 as well (Truneh *et al*., 1985; van Lier *et al*., 1988). IL-13 protein levels in the supernatant correlated very well ($r^2=0.94$) with mRNA levels excluding post-transcriptional effects during the induction of IL-13. The combination of TPA and ionomycin caused the highest level of IL-13. In contrast, TPA alone is a poor stimulus. It has been shown that TPA is also insufficient to

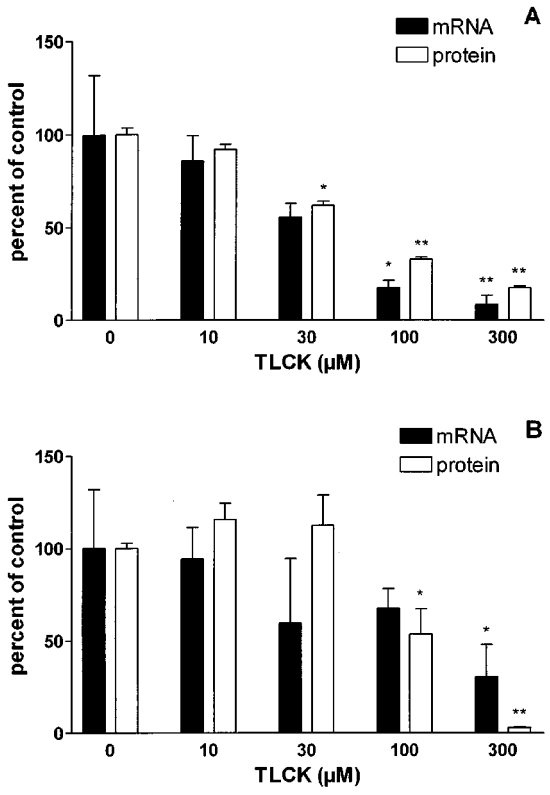

Figure 5 Effect of TLCK on IL-13 induction. PBMCs were preincubated with different doses of TLCK for 30 min. Then cells were stimulated either with TPA/ionomycin (**a**) or with α-CD3/α-CD28 (**b**) for 4 h for mRNA determination and 24 h for protein determination respectively. Cytokine mRNA level were determined using real-time RT–PCR and were normalized to β-actin. Protein was determined in the supernatant by ELISA. Stimulated cells treated with DMSO were used as 100% control. Each column represents mean ± s.e.mean of three replicate measurements of one donor. This experiment is one representative for three different donors. $*P<0.05$, $**P<0.01$ (vs control).

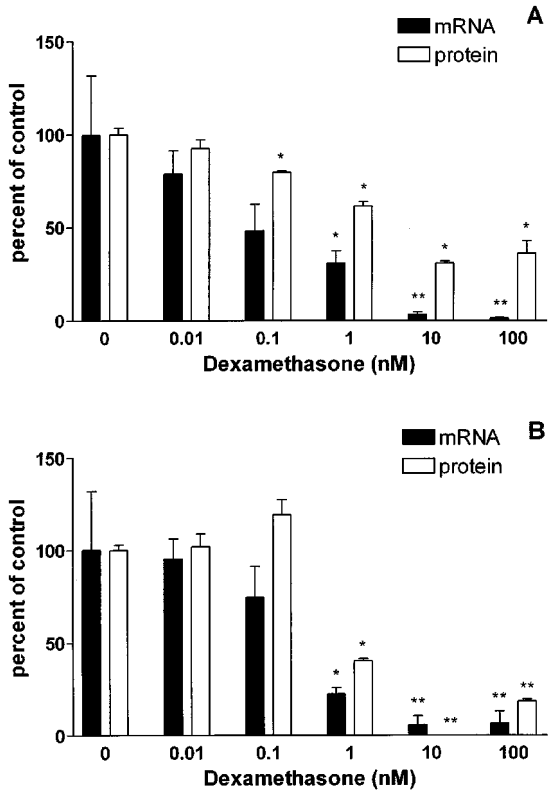

Figure 6 Effect of dexamethasone on IL-13 induction. PBMCs were preincubated with different doses of dexamethasone for 30 min. Then cells were stimulated either with TPA/ionomycin (**a**) or with α-CD3/α-CD28 (**b**) for 4 h for mRNA determination and 24 h for protein determination respectively. Cytokine mRNA level were determined using real-time RT–PCR and were normalized to β-actin. Protein was determined in the supernatant by ELISA. Stimulated cells treated with DMSO were used as 100% control. Each column represents mean ± s.e.mean of three replicate measurements of one donor. This experiment is one representative for three different donors. $*P<0.05$, $**P<0.01$ (vs control).

stimulate IL-4 synthesis in T cells (Boothby *et al*., 2001). In contrast, ionomycin is a very potent IL-4 inducer (Paliogianni *et al*., 1996) whereas it was insufficient to induce IL-13 (not shown). In contrast, using ionomycin together with α-CD3 proved to be very potent, reaching similar levels to those attained by stimulation with α-CD3/α-CD28.

Time course experiments revealed that TPA/ionomycin induced a rapid and transient increase of IL-13 mRNA and protein levels. With TPA/ionomycin, IL-13 mRNA peaked at 4 h, whereas the protein peak occurred at 8 h. Stimulation with α-CD3/α-CD28 caused a much slower increase which remained at high levels throughout the observation period. Similar kinetics have been observed for IL-2 (Herold *et al*., 1986).

CsA and FK506 strongly inhibited IL-13 induced by TPA/ionomycin both at mRNA and protein level. However, these substances enhanced gene expression and protein production after stimulation with α-CD3/α-CD28. The enhancing effect of CsA on IL-13 synthesis after this kind of stimulation has been reported earlier for a drug concentration of 100 ng ml^{-1} (van der Pouw Kraan *et al*., 1996). Dumont, (1997) reports a stimulatory effect of FK-506 on PBMCs stimulated by α-CD3/α-CD28. We also observe a stimulatory effect of FK-506 at similar concentrations. Since both drugs cause the same effect, the common target of CsA and FK-506, calcineurin, probably mediates this effect. Supporting this hypothesis, we found that rapamycin, while binding to the same receptor as FK-506, does not enhance IL-13 levels (data not shown). Surprisingly, this stimulation could not be observed in pure human $CD4^+$ T cells. In these cells, CsA and FK-506 were effective inhibitors. These findings lead to the hypothesis that $CD8^+$ T cells, NK cells or B cells are responsible for the induction of IL-13 after CsA or FK-506 treatment and stimulation with α-CD3/α-CD28. In contrast, the calcium→calcineurin→NF-AT pathway seems to be important for induction of IL-13 in $CD4^+$ T cells. IL-2 stimulation with α-CD3/α-CD28 has been reported to be resistant to CsA (June *et al*., 1987). IFNγ production is also enhanced by CsA after α-CD3/α-CD28 stimulation (Rafiq *et al*., 1998). Testing a single dose of FK-506 Dumont *et al*. (1998b) reported after α-CD3/α-CD28 stimulation the enhancement of IFNγ, IL-5 and GM-CSF synthesis and the inhibition of IL-2, IL-3 and IL-4 synthesis by this drug. These results indicate that α-CD3/α-CD28 stimulation activates a number of different signalling pathways of which only some are necessary for activation of cytokines such as IL-2, IFNγ and IL-13.

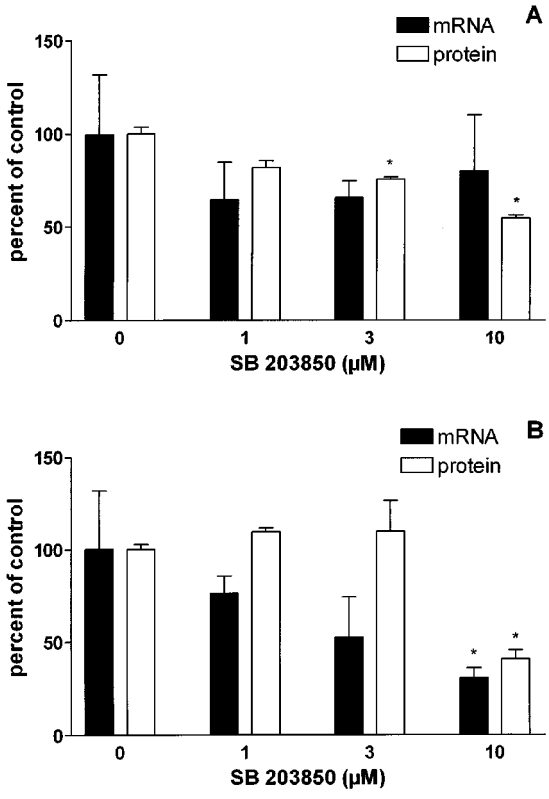

Figure 7 Effect of SB 203580 on IL-13 induction. PBMCs were preincubated with different doses of SB 203580 for 30 min. Then cells were stimulated either with TPA/ionomycin (**a**) or with α-CD3/α-CD28 (**b**) for 4 h for mRNA determination and 24 h for protein determination respectively. Cytokine mRNA level were determined using real-time RT–PCR and were normalized to β-actin. Protein was determined in the supernatant by ELISA. Stimulated cells treated with DMSO were used as 100% control. Each column represents mean ± s.e.mean of three replicate measurements of one donor. This experiment is one representative for three different donors. $*P<0.05$, $**P<0.01$ (vs control).

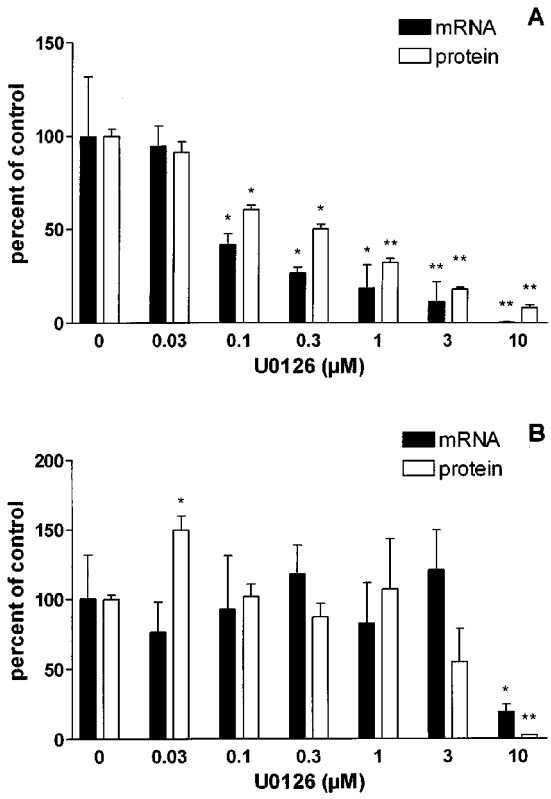

Figure 8 Effect of U0126 on IL-13 induction. PBMCs were preincubated with different doses of U0126 for 30 min. Then cells were stimulated either with TPA/ionomycin (**a**) or with α-CD3/α-CD28 (**b**) for 4 h for mRNA determination and 24 h for protein determination respectively. Cytokine mRNA level were determined using real-time RT–PCR and were normalized to β-actin. Protein was determined in the supernatant by ELISA. Stimulated cells treated with DMSO were used as 100% control. Each column represents mean ± s.e.mean of three replicate measurements of one donor. This experiment is one representative for three different donors. $*P<0.05$, $**P<0.01$ (vs control).

Table 1 IC_{50} values for the inhibition of IL-13 mRNA and protein synthesis by different drugs

	PBMC		*CD4*$^+$	*PBMC*		*CD4*$^+$
	TPA/Ionomycin		*TPA/Ionomycin*	*α-CD3/α-CD28*		*α-CD3/α-CD28*
Stimulation	*mRNA*	*Protein*	*mRNA*	*mRNA*	*Protein*	*mRNA*
CsA (nM)	0.80 ± 0.46	3.69 ± 2.70	0.23 ± 0.01	stimulation	stimulation	2.18 ± 1.28
FK-506 (nM)	0.045 ± 0.004	0.05 ± 0.03	0.045 ± 0.010	stimulation	stimulation	0.180 ± 0.014
Dexamethasone (nM)	1.46 ± 0.37	1.02 ± 0.83	1.20 ± 0.02	2.30 ± 2.24	0.89 ± 0.14	0.54 ± 0.41
U0126 (nM)	812 ± 1065	515 ± 189	200.6 ± 100.5	> 5000	646 ± 364	63 ± 57

Data shown are mean IC_{50} ± s.e.mean of at least three different donors.

TLCK is a proteasome inhibitor which inhibits the NF-κB activation *via* the inhibition of I-κB degradation (Kim *et al*., 1995). It inhibited TPA/ionomycin induced IL-13 effectively but only at highest concentration after stimulation with α-CD3/α-CD28. Our findings that TPA is an efficient IL-13 stimulus and that TLCK inhibits this induction is consistent with reports describing the activation of NF-κB by TPA in T cells (Lin *et al*., 2000; Sun *et al*., 2000). Stimulation with α-CD3/α-CD28 also activates NF-κB at least *via* PKCs (Lin *et al*., 2000; Sun *et al*., 2000). The resistance of this kind of stimulation to TLCK hints at activation of further signalling cascades leading to NF-κB activation compared to stimulation by TPA/ionomycin.

Dexamethasone was also used as an NF-κB inhibitor, because two of its major mechanisms are induction of I-κB and direct inhibition of NF-κB (Dumont *et al*., 1998a). Nevertheless, other mechanisms of action have been reported (Beato & Klug, 2000). This is supported by our study: dexamethasone was able to inhibit α-CD3/α-CD28-induced IL-13 synthesis whereas TLCK could not. Dexamethasone

Table 2 Inhibition of late phase eosinophilia in Brown–Norway rats

Substance	*Dose* (mg kg^{-1})	*% Inhibition* *x*
U0126	5	18
	10	44*
Cyclosporin A	1	12
	5	70*
	30	99*

Actively sensitized rats were challenged by ovalbumin inhalation. Compounds were given intraperitoneally 2 h prior to ovalbumin challenge. After 48 h, numbers of eosinophils in broncheoalveolar lavage were determined. Vehicle-treated animals were set to 100%, x = mean of at least five animals. *$P<0.05$ as compared to vehicle-treated animals.

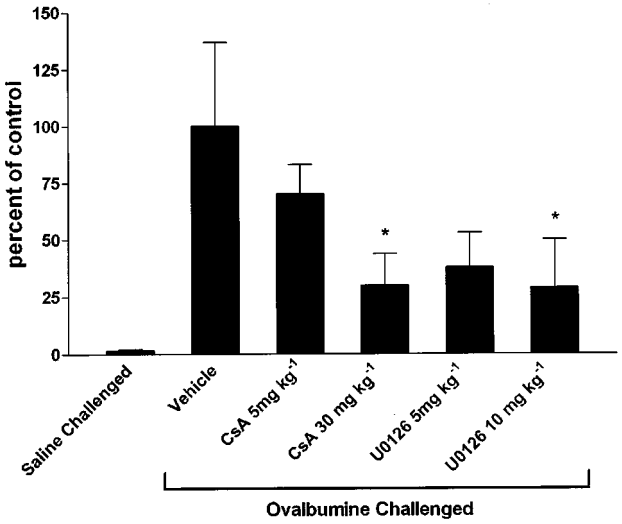

Figure 9 Effect of U0126 on IL-13 production in BAL cells. Actively sensitized rats were challenged by ovalbumin or saline inhalation. Compounds were given intraperitoneally 2 h prior to ovalbumin challenge. After 48 h, cells were recovered from broncheoalveolar lavage by centrifugation and RNA was prepared from the cell pellet. IL-13 mRNA level were determined using real-time RT–PCR and were normalized to β-actin. OVA challenged vehicle-treated animals were set to 100%. Each column represents mean ± s.e.mean of at least five animals. *$P<0.05$ (versus OVA challenged vehicle control).

seems to inhibit further signalling pathways induced by this kind of stimulation. The transcription of IL-2 is inhibited by dexamethasone *via* the inhibition of AP-1 (Paliogianni *et al*., 1993). Stimulation by α-CD28 has been reported to activate a CD28RE in the IL-2 promoter. Conflicting results have been published concerning transcription factors binding to this element (Harhaj & Sun, 1998; Kempiak *et al*., 1999). It is most likely that binding of transactivators to this element is inhibited by dexamethasone, which explains its inhibitory effect on both kinds of stimulation. The IC_{50} for dexamethasone is similar for PBMCs and $CD4^+$ T cells, indicating that the mechanism in the two cell systems is similar.

Three different kinds of MAPKs have been described in mammalian cells. These are ERK, JNK and p38 kinase (Su *et al*., 1996). ERK, JNK and p38 pathways have all been shown to play a critical role in the events leading to activation and increased IL-2 production in T cells stimulated by TPA/ionomycin or α-CD3/α-CD28 (DeSilva *et al*., 1997; Su *et al*., 1996; Whitehurst & Geppert, 1996). Unfortunately, no JNK inhibitor is publicly available. Previous studies of p38 MAP kinase in Jurkat human T cells line (Matsuda *et al*., 1998), human purified T cell (Koprak *et al*., 1999), $CD4^+$ subset (Schafer *et al*., 1999) and mouse T cell clones (Zhang *et al*., 1999) clearly demonstrated the involvement of p38 MAP kinase in the cell activation through TCR and CD28 costimulation signal pathways. However, little is known about this MAP kinase in human primary peripheral blood leukocytes. In $CD45RO^+$ T cells the p38 inhibitor SB 203580 inhibited only IL-4 production whereas IL-2 was unaffected (Schafer *et al*., 1999). We observed that SB 203580 inhibited IL-13 gene expression by 40% in human PBMC stimulated with TPA/ionomycin or α-CD3/α-CD28. Therefore, IL-13 production is largely p38-independent but this kinase is required for maximal expression. Koprak *et al*. (1999) reported that SB 203850 inhibited IL-13 only on protein level, but had no effect on mRNA level. This difference is most likely due to different experimental protocols and the use of a different T cell population.

U0126 is a specific MEK kinase inhibitor. MEK phosphorylates ERK and by this U0126 is a specific inhibitor of the ERK pathway. We determined the influence of this drug on IL-13 gene expression and protein production. Our results indicate that U0126 inhibited IL-13 very potently after stimulation with TPA/ionomycin. In contrast, stimulation by α-CD3/α-CD28 was resistant to U0126. Inhibition at 10 μM may be due to non-specific actions on other kinases. Only a few studies have been published about the effect of this drug on other cytokines. At 10 μM, it inhibited IL-2 mRNA induction in a murine T cell clone (DeSilva *et al*., 1998). Surprisingly, investigation of pure human $CD4^+$ T cells revealed that in these cells U0126 was 4 fold more effective in inhibiting IL-13 synthesis after TPA/ionomycin stimulation. Furthermore, α-CD3/α-CD28 stimulated IL-13 synthesis, resistant in PBMCs to this drug, was highly sensitive to U0126 in these cells. These results indicate that the MEK→ERK pathway plays a pivotal role in regulating IL-13 production after TPA/ionomycin and α-CD3/α-CD28 stimulation in human $CD4^+$ T cells. PBMCs have to contain other cells which are responsible for the resistance to this drug. Hence these cells must possess partially different IL-13 signalling pathways.

The high sensitivity of $CD4^+$ T cells to U0126 in respect of IL-13 synthesis prompted us to analyse this drug in an animal model of asthma. It effectively reduced the induction of IL-13 mRNA in BAL cells and the influx of eosinophils into the lungs of sensitized and challenged rats. Only inhibitors of p38 MAPK have been analysed in asthma models so far. The effect of the p38 inhibitor SB 203580 was recently tested in the same animal model. No influence on antigen-induced airway eosinophilia could be observed (Escott *et al*., 2000). In another study, a second generation p38 inhibitor was found to be effective (Underwood *et al*., 2000). CsA was a little more effective in reducing airway eosinophilia, but compared to U0126 the affinity of CsA to its receptor is approximately 100 fold higher. Therefore U0126 is more effective on a molecular basis. Although there is no doubt that immunosuppressants such as FK506 and CsA are effective in asthma

treatment, they cannot find a broad practice due to their unacceptable side effects (Corrigan *et al*., 1996; Khan *et al*., 2000; Lock *et al*., 1996; Mori *et al*., 2000; Sperr *et al*., 1997). These agents interfere with multiple T cell functions, thereby causing generalized immunosuppression. An agent capable of selectively regulating cytokine synthesis with little effect on other major T cell cytokines would provide an ideal treatment for inflammation without severe side effects including general immunosuppression.

In conclusion, regulation of IL-13 in T cells differs from that of other cytokines such as IL-2 and IL-4. The calcium→calcineurin→NF-AT pathway is important for TPA/ionomycin – as well as α-CD3/α-CD28-induced IL-13 synthesis. NF-κB is only important after induction by TPA/ionomycin. The MAP kinase ERK is important for both stimuli while p38 MAPK activation is dispensable. The different regulation can be exploited to find new specifically targeted drugs aimed for diseases where IL-13 plays an important pathophysiological role. We could demonstrate for the first time that inhibition of the MEK – ERK cascade is a therapeutic option for asthma. The specific MEK inhibitor U0126 reduced the influx of eosinophils in an animal model of asthma. The development of more specific IL-13-targeted drugs would support this approach to treating diseases such as asthma.

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