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Short communication

Therapeutic blockade of the interleukin-6 receptor (IL-6R) allows sIL-6R generation by proteolytic cleavage

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ABSTRACT

Blockade of the interleukin-6 receptor (IL-6R) is a successful therapeutic strategy in various inflammatory diseases. IL-6 can signal via membrane-bound (classic signaling) and soluble forms (sIL-6R, trans-signaling) of the IL-6R. Trans-signaling is causative for the pro-inflammatory properties of IL-6, and the selective inhibition of this pathway holds the promise to cause less side effects than the global blockade of IL-6 signaling. We have recently shown that the majority of sIL-6R in humans is generated by proteolytic cleavage of the membrane-bound IL-6R, but whether this process is influenced by therapeutic blockade of the IL-6R is unknown. In this study, we show that the monoclonal antibody tocilizumab and a single chain antibody directed against the IL-6R efficiently block IL-6 signaling, but do not prevent the proteolytic generation of sIL-6R.

1. Introduction

The cytokine interleukin-6 (IL-6) has pleiotropic functions in health and disease [1]. It is a small glycoprotein that can be secreted by a number of different cell types, e.g. macrophages, monocytes and fibroblasts [2]. While IL-6 is usually present in pictogram per milliliter amounts in healthy human individuals, its serum levels rise in basically all inflammatory diseases and can reach quantities of microgram per milliliter in drastic conditions like meningococcal septic shock [3]. Monoclonal inhibitory antibodies that bind either IL-6 or the IL-6 receptor (IL-6R) have been developed and are approved for clinical use [4]. One example is tocilizumab, which targets the IL-6R and is used in more than 100 countries for the treatment of rheumatoid arthritis [5]. Additionally, single domain antibodies, also known as nanobodies or V_HH, directed against the IL-6R have been successfully tested in clinical trials with rheumatoid arthritis patients [4,6].

IL-6 is a member of the IL-6 family of cytokines. In order to provoke a biological response it first binds to the non-signaling IL-6 receptor (IL-6R), which is only expressed on a small number of cell types, including hepatocytes and several leukocyte subsets [2]. The formation of the IL-6/IL-6R complex (termed classic signaling) results in the subsequent recruitment of a homodimer of the signal-transducing β-receptor gp130 and the activation of intracellular signaling cascades, e.g. the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway. Interestingly, soluble forms of the IL-6R (sIL-6R) exist that form agonistic IL-6/sIL-6R complexes, which in turn can activate cells via gp130 homodimers. This pathway has been termed IL-6 trans-signaling and can activate in principle all cells, because gp130 is expressed ubiquitously and this pathway does not require IL-6R expression on the target cell [7].

The sIL-6R can either be generated by alternative splicing of the IL-6R mRNA, which results in the excision of the exon encoding the transmembrane region, or by proteolytic processing of the membrane-bound IL-6R and thus the cellular release of the sIL-6R ectodomain [7]. We have recently shown that the majority of sIL-6R found in human serum is generated by proteolytic cleavage, most probably by the metalloproteases ADAM10 and ADAM17 [8]. Whether this mechanism is influenced by therapeutics targeting the IL-6R is unknown.

In this study, we show that tocilizumab and a single-chain only antibody (V_HH) both block IL-6 signaling, but do not influence proteolytic sIL-6R generation, suggesting that this mechanism is unaltered in patients undergoing anti-IL-6R therapy.

2. Materials and methods

2.1. Cells and reagents

Ba/F3-gp130 cells were obtained from Immunex (Seattle, WA, USA

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[9]). Ba/F3-gp130-IL-6R cells have been described previously [10]. All cells were cultured in DMEM high glucose culture medium (Gibco, Thermo Fisher Scientific, Bonn, Germany) supplemented with 10% fetal bovine serum, penicillin (60 mg/l) and streptomycin (100 mg/l) under standard conditions (37 °C, 5% CO_2, water saturated atmosphere). Ba/F3-gp130 cells were cultured with 10 ng/ml Hyper-IL-6, an IL-6/IL-6R fusion protein, and Ba/F3-gp130-IL-6R cells with 10 ng/ml IL-6. Hyper-IL-6 and IL-6 were produced in house. Human PBMCs were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA). Tocilizumab (RoActemra) was from Roche Applied Science (Penzberg, Germany) and phorbol-12-myristate-13-acetate (PMA) from Sigma-Aldrich. Anti-phospho-STAT3 (pTyr705), anti-STAT3 (124H6), anti-Myc (71D10) and anti-GAPDH (14C10) antibodies were purchased from Cell Signaling Technology (Frankfurt/M., Germany). Alexa Fluor 647-conjugated anti-rabbit secondary antibody was obtained from Thermo Fisher Scientific (Bonn, Germany) and HRP-conjugated secondary anti-rabbit and anti-mouse antibodies from Dianova (Hamburg, Germany).

2.2. Expression and purification of the anti-IL-6R V_HH

A plasmid encoding the anti-IL-6R V_HH in frame with a N-terminal pelB leader sequence and a C-terminal Myc- and His-tag was transformed into *E. coli* BL21 (DE3, Merck, Darmstadt, Germany). The plasmid was kindly provided by Jürgen Scheller (Institute of Biochemistry and Molecular Biology II, HHU Düsseldorf, Germany). When OD600 reached 0.6–0.8, expression was induced by addition of 1 mM isopropyl 1-thio-β-d-galactopyranoside (IPTG) for 4 h at 37 °C. Cells were removed by centrifugation (4 °C, 5000*g*, 10 min) and filtration. The supernatant was loaded on a 1 ml His-Trap HP column (GE Healthcare, Munich, Germany) using a peristaltic pump. The column was washed with PBS and recombinant protein was eluted with 250 mM imidazole in PBS. Imidazole was removed from the buffer using a NAP-25 Column (GE Healthcare, Munich, Germany). Purity of expressed anti-IL-6R V_HH was analyzed by Coomassie Blue staining after SDS-PAGE under reducing conditions.

2.3. Cell viability assay

$5x10^3$ Ba/F3-gp130-IL-6R cells per well were seeded into 96well plates and cultured in the presence of IL-6 and IL-6R inhibitors as indicated for 48 h. Cell viability was determined using the Cell Titer Blue cell viability assay reagent (Promega) according to the manufacturer's protocol. All conditions were analyzed in triplicates, and relative light units (RLU) after 60 min were normalized by subtraction of values measured at 0 min.

2.4. Isolation of PBMCs

PBMCs were isolated from leukocyte concentrates by density gradient centrifugation. The concentrates originated from the Institute of Transfusion Medicine of the University Hospital Schleswig-Holstein Kiel and were obtained from the Institute of Immunology (Kiel University). Ethics approvals was obtained from the review board of the medical faculty of Kiel University (study #D 556/15).

2.5. Ectodomain shedding assay

1×10^6 Ba/F3-gp130-IL-6R cells or 2×10^6 PBMCs per well were seeded into 24well plates and treated with PMA, Tocilizumab or the anti-IL-6R V_HH as indicated. The supernatants were collected, cleared from debris and either used for ELISA or for stimulation of Ba/F3-gp130 cells as described below.

2.6. Flow cytometry

1×10^6 Ba/F3-gp130-IL-6R were incubated with anti-IL-6R V_HH diluted 1:100 in FACS buffer (PBS, 0.5% BSA) for 30 min on ice. Cells were washed with FACS buffer and incubated with an anti-myc antibody (1:100 in FACS buffer) for 30 min. Following two more washing steps, the cells were stained with 1:100 diluted Alexa Fluor 647-conjugated anti-rabbit antibody for 30 min. After a final washing step, cells were analyzed on a BD FACS Canto II using the Diva Software (BD Biosciences, Heidelberg, Germany) and FlowJo V10.1 (Tree Star, Ashland, OR, USA).

2.7. Stimulation of cells and Western blotting

Prior Stimulation, Ba/F3-gp130 and Ba/F3-gp130-IL-6R cells were washed three times with PBS and serum-starved for 2 h. Ba/F3-gp130 cells were stimulated with the supernatants from the ectodomain shedding assays with or without IL-6 for 15 min. Ba/F3-gp130-IL-6R cells were incubated with 10 µg/ml anti-IL-6R V_HH for 30 min, and afterwards stimulated with 10 ng/ml IL-6 for 15 min. Cells were harvested by centrifugation and directly boiled in 2.5× reducing Laemmli buffer. Western blots were analyzed with the ChemoCam Imager (Intas, Göttingen, Germany).

2.8. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Concentration of sIL-6R secreted into the cell culture supernatant was measured using the Human IL-6 R alpha DuoSet ELISA kit (R&D Systems) as recommended by the manufacturer. Absorbance was detected using a Tecan Spectra Rainbow Platereader (Tecan, Maennedorf, Switzerland). Supernatants were diluted when necessary and the concentration calculated accordingly.

3. Results

3.1. Therapeutic inhibition of the IL-6R by tocilizumab or an anti-IL-6R V_HH blocks IL-6 signaling

In order to analyze whether therapeutic inhibition of the IL-6R influences its proteolytic cleavage, we first expressed an anti-IL-6R V_HH with a myc- and his-tag at the C-terminus in E. coli. We used a N-terminal pelB leader sequence to ensure periplasmic expression of the protein. The secreted anti-IL-6R V_HH was collected using the his-tag and its purity verified by SDS-PAGE followed by Coomassie staining (Fig. 1A). To ensure that the anti-IL-6R V_HH was correctly folded and capable of binding to the IL-6R, we incubated Ba/F3-gp130-IL-6R cells with the V_HH followed by staining with an anti-myc antibody. As shown in Fig. 1B, the anti-IL-6R V_HH successfully bound to the IL-6R as judged by flow cytometry. Afterwards, we analyzed whether the V_HH was able to block IL-6 signaling. We incubated Ba/F3-gp130-IL-6R cells with a constant amount of 1 ng/ml IL-6 and added increasing amounts (10–10,000 ng/ml) of either the anti-IL-6R V_HH or the monoclonal antibody tocilizumab to the cells. Both V_HH and tocilizumab significantly blocked IL-6-dependent proliferation in a dose-dependent manner (Fig. 1C). Afterwards, we stimulated Ba/F3-gp130-IL-6R cells with 10 ng/ml IL-6 for 15 min and analyzed phosphorylation of the transcription factor STAT3 via Western blot. STAT3 phosphorylation was completely abolished when the cells were pre-treated with the V_HH, letting us conclude that the V_HH was able to block IL-6 classic signaling via the membrane-bound receptor (Fig. 1D).

In order to investigate whether the V_HH would also block IL-6 trans-signaling via the soluble receptor, we stimulated Ba/F3-gp130-IL-6R cells with the phorbol ester PMA, which activates ADAM17 and results in the release of sIL-6R, or with DMSO as control, which does not activate proteolytic cleavage. The supernatants were mixed with tocilizumab, V_HH or were left untreated and further mixed with IL-6 or not, and then added to Ba/F3-gp130 cells (Fig. 1E). Only supernatant from PMA-stimulated cells, which contained sIL-6R, was able to induce STAT3 phosphorylation when IL-6 was added, while the

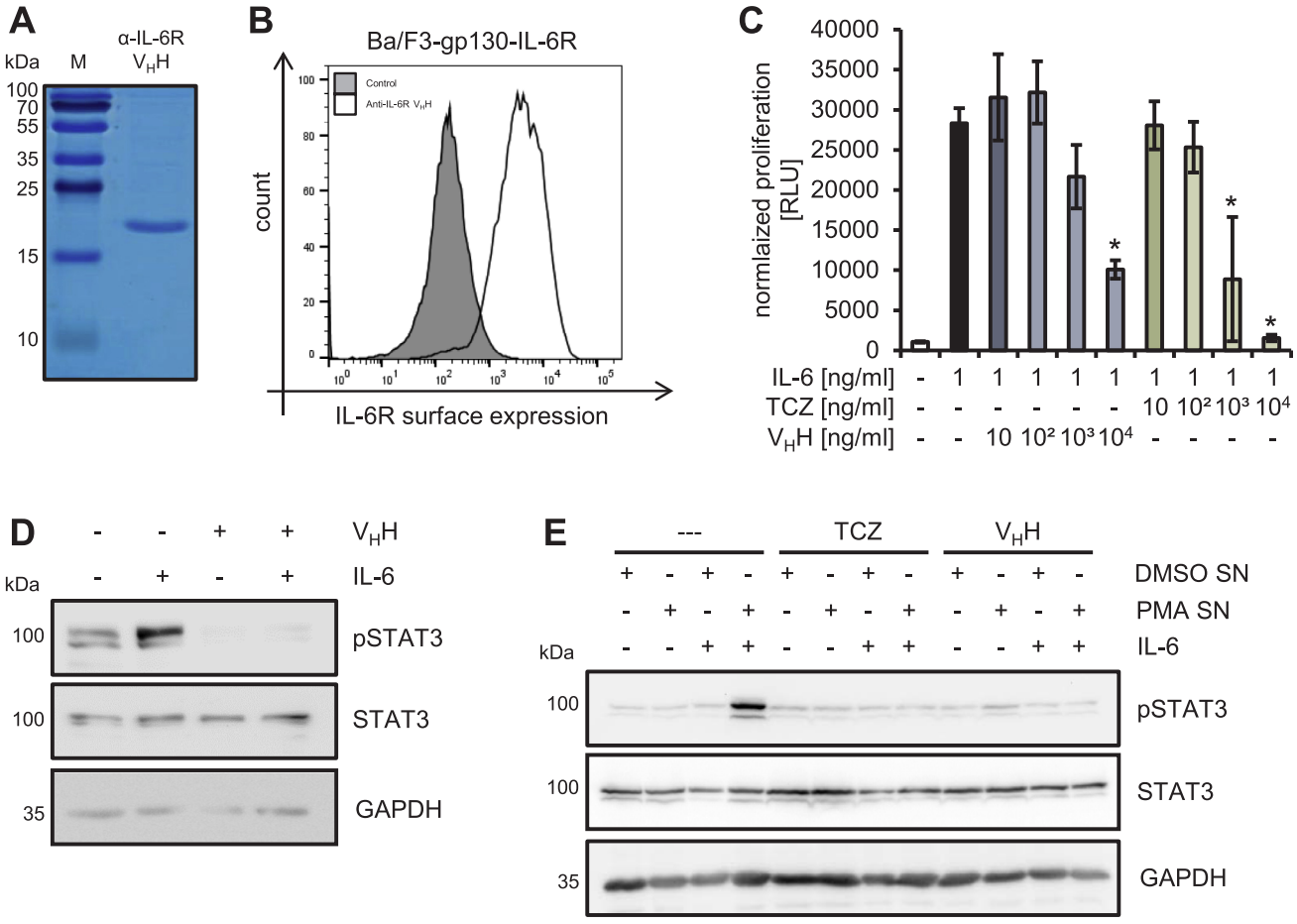

Fig. 1. An anti-IL-6R V_HH blocks IL-6 signaling. (A) Representative Coomassie-stained SDS gel of the expressed and purified anti-IL-6R V_HH. A molecular weight marker (M) is shown on the left. (B) Staining of the IL-6R on Ba/F3-gp130-IL-6R cells using the anti-IL-6R V_HH, an anti-myc antibody and a fluorophore-labelled secondary antibody via flow cytometry. (C) Viability assay of Ba/F3-gp130-IL-6R cells. 5000 cells were incubated with the indicated amounts of IL-6, tocilizumab (TCZ) and the anti-IL-6R V_HH for 48 h. Data shown are the mean ± SD (n = 3). Asterisks indicate significant reduction ($p < 0.05$) in cell viability compared to the cells stimulated with IL-6 alone (one-way analysis of variance and Dunnett's Multiple Comparison Test). (D) Ba/F3-gp130-IL-6R cells were serum-starved for 2 h, incubated with 10 μg/ml anti-IL-6R V_HH for 30 min where indicated, and stimulated afterwards with 10 ng/ml IL-6 for 15 min where indicated. Phosphorylation of STAT3 was analyzed via Western blot, STAT3 and GAPDH were visualized to ensure equal loading of the samples. (E) Ba/F3-gp130-IL-6R cells were stimulated with either 100 nM PMA or DMSO for 2 h and the supernatants separated from the cells via centrifugation. Where indicated, tocilizumab (TCZ, 10 μg/ml), anti-IL-6R V_HH (10 μg/ml) and IL-6 (10 ng/ml) were added to the supernatants. Ba/F3-gp130 cells were serum-starved for 2 h, stimulated with the supernatants for 15 min, and phosphorylation of STAT3 was analyzed via Western blot. STAT3 and GAPDH were visualized to ensure equal loading of the samples. All experiments are representative of three independent experiments with similar outcome.

supplementation of either V_HH or tocilizumab completely prevented activation of STAT3 via trans-signaling (Fig. 1E).

3.2. IL-6R blockade by tocilizumab or V_HH allows proteolytic cleavage of the IL-6R

The proteolytic generation of the sIL-6R by ADAM17 is a critical step that initiates IL-6 trans-signaling [8]. In order to analyze a possible influence of the V_HH or tocilizumab on IL-6R proteolysis, we used again Ba/F3-gp130-IL-6R cells and activated ADAM17 with PMA. As shown in Fig. 2A, PMA led to a 2-fold increase in sIL-6R generation compared to the unstimulated cells. Pre-treatment of the cells with increasing amounts (10–10,000 ng/ml) of tocilizumab or the V_HH did not significantly alter IL-6R proteolysis (Fig. 2A). We substantiated this finding in human peripheral blood mononuclear cells (PBMCs, Fig. 2B).

Additionally, we sought to prove that the shed sIL-6R, which was bound by either the V_HH or tocilizumab, remained biologically inactive. Therefore, we treated Ba/F3-gp130-IL-6R cells with either tocilizumab, the V_HH or left them untreated, and stimulated the cells with PMA or DMSO as control. We collected the supernatants, added IL-6 or left them untreated, and stimulated Ba/F3-gp130 cells with them. In accordance with our previous results, supernatant from PMA-stimulated cells in combination with IL-6 induced STAT3 phosphorylation, which was largely absent when the cells had been pre-treated with tocilizumab or the V_HH (Fig. 2C).

4. Discussion

Therapeutic targeting of the IL-6R is a promising therapeutic option to treat a variety of inflammatory diseases. The monoclonal antibody tocilizumab, which binds to the cytokine-binding region of the IL-6R and thereby blocks its biological function, is currently approved in more than 100 countries for the treatment of rheumatoid arthritis [5]. Other approved indications include Castleman disease, cytokine release syndrome and giant cell arteritis [4]. A number of antibodies and other compounds, including an anti-IL-6R V_HH, are currently in different phases of clinical studies or already approved [4].

The generation of the sIL-6R is a crucial step for the initiation of the IL-6 trans-signaling pathway, and recent evidence shows that the majority of the 20–80 ng/ml sIL-6R in the human circulation originates from membrane-bound IL-6R that has been proteolytically cleaved off, while only around 15% is generated by alternative splicing of the IL-6R

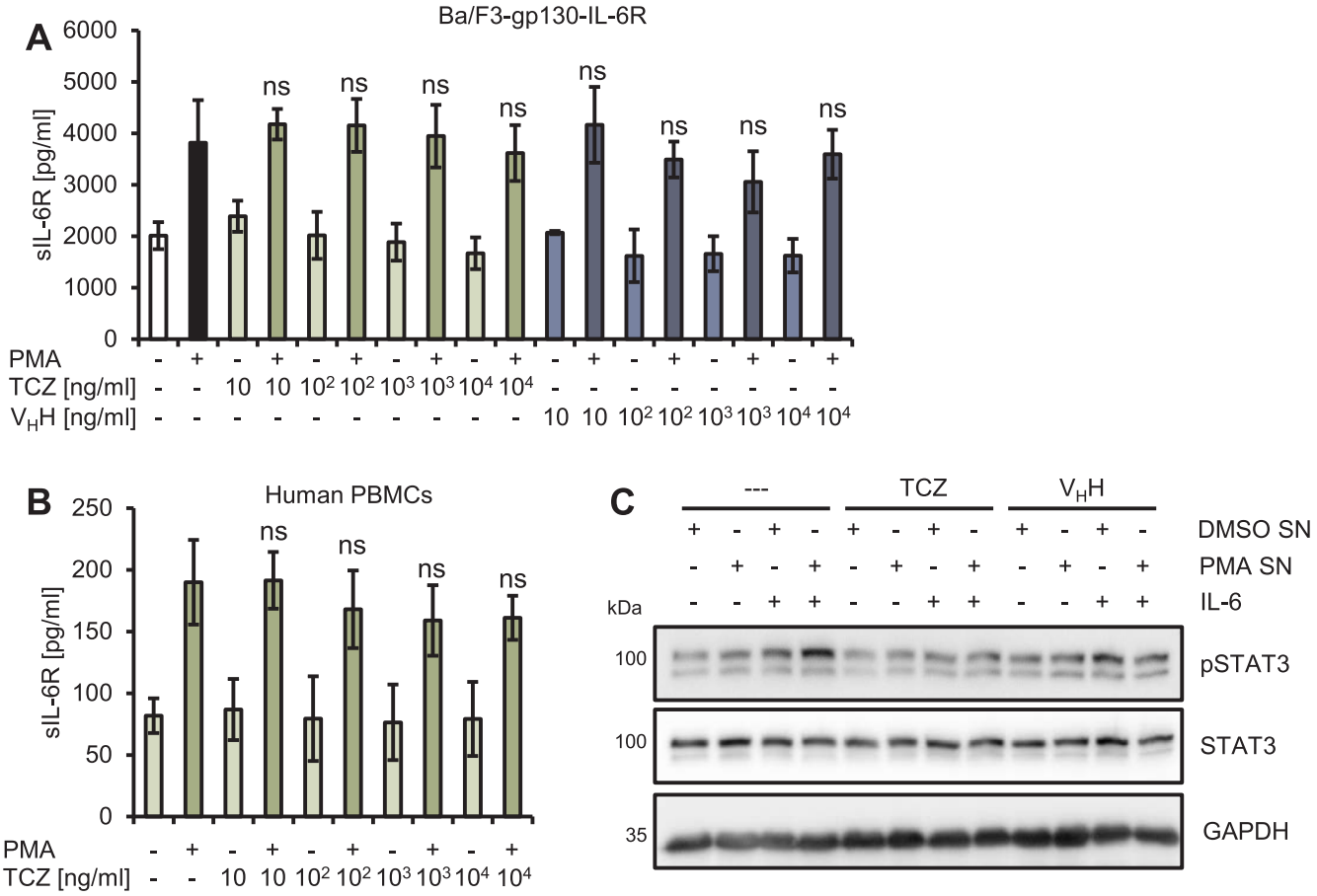

Fig. 2. Therapeutic blockade of the IL-6R allows proteolytic sIL-6R generation. (A) Ba/F3-gp130-IL-6R cells were pre-treated with the indicated amounts of either tocilizumab (TCZ) or the anti-IL-6R V_HH for 30 min or left untreated. Afterwards, the cells were stimulated with 100 nM PMA or DMSO as control for 120 min. The supernatants were harvested and the amount of sIL-6R determined via ELISA. Data are shown as mean ± SD, n = 3. (B) Human PBMCs were pre-treated with the indicated amounts of tocilizumab (TCZ) for 30 min or left untreated. Afterwards, the cells were stimulated with 100 nM PMA or DMSO as control for 2 h. The supernatants were harvested and the amount of sIL-6R determined via ELISA. Data are shown as mean ± SD, n = 3. (C) Ba/F3-gp130-IL-6R cells were pre-treated with the indicated amounts of either tocilizumab (TCZ) or the anti-IL-6R V_HH for 30 min or left untreated. Afterwards, the cells were stimulated with 100 nM PMA or DMSO as control for 2 h. The cells were removed via centrifugation and IL-6 added to the supernatants where indicated. Ba/F3-gp130 cells were serum-starved for 2 h and then stimulated with the supernatants for 15 min. Phosphorylation of STAT3 was analyzed via Western blot. STAT3 and GAPDH were visualized to ensure equal loading of the samples. All experiments are representative of three independent experiments with similar outcome. "ns" indicate no significant reduction in sIL-6R generation compared to the cells stimulated with PMA alone (one-way analysis of variance and Dunnett's Multiple Comparison Test).

mRNA [8]. ADAM17 is considered as the major protease for this cleavage event, although a definitive proof is still missing and hypomorphic $ADAM17^{ex/ex}$ mice, which show drastically decreased ADAM17 levels, display unaltered steady-state sIL-6R serum levels [11]. In contrast, under inflammatory conditions, ADAM17 was shown to be the responsible protease for sIL-6R release in mice [12]. It was unclear, however, whether proteolysis in patients treated with anti-IL-6R therapeutics would still function or whether the therapeutics would somehow interfere with the interaction of ADAM17 and the IL-6R. This is not unlikely, given the fact the so-called exosites of substrates are known to modulate proteolytic cleavage events. This has been shown e.g. for CD30 [13], but also for the IL-6R itself [8]. In this study, we found no evidence that tocilizumab or the anti-IL-6R V_HH affect sIL-6R generation by ADAM17-mediated cleavage using a cell line that overexpresses the IL-6R as well as primary cells with endogenous IL-6R expression. It is therefore likely to assume that sIL-6R generation is unaltered in patients, although the liberated sIL-6R will be biologically inactive due to the blocking antibody.

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