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Due to interleukin-6 type cytokine redundancy only glycoprotein 130 receptor blockade efficiently inhibits myeloma growth

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Haematologica 2017
Volume 102(2):381-390

ABSTRACT

Interleukin-6 has an important role in the pathophysiology of multiple myeloma where it supports the growth and survival of the malignant plasma cells in the bone marrow. It belongs to a family of cytokines which use the glycoprotein 130 chain for signal transduction, such as oncostatin M or leukemia inhibitory factor. Targeting interleukin-6 in plasma cell diseases is currently evaluated in clinical trials with monoclonal antibodies. Here, efforts were made to elucidate the contribution of interleukin-6 and glycoprotein 130 signaling in malignant plasma cell growth *in vivo*. In the xenograft severe combined immune deficiency model employing our interleukin-6-dependent plasma cell line INA-6, the lack of human interleukin-6 induced autocrine interleukin-6 production and a proliferative response to other cytokines of the glycoprotein 130 family. Herein, mice were treated with monoclonal antibodies against human interleukin-6 (elsilimomab/B-E8), the interleukin-6 receptor (B-R6), and with an antibody blocking glycoprotein 130 (B-R3). While treatment of mice with interleukin-6 and interleukin-6 receptor antibodies resulted in a modest delay in tumor growth, the development of plasmacytomas was completely prevented with the anti-glycoprotein 130 antibody. Importantly, complete inhibition was also achieved using F(ab')2-fragments of monoclonal antibody B-R3. Tumors harbor activated signal transducer and activator of transcription 3, and *in vitro*, the antibody inhibited leukemia inhibitory factor stimulated signal transducer and activator of transcription 3 phosphorylation and cell growth, while being less effective against interleukin-6. In conclusion, the growth of INA-6 plasmacytomas *in vivo* under interleukin-6 withdrawal remains strictly dependent on glycoprotein 130, and other glycoprotein 130 cytokines may substitute for interleukin-6. Antibodies against glycoprotein 130 are able to overcome this redundancy and should be explored for a possible therapeutic window.

Introduction

Multiple myeloma (MM) is characterized by the expansion of clonal plasma cells within the bone marrow (BM), leading to an M-protein in serum, antibody deficiency, and skeletal destruction. Despite stem cell transplantation and novel therapies, the vast majority of patients with MM will eventually relapse and become refractory to standard therapy. Treatment strategies specifically targeting mechanisms of tumor growth and survival are being intensely explored in MM in order to improve

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Received: February 25, 2016.
Accepted: September 14, 2016.
Pre-published: September 22, 2016.

doi:10.3324/haematol.2016.145060

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/102/2/381

patient outcome.[1]

In the pathogenesis of MM, genetic changes drive the development of the malignant clone, but the interaction between the malignant plasma cells and the BM microenvironment has been shown to be equally important in mediating myeloma cell survival and progression.[2] One of the established pathogenic key factors produced in the BM milieu is interleukin(IL)-6, which promotes the growth and survival of the malignant plasma cells and mediates drug resistance.[3] While some myeloma cells produce their own IL-6,[4] bone marrow stromal cells (BMSCs) are the main source, establishing a strong paracrine growth stimulation.[5] Other sources of IL-6 in MM are macrophages, osteoblasts and osteoclasts;[2] eosinophils and megakaryocytes may also contribute.[6]

The receptor for IL-6 comprises a specific α-receptor, glycoprotein (gp) 80 (CD126), which, after ligand binding, recruits the gp130 receptor (IL6ST, CD130). Gp130 is the common signal transducer for a family of cytokines with pleiotropic and partly redundant activities.[7] While signaling *via* IL-6 and IL-11 is initiated *via* gp130 homodimerization, the receptor complexes of other family members consist of heterodimers of gp130 with a second signaling molecule, most of which use the leukemia inhibitory factor receptor (LIFR). Leukemia inhibitory factor (LIF) and oncostatin M (OSM) directly induce gp130/LIFR heterodimerization without the involvement of other receptor components. Upon dimerization, associated Janus kinases (JAKs) become activated and phosphorylate specific tyrosine residues on the receptors, which serve as docking sites for transcription factors and adaptor proteins. The main signaling pathways induced by gp130 are the activation of STAT (signal transducer and activator of transcription)-3, the Ras-dependent mitogen-activated protein kinase (MAPK) cascade, and the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) pathway.[7,8]

The human plasma cell line INA-6 was generated in our laboratory from the pleural effusion of a patient with advanced plasma cell disease.[9] The survival of INA-6 cells *in vitro* is strictly dependent on exogenous IL-6 without growth response to other gp130 cytokines. With the establishment of a xenograft model in severe combined immune deficiency (SCID) mice using INA-6, a non-optimal environment devoid of human IL-6 was provided. Despite the fact that murine IL-6 is not active on human cells, plasma cell tumors developed over a period of up to five months. In serum and ascites of tumor-bearing mice, tiny amounts of human IL-6 were detected, suggesting an autocrine growth mechanism. Even more exciting, some of the plasmacytomas that evolved were responsive not only to IL-6, but also to other gp130 cytokines, such as LIF and OSM, by virtue of emerging LIFR expression.[9,10] These studies were performed *in vitro* after explantation of the tumor cells.

The aim of the study herein was to evaluate the contribution of IL-6 and the potential role of other gp130 family cytokines for INA-6 cell growth *in vivo*. Monoclonal antibodies (mAbs) against human IL-6, the human interleukin-6 receptor (IL-6R), and against gp130 were compared for their anti-myeloma activity in the INA-6 xenograft model.

Table 1. Numerical and structural chromosomal changes in INA-6 and subline INA-6.Tu1.

	Shared	Not shared	Cell line
Numerical	+1	none	INA-6
	−4	−2	
	+7	−5	
	+8	−9	
		−13	INA-6.Tu1
		−15	
		−17	
		−18	
Structural	del(7)(p13)x2	add(4)(p16)	
	dup(8)(q22q23)x2	del(5)(q13q35)	
	der(14)t(11;14)(q13;q32)x2	+mar2	INA-6
	+mar1	del(1)(p22)x3	
		der(10)t(10;15)(q23;q22)	
		+mar3	INA-6.Tu1
		+mar4	
		+mar5	

mar: marker chromosome; del: deletion; dup; duplication; der: derivative; add: additional material.

Methods

Cell lines

The human (hu) IL-6-dependent INA-6 plasma cell line was established in our laboratory as described.[9] Subline INA-6.Tu1 is derived from an engrafted tumor of INA-6, grown in the peritoneum of a SCID mouse, and described earlier.[9] The murine (mu) IL-6-dependent B9 hybridoma was a kind gift of Dr. L. A. Aarden (Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands).[11] Cells were routinely cultivated in the presence of recombinant huIL-6 at 2.5 ng/ml (Gibco®/Life Technologies GmbH, Darmstadt, Germany). Additional details are provided in the *Online Supplementary Methods.*

Antibodies and fusion proteins

Mouse mAbs against huIL-6 (B-E8/elsilimomab),[12] the huIL-6R/gp80 (B-R6),[13] and hugp130 (B-R3)[14] were developed by Dr. J. Wijdenes (Diaclone, Besançon, France). Antibodies were carrier and preservative free. All antibodies, with the exception of B-R3 (immunoglobulin (Ig) G2a), are of IgG1 isotype. Fusion proteins Hyper-IL-6 and sgp130Fc were developed as described.[15,16] Cytokines and other reagents are described in the *Online Supplementary Methods*.

Cytogenetic analysis

Karyotyping and interphase fluorescence *in situ* hybridization (FISH) analyses were performed as described.[17] Details are provided in the *Online Supplementary Methods.*

Cell growth assay

Cell growth was determined using a colorimetric, tetrazolium salt (MTS)-based assay (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA) as described.[10] Details are described in the *Online Supplementary Methods.*

Cell lysis and Western blotting

To evaluate the effects of antibodies on signaling pathways, INA-6 and INA-6.Tu1 cells were extensively washed and then resuspended in R10$^+$ medium in the absence or presence of antibodies (20 μg/ml) or the JAK inhibitor (4 μM). After 2 h at 37°C,

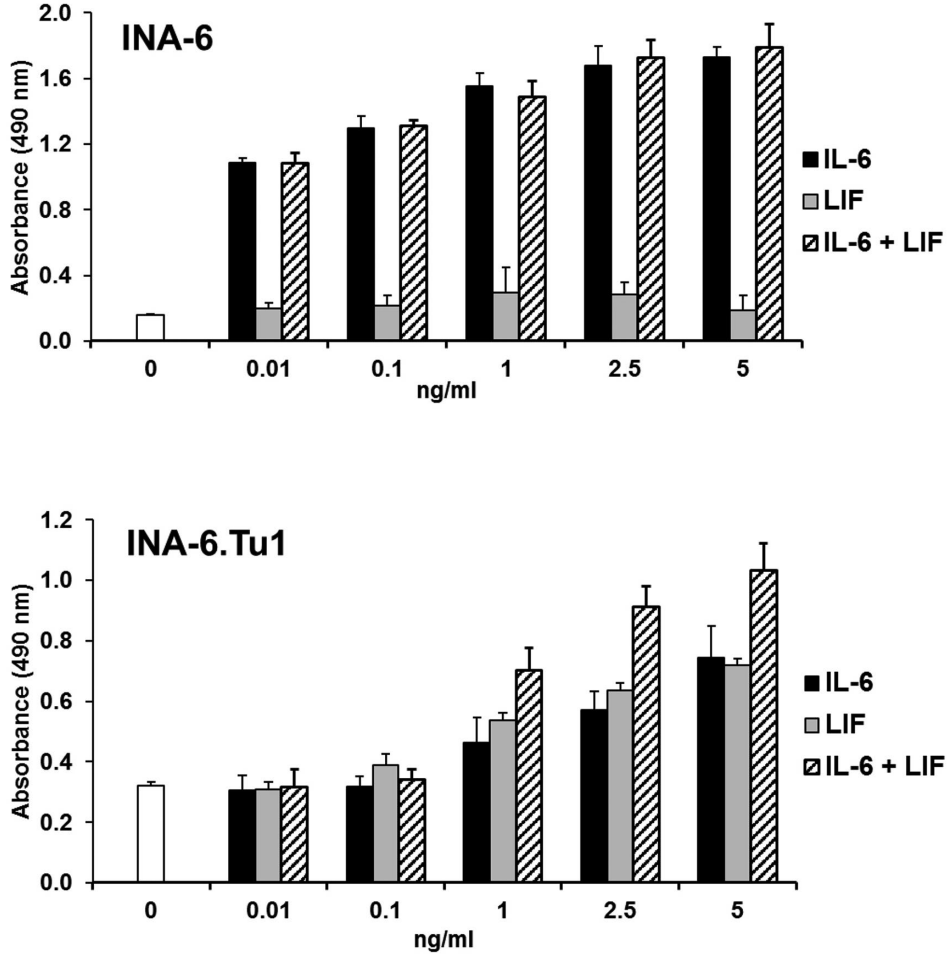

Figure 1. Growth response of INA-6 and INA-6.Tu1 to human recombinant IL-6 and LIF. Cells were cultivated at indicated concentrations of cytokines alone or in combination for 72 h, and viable cell mass evaluated by measuring metabolic activity in a tetrazolium (MTS)-based colorimetric assay. Values represent the mean of triplicate cultures. Error bars, standard deviation (SD). IL-6: interleukin-6; LIF: leukemia inhibitory factor.

cells were stimulated with 10 ng/ml IL-6 or LIF for 10 min, washed in cold phosphate buffered saline (PBS), and subjected to lysis. Equal amounts of protein were resolved by standard sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking, membranes were stained with antibodies against phosphorylated and total STAT3, extracellular signal-regulated kinases 1/2 (ERK1/2) and S6 ribosomal protein and processed as described in the *Online Supplementary Methods.*

Animal Studies

In general, 25 x 10^6 INA-6.Tu1 plasma cells were injected intraperitoneally (IP) into 7 to 9 week-old SCID or SCID/beige mice. Antibody treatment started one day after cell inoculation by IP or, in the case of F(ab')$_2$ fragments, alternating IP/intravenous (IV) injections. Seven injections and a total of 0.85 mg antibody per mouse were administered within 14 days. In the study with sgp130Fc, 150 μg protein were injected IP once a week for 4 consecutive weeks. Mice were monitored on a routine basis for signs of tumor development. The survival time was defined as the time between cell inoculation and the day of sacrifice, when tumor burden caused paraplegia, cachexia, or any other signs of suffering. Animals without any signs of tumors were sacrificed at the end of the experiment, on day 136 at the latest.

Additional details are provided in the *Online Supplementary Methods.*

Statistical analysis

Details are given in the *Online Supplementary Methods*.

Results

Cytogenetic characterization of INA-6 and its subline INA-6.Tu1

For studies in SCID mice, we used a subline of the human IL-6 dependent INA-6 cell line, INA-6.Tu1.

This subline was derived from an engrafted tumor of INA-6 grown in the abdominal cavity of a SCID mouse. INA-6.Tu1 yields reproducible and faster engraftment rates than the original INA-6, while having maintained its dependency on human IL-6.[9] A comprehensive cytogenetic analysis was performed of INA-6 and, for the first time, of INA-6.Tu1 cells.

Both cell lines harbored a unique R-banding karyotype, which correlates with previously reported karyotypes and

excludes cross-contamination with other cell lines (*Online Supplementary Figure S1*). They showed a male tetraploid karyotype with the previously described derivative chromosome der(14)t(11;14)(q13;q32), resulting in an atypical *CCND1-IGH* fusion with loss of the derivative chromosome 11. Subline INA6.Tu1 with 11 numerical and 9 structural aberrations has a higher complexity score than the original INA-6 with 4 numerical and 7 structural aberrations (Table 1). A number of shared common aberrations such as a deletion in 7p, a duplication involving 8q, one marker chromosome as well as various numerical aberrations confirm the common origin of these cell lines. Interestingly, INA-6 harbors a duplication of the *MMSET/FGFR3* locus on the aberrant chromosome add(4)(p16), and INA-6.Tu1 presents with a deletion in 1p, which is absent in INA-6 (Table 1).

Cytokine activation of INA-6.Tu1 cells in vitro *and* in vivo

Of note, INA-6 and INA-6.Tu1 grow in SCID mice without an exogenous supply of human IL-6. While mouse IL-6 is not active on human cells, some other gp130 cytokines such as LIF display cross-species activity.[9] Signaling *via* LIF involves a heterodimer of gp130 and the LIFR. While the LIFR is absent in INA-6, it is expressed in INA-6.Tu1 cells[9] (*Online Supplementary Figure S2*), and results in growth responsiveness to LIF (Figure 1). Moreover, INA-6.Tu1 cells produce IL-6 in an autocrine fashion not seen *in vitro*.[9] In plasmacytomas derived from INA-6.Tu1, important IL-6/gp130 mediated signaling pathways are activated *in vivo*, as indicated by phosphorylation of STAT3 and ERK1/2 in freshly explanted tumor cells (Figure 2).

IL-6 and LIF inhibition in vitro

Three antibodies targeting IL-6 or the IL-6R/gp130 complex were compared regarding their potential to block IL-6- and LIF-induced INA-6.Tu1 cell growth: antibody B-E8 neutralizes human IL-6,[12] the B-R6 antibody binds to the human gp80 α-receptor,[13] and antibody B-R3 binds to human gp130 (Figure 3A).[14]

While B-E8 and B-R6 antibodies specifically inhibit IL-6- but not LIF-induced cell growth, antibody B-R3 preferentially blocks the response mediated by LIF (Figure 3B). No differences in the degree of inhibition were observed when the receptor antibodies B-R6 and B-R3 were used over a concentration range of 1 μg/ml up to 100 μg/ml (*data not shown*). Nearly complete inhibition of IL-6-induced growth can be achieved when B-R3 is combined with B-R6 (Figure 3B). Antibodies B-R6 and B-R3 do not cross-react with mouse receptors, as verified on the murine IL-6-dependent B9 hybridoma cell line. B9 cells were stimulated with human IL-6, which was effectively neutralized by antibody B-E8 (Figure 4).

The main signaling pathway induced by IL-6 in INA-6 cells is the activation of STAT3, which is essential for these cells to grow and survive.[18] Cytokine-starved INA-6 and INA-6.Tu1 cells were pre-treated with antibodies or a JAK inhibitor for two hours prior to stimulation with IL-6 or LIF, respectively. Both IL-6 and LIF induced a strong STAT3 phosphorylation signal, which was completely abrogated by the JAK inhibitor (Figure 5). Remarkably, the anti-gp130 antibody B-R3 was able to inhibit IL-6 as well as LIF induced STAT3 phosphorylation to an almost full extent. Antibody B-R6 prevented STAT3 phosphorylation only when cells were stimulated with IL-6, as expected given its specificity for the human IL-6R (Figure 5). Activation of the PI3 kinase/AKT and the MAPK pathway as shown by phosphorylation of the S6 protein and ERK1/2, occurs independently from gp130 stimulation without differences between INA-6 and INA-6.Tu1 cells, namely by serum factors and a mutation in the *N-RAS* gene.[9] Consequently, these two pathways were not affected by IL-6R or gp130 antibodies (Figure 5).

Monoclonal antibodies against the IL-6/IL-6R complex in the xenograft model

Antibodies B-E8, B-R6 and B-R3 were compared regarding their ability to inhibit plasma cell growth *in vivo*. SCID mice grafted with INA-6.Tu1 cells were treated with antibodies starting one day after cell inoculation. Antibodies

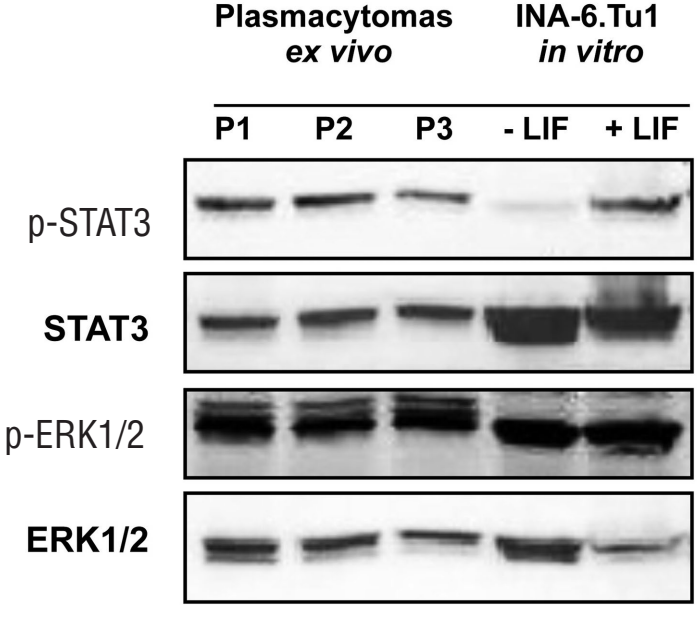

Figure 2. Activated signaling pathways in INA-6.Tu1 plasma cell tumors. Western blot analysis of whole cell lysates freshly prepared from explanted plasmacytomas of three individual animals (P1-P3). Lysates of INA-6.Tu1 cells unstimulated and stimulated with LIF at 10 ng/ml for 10 min were included for comparison. Membranes were stained with antibodies against phosphorylated and total STAT3 and ERK1/2 as indicated. p: phosphorylated; STAT3: signal transducer and activator of transcription 3; ERK 1/2: extracellular signal-regulated kinases 1/2; LIF: leukemia inhibitory factor.

were administered with 7 injections within 14 days. In PBS treated control groups, all animals but one developed tumors and had to be sacrificed in less than 60 days (Figure 6). In mice treated with anti-IL-6-antibody B-E8 (Figure 6A) and with the IL-6R-specific antibody B-R6 (Figure 6B), plasmacytoma formation was slightly delayed compared to the control group. In contrast, no plasmacytomas were observed in mice that received the anti-gp130 antibody B-R3, nor did they show any signs of illness (Figure 6A,B). Importantly, complete tumor prevention was also achieved using F(ab')2 fragments of antibody B-R3 (Figure 6C), arguing against tumor cell eradication by opsonization or antibody-dependent cell-mediated cytotoxicity.

IL-6 trans-signaling

IL-6 trans-signaling is mediated by the complex of IL-6 and the soluble (s) IL-6R on cells that lack the IL-6R but express gp130.[7,15] While muIL-6 is not active on human cells,

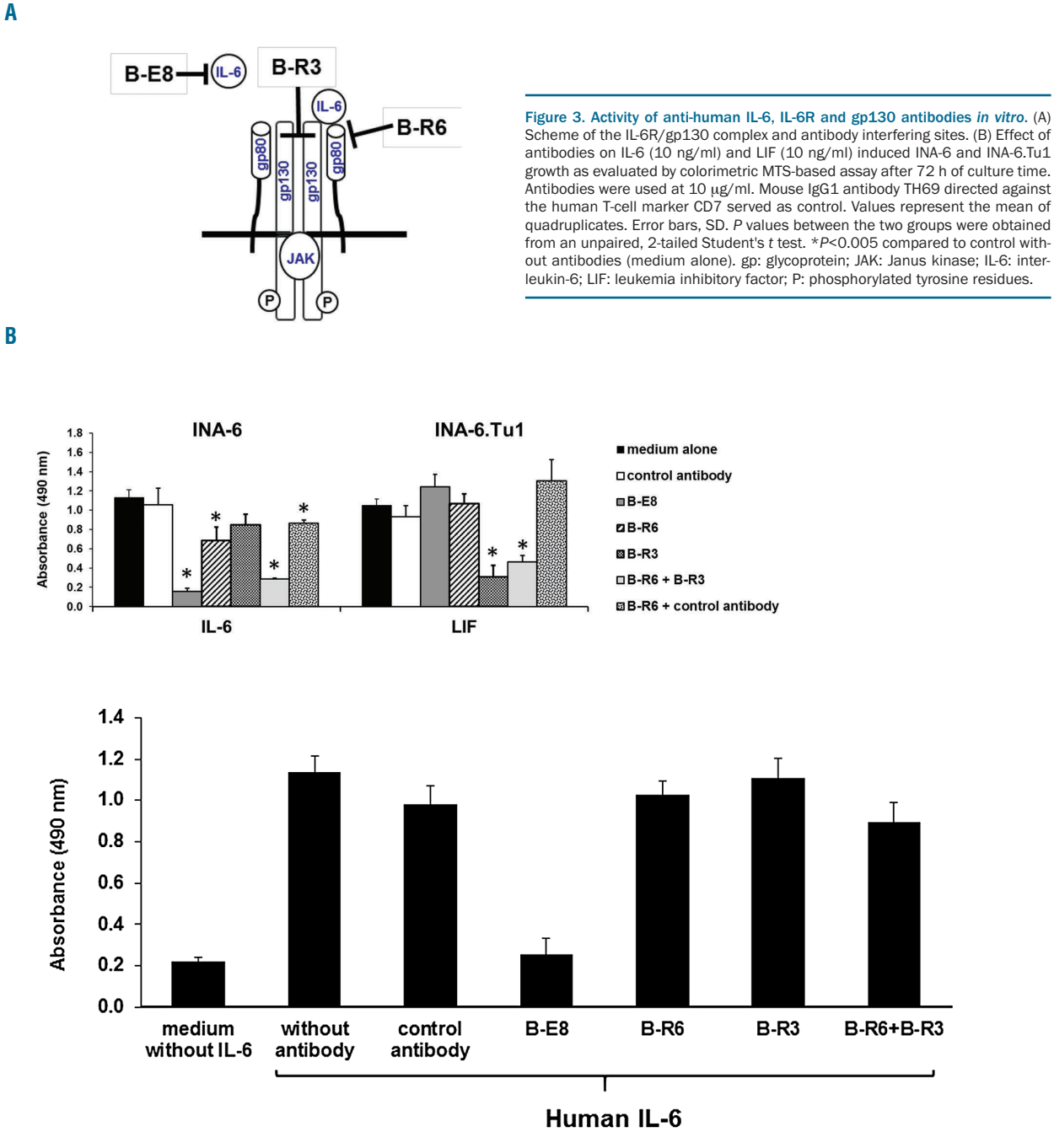

Figure 3. Activity of anti-human IL-6, IL-6R and gp130 antibodies *in vitro*. (A) Scheme of the IL-6R/gp130 complex and antibody interfering sites. (B) Effect of antibodies on IL-6 (10 ng/ml) and LIF (10 ng/ml) induced INA-6 and INA-6.Tu1 growth as evaluated by colorimetric MTS-based assay after 72 h of culture time. Antibodies were used at 10 μg/ml. Mouse IgG1 antibody TH69 directed against the human T-cell marker CD7 served as control. Values represent the mean of quadruplicates. Error bars, SD. *P* values between the two groups were obtained from an unpaired, 2-tailed Student's *t* test. **P*<0.005 compared to control without antibodies (medium alone). gp: glycoprotein; JAK: Janus kinase; IL-6: interleukin-6; LIF: leukemia inhibitory factor; P: phosphorylated tyrosine residues.

Figure 4. Antibodies B-R6 and B-R3 do not interfere with mouse receptors. Murine B9 cells (2 x 10^4 per well) were cultivated with 10 ng/ml human IL-6 in the presence or absence of antibodies (20 μg/ml) for 3 days, and viable cell mass evaluated by MTS assay. Antibody B-E8 neutralizes human IL-6, while receptor antibodies have no effect. Mouse IgG1 antibody TH69 against the T-cell antigen CD7 was used as a control. Values represent the mean of triplicate cultures. Error bars, SD. IL-6: interleukin-6.

the combination of muIL-6 and murine sIL-6R induced significant growth of INA-6 cells (Figure 7A). In order to evaluate if IL-6 trans-signaling *via* the murine IL-6/sIL-6R complex could play a role in the INA-6 xenograft model, we used a fusion protein of the human gp130 ectodomain linked to the fragment crystallizable (Fc)-portion of a human IgG antibody, sgp130Fc.[16] This protein selectively blocks IL-6 trans-signaling and does not interfere with IL-6 responses *via* the membrane-bound IL-6R. In addition to inhibiting the human IL-6/sIL-6R complex, sgp130Fc had been shown to block the activity of murine IL-6/sIL-6R on cells transfected with human gp130.[19] Consistently, sgp130Fc was capable of inhibiting INA-6.Tu1 cell growth when stimulated with muIL-6 together with the murine sIL-6R (Figure 7B). As a control for sgp130Fc functionality, IL-6 trans-signaling by Hyper-IL-6, a fusion protein of huIL-6 and human sIL-6R,[15] was also significantly decreased, while stimulation of INA-6.Tu1 cells with huIL-6 *via* the membrane IL-6R was not affected (Figure 7B).

The treatment of mice with sgp130Fc did not inhibit the development of plasmacytomas, nor did it prolong survival compared to the control group (Figure 7C). In conclusion, trans-signaling by the murine IL-6/sIL-6R complex does not seem to play a role in INA-6.Tu1 plasmacytoma growth *in vivo*.

Discussion

Growth of the IL-6-dependent human plasma cell line INA-6 in SCID mice induced autocrine IL-6 production and a proliferative response to other cytokines of the gp130 family.[9] The mechanism of INA-6 growth *in vivo*, however, remained to be elucidated. Herein, the relative contribution of IL-6 as opposed to other gp130 cytokines was evaluated by treating INA-6.Tu1 grafted SCID mice with antibodies against human IL-6, the human IL-6R and against gp130. While the blockade of IL-6 only resulted in some delay of plasma cell growth, the development of plasmacytomas was completely prevented with the anti-gp130 antibody B-R3.

Gp130 is a ubiquitously expressed signaling receptor shared by a number of cytokines.[7] It signals by homodimerization in the case of IL-6 and IL-11, or by heterodimerization with a second signaling chain, often the LIFR, which serves to bind LIF, OSM, ciliary neurotrophic factor (CNTF), cardiotrophin (CT)-1, and cardiotrophin-like cytokine. The extracellular part of gp130 is composed of six domains with an Ig-domain at the N-terminus, designated D1, followed by the cytokine-binding homology region (CHR) D2 and D3, and three fibronectin III-like domains (D4-D6). With regard to IL-6, the Ig-domain and the CHR domain bind to the ligand at site III and site II, respectively, to form a hexamer with two nonsignaling α-receptors. In contrast, heterodimeric gp130/LIFR assembly involves contacts between the gp130-CHR and site II of the ligand, and of the Ig-domain (D3) of the LIFR with site III of the ligand, resulting in trimeric (e.g., LIF/LIFR/gp130) or quaternary (CNTF/CNTFRα/LIFR/gp130) complexes.[8]

Antibody B-R3 binds to D2 of gp130, which involves the relative non-specific cytokine-binding module.[14]

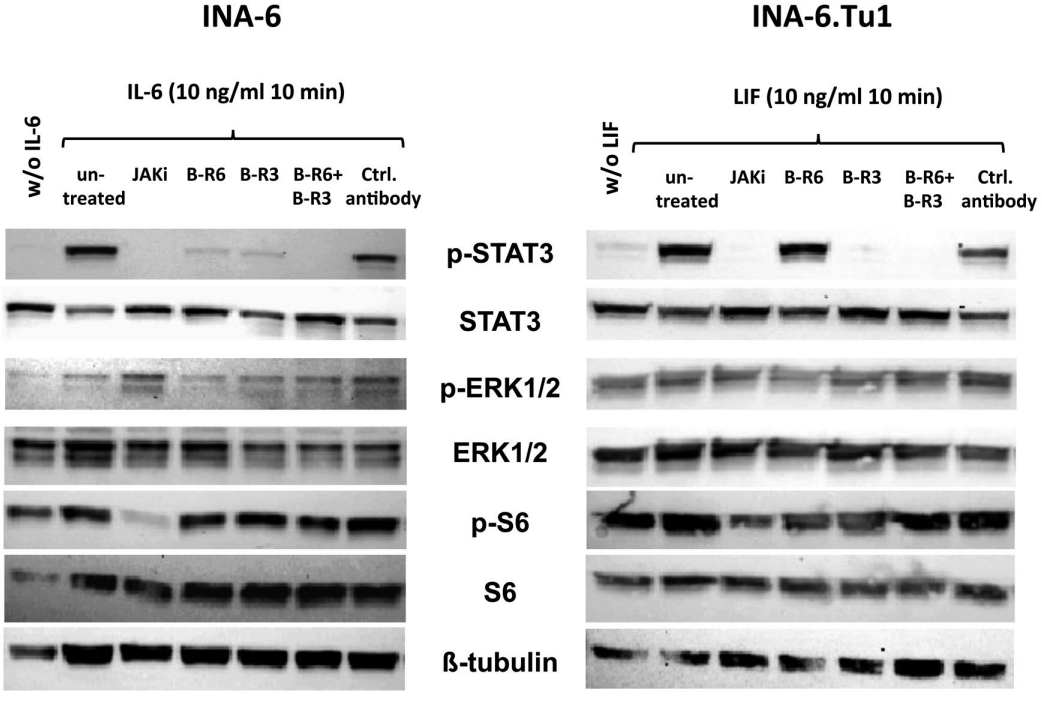

Figure 5. Inhibition of cytokine-induced STAT3 phosphorylation by gp130 antibody B-R3 in INA-6 and INA-6.Tu1 plasma cells. INA-6 and INA-6.Tu1 cells were left untreated or incubated for 2 h in complete medium containing 10% serum in the absence or presence of the pan-JAK inhibitor-I (4 μM), anti-IL-6R antibody B-R6 (20 μg/ml), anti-gp130 antibody B-R3 (20 μg/ml) or a combination of both (10 μg/ml each). An irrelevant mouse IgG1 antibody (TH69) served as a control. After stimulation with IL-6 (INA-6) or LIF (INA-6.Tu1), cells were washed in cold PBS and whole cell lysates prepared. Membranes were stained with antibodies against phosphorylated and total proteins as indicated. β-tubulin served as a loading control. IL-6: interleukin-6: w/o: without; LIF: leukemia inhibitory factor; JAKi: Janus kinase inhibitor; STAT3: signal transducer and activator of transcription 3; ERK 1/2: extracellular signal-regulated kinases 1/2; Ctrl: control; p: phosphorylated.

However, with the exception of OSM, the antibody does not interfere with gp130-ligand binding to the respective receptors, instead, it inhibits the association with the second signaling chain.[20] Thus, B-R3 has been described as a pan-blocking antibody, which is able to antagonize the biological activities of several gp130 cytokines.[14,20] The antibody markedly inhibited proliferation of a cytokine-dependent INA-6 subline stimulated with OSM, LIF, CNTF, CT-1, or B cell-stimulating factor (BSF)-3.[10] B-R3 also blocked INA-6 cell proliferation induced by viral IL-6 encoded by human herpesvirus type-8 (HHV-8).[21] With regard to human IL-6, the antibody is less active.[10,21]

All gp130 cytokines potently activate STAT3 *via* receptor-associated protein tyrosine kinases JAK1, JAK2 and TYK2.[8] STAT3 phosphorylation induced by IL-6 and LIF in INA-6 and INA-6.Tu1 cells was abrogated in the presence of the B-R3 antibody. This is in line with previous observations that the antibody antagonizes receptor homo- or heterodimerization and phosphorylation, independently of the nature of the cytokine.[20] Activated STAT3 is essential for INA-6 cell growth and survival,[18] and is present in xenografted tumors. This leads to the conclusion that the abrogation of gp130-mediated STAT3 signaling is the major mechanism by which the antibody prevents plasmacytoma cell growth *in vivo*. The existence of other signaling pathways in INA-6 and INA-6.Tu1, that are activated independently from gp130 stimulation and are not affected by the B-R3 antibody, strongly support our hypothesis. Importantly, complete tumor prevention in mice with F(ab')2 fragments of B-R3 demonstrates that the observed effect was by receptor inhibition rather than Fcγ-receptor-mediated immune effector mechanisms such as antibody-dependent cell-mediated cytotoxicity. Moreover, the difference in anti-myeloma activity between anti-gp130 and anti-IL-6R antibodies in our model seems not to be caused by differences in plasma clearance rates, which are similar for mouse IgG1 and IgG2a in SCID mice,[22] but instead points to an *in vivo* growth support by gp130 cytokines other than IL-6. While the lack of STAT3 phosphorylation in unstimulated INA-6.Tu1 cells indicates that *in vitro* autocrine IL-6 does not play a role, some contribution of autocrine IL-6 to INA-6.Tu1 growth *in vivo* cannot be ruled out. However, human IL-6 levels in serum or ascites of INA-6.Tu1 grafted SCID

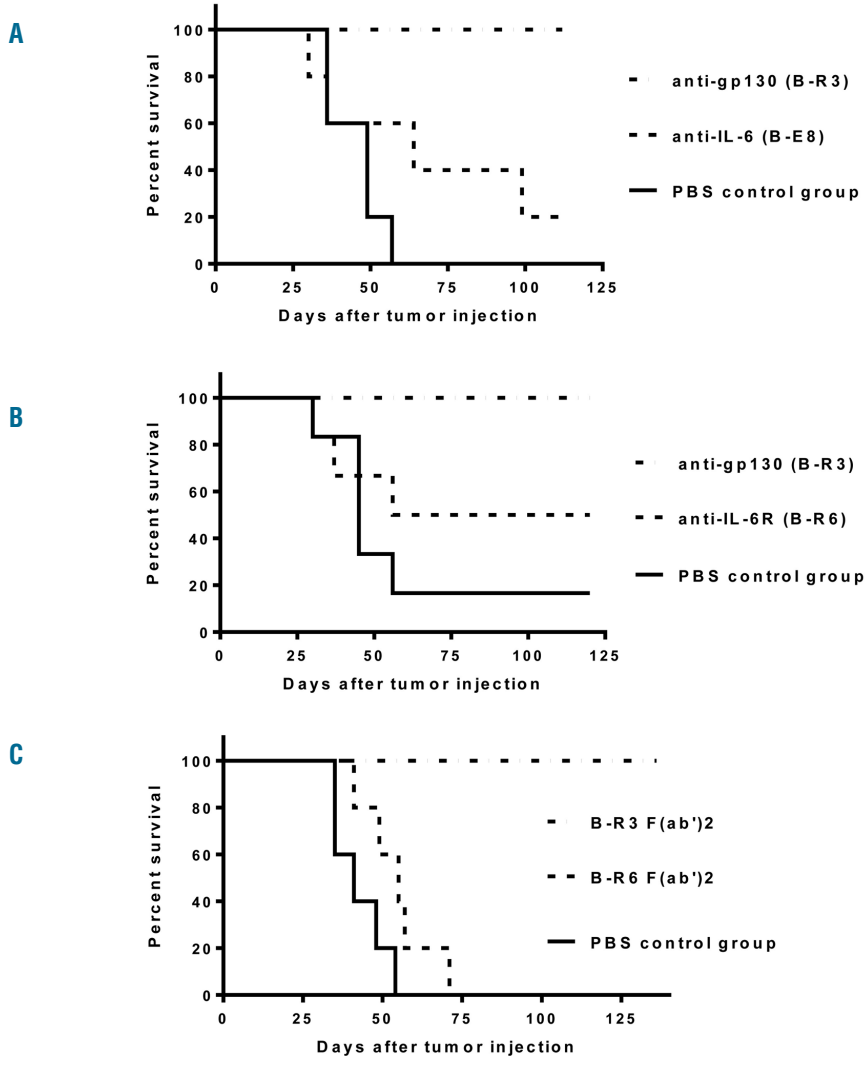

Figure 6. Anti-gp130 antibody B-R3 prevents plasmacytoma growth *in vivo*. Mice were inoculated IP with 25 x 10^6 INA-6.Tu1 cells and treated with antibodies as indicated, starting one day after cell inoculation. Antibodies were administered with 7 injections within 14 days, with a total amount of 0.85 mg antibody per animal. PBS was used as vehicle control. *P*-values were calculated by log-rank test. (A) Survival of mice treated with IL-6 neutralizing antibody B-E8 and anti-gp130 antibody B-R3. $P<0.18$ for B-E8, $P<0.005$ for B-R3, compared to the control group (n=5 per group). (B) Survival of mice treated with the anti-IL-6R antibody B-R6 and anti-gp130 antibody B-R3. $P=0.36$ for B-R6, $P<0.005$ for B-R3, compared to the control group (n=6 per group). (C) Survival of mice treated with F(ab')2 fragments of antibodies B-R6 (n=5) and B-R3 (n=3). $P<0.05$ for B-R3, $P<0.05$ for B-R6, compared to the control group (n=5). gp: glycoprotein; IL-6: interleukin-6; PBS: phosphate buffered saline.

mice were very low.[9] Interestingly, in a disseminated myeloma xenograft model with an autocrine IL-6 growth mechanism, an anti-IL-6R antibody achieved significant suppression of tumor growth. However, this effect was only transient, and eventually all of the mice succumbed to the disease.[23] In a B-cell lymphoma xenograft model with high autocrine IL-6 production, blocking IL-6 with B-E8 and B-R6 antibodies had little effect on survival, while treatment with anti-gp130 antibody B-R3 resulted in the survival of a significant number of animals. The authors raised the possibility that other cytokines of the gp130 family participated in the *in vivo* growth of the lymphoma cells.[24]

Murine IL-6 does not bind to the human IL-6R, but it can act on cells expressing human gp130 in conjunction with the soluble mouse IL-6R, i.e., by trans-signaling.[19] Therefore, the stimulation of INA-6.Tu1 cells by mouse IL-6/sIL-6R could potentially represent a paracrine growth mechanism *in vivo*. Since the interaction between the IL-6/sIL-6R complex and gp130 is species independent, the human sgp130Fc fusion protein could function as an antagonist to block mouse IL-6 trans-signaling.[19] Sgp130Fc is specific for IL-6/sIL-6R and does not inhibit CNTF activity, and only poorly limits LIF and OSM.[16] While sgp130Fc was able to block INA-6.Tu1 cell growth induced by murine IL-6/sIL-6R *in vitro*, it provided no protective effects in mice. The amounts administered were sufficient to block IL-6 trans-signaling mediated processes in other mouse models.[25,26] In summary, IL-6 trans-signaling does not seem to play a role in this model of plasmacytoma development, however, it could be of importance in addressing other gp130 positive cells in the bone marrow, such as stromal cells and osteoclasts.[27]

The activation of gp130 is a major event in controlling the growth and survival of malignant plasma cells. Interestingly, some antibodies binding to gp130 have agonistic activity and support the growth of IL-6 dependent myeloma cell lines *in vitro* and *in vivo*.[14,28,29] These antibodies induce receptor dimerization, thereby mimicking the effect of the natural ligand.[30] The critical role for gp130 in early plasmacytoma development was recently demonstrated in a retroviral bone marrow transduction-trans-

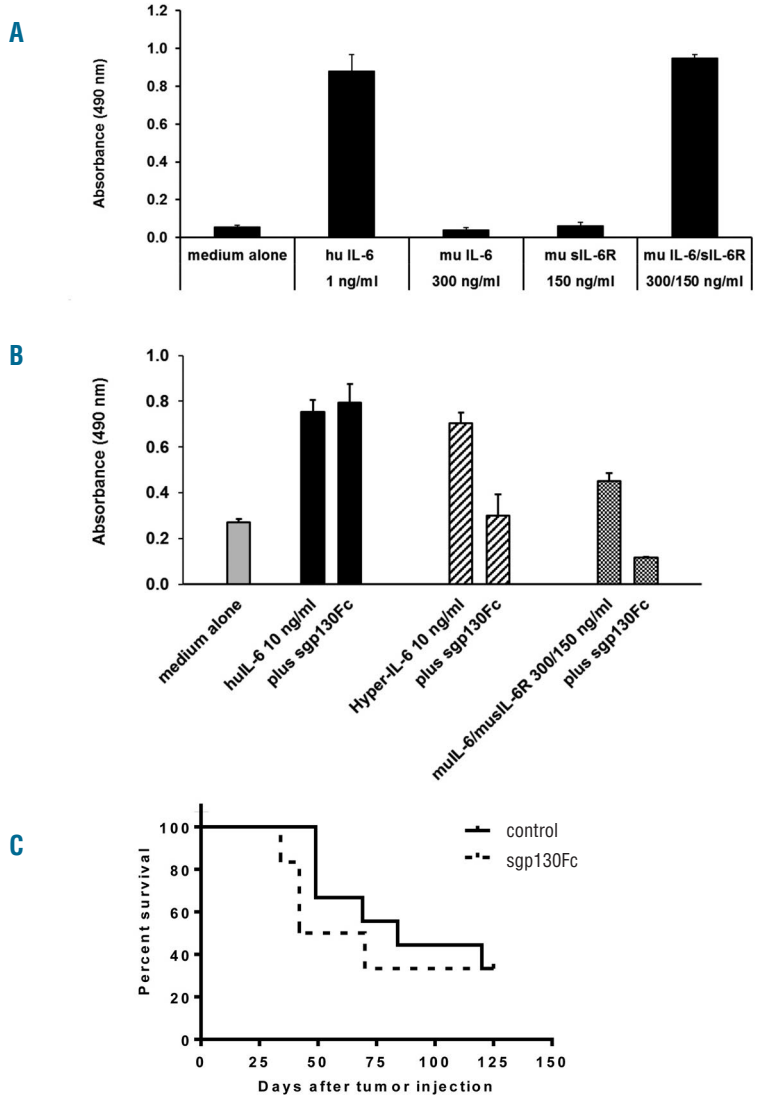

Figure 7. Trans-signaling by the murine IL-6/sIL-6R complex. (A) The complex of murine IL-6/sIL-6R stimulates INA-6 cell growth. Cells were cultivated with IL-6 and sIL-6R as indicated for 72 h, and viable cell mass evaluated by an MTS-based colorimetric assay. Values represent the mean of quadruplicate cultures. Error bars, SD. (B) The designer protein sgp130Fc inhibits IL-6 trans-signaling by Hyper-IL-6 and the murine IL-6/sIL-6R complex, but not signaling by human IL-6. sgp130Fc was used at a concentration of 10 μg/ml. Cell growth was measured as above. Values represent the mean of quadruplicate cultures. Error bars, SD. (C) Effect of sgp130Fc on INA-6.Tu1 plasmacytoma formation and survival. Mice (n=6) were treated with sgp130Fc by IP injections of 150 μg protein weekly for 4 consecutive weeks, starting one day after cell inoculation. Mice in the control group (n=9) were treated with PBS. *P*=0.56 by log-rank test. hu: human; IL-6: interleukin-6; mu: murine; mus: murine soluble; sIL-6R: soluble interleukin-6 receptor; gp: glycoprotein; sgp130Fc: soluble gp130Fc; Fc: fragment crystallizable.

plantation model, in which mouse bone marrow cells were infected with retroviruses carrying an expression plasmid for a constitutively active form of gp130. After transplantation of the cells into syngeneic recipients, mice developed myeloma with characteristics of the human disease.[31]

Tumor samples from myeloma patients harbor activated STAT3,[32,33] which is mediated by cytokines of the gp130 family and not by other myeloma growth factors.[34] In fact, human plasma cell lines as well as patient myeloma cells have been shown to respond to LIF, OSM, IL-11 and CNTF.[35,36] Importantly, receptors for OSM, LIF and IL-11 were found to be expressed on MM cells in a significant portion of patients, and IL-6, LIF and OSM in the myeloma bone marrow environment.[37] Moreover, elevated levels of gp130 cytokines, including OSM, were detected in patients with MM.[38] Interestingly, transplantation of a human myeloma cell line in SCID mice induced the expression of several gp130 cytokines in the spleens, in addition to the constitutive expression of IL-6, IL-11, and OSM in the murine bone marrow.[39] In the presence of a human bone marrow environment like that provided in the SCID-hu mouse model of MM, the humanized version of B-E8, mAb 1339, had inhibitory activity *in vivo*, however, it could not completely block myeloma cell growth.[40]

Despite the early recognition of the importance of IL-6 for MM growth and survival, clinical studies aimed at blocking IL-6 in MM have not yet been translated into reliable long-term benefits. Ranging from the IL-6 inhibitor dexamethasone,[41,42] to specific IL-6[43-47] or IL-6 receptor[48] blockade, therapeutic effects often vanish during prolonged treatment. In an environment devoid of IL-6, myeloma cells can adapt their receptor expression profile and switch to other gp130 cytokines. The data presented herein strongly support the idea that targeting the gp130 receptor would overcome this redundancy. Clearly, the broad expression of gp130 has to be considered, but there may be a therapeutic window, rendering gp130 a promising target for cancer therapy.[49] Remarkably, a novel first-in-class small molecule gp130 inhibitor showed activity against ovarian cancer,[50] and strategies to block gp130 in pain and inflammation are currently being developed.[51]

Acknowledgments

The authors would like to thank Kathrin Richter, Tanja Ahrens and Wolfgang Baum for their excellent technical assistance. We also thank the staff of the animal facility for their support.

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