

## Original articles

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## DIFFERENTIAL RESPONSES OF HEMATOPOIETIC AND NON-HEMATOPOIETIC CELLS TO ANTI-INFLAMMATORY CYTOKINES: IL-4, IL-13 and IL-10

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Recombinant preparations of human anti-inflammatory cytokines: IL-4, IL-13 and IL-10, inhibited LPS-induced synthesis of TNF $\alpha$  and IL-6 in the whole human blood tested *in vitro*. These cytokines also inhibited LPS-induced IL-6 and TNF mRNA accumulation in isolated human blood monocytes/macrophages. On the other hand, similar concentrations of IL-4 and IL-13 (but not IL-10) enhanced synthesis of IL-6 in cultured human umbilical vein endothelial cells (HUVEC). In human hepatoma HepG2 cells IL-4 and IL-13 (but not IL-10) inhibited IL-6-induced synthesis of haptoglobin. These differential responses to the tested anti-inflammatory cytokines were observed at mRNA and protein levels and may reflect cell specificities in signalling pathways and gene expression.

When HUVEC and HepG2 cells were cultured together and stimulated with LPS the addition of IL-4 or IL-13 resulted in the reduction of LPS-induced and IL-6-mediated haptoglobin synthesis. Thus in co-culture the inhibitory effects of IL-4 or IL-13 on HepG2 cells prevail over stimulation of IL-6 synthesis in HUVEC.

**Key words:** *macrophages, HepG2, HUVEC, IL-4, IL-13, IL-10, IL-6*

## INTRODUCTION

It is well established that IL-10, as well as IL-4 and related IL-13, inhibit synthesis of proinflammatory cytokines, such as TNF $\alpha$ , IL-1 and IL-6 in monocytes/macrophages (1,2,3). However, in distinction to hematopoietic cells, other cell types including fibroblasts (1), keratinocytes (4) or human endothelial cells (5) respond to IL-4 or IL-13 with increased IL-6 expression. IL-6 is crucial for regulating various aspects of acute phase response, including synthesis of acute phase proteins in liver cells (6). Thus, a question arises what are the real functions of IL-4, IL-13 or IL-10 in the complex cytokine network during the development of inflammatory processes. The aim of the present study was to compare the effects of these three cytokines on LPS-induced synthesis of TNF $\alpha$  and IL-6 in human blood cells and human umbilical vein endothelial cells (HUVEC), as well as on synthesis of some acute phase proteins regulated by IL-6 in human hepatoma HepG2 cells.

## MATERIALS AND METHODS

*Reagents*

Human recombinant IL-4, IL-13 and IL-10 were generously supplied by DNAX (Palo Alto, CA, USA) and human recombinant IL-6 by Dr. P.C. Heinrich (RWTH Aachen, Germany). Antisera to human haptoglobin and fibrinogen were purchased from Atlantic Antibodies (Stillwater, MN, USA). Cell culture media (DMEM, RPMI and M.199), fetal bovine serum, TRIZOL reagent, RT-PCR primers, H-Reverse transcriptase and Taq polymerase were from Gibco BRL (Grand Island, NY, USA). Human epidermal growth factor (EGF), human fibroblast growth factor (FGF), endotoxin (lipopolysaccharide from *E. coli* 026:B6), human serum AB and gentamycin were from Sigma (St. Louis, MO, USA). Labelled  $^{32}\text{P}\alpha\text{dCTP}$  was from ICN Pharmaceuticals Inc. (Irvine, CA, USA).

*Cell culture and assay of TNF, IL-6 and haptoglobin*

The whole heparinized blood obtained from healthy volunteers was diluted 1:4 with RPMI containing heparin (10 units/ml). Fresh samples of diluted blood (0.6 ml) were incubated in 24-well cluster plates (Nunc, Denmark) in 5%  $\text{CO}_2$  for 24 h. For cell stimulation 500 ng/ml LPS was added alone or with IL-4, IL-13 or IL-10 at concentrations ranging between 50 and 150 Units/ml. After incubation the diluted blood was collected, centrifuged and the supernatant used for measuring IL-6 or TNF $\alpha$  with commercially available ELISA kits (Genzyme, Cambridge, MA, USA) according to the manufacturer's instructions.

Monocytes/macrophages were isolated from peripheral blood mononuclear cells (PBMC) by the modified adherence method (7). Firstly, PBMC were isolated by standard Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) gradient centrifugation from heparin- or EDTA-treated blood from healthy donors. PBMC ( $1.5 \times 10^7$ ) were placed in 35 mm  $\phi$  tissue culture wells (Nunc, Denmark) in 2 ml of RPMI1640 supplemented with 10% human serum AB, 2 mM L-glutamine, and 50  $\mu\text{g}/\text{ml}$  gentamycin. After 18 hrs of incubation at 37°C in 5%  $\text{CO}_2$  humidified atmosphere the non-adherent cells were removed and the adherent cells were cultured for 7 days prior to the experiment. They were stimulated with 100 ng/ml LPS for 6 or 18 h and then total cellular RNA was isolated.

HUVEC cells were isolated from human umbilical vein by a typical procedure (8) and cultured in M.119 with 20% of FBS, the mixture of growth factors (EGF 10 ng/ml, FGF 10 ng/ml) and heparin (10 U/ml). For experiments the cells between 4<sup>th</sup> and 8<sup>th</sup> passages were used. The subconfluent monolayer of HUVEC seeded on 12-well cluster plates (for protein estimation), or on 100 mm dishes (for RNA isolation), was treated with LPS (200 ng/ml) or with the mixture of LPS + IL-4, LPS + IL-10 or LPS + IL-13 in M.199 medium without growth factors but containing 5% FBS. In all experiments anti-inflammatory cytokines were used in the concentration of 100 U/ml, unless indicated otherwise.

HepG2 cells (obtained from Dr. B. Knowles, Wistar Institute, Philadelphia, PA) cultured in DMEM were stimulated by interleukin-6 (40 ng/ml) to induce acute phase protein synthesis. The cells were cultured for 24 h with IL-6, or the mixture of IL-6 + IL-4, IL-6 + IL-10 or IL-6 + IL-13 in 6-well plates, and the amount of haptoglobin and fibrinogen released to the culture medium was estimated by rocket immunoelectrophoresis (9). In some experiments HepG2 cells were stimulated by the conditioned medium from HUVEC cultured with the tested cytokines. In experiments with an insert, HepG2 cells were seeded in the insert, cultured overnight in DMEM and the insert was transferred to the well already covered with HUVEC. After addition of the tested factors culture in M.199 was continued for 24 h.

*Northern blotting*

For RNA isolation HUVEC and HepG2 cells were cultured for 18 h in 100 or 60 mm dishes, respectively. Total RNA was prepared using the phenol extraction method and LiCl precipitation

(10). Electrophoresis was performed in 1% agarose and the separated RNA was transferred to Hybond-N membranes (Amersham, UK). Specific mRNAs were detected by hybridization with corresponding probes labelled with  $^{32}\text{P}$ -dCTP by random priming.

### Reverse transcriptase-PCR

Total cellular RNA was isolated from adherent monocytes ( $1.5 \times 10^6$  cells) using Trizol Reagent strictly following the manufacturer's instruction. Precipitated RNA was dissolved in 10  $\mu\text{l}$  of sterile, RNase-free water, and stored at  $-30^\circ\text{C}$  when necessary.

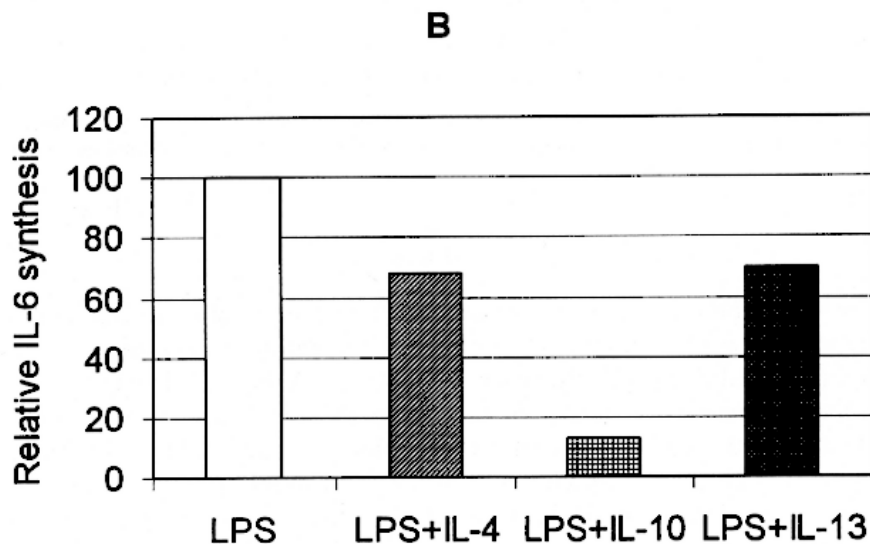
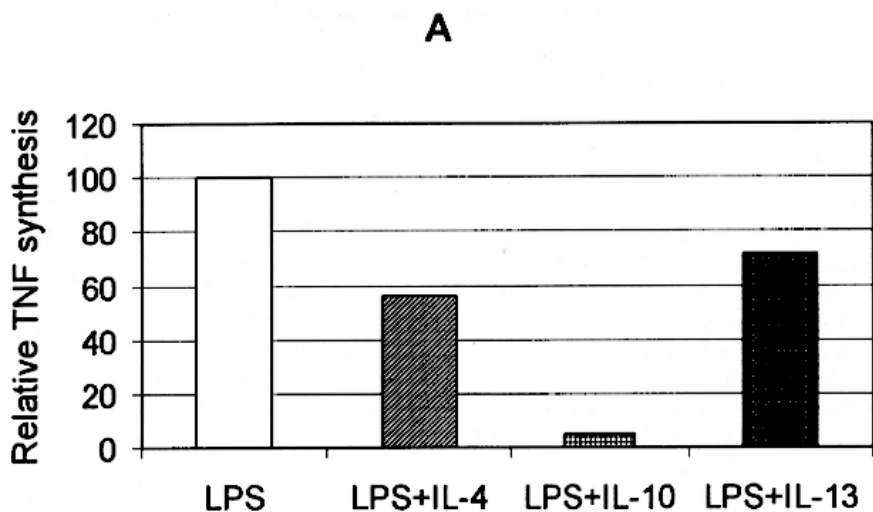
cDNA synthesis reactions were carried out in a total volume of 20  $\mu\text{l}$  containing 10  $\mu\text{l}$  of each RNA sample, 0.5  $\mu\text{g}$  oligo(dT)<sub>12-18</sub> primer and 200 U of SuperScript™ II RNase H-Reverse Transcriptase according to the protocol provided with the enzyme. PCRs were set up in a total volume of 50  $\mu\text{l}$  containing 2  $\mu\text{l}$  of the cDNA, 0.2 mM deoxynucleoside triphosphates, 0.5  $\mu\text{M}$  of each oligonucleotide primer, 50 mM KCl, 1.5 mM  $\text{MgCl}_2$ , and 2.5 U of Taq polymerase. PCRs were run in 0.5 ml tubes, in OmniGene thermal cycler (Hybaid, Teddington, UK) equipped with heated lid. IL-6 cDNA was amplified simultaneously with  $\beta$ -actin (duplex PCR) to standardise the signal. Reactions (30 cycles) were carried out according to the following thermal profile:  $94^\circ\text{C}$  for 30 s,  $60^\circ\text{C}$  for 60 s, and  $72^\circ\text{C}$  for 90 s. Each amplification was ended with final extension at  $72^\circ\text{C}$  for 15 min. The reaction products were then resolved on nondenaturing 2% agarose gel and visualized by staining with ethidium bromide. The following PCR primers were custom-synthesized by GibcoBRL:

$\beta$ -actin (sense) 5'-AGCGGGAAATCGTGCGTG-3',  
 (anti) 5'-GGGTACATGGTGGTGCCG-3';  
 IL-6 (sense) 5'-CTTTTGAGTTTGAGGTATACCTAG-3'  
 (anti) 3'-GCTGCGCAGAATGAGATGAGTTGTC-3'

All primers were designed to match sequences in separate exons to avoid the contribution of genome-templated product in the analyzed signal. The expected product lengths are: 307bp for  $\beta$ -actin and 237bp for IL-6.

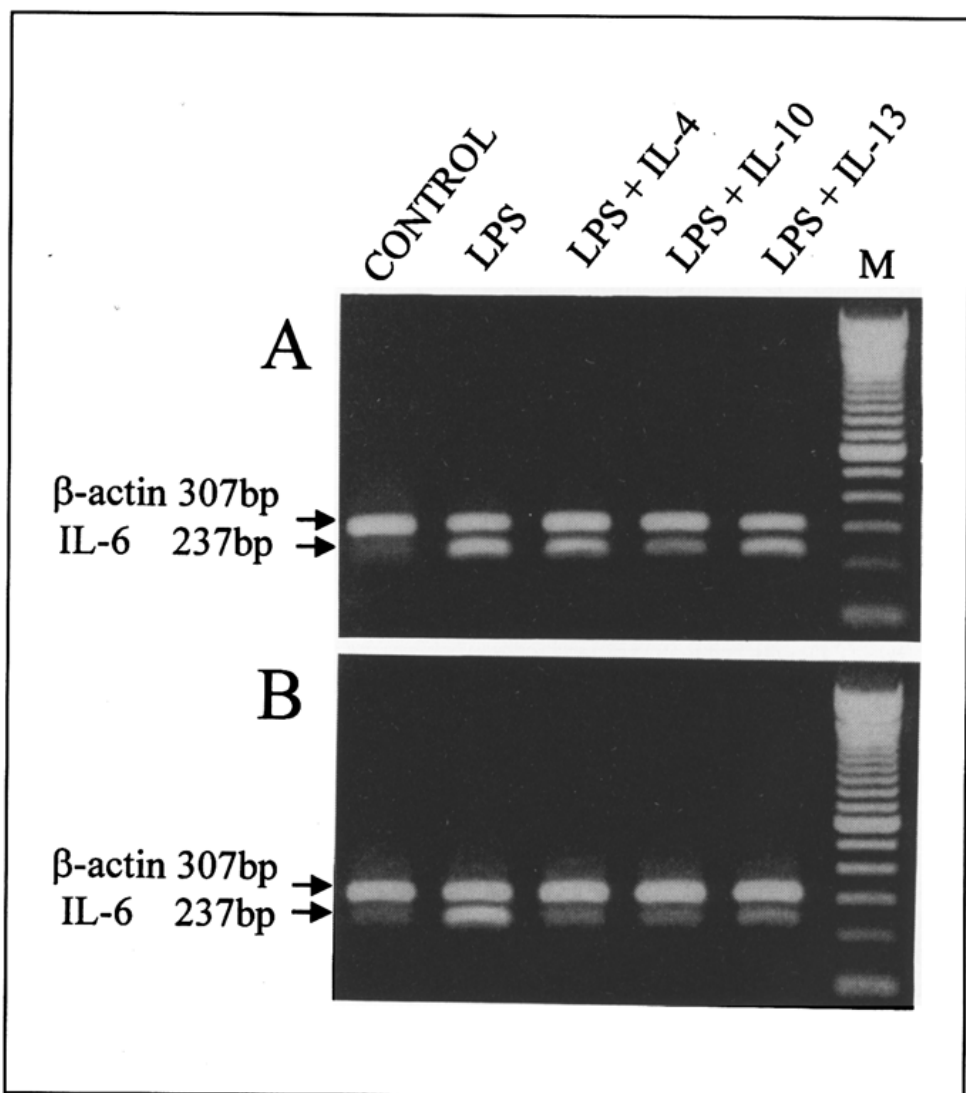
## RESULTS

The whole blood tested *ex vivo* mimics the natural cell-to-cell interactions and represents a physiologic environment for examining cytokine production in response to LPS (11, 12). However, in such system the results may be affected by individual responsiveness (13). As shown in Fig. 1, preparations of IL-4, IL-13 and IL-10 used in our experiments inhibited synthesis of  $\text{TNF}\alpha$  and IL-6 by human whole blood stimulated with LPS. Although inhibition was always observed, considerable individual variations were noted and for this reason statistical analysis was not carried out. Amounts of  $\text{TNF}\alpha$  produced in the blood sample during 24 h in the presence of IL-4 and LPS were in the range of 36–72% of LPS alone. Corresponding figures for IL-13 and IL-10 ranged between 45–98% and 2–12%, respectively. It is thus clear that in full blood IL-10 was the most effective inhibitor of cytokine synthesis. This is in agreement with observations of Minty *et al.* (14) who reported that also in isolated human blood monocytes IL-10 was a more potent inhibitor of  $\text{TNF}\alpha$  production than IL-13.



*Fig. 1.* Inhibition of production of TNF $\alpha$  (A) and IL-6 (B) by IL-4, IL-10 and IL-13 in LPS-stimulated whole human blood after 24 h incubation. The mean values of 3 independent experiments are expressed in relative terms assuming LPS-stimulated sample as 100%. Unstimulated blood had no detectable IL-6 or TNF. Treatment with LPS induced the following amounts of cytokines (ng/100  $\mu$ l of undiluted blood/24 h): TNF $\alpha$  —  $7.86 \pm 2.88$ ; IL-6 —  $20.35 \pm 5.43$ .

We found that after 6 h exposure of adherent monocytes to LPS and IL-4, IL-13 or IL-10 only the latter strongly inhibited IL-6 mRNA accumulation whereas the effects of IL-4 and IL-13 were barely detectable (*Fig. 2A*).



*Fig. 2.* Modulation of LPS-induced IL-6 mRNA accumulation by IL-4, IL-10 and IL-13 in human adherent monocytes. RNA was isolated 6 h (A) or 18 h (B) after the treatment of culture as indicated in the upper description: Control — untreated monocytes, LPS — 100 ng/ml; LPS + IL-4 — 100 ng/ml of LPS and 100 U/ml of IL-4; LPS + IL-10 — 100 ng/ml of LPS and 100 U/ml of IL-10; LPS + IL-13 — 100 ng/ml of LPS and 100 U/ml of IL-13; M. — 100 bp DNA ladder. The results show a typical experiment.

However, after 18 h all three tested cytokines caused reduction of IL-6 mRNA almost to the control levels (*Fig. 2B*). A similar pattern was observed with TNF mRNA, although the inhibition by IL-4, IL-13 and especially by IL-10, was clearly visible already after 6 h (data not shown).

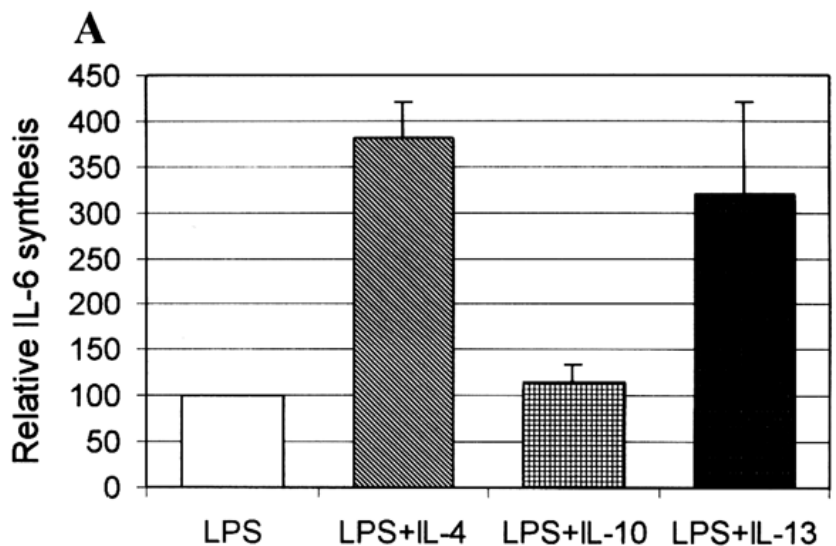
Cultured human endothelial cells synthesize small amounts of IL-6 (but not TNF) and they can be further stimulated by LPS to produce IL-6. In these cells, however, IL-4 or IL-13 enhanced both basal and LPS-induced IL-6 expression (*Table 1*). In distinction, IL-10 had no effect on IL-6 synthesis in HUVEC cells (*Fig. 3A*). Stimulatory effects of IL-4 and IL-13 on IL-6 production in endothelial cells were observed also at the mRNA level, and 100 units of IL-4 or IL-13 gave a slightly higher response than 20 units (*Fig. 3B*). These effects cannot be attributed to a possible contamination with endotoxin of IL-4 and IL-13 preparations since they were unable to induce TNF $\alpha$  expression in the whole blood when tested alone without addition of LPS (data not shown).

*Table 1.* Effects of IL-4 (100 U/ml) and IL-13 (100 U/ml) on basal and LPS-induced IL-6 production in HUVEC cultured for 24 h with the tested factors. The results are the means of 4 experiments,  $\pm$ SD.

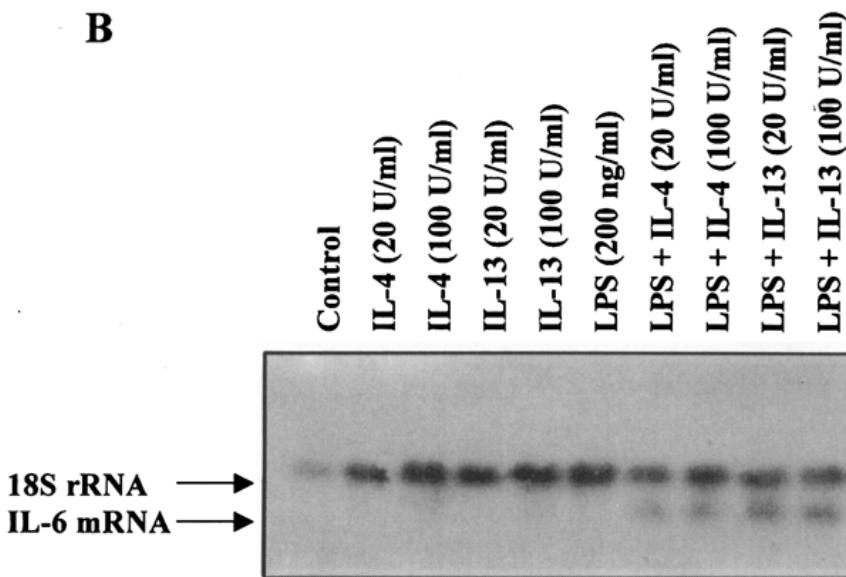
	IL-6 (ng/ml)
Control	5.53 $\pm$ 1.41
IL-4	16.6 $\pm$ 2.83
IL-13	16.2 $\pm$ 2.11
LPS	17.45 $\pm$ 6.16
LPS+IL-4	25.66 $\pm$ 7.58
LPS+IL-13	24.46 $\pm$ 7.22

It may be of interest that in our experiments IL-4 and IL-13 (but not IL-10) augmented also the LPS-induced expression of VCAM-1 gene coding for adhesion molecules produced during inflammatory reactions (*Fig. 4*). Stimulation of VCAM-1 synthesis by treatment of HUVEC with IL-4 or IL-13 alone was already reported by Kotowicz *et al.* (15).

IL-6 is held responsible for induced synthesis of acute phase proteins in liver cells although other cytokines, hormones and growth factors contribute to the regulatory network (6). We stimulated human hepatoma HepG2 cells, a commonly used model to study acute phase protein synthesis, with IL-6 alone or in combination with IL-4, IL-13 and IL-10. It was found that IL-4 and IL-13 inhibit IL-6-induced haptoglobin synthesis (*Fig. 5A*) whereas IL-10 had no effect (data not shown). The down-regulation of haptoglobin synthesis by



**B**



*Fig. 3.* Effects of IL-4, IL-10 and IL-13 (each cytokine 100 U/ml) on IL-6 production by LPS-stimulated (200 ng/ml) HUVEC cells. Results are the mean  $\pm$  SD of 4 experiments and are expressed in relative terms assuming LPS-stimulated sample as 100%. (A) Control HUVEC culture produced  $5.53 \pm 1.41$  ng IL-6/ml during 24 h. After LPS treatment this value increased to  $17.45 \pm 6.16$  ng/ml/24 h. (B) Abundance of IL-6 mRNA in HUVEC cells cultured for 8 h with indicated amounts of LPS, IL-4, IL-13 or the mixture of LPS+IL-4 or LPS+IL-13. For comparison, the hybridization pattern of 18S rRNA is shown.

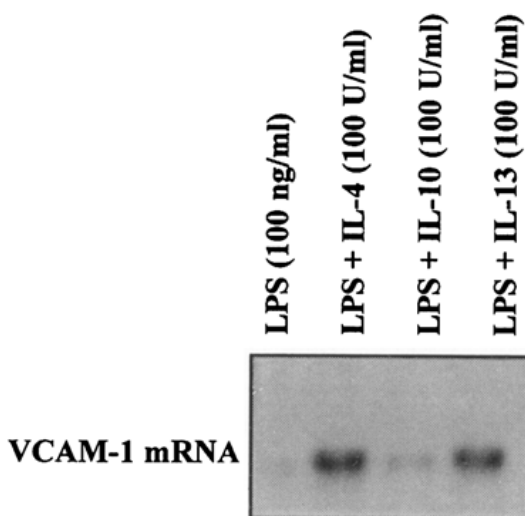
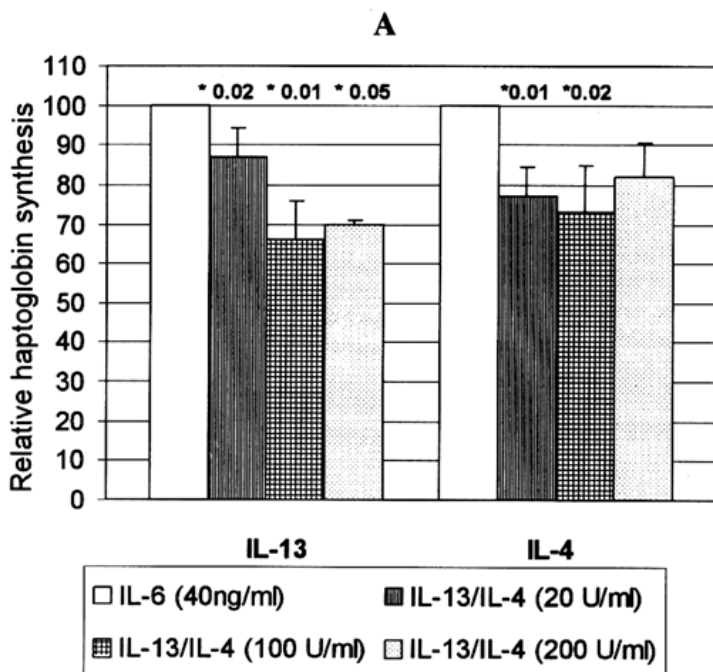


Fig. 4. Abundance of VCAM-1 mRNA in HUVEC cells cultured for 8 h after addition of LPS, IL-4, IL-10 and IL-13.

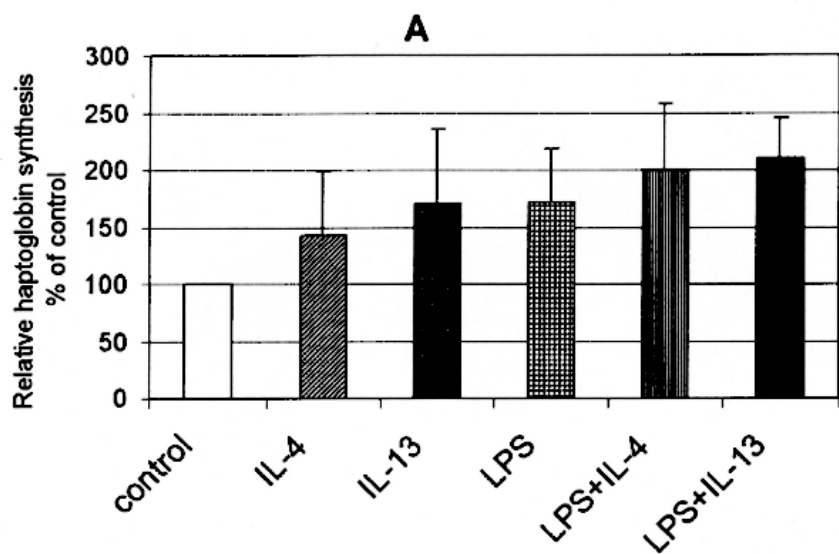
IL-4 and IL-13 was also clearly visible at the level of mRNA (Fig. 5B). Other acute phase proteins, such as fibrinogen, were only marginally affected by IL-4, IL-13 or IL-10 (data not shown). Inhibition of haptoglobin synthesis by IL-4 in human primary hepatocytes was reported earlier by Loyer *et al.* (16).

Since several types of cells participate in the acute phase response *in vivo*, and endothelial cells are abundant in inflammatory regions, we tested a co-culture of HUVEC and HepG2 cells. When HUVEC were cultured separately and exposed to LPS, IL-4, IL-13, or a mixture of LPS and these cytokines, and then the collected medium was added to HepG2 cells, stimulation of haptoglobin synthesis was observed (Fig. 6A). On the other hand, if HepG2 cells were cultured on an insert in a plate with HUVEC, addition of IL-4 or IL-13 reduced synthesis of haptoglobin in comparison to treatment with LPS alone (Fig. 6B). This indicates that in a co-culture of HepG2 and HUVEC cells the inhibitory effect of IL-4 and IL-13 on HepG2 cells prevails over stimulation of IL-6 synthesis in HUVEC.

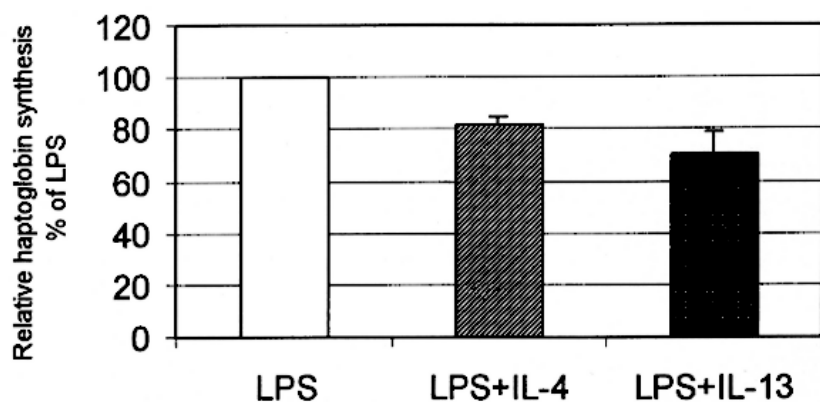




*Fig. 5.* Modulatory effects of IL-4 and IL-13 on IL-6-stimulated haptoglobin production by HepG2 cells cultured for 48 h. Results are the means  $\pm$  SD of 4 experiments and are expressed in relative terms assuming IL-6-induced haptoglobin synthesis as 100%. (A) Without IL-6 HepG2 cells produced on the average 0.86  $\mu$ g haptoglobin during 48 h, and after stimulation with IL-6 this value was increased almost 8-fold. (B) Abundance of human haptoglobin (hHPT) mRNA in HepG2 cells cultured for 18 h with IL-6, or the mixture of IL-6+IL-4 or IL-6+IL-13. For comparison, amounts of 18S and 28S rRNA in the ethidium bromide stained gel are shown.



**B**



*Fig. 6.* Stimulation of haptoglobin synthesis by the media from HUVEC culture: control (untreated cells) or cells exposed to IL-13, IL-4, LPS (200 ng/ml) or the mixture of LPS and these cytokines. (A) The results are the means  $\pm$  SD of 3 experiments and are reported in relative term assuming as 100% haptoglobin synthesis by HepG2 cells treated with the media from control HUVEC culture. (B) Effects of IL-4 and IL-13 on haptoglobin synthesis by HepG2 cells cultured in the insert with HUVEC in the main well.

## DISCUSSION

The experiments presented here permit to compare directly the effects of three cytokines regarded as anti-inflammatory (IL-4, IL-13 and IL-10) on the expression of some genes in the hematopoietic (whole blood, monocytes) and non-hematopoietic (HUVEC, HepG2) cells. The difference in response is striking: while in the whole blood tested *ex vivo*, and in isolated mononuclear cells, all these cytokines inhibited synthesis of TNF $\alpha$  and IL-6 (Figs 1 and 2), in human endothelial cells (HUVEC) IL-4 and IL-13 stimulated IL-6 production, both at mRNA and protein levels (Fig. 3 and Table 1).

This tissue-specific difference may reflect variations in the intracellular signalling pathways and/or involvement of certain transcription factors. It seems that in the cells of hematopoietic lineage NF $\kappa$ B activation is the central target for anti-inflammatory cytokines, (IL-10, IL-4 and IL-13), but no agreement exists on the mechanism involved (1, 2, 14, 17, 18). In distinction to monocytes, IL-4 was reported to enhance NF- $\kappa$ B activity in human dermal fibroblasts stimulated by IL-1 to produce IL-6 (1). The opposite regulation of NF- $\kappa$ B by IL-4 and IL-13 or IL-10 in hematopoietic and non-hematopoietic cells and its relevance to synthesis of proinflammatory cytokines remains to be elucidated.

Complex effects of the tested cytokines on acute phase response are also evident with human hepatoma HepG2 cells used in our experiments. IL-10 had no influence on protein synthesis, but as demonstrated by elegant studies of Lai *et al.* (19) HepG2 cells can respond to this cytokine after transfection of functional receptor IL-10R. On the other hand, we found that IL-4 and IL-13 were active in HepG2 cells and similarly inhibited IL-6-induced synthesis of haptoglobin, both at the protein (Fig. 5A) and mRNA levels (Fig. 5B).

It is known that despite differences in receptor structure of IL-4, IL-13 and IL-6 these cytokines share JAK-STAT signalling pathway in a variety of cells. However, IL-6 requires JAK1, JAK2, STAT1 and STAT3 (20) while JAK1, JAK2, JAK3 and STAT6 are engaged in IL-4/IL-13 response (21, 22). As demonstrated by Murata *et al.* (22) the phosphorylation and activation of JAK3 tyrosine kinase is crucial for IL-4 activation of STAT6 in hematopoietic cells. In distinction, the non-hematopoietic cells lack JAK3 expression and STAT6 activation in these cells is mediated through JAK1 and JAK2 tyrosine kinases. This is due to the fact that major differences exist between these two types of cells in respect of structure and signal transduction through IL-4R and IL-13R systems (22). The classical IL-4R consisting of IL-4R $\beta$  and IL-4R $\gamma$ c chains is predominantly expressed in hematopoietic cells. The alternative form of IL-4R occurs in non-hematopoietic cells and consists of IL-4R $\beta$  and IL-13R $\alpha'$  chains. Thus it can be speculated that differences between hematopoietic and non-hematopoietic cells in expression of certain genes affected by

IL-4/IL-13 result from variations of JAK-STAT transduction pathway. Recently Wery-Zennaro and co-workers (23) reported that in keratinocyte-deriving cell line binding of IL-4 to its receptor leads to STAT3 phosphorylation but not to its nuclear translocation. Preliminary results obtained in our laboratory indicate, however, that IL-4 and IL-13 have no direct effect on STAT1 and STAT3 activation by IL-6 in HepG2 cells (A. Kasza, unpublished observations).

Our experiments with co-cultures of HUVEC and HepG2 cells (Fig. 6A and 6B) suggest that *in vivo* response to LPS and anti-inflammatory cytokines may be different than in a model of single-type cells. The final outcome will depend on several factors, including the number and affinities of specific cytokine receptors, and the receptors cross-talk in target cells. These possibilities should be taken into account when evaluating biological actions of cytokines and relevance to pathological phenomena.

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