

Interleukin-6 trans-signalling differentially regulates proliferation, migration, adhesion and maspin expression in human prostate cancer cells

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Abstract

Interleukin-6 (IL-6) is suggested to have a pathogenic role in the progression of prostate cancer (PC), therefore representing an attractive target for new therapies. However, due to the pleiotropy of this cytokine, targeting IL-6 results in different and unpredictable responses. In order to better understand the mechanisms underlying the different responses to the cytokine, we focused our attention on IL-6 receptors (IL-6Rs) that represent the first element in the cascade of cytokine-activated signalling pathways. IL-6 signal transduction may indeed occur through the membrane IL-6R (classical signalling) and/or through the less studied soluble IL-6R (sIL-6R; IL-6 trans-signalling (IL-6TS)). We provide the first evidence how responses to IL-6 may depend on the different content of IL-6Rs in PC. In particular, the studies of ³H-thymidine incorporation and exploitation of different approaches (i.e. activation or inhibition of IL-6TS in sIL-6R-negative and -positive cell lines and transfection of IL-6R siRNA) allowed us to demonstrate that IL-6TS specifically accounts for an anti-proliferative effect of the cytokine in three PC cell lines that are known to respond differently to IL-6. Additionally, by applying migration-, scratch- and adhesion assays, we show that IL-6TS increases motility and migration and decreases adhesion of prostate cells facilitating thereby processes that determine metastasis initiation and spread. Finally, by western analyses, we uncovered an IL-6- and sIL-6R-dependent downregulation of the tumour suppressor maspin. Collectively, these data suggest that selective targeting of IL-6TS might allow to refine the currently available experimental anti-IL-6 therapies against PC.

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Introduction

Prostate cancer (PC) is one of the most common cancers in the western world. When the tumour is organ confined, radical prostatectomy or radiotherapy can in most cases cure the disease. In contrast, advanced stages of the tumour, after regression obtained with androgen ablation therapies, eventually develop a therapy-refractory phenotype. Median survival of men with therapy-resistant metastatic PC is limited to only 1–2 years; at this stage of the disease, the attempts to delay tumour progression have so far resulted in only a

few months prolongation of survival (Petrylak 2007, Friedman *et al.* 2008, Lassi & Dawson 2009). A better understanding of the mechanisms responsible for recurrence of the tumour is the basis for any possible therapeutical improvement.

Interleukin-6 (IL-6), a cytokine known as mediator of immunological and inflammatory events, has been shown to have a role in the development and progression of several types of tumours (Tripathi *et al.* 2003, Mumm & Oft 2008). In particular, clinical observations have documented increased IL-6 levels in plasma from patients with therapy-resistant metastatic

disease compared to patients with earlier stages of the disease and healthy individuals (Twillie *et al.* 1995, Shariat *et al.* 2001, George *et al.* 2005). *In vitro* and *in vivo* studies suggested a pathogenic role of this cytokine in the progression of PC (Lin *et al.* 2001, Steiner *et al.* 2003, 2006, Cavarretta *et al.* 2007, Malinowska *et al.* 2009). In particular, an anti-IL-6 therapy was shown to be effective in inducing apoptosis and regression of androgen-dependent prostate xenografts in nude mice and the following inhibition of the conversion to an androgen-independent phenotype (Wallner *et al.* 2006). Although IL-6 represents a promising potential therapeutic target, biological responses to IL-6 and/or anti-IL-6 treatment are pleiotropic in various PC cell lines and xenografts confirming the complexity of the IL-6 action (Naka *et al.* 2002).

IL-6 binds to its specific receptor, IL-6 receptor α (IL-6R), a 80 kDa glycosylated transmembrane protein belonging to the cytokine receptor superfamily. The complex IL-6/IL-6R associates with the protein gp130, a transmembrane cytokine receptor, thereby inducing gp130 dimerisation and initiation of signal transduction mainly through the Janus kinase/signal transducers and activators of transcription (STAT), phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways.

Neither IL-6 nor IL-6R alone is capable of binding to and activating gp130. However, a naturally occurring soluble form of IL-6R (sIL-6R), derived either by alternative splicing or by shedding of the membrane-bound IL-6R (mIL-6R), is able to bind the cytokine. The complex IL-6/sIL-6R activates gp130 also in the absence of the mIL-6R. This kind of signal transduction is named IL-6 trans-signalling (IL-6TS). The IL-6/sIL-6R complex may therefore potentiate the signal transduced by the mIL-6R or activate it in cells that do not express the receptor (Rose-John 2003). The gp130 subunit can also exist in a soluble form named sgp130. sgp130 has been shown to exclusively inhibit activation of the complex IL-6/sIL-6R without interfering with the complex IL-6/mIL-6R (Jostock *et al.* 2001). Classical and IL-6TS are thought to act in concert in mediating the response to IL-6. However, lately, it became evident that IL-6TS has a major relevance in the pathogenesis and development of chronic inflammatory diseases (Scheller & Rose-John 2006, Nowell *et al.* 2009), as well as in colon cancer and melanoma (Jones *et al.* 2001, Kallen 2002, Becker *et al.* 2004, Mitsuyama *et al.* 2006b, Kang *et al.* 2007, Wagley *et al.* 2007, Nowell *et al.* 2009). Preclinical data suggest the possibility to improve current therapies by specifically inhibiting the sIL-6R (Nowell *et al.* 2003,

Mitsuyama *et al.* 2006a, Rose-John *et al.* 2007). Whether classical and IL-6TS pathways may be able to transduce specific events in PC is not known yet.

It has been reported that in a large cohort of patients with clinically localised PC, the preoperative serum levels of IL-6 and sIL-6R are higher in those who will develop metastases compared to individuals who will not experience recurrence of disease in the following 5 years (Kattan *et al.* 2003, Shariat *et al.* 2008). Interestingly, sIL-6R showed a stronger association with disease progression than IL-6, suggesting a role for sIL-6R in mediating specific cytokine effects and the spread of metastases. However, no data proving this hypothesis are so far available in PC.

In this study, we asked whether the effects of IL-6 on PC cell proliferation are dependent on the preferential activation of its membrane or the soluble receptor and whether IL-6TS may play a role in processes that facilitate metastasis formation.

Materials and methods

Cell lines and chemicals

EP156T cells (human normal prostate epithelium, immortalised with hTERT) (Kogan *et al.* 2006) were grown in modified MCDB153 (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 5% FCS (PAA Laboratories, Pasching, Austria), 50 mg/l bovine pituitary extract (Invitrogen), penicillin/streptomycin, 5 μ g/l epidermal growth factor (Pepro-Tech, Rocky Hill, NJ, USA) and 10 nM of the synthetic androgen methyltrienolone R1881 (New England Nuclear, Dreieichenhein, Germany). LNCaP, PC3, Du145 (PC cells derived respectively from lymph node, bone and brain metastases), 22Rv1 (Sramkoski *et al.* 1999) and BPH-1 (Hayward *et al.* 1995) were grown in RPMI 1640 (PAA Laboratories) supplemented with 10% FCS, penicillin/streptomycin and 2 mM GlutaMax. LAPC4 (lymph node metastatic PC cells) were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 15% foetal bovine serum (HyClone, Szabo Scandic, Vienna, Austria) and 10 nM R1881. LNCaP, PC3 and Du145 were obtained by the American Type Culture Collection (Manassas, VA, USA). BPH-1 and 22Rv1 cells were a kind gift from Dr Jack Schalken (University Medical Center St Radboud, Nijmegen, The Netherlands), and LAPC4 cells were kindly provided by Dr Charles Sawyers (University of California, Los Angeles, CA, USA). Recombinant human IL-6 and gp130/Fc Chimera (i.e. sgp130) were purchased from R&D Systems (Wiesbaden, Germany), and CD126 (sIL-6R) was

purchased from PromoKine (Heidelberg, Germany). Polyclonal goat maspin (C-20) antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and monoclonal mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6C5) antibody was purchased from Chemicon (Millipore, Billerica, MA, USA).

siIL-6R and sgp130 ELISA

Cells were seeded onto 6-well plates in their respective medium containing 3% (v/v) charcoal-stripped serum (CSS). After 72 h, sgp130 and siIL-6R levels were assessed with the human CD130/gp130 ELISA Kit and the CD126/IL-6R/gp80 ELISA Kit (both from Diaclone, Stamford, CT, USA) according to the manufacturer's protocol. Results were normalised to cell number in the respective well.

RT-PCR

Experimental conditions for RT-PCR were previously described (Malinowska *et al.* 2009).

Treatments

Treatments were performed in medium containing 3% CSS. Soluble receptors were added at the concentrations indicated in each figure prior to exposure of cells to the cytokine. Du145, LNCaP and EP156T cells were treated with 5 ng/ml IL-6. Based on a previous publication (Malinowska *et al.* 2009), LAPC4 were treated with 25 ng/ml IL-6.

siRNA transfections

Cells were seeded onto 96-well or 6-well plates and transfected on the next day with 5 or 25 nM IL-6R siRNA On-target Plus Smart Pool IL-6Ralpha or negative siRNA Control On-Target Plus Non-targeting Pool (Dharmacon, Lafayette, CO, USA) by using Lipofectamine 2000 (Invitrogen) in 3% CSS.

Proliferation assay

Proliferation assay was previously described (Puhr *et al.* 2009).

Cell motility assay

Cells were cultured in full-growth medium in 24-well cell culture inserts consisting of a transparent, 8 µm pore diameter polyethylene terephthalate (PET) membrane (BD Biosciences, Schwechat, Austria). On the next day, medium was replaced with RPMI containing 3% CSS. Soluble receptors or vehicle and IL-6 were added in the lower chamber. After 48 h, cells in the upper chamber were removed with a cotton swab,

whereas the cells that traversed the membrane pores were fixed with cold methanol for 10 min. Membranes were then mounted on microscope slides with Vectashield Mounting Medium with 4'-6'-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA). Five fields of views per membrane were analysed by fluorescence microscopy with automated cell counting (TissueGnostics, Vienna, Austria).

Scratch assay

Cells were seeded to near confluence onto 6-well plates and treated as indicated in figure legend. A 10 µl tip was used to make a scratch in the confluent layer of cells. After 24 h, pictures at 40-fold magnification were taken and the remaining free area was measured using the Magic Wand Tool of Adobe Photoshop 7.0.

Adhesion assay

Ninety-six-well plates were coated with human fibronectin (BD Biosciences) at a final concentration of 2 µg/cm². Cells were seeded in 3% CSS RPMI in the presence of the soluble receptors or vehicle and IL-6. After up to 2 h of incubation, cells were washed up to three times with PBS. As an index of cell number, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Biomedica, Vienna, Austria) was performed on the remaining cells according to the manufacturer's protocol. To avoid artefacts due to changes in cell viability, values were normalised to the values obtained from cells equally treated but not washed.

Western blot analysis

Cells were processed for western blot analyses as previously described (Cavarretta *et al.* 2007).

Statistical analysis

All experiments were repeated at least three times. RT-PCR and ELISA assays were repeated twice. The SPSS 12.0 program (Chicago, IL, USA) was used for statistical evaluation and the Mann–Whitney *U* test was used for the assessment of statistical significance.

Results

siIL-6R and sgp130 levels in PC cell lines

We initially tested different prostate cell lines for their ability to release the siIL-6R and the soluble gp130 receptors. We included the non-cancerous cell lines EP156T and BPH-1, the androgen-sensitive LNCaP, LAPC4 and 22Rv1, and the androgen-independent PC3 and Du145 PC cell lines.

All these cells expressed the mIL-6R, as verified by RT-PCR (data not shown). We found that all cell lines secreted both soluble receptors to a different extent (Tables 1 and 2). Amounts of sIL-6R released by the LAPC4 cells are extremely low, sometimes in the same range of those measured in the culture medium (data not shown). Therefore, we considered LAPC4 cells basically sIL-6R-negative. The addition of IL-6 increased the levels of sgp130 in LNCaP but not in LAPC4.

Interestingly, LNCaP-high passage (LNCaP-HP) cells released a much higher amount of both soluble receptors. We chose for our next experiments two sIL-6R-positive cell lines known to respond differently to the cytokine: LNCaP cells that are growth-inhibited (Hobisch et al. 2001) and Du145 cells that are growth-stimulated by the cytokine (Onuma et al. 2003).

sIL-6R mediates an anti-proliferative effect of IL-6 in different PC cells

To unmask a possible specific role of the different IL-6Rs on cell proliferation, we applied four approaches, illustrated in Figs 1–4.

We first analysed the effect of the cytokine on ³H-thymidine incorporation after specifically inhibiting IL-6TS by pretreating the cells with increasing doses of sgp130. In this model, IL-6 increased proliferation of both LNCaP and Du145 cells by 20 and 40% (*P<0.05) respectively (Fig. 1A and B). When applying this method to LNCaP-HP cells, the effect of inhibiting IL-6TS was even more pronounced (60%), probably due to the higher levels of sIL-6R released by the cell line at this stage (Fig. 1C). When the values of ³H-thymidine incorporation from sgp130/IL-6-treated cells were expressed as a percentage of values from vehicle/IL-6-treated cells, it became evident that suppression of IL-6TS abrogates the

Table 1 Soluble interleukin-6 receptor (sIL-6R) levels in prostate cell lines

Cell line	pg sIL-6R/2 ml per 3 days per 10 ⁶ cells (mean ± s.e.m.) ^a
LAPC4	19.88 ± 10.04
22Rv1	35.64 ± 13.51
Du145	170.18 ± 120.57
BPH-1	208.53 ± 41.61
EP156T	246.42 ± 8.12
LNCaP	323.63 ± 73.42
PC3	445.17 ± 282.58
LNCaP-HP	1363.16 ± 183.11

^aSensitivity: 5 pg/ml.

Table 2 sgp130 levels in prostate cell lines under basal conditions and after interleukin-6 (IL-6) treatment

Cell line	pg sgp130/2 ml per 3 days per 10 ⁶ cells (mean ± s.e.m.) ^a
22Rv1	45.76 ± 23.95
LAPC4	94.68 ± 11.87
EP156T	143.63 ± 8.93
PC3	391.07 ± 95.44
LNCaP	452.74 ± 33.42
Du145	528.28 ± 77.15
LNCaP-HP	930.86 ± 129.70

Cell line	Treatment	Relative expression (%) of sgp130/2 ml per 3 days per 10 ⁶ cells (mean ± s.e.m.) ^a
LNCaP		100 ± 8.46
LNCaP	5 ng/ml IL-6	147.24 ± 9.60
LAPC4		100 ± 1.64
LAPC4	25 ng/ml IL-6	98.40 ± 1.89

^aSensitivity: 15 pg/ml.

anti-proliferative effect exerted by the cytokine on LNCaP-HP cells (Fig. 1D). The effect is sgp130 dose-dependent.

In the second approach, we investigated the effect of adding increasing doses of sIL-6R to both LNCaP and Du145 cells prior to exposure to IL-6. In both cases, we measured an increased IL-6-induced proliferation (Fig. 2A and B). Concentrations up to 100 ng/ml sIL-6R did not further increase proliferation (data not shown). Thus, both inhibition of IL-6TS and increase of sIL-6R levels caused an increase of proliferation.

In order to facilitate the interpretation of these results, we decided to remove any contribution of the mIL-6R, therefore unmasking the effect of sIL-6R. In this approach, LNCaP and Du145 cells were transfected with IL-6R siRNA or control siRNA and exposed to sIL-6R and IL-6. Efficacy of siRNA transfection was demonstrated by RT-PCR (Fig. 3A) and sIL-6R ELISA (Fig. 3B). sIL-6R secretion decreased by 70 and 90% in LNCaP and Du145 cells respectively, transfected with 25 nM IL-6R siRNA compared to cells transfected with control siRNA. Residual receptors were not sufficient to mediate IL-6 effects since the cytokine failed to decrease proliferation of LNCaP cells (Fig. 3C). Transfected cells were thus treated with increasing doses of sIL-6R and IL-6. Again, in the presence of the mIL-6R, i.e. in cells transfected with control siRNAs, we could observe an increase of proliferation when the cells were exposed to the sIL-6R. However,

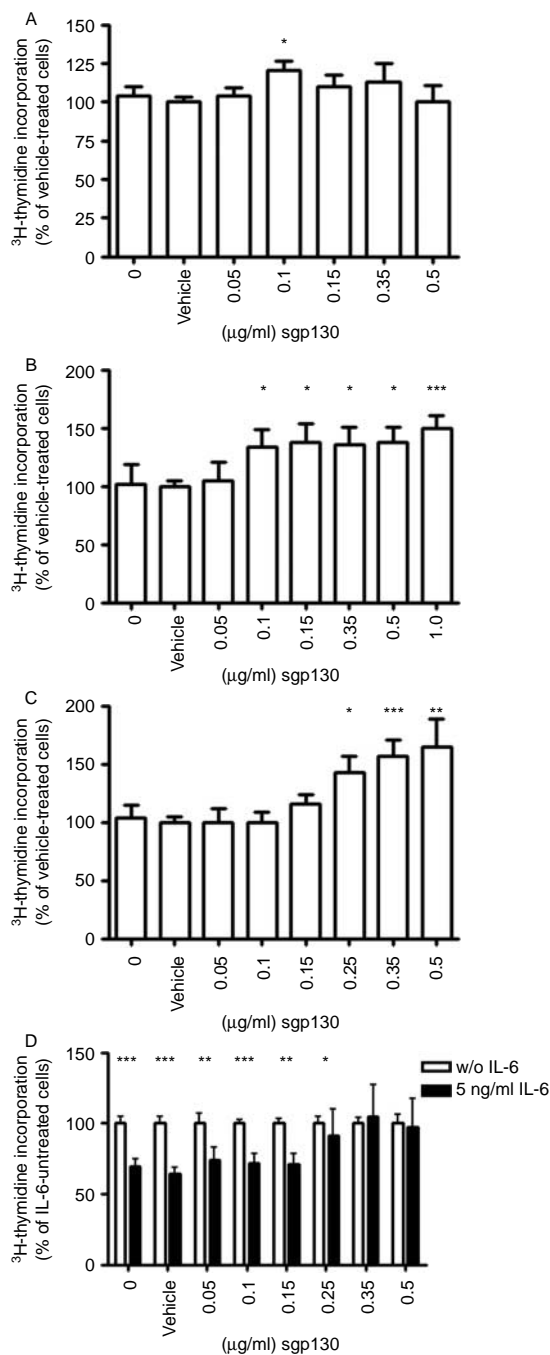


Figure 1 Addition of sgp130 reveals an anti-proliferative effect of IL-6TS in LNCaP and Du145 cells. LNCaP (A), Du145 (B) and LNCaP-HP (C) cells were treated with increasing doses of sgp130, as indicated, and exposed to IL-6. Proliferation was measured by ³H-thymidine incorporation and values, expressed as mean \pm S.E.M., were referred as a percentage of vehicle-treated samples. (D) The effect of IL-6 on LNCaP-HP cell proliferation was assessed in the absence or presence of increasing doses of sgp130. Values, expressed as mean \pm S.E.M., are referred as a percentage of IL-6-untreated samples * P <0.05; ** P <0.01; *** P <0.001.

after transfection with IL-6R siRNA and exposure to sIL-6R, IL-6 treatment decreased proliferation by 30% in LNCaP cells (** P <0.01) and failed to increase proliferation of Du145 cells (Fig. 3D and E). Thus, it seems that sIL-6R mediates an anti-proliferative effect of the cytokine.

To further confirm this hypothesis, we used LAPC4 cells as a negative control for sIL-6R, as mentioned above. These cells also express lower levels of IL-6R mRNA compared to the other PC cell lines considered (data not shown). Addition of IL-6 to sIL-6R-pretreated LAPC4 cells resulted in a 25% decrease of proliferation (** P <0.01; Fig. 4). The results were confirmed by counting the number of cells (data not shown).

From this set of experiments, we conclude that sIL-6R mediates an anti-proliferative effect of the cytokine in at least three PC cell lines that are known to respond differently to IL-6. This effect can be masked by the presence and activation of the mIL-6R.

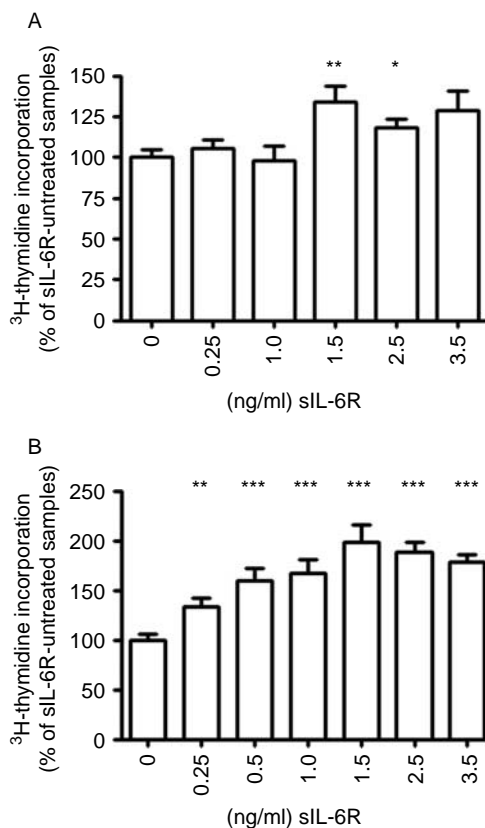


Figure 2 Addition of sIL-6R and IL-6 induces proliferation in LNCaP and Du145 cells. LNCaP (A) and Du145 (B) cells were exposed to increasing doses of sIL-6R, as indicated, prior to treatment with IL-6. Proliferation was measured by ³H-thymidine incorporation and values, expressed as mean \pm S.E.M., were referred as a percentage of sIL-6R-untreated samples. * P <0.05; ** P <0.01; *** P <0.001.

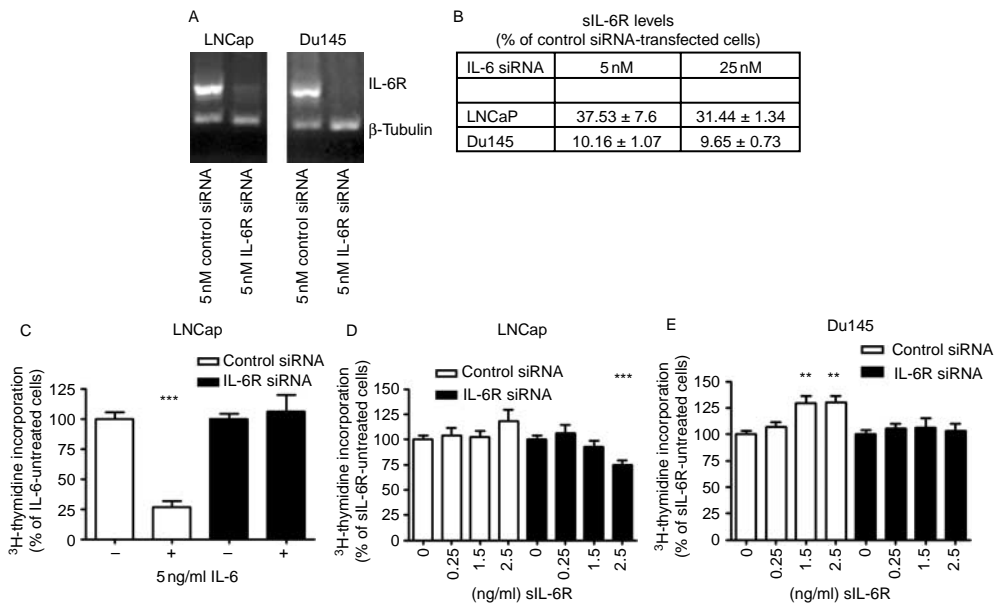


Figure 3 Removal of the contribution of membrane IL-6R by siRNA confirms the anti-proliferative effect of IL-6TS. (A) LNCaP and Du145 were transfected with 5 nM siRNA against IL-6R or equal amounts of control siRNA. RT-PCR was performed to confirm knockdown of IL-6R mRNA. β -Tubulin was used as a loading control. (B) LNCaP and Du145 were transfected with 5 or 25 nM siRNA against IL-6R or control siRNA, as indicated. Reduction of sIL-6R protein levels was assessed by sIL-6R ELISA. (C) LNCaP were transfected with 25 nM siRNA against IL-6R or control siRNA. Proliferation was measured by ^3H -thymidine incorporation in the absence or presence of IL-6. LNCaP (D) and Du145 (E) were transfected with 25 nM siRNA against IL-6R or control siRNA and exposed to increasing doses of sIL-6R prior to treatment with IL-6. Proliferation was measured by ^3H -thymidine incorporation and values, expressed as mean \pm s.e.m., were referred as a percentage of sIL-6R-untreated samples. $^{**}P < 0.01$; $^{***}P < 0.001$.

Addition of IL-6 and sIL-6R increases motility and migration of PC cells and decreases cell adhesion

Based on the higher incidence of PC relapse in patients with increased preoperative levels of sIL-6R and IL-6, we considered a possible involvement of IL-6TS in

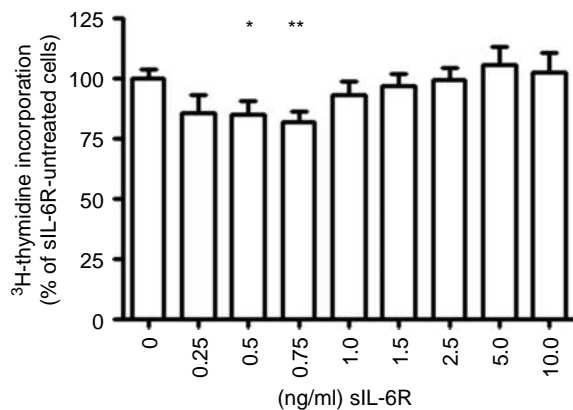
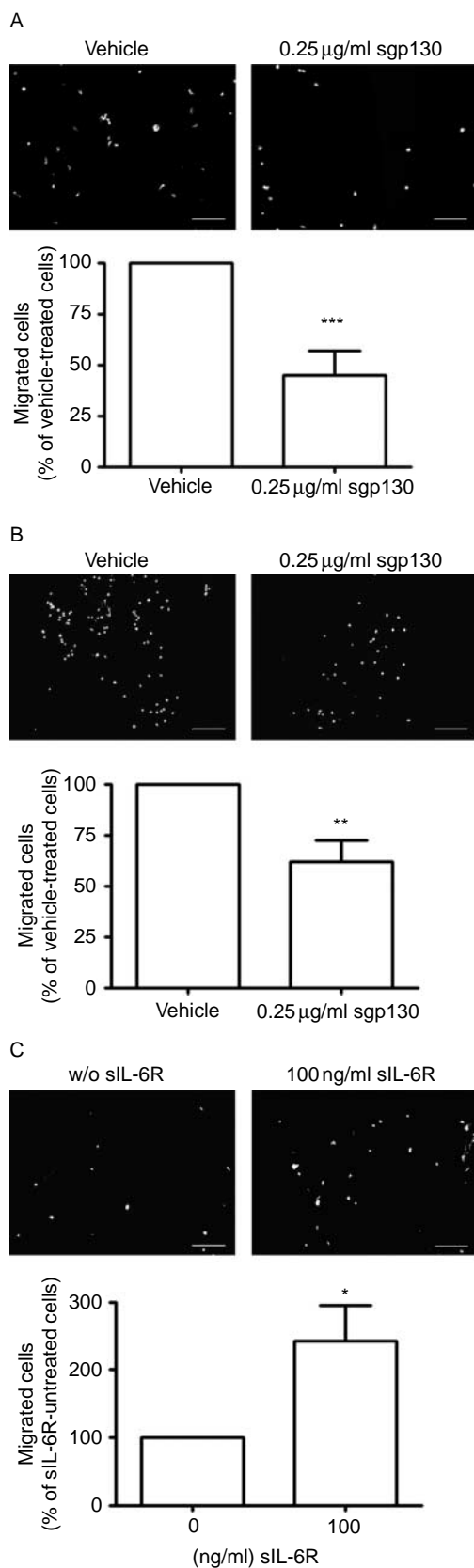


Figure 4 Addition of sIL-6R to LNCaP mediates an anti-proliferative effect of IL-6. LNCaP were treated with increasing doses of sIL-6R and exposed to IL-6, as indicated. Proliferation was measured by ^3H -thymidine incorporation. The values were referred as a percentage of sIL-6R-untreated samples (mean \pm s.e.m.). $^{*}P < 0.05$; $^{**}P < 0.01$.

processes relevant to tumour metastases. To this aim, as an index of cell motility, we tested the ability of LNCaP, Du145 and LAPC4 cells to migrate through an 8 μm pore diameter membrane after exposure to IL-6 in the presence of sgp130, vehicle or sIL-6R. In particular, we found that the number of migrated cells was significantly decreased when LNCaP and Du145 cells were exposed to sgp130 and IL-6 compared to cells exposed to the vehicle and IL-6 (Fig. 5A and B). Consistent with this finding, migration of LAPC4 cells was facilitated in the presence of sIL-6R and IL-6 compared to cells exposed to IL-6 only (Fig. 5C). Afterwards, we applied the scratch assay. Consistently, Du145 cells migrated less after inhibition of IL-6TS (Fig. 6). Unfortunately, LNCaP and LAPC4 cells could not be used in this kind of assay due to their characteristic to form clusters.

Then we investigated the involvement of the sIL-6R on cell adhesion. To this aim, Du145 cells were seeded on fibronectin substrate in the presence of IL-6 alone or along with increasing doses of sIL-6R. We found that the presence of sIL-6R caused a 25% decrease in adhesion to the substrate (Fig. 7). However, when the cells were pre-exposed to the IL-6TS inhibitor sgp130, there was no significant difference between sIL-6R-untreated



and -treated cells. A significantly decreased adhesion in the presence of IL-6 and sIL-6R was also observed when LAPC4 were used (data not shown).

We concluded that sIL-6R has a role in decreasing adhesion and increasing motility and migration of PC cells, events that represent crucial initial steps in the formation of metastases.

IL-6TS decreases maspin expression in PC cell lines

In order to investigate the mechanism by which IL-6TS may promote the spread of PC cells, we looked at the expression of maspin, a tumour suppressor recently shown to be involved in regulation of cell adhesion and motility in PC (Sheng *et al.* 1996, Abraham *et al.* 2003, Ngamkitidechakul *et al.* 2003). We found that the non-cancerous epithelial prostate cells BPH-1 and EP156T express higher basal levels of the protein compared to the PC cell lines tested (Fig. 8). Interestingly, we found that IL-6 decreased the expression of maspin in Du145, LNCaP and LAPC4 cells, but not in EP156T cells (Fig. 9A–C and F respectively). Inhibition of IL-6TS prior to exposure to IL-6 antagonised the decrease of maspin in LNCaP (Fig. 9E) and abrogated it in Du145 cells (Fig. 9D). Finally, a further decrease of maspin was observed in LAPC4 cells after exposure to sIL-6R and to IL-6. The effect is sIL-6R dose-dependent (Fig. 10). These results contribute to understanding the mechanisms through which the cytokine may promote PC progression and demonstrate the involvement of IL-6TS in the downregulation of the tumour suppressor maspin.

Discussion

A pathophysiological role of IL-6 in the progression of PC has been suggested and supported by several *in vitro* and *in vivo* investigations leading to the idea that anti-cytokine therapies could represent a promising attempt to slow down the evolution of this disease. However, *in vitro* and preclinical studies have yielded contrasting responses to IL-6 or anti-IL-6 antibodies. Thus, the outcome of IL-6-targeting therapies may be unpredictable also in PC patients, ranging from a lack of response to beneficial or detrimental effects.

Figure 5 IL-6TS increases cell motility. Cells were seeded onto cell culture inserts, as described in materials and methods. LNCaP (A) and Du145 (B) were exposed to IL-6 after treatment with sgp130 or vehicle. LAPC4 (C) were exposed to IL-6 in the presence or absence of 100 ng/ml sIL-6R. Motility was measured by counting cells that migrated through the membrane. Scale bar is 200 µm. Values are expressed as mean ± s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

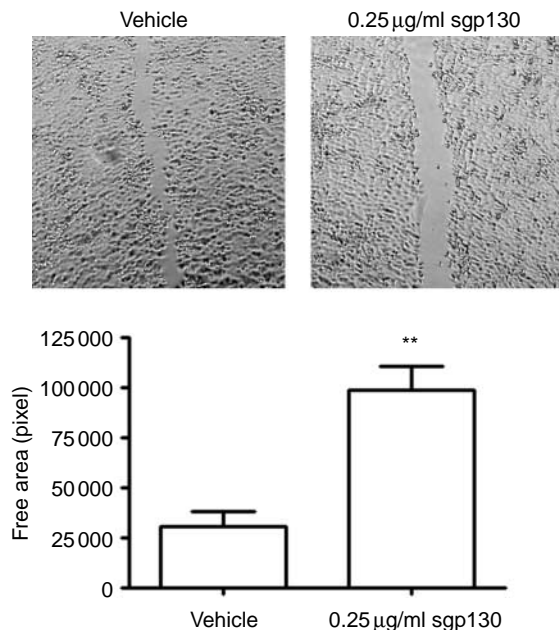


Figure 6 Blocking of IL-6TTS decreases Du145 cell migration. Du145 cells were treated with IL-6 in the presence of sgp130 or vehicle. At cell confluence, a scratch was performed using a 10 µl tip. After 24 h remaining free area was measured. Values are expressed as mean ± s.e.m. ** $P < 0.01$.

In order to better understand the mechanisms underlying the different responses to the cytokine, we focused our attention on IL-6Rs that represent the first element in the cascade of signalling activated by IL-6. In particular, we investigated which roles of IL-6 may be specifically ascribed to IL-6TTS rather than to the classical IL-6 signalling in PC cells.

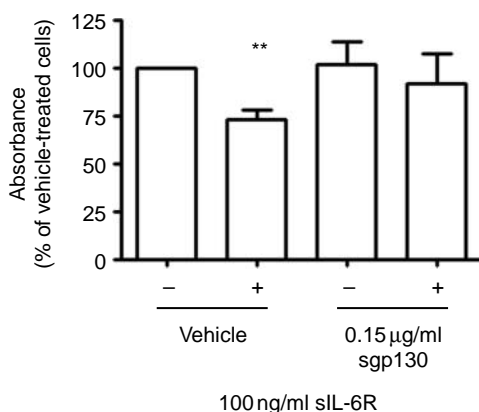


Figure 7 Addition of sIL-6R and IL-6 decreases adhesion to fibronectin, which can be inhibited by sgp130. Du145 cells were seeded on fibronectin substrate in the presence of sgp130 or vehicle and treated with IL-6 in the absence or presence of sIL-6R. Values are expressed as mean ± s.e.m. ** $P < 0.01$.

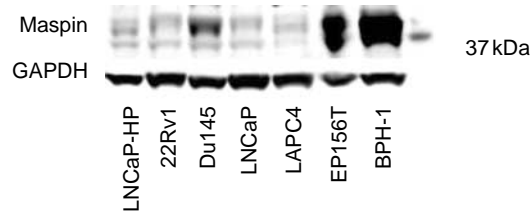
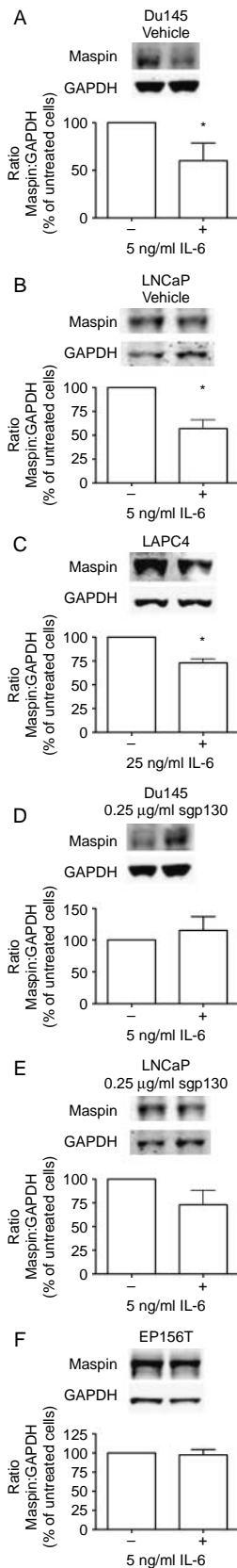


Figure 8 Expression levels of maspin in cancerous and non-cancerous prostate cells. Equal amounts of protein lysates from the non-cancerous BPH-1 and EP156T and the cancerous LAPC4, LNCaP, Du145, PC3, 22Rv1 and LNCaP-HP cells were analysed for maspin expression levels by western blotting. GAPDH served as a loading control.

We used PC cells that express both membrane and soluble receptors as a natural mIL-6R +/sIL-6R + model and cells that possess virtually only the mIL-6R as a natural mIL-6R +/sIL-6R - model. In addition, by modifying the levels of the receptors, we created two additional models: mIL-6R +/sIL-6R -, obtained by specifically inhibiting IL-6TTS in LNCaP and Du145 cells by sgp130; and mIL-6R -/sIL-6R +, in which the sIL-6R has been added to the cells after knockdown of both soluble and membrane IL-6Rs. By using these different approaches, we were able to attribute the capacity to mediate an anti-proliferative effect of IL-6 to the sIL-6R, regardless the cell line used. In particular, proliferation was increased by IL-6 in both LNCaP and Du145 cells after inhibition of IL-6TTS by sgp130. LAPC4 cells were growth inhibited by IL-6 in the presence of sIL-6R. Based on this finding, we expected that the addition of IL-6 and sIL-6R would have caused a decrease of proliferation also in LNCaP and Du145 cells; instead, we measured a significant increase of proliferation. Several hypotheses might explain this apparent discrepancy; the possible existence of a still unknown complex containing both mIL-6R and sIL-6R is intriguing. This complex would be responsible for an increase of proliferation whenever the sIL-6R is increased. On the other hand, the complex IL-6/sIL-6R/gp130, already known to activate only IL-6TTS, would be responsible for a decreased proliferation. The final result would be a sum of the two events. Thus, the mIL-6R would mediate a proliferative effect of the cytokine in competition with IL-6TTS.

Further complexity was found by the fact that IL-6 increases the levels of sgp130 released from LNCaP cells. This effect is probably mediated by the sIL-6R in that IL-6 is not effective in LAPC4 (Table 2) nor in cells with low levels of sIL-6R (data not shown). These data further underline the relevance of the relative content of sIL-6R and mIL-6R for a specific effect of the cytokine. Consistent with our finding, a decrease of proliferation



by IL-6TS has also been recently observed in a pleomorphic malignant fibrous histiocytoma cell line (Nakanishi *et al.* 2004). A fusion protein consisting of human IL-6 and human sIL-6R was also shown to reduce proliferation of B16 melanoma (Ozbek *et al.* 2001) and F10.9 cells (Wagley *et al.* 2007).

At this stage, the possibility of interactions between sIL-6R and other not yet identified factors cannot be excluded and the situation *in vivo* seems to be more complex. In this regard, we cross-refer to Knupfer & Preiss (2008) for an interesting discussion on the possible agonistic and non-agonistic properties of sIL-6R.

Although high levels of IL-6 are considered indicators of bad prognosis and consistently found associated with metastases, very little data showing a direct link between IL-6 and metastatogenesis in different malignancies are known (Kanazawa *et al.* 2007, Studebaker *et al.* 2008); surprisingly, no reports are so far available in the field of PC. As previously mentioned, an association between the levels of sIL-6R in PC patients before prostatectomy and higher probability to develop metastases has been found (Kattan *et al.* 2003, Shariat *et al.* 2004, 2008). We therefore turned our attention to the possible involvement of IL-6/sIL-6R in mechanisms responsible for cancer dissemination to secondary organs. One event relevant to invasion and metastasis formation is the increased motility. Interestingly, we found that activation of IL-6TS resulted in increased motility and migration of PC cells. In accordance with this, a role for IL-6 in promoting migration and invasion has been very recently reported also in breast tumour cells (Walter *et al.* 2009).

Changes in adhesive properties to extracellular matrix (ECM) components also play a critical role in the process of tumour dissemination being an essential prerequisite for increased acquisition of movement of the cells. We measured an IL-6/sIL-6R-dependent decrease of attachment of Du145 and LAPC4 cells to one of the major components of the prostate ECM. This is the first evidence that IL-6TS activation may promote PC dissemination.

In order to clarify through which mechanism IL-6 could facilitate the spread of PC cells, we considered the tumour suppressor gene maspin, a 42 kDa serine protease inhibitor, which has been recently proven to

Figure 9 Maspin expression is decreased by IL-6. This effect can be antagonised by the addition of sgp130. Cells were treated for 48 h with or without IL-6 in the absence or presence of sgp130, as indicated. Maspin and GAPDH (loading control) expression levels were analysed by western blotting and quantified densitometrically. Values are expressed as mean \pm S.E.M. * $P < 0.05$.

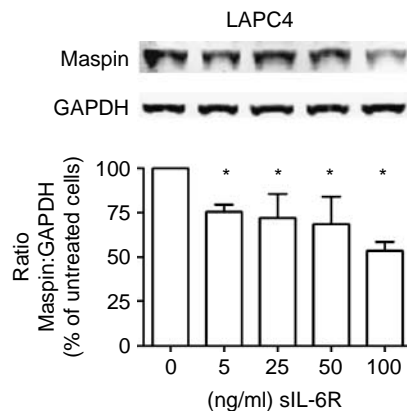


Figure 10 Addition of sIL-6R and IL-6 reduces maspin expression levels in LAPC4. LAPC4 were treated with increasing doses of sIL-6R prior to exposure to IL-6. Treatment lasted for 48 h. Equal amounts of protein lysates were analysed for maspin and GAPDH (loading control) expression by western blotting. Values are expressed as mean \pm S.E.M. * $P < 0.05$.

block tumour invasion, stimulates cell adhesion and blocks angiogenesis in breast and PCs (Zou *et al.* 1994, Sheng *et al.* 1996, Zhang *et al.* 2000, Abraham *et al.* 2003, Cher *et al.* 2003, Ngamkitidechakul *et al.* 2003, Denk *et al.* 2007). In particular, Du145, LNCaP and PC3 cells were shown to be inhibited in terms of invasion and motility when treated with exogenous recombinant maspin (Schaefer & Zhang 2003). Most importantly, *in vivo* evidence that maspin inhibits metastatogenesis was provided by Luo *et al.* (2007); they demonstrated that maspin-negative PC cells from TRAMP mice give rise to numerous liver metastases in nude male mice, whereas forced maspin expression in the same cells dramatically reduced their metastatic activity. Yet, athymic nude mice inoculated with PC3-overexpressing maspin developed significantly fewer and less destructive bone metastases than parental PC3 (Hall *et al.* 2008). We demonstrate that IL-6 decreases the expression of maspin in Du145, LAPC4 and LNCaP PC cells but not in the non-cancerous prostate cells. This finding provides a mechanistic explanation for the pathogenic role of IL-6 in PC progression.

Interestingly, inhibition of IL-6TTS antagonised the effect of the cytokine on maspin levels suggesting the main role for the sIL-6R in mediating this effect of IL-6. The soluble gp130 may regulate maspin expression also in the absence of the IL-6 (data not shown) confirming the already proposed binding of sgp130 to other ligands/soluble receptors (Montero-Julian *et al.* 1997, Jones *et al.* 2001). However, in the presence of the cytokine, affinity of sgp130 for IL-6TTS prevails. The role of IL-6TTS on maspin regulation is further supported by experiments showing that the

levels of maspin decrease in LAPC4 cells after exposure to sIL-6R and IL-6. Interestingly, an increased attachment on fibronectin-coated plates by Du145 cells overexpressing maspin was also reported (Abraham *et al.* 2003, Tahmatzopoulos *et al.* 2005).

Maspin expression is reduced in prostate tumours and cancer cell lines compared to normal prostate epithelial cells (Sheng *et al.* 1996). Consistent with this, we also measured dramatically higher basal levels of maspin in the non-cancerous prostate epithelial cells compared to PC cells. Decreased expression of maspin has been shown to correlate with breast and PC progression (Maass *et al.* 2000, Pierson *et al.* 2002). In particular, in human primary PCs, maspin expression consistently appears to be down-regulated at the critical transition from non-invasive, low-grade disease to highly invasive, high-grade PC (Pierson *et al.* 2002).

With respect to possible signalling pathways activated by IL-6TTS, we have considered phosphorylation of STAT3, ERK1/2 and STAT1 in cells in which IL-6TTS had been previously enhanced or inhibited. Neither signalling pathway was found to be specifically activated by IL-6TTS (data not shown). To the best of our knowledge, there is so far nothing known about a signalling pathway that can be switched on/off only by IL-6/sIL-6R. We believe that specificity to the IL-6TTS may be given by other cytokines and/or receptors. Future investigations in these directions are planned.

The current work showing a role of IL-6TTS in decreasing adhesion and increasing motility and migration along with its effect on the inhibition of maspin strongly supports the hypothesis that the sIL-6R may have an important role in metastasis formation. Therefore, specific targeting of IL-6TTS in PC patients might represent an elegant way to refine the currently available experimental anti-IL-6 therapies. It would be worth in the future to determine whether the sgp130 plasma levels, in addition to those of sIL-6R and IL-6, are altered in PC patients with a worse prognosis. This information may help to identify those patients who could benefit from the anti-cytokine therapy.

Declaration of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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