

**Background:** In our previous study we found that sIL-6R, along with recombinant human interleukin-6, plays a regulatory role in the immune response by modulating the tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression and its production by peripheral blood mononuclear cells (PBMC). We also suggested that sIL-6R with IL-6 secreted by human PMN (neutrophils) influenced the TNF- $\alpha$  expression and its production by autologous PBMC.

**Aims:** Since soluble gp130 (sgp130) is a natural inhibitor for sIL-6R/interleukin-6 responses, in the present study we estimated an effect of exogenous recombinant human sgp130 and sgp130 secreted by PMN on the TNF- $\alpha$  expression and its production by PBMC.

**Methods:** Cells were isolated from whole blood of healthy persons. The PMN were cultured in 96-well plates for 1 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>. After the incubation, the culture supernatant of PMN was removed and added to the PBMC. PBMC were incubated for 1 h at 37°C in the same conditions. Cytoplasmic protein fractions of PMN and, for comparative purpose of PBMC, were analysed for presence of sgp130 by western blotting with the use of monoclonal antibody capable of detecting this protein. In the culture supernatants of PMN we examined the concentrations of sgp130 by human enzyme-linked immunosorbent assay. TNF- $\alpha$  was measured at the protein levels as well as the mRNA levels.

**Results and conclusions:** The present results revealed that exogenous recombinant human sgp130 modulates the TNF- $\alpha$  expression and production by PBMC. In contrast, we did not find any effect of sgp130 secreted by PMN on the TNF- $\alpha$  expression and its production by autologous PBMC.

**Key words:** Soluble gp130, Tumour necrosis factor- $\alpha$ , Neutrophils, Peripheral blood mononuclear cells

## Role of soluble gp130 in the tumour necrosis factor- $\alpha$ expression and its production by peripheral blood mononuclear cells

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## Introduction

Interleukin (IL)-6 belongs to a cytokine family that includes IL-11, ciliary neurotrophic factor, oncostatin M, leukemia inhibitory factor (LIF) and cardiotropin 1, granulocyte colony-stimulating factor, IL-12, IL-23, and leptin.<sup>1–3</sup> This family is defined by the fact that all its members use the transmembrane protein gp130 as a signal transducing receptor component. IL-6 and IL-11, after binding to their specific  $\alpha$ -receptors (IL-6R $\alpha$ ), induce gp130 homodimerization, whereas LIF, oncostatin M, ciliary neurotrophic factor and cardiotropin 1 signal via a heterodimer of gp130 and a second signal transducing protein, the LIF receptor.<sup>2,4,5</sup> Expression of the transmembrane gp130 was found in almost all organs, including the heart, kidney, spleen, liver, lung, placenta and brain.<sup>6,7</sup> In contrast, cellular distribution of the cognate IL-6R is

limited and its expression is predominantly confined to hepatocytes and the leukocyte subpopulation (monocytes, neutrophils, T cells, and B cells).<sup>6,7</sup>

gp130 activation leads to the activation of the Janus family of tyrosine kinases (JAK-1, JAK-2 and TYK-2) and subsequently to the activation of signal transducer proteins and proteins that activate transcription (STAT proteins). Other signal transduction pathways such as the mitogen-activated protein kinase pathway and the phosphoinositol-3-kinase pathway are also activated by gp130.<sup>3,7,8</sup>

It was shown that membrane-bound receptors of IL-6 exist in a soluble form: soluble IL-6R $\alpha$  (sIL-6R $\alpha$ ) and soluble gp130 (sgp130). sIL-6R $\alpha$  binds IL-6 with an affinity similar to that of the membrane-bound receptor, and also prolongs its plasma half-life. Moreover, the sIL-6R/IL-6 complex is capable of activating cells via interaction with the membrane-

bound gp130. It is known that sIL-6R has a potential to regulate both local and systemic IL-6-mediated responses.<sup>1,2,6,9</sup>

In our previous study we revealed that rhIL-6R with recombinant human interleukin-6 (rhIL-6) appear to play a regulatory role in the immune response by modulating the tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression and its production by peripheral blood mononuclear cells (PBMC).<sup>10</sup>

sgp130 is the natural inhibitor of IL-6 responses that is dependent on sIL-6R. sgp130, by interacting with the IL-6/sIL-6R complex (but not with IL-6 alone), specifically inhibits IL-6 responses mediated by the sIL-6R trans signalling pathway, whereas IL-6 responses through the membrane-bound IL-6R remained unaffected.<sup>4,6</sup>

In the present study we estimated a role of recombinant human sgp130 (rhsgp130) and sgp130 secreted by PMN in the TNF- $\alpha$  expression and production by autologous PBMC. Examination of the biological function of sgp130 may provide new information about a potential activity of this receptor in the immune response mediated by IL-6 and TNF- $\alpha$ .

## Materials and methods

Cells were isolated from heparinized (10 U/ml) whole blood of 15 healthy persons by Gradisol G gradient (1.115 g/ml). The purity of isolated PMN and PBMC was determined by May–Grunewald–Giemsa staining; PBMC contained 95% lymphocytes and polymorphonuclear cells contained 94% PMN. PMN were suspended in the culture medium (RPMI-1640, autologous serum, penicillin and streptomycin) to provide  $5 \times 10^6$  cells/cm<sup>3</sup>. The PMN were cultured in 96-well plates (Falcon, Franklin Lakes, NY, USA) for 1 h at 37°C in a humidified incubator with 5% CO<sub>2</sub> (Nuair<sup>TM</sup>; Nu Aire, Inc., Minnesota, USA). After incubation, the culture supernatant of PMN was removed and added to PBMC ( $5 \times 10^6$  cells/cm<sup>3</sup>). The viability of PMN was > 92% as determined by trypan blue exclusion. PBMC were incubated for 1 h at 37°C in the same conditions. PBMC with the culture supernatants of PMN and monoclonal antibody (mAb) anti-sgp130 (20 ng/ml) (R&D Systems, Minneapolis, MN, USA), and anti-IL-6 (20 ng/ml) (Biosource, Nivelles, Belgium) were the negative control, whereas PBMC with rhsgp130 (1  $\mu$ g/ml) (R&D Systems) and rhIL-6 (50 ng/ml) (R&D Systems) were the positive control.

Cytoplasmic protein fractions of PMN and, for comparative purposes, of PBMC were analysed for the presence of sgp130 by western blotting using mAb capable of detecting this protein anti-sgp130 (R&D Systems) (Jackson ImmunoResearch Inc., from BIOKOM, Warsaw, Poland). Cells were lysed directly

by sonication and cytoplasmic proteins were electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The resolved proteins were transferred onto nitrocellulose and incubated with the primary mAb anti-sgp130. The membranes were incubated at room temperature with alkaline phosphatase anti-mouse immunoglobulin G. Immunoreactive protein bands were visualized by the AP Conjugate Substrate Kit.

In the culture supernatants of PMN we examined the concentrations of sgp130 by human enzyme-linked immunosorbent assay (ELISA) (Biosource and R&D Systems).

TNF- $\alpha$  was measured at both the protein and mRNA levels. Protein levels were determined by a human TNF- $\alpha$  ELISA.

To examine TNF- $\alpha$  mRNA expression we isolated mRNA from PBMC after culture, using TRIZOL Reagent (Gibco, Paisley, UK) according to the manufacturer's instruction. The quantity of mRNA TNF- $\alpha$  was determined by Quantikine mRNA assay – Human TNF- $\alpha$  (R&D Systems).

## Results

### sgp130 protein expression in human PMN and PBMC detected by western blot

We used a specific mAb directed against intracellular forms of sgp130 for western blot in PMN and, for comparative purpose, in PBMC. As shown in Fig. 1 the antibody in human unstimulated and lipopolysaccharide (LPS)-stimulated PMN and PBMC identified bands of ~110 kDa from each donor.

Western blot analysis showed that the samples of unstimulated PMN and PBMC contained a 110 kDa protein, which was stained by an anti-sgp130 monoclonal antibody. The LPS-stimulated PMN and PBMC expressed a slightly increased sgp130 protein in comparison with the unstimulated cell lines.

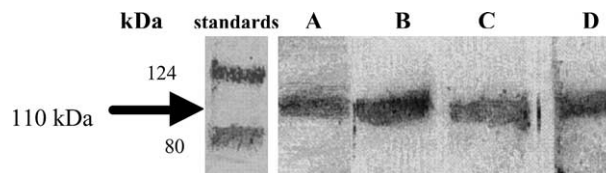


FIG. 1. Western blot analysis of sgp130 protein expression in human PMN and PBMC. We showed detection of sgp130 protein from human unstimulated PMN (line A) and LPS-stimulated PMN (line B), unstimulated PBMC (line C), and unstimulated PBMC (line D). Whole cell lysates were obtained from fresh PMN of healthy donors. One hundred micrograms of total proteins from PMN was loaded onto 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The filter was incubated with 1:1000 dilution of the respective antibody. Sizes of protein standards are given in kilodaltons. One representative western blot of few independent experiments is shown.

### sgp130 concentrations in the culture supernatants of PMN detected by ELISA

In the culture supernatants of unstimulated PMN we observed a significant amount of sgp130 (mean  $\pm$  standard deviation,  $27.6 \pm 14.2$  ng/ml). LPS stimulation led to an increase in the sgp130 production by PMN (mean  $\pm$  standard deviation,  $41.8 \pm 22.3$  ng/ml) in comparison with the unstimulated cells.

### TNF- $\alpha$ mRNA in PBMC

In the culture supernatants of PBMC that were cultured in the presence of supernatants of unstimulated PMN, concentrations of TNF- $\alpha$  mRNA were lower than those in the supernatants of PBMC cultured without PMN supernatants (Fig. 2A). The supernatants of LPS-stimulated PMN led to a slightly enhanced TNF- $\alpha$  expression in PBMC. TNF- $\alpha$  expression in the culture supernatants of PBMC cultured

with rhsgp130 and rhIL-6 was higher than in PBMC cultured with unstimulated PMN.

Anti-sgp130 monoclonal antibody with rhIL-6 was used to confirm whether actions of PMN supernatant has been generated by this protein. The presence of anti-sgp130 antibody in the culture of PBMC with PMN supernatants induced lower levels of TNF- $\alpha$  expression in comparison with PBMC cultured without PMN.

### TNF- $\alpha$ concentrations in the culture supernatants detected by ELISA

Concentrations of TNF- $\alpha$  in the culture supernatants of PBMC culture in the presence of supernatants of unstimulated PMN were lower than those in the supernatants of PBMC cultured without PMN supernatants (Fig. 2B). In the presence of LPS-stimulated PMN supernatants we found non-significantly higher amounts of TNF- $\alpha$  in the supernatants of PBMC in

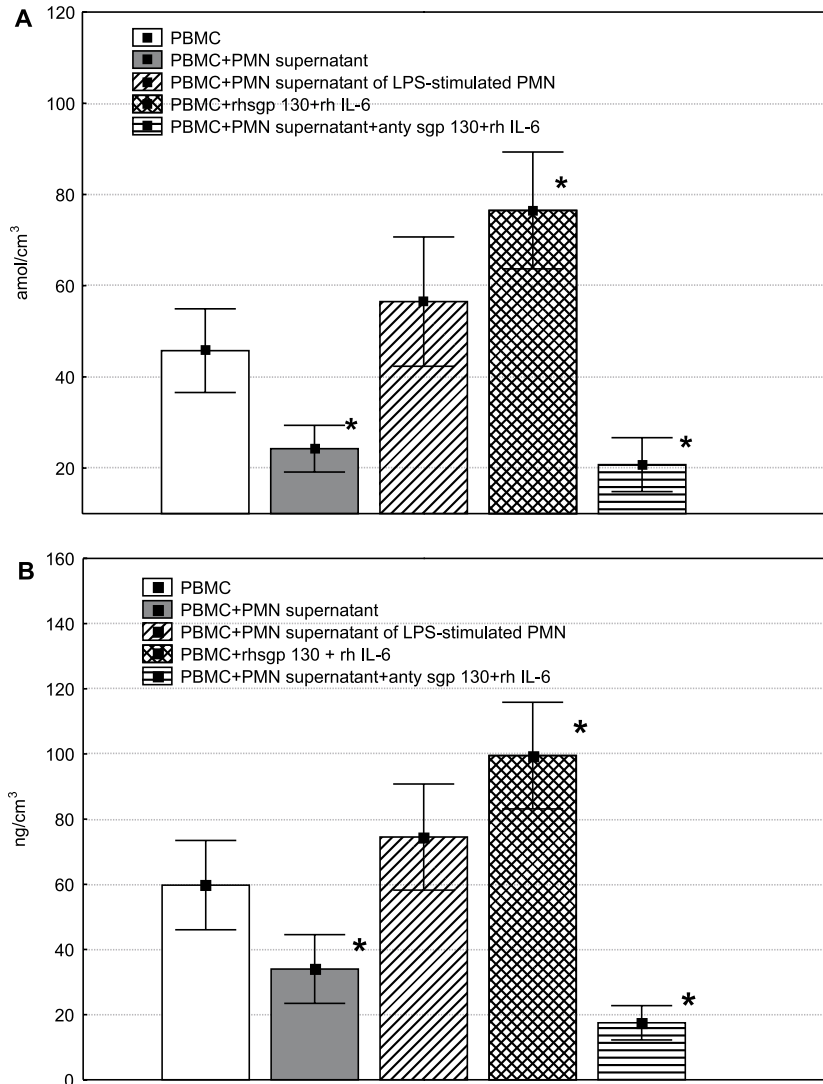


FIG. 2. TNF- $\alpha$  expression and its production by unstimulated and stimulated PBMC: (A) TNF- $\alpha$  mRNA expression (amol/cm<sup>3</sup>); (B) TNF- $\alpha$  concentration in the culture supernatant of PBMC (pg/ml). \* Statistical difference with PBMC alone ( $p < 0.05$ ).

comparison with PBMC culture with unstimulated PMN. PBMC cultured with rhsgp130 and rhIL-6 secreted higher concentrations of TNF- $\alpha$  than PBMC alone. The presence of anti-sgp130 and rhIL-6 induced a lower level of TNF- $\alpha$  than in the culture of PBMC alone.

## Discussion

Soluble cytokine receptors through the control of cytokine bioactivity play a significant role in the immune response. There are different data referring to the biological activity of soluble receptors alone or complexed with respective ligand.<sup>9,11</sup> It was well established that the soluble IL-6 receptors (sIL-6R $\alpha$  and sgp130) have opposite biologic effects. However, sgp130 antagonistic activity is markedly enhanced in the presence of sIL-6R $\alpha$ .<sup>1</sup> It has recently been shown that sIL-6R $\alpha$  and sgp130 could be effective stimulators of the cell activity.<sup>9,12,13</sup> For instance, Modur *et al.* demonstrated that PMN exposed to fMLP shed IL-6R in sufficient quantities to activate the inflammatory response of endothelial cells.<sup>9</sup> sgp130 has been shown to inhibit sIL-6R-mediated proliferation of Kaposi sarcoma cells, STAT activation and the expression of  $\alpha_1$ -antichymotrypsin.<sup>6</sup> Aukrust *et al.* demonstrated that sgp130 correlates with variables reflecting a deranged haemodynamic status. This indicate the involvement of sgp130 in the pathophysiology of cardiovascular disease.<sup>14</sup>

The present study indicate that exogenous rhsgp130 has the ability to enhance the TNF- $\alpha$  expression and its production by human PBMC. In contrast, we did not find any significant effect of sgp130 released by human PMN on the TNF- $\alpha$  expression and its production by autologous PBMC. One possible explanation of this situation may be the presence of other mediators in the culture supernatants of PMN as well as of PBMC. PMN have the ability to synthesize pro-inflammatory and anti-inflammatory cytokines that co-stimulate each other's release and activity. In our previous study we found that IL-6 together with sIL-6R secreted by PMN inhibits the TNF- $\alpha$  expression and production by PBMC.<sup>10</sup> Moreover, neutrophils may affect the IL-6 function through the release proteases playing a crucial role in regulation of the biological potency of this cytokine at sites of inflammation.<sup>15</sup> Among the known anti-inflammatory mediators secreted by PMN, IL-10 was shown to inhibit the production of TNF- $\alpha$ .<sup>16</sup> Furthermore, cytokines produced by PBMC, such as IL-4, IL-6 and IL-10, in an autocrine way may influence the TNF- $\alpha$  expression.

Although, we did not observe any significant effect of sgp130 on TNF- $\alpha$  expression in PBMC, the secretion of a relatively large amount of sgp130 by PMN

might directly affect the other biological function of IL-6. It is known that the lack of sgp130 may lead to overstimulation of reactions mediated by the IL-6/sIL-6R complex. Jones and Rose-John showed that sgp130 can modulate the leukocyte recruitment in a murine model of acute inflammation and can suppress the clinical indices of experimental colitis.<sup>6</sup> Thus, sgp130 as natural inhibitor of the IL-6/sIL-6R complex can be useful in blocking the sIL-6R responses in different diseases.<sup>4</sup>

In estimation of the biological function of sgp130 released by neutrophils, as well as by other cells, the presence of three isoforms of this protein should be considered. Soluble sgp130 is generated through either proteolytic shedding or as a product of differential mRNA splicing. The 50, 90 and 110 kDa forms of sgp130 are secreted by shedding of membrane-bound gp130. A spliced variant of sgp130 containing an additional 85 base pair insertion was also found. Recently, a third isoform of sgp130 (the 55 kDa molecule) was identified as the autoantigen in rheumatoid arthritis.<sup>6</sup> It is possible that these isoforms, like isoforms of sIL-6R $\alpha$ , may have various biological effects. Additionally, recent data indicate that an inhibitory action of sgp130 is not restricted to suppressing sIL-6R signalling and may also block LIF-mediated and oncostatin M-mediated events.<sup>4</sup> Thus, sgp130 may influence a wider range of reactions in the immune response than recognized to date.

Summarizing, the relationship between both soluble IL-6 receptors should be considered in the estimation of reactions mediated by the IL-6 family and TNF- $\alpha$ . Neutrophils, abundant cells of an early phase of inflammation, through the simultaneous release of sIL-6R and sgp130 appear to play a significant role in this response. However, further examinations involving a wider range cytokines are required to explain the lack of effect of sgp130 secreted by PMN on TNF- $\alpha$  expression in autologous PBMC.

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