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The interleukin-6 receptor Asp358Ala single nucleotide polymorphism rs2228145 confers increased proteolytic conversion rates by ADAM proteases

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ABSTRACT

The pleiotropic activities of Interleukin (IL-)6 are controlled by membrane-bound and soluble forms of the IL-6 receptor (IL-6R) in processes called classic and trans-signaling, respectively. The coding single nucleotide polymorphism (SNP) rs2228145 of the Interleukin 6 receptor (IL-6R Asp358Ala variant) is associated with a 2-fold increase in soluble IL-6R (sIL-6R) serum levels resulting in reduced IL-6-induced C-reactive protein (CRP) production and a reduced risk for coronary heart disease. It was suggested that the increased sIL-6R level leads to decreased IL-6 classic or increased IL-6 trans-signaling. Irrespective of the functional outcome of increased sIL-6R serum level, it is still under debate, whether the increased sIL-6R serum levels emerged from differential splicing or ectodomain shedding. Here we show that increased proteolytic ectodomain shedding mediated by the A Disintegrin and metalloproteinase domain (ADAM) proteases ADAM10 and ADAM17 caused increased sIL-6R serum level in vitro as well as in healthy volunteers homozygous for the IL-6R Asp358Ala allele. Differential splicing of the IL-6R appears to have only a minor effect on sIL-6R level. Increased ectodomain shedding resulted in reduced cell-surface expression of the IL-6R Asp358Ala variant compared to the common IL-6R variant. In conclusion, increased IL-6R ectodomain shedding is a mechanistic explanation for the increased serum IL-6R levels found in persons homozygous for the rs2228145 IL-6R Asp358Ala variant.

1. Introduction

Interleukin (IL-)6 plays an important role in health and disease [1,2]. The duration and strength of IL-6 cytokine-mediated signaling are tightly regulated to avoid overshooting activities, e.g. acute-phase response [3]. IL-6 activates signal transduction via homo-dimerization of the ubiquitously expressed trans-membrane gp130 β-receptor, which leads to subsequent activation of intracellular signaling pathways, namely the Janus kinase/signal transducer and activator of transcription (Jak/STAT), the phosphatidylinositide-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK)-cascade pathway. To enable binding to gp130, IL-6 needs an additional non-signaling IL-6 α-receptor (IL-6R) [2,4], whose expression is restricted to hepatocytes and some leukocyte populations. Consequently, IL-6 signaling is a priori limited to membrane-bound IL-6R expressing cells, in a process referred to as classic signaling [1,4].

However, a soluble form of the IL-6R (sIL-6R) is found in many body fluids. In concert with soluble gp130, also found in the serum, sIL-6R might act as an IL-6 buffer system, limiting overshooting systemic IL-6 actions [5]. Paradoxically in trans-signaling, the sIL-6R in a complex with IL-6 is also able to induce signal transduction on cells which do not express IL-6R but only gp130 [3]. In recent years, trans- but not classic signaling was associated with chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease [3]. The sIL-6R is generated by two separate mechanisms: Limited proteolysis of the membrane-bound IL-6R precursor (also called ectodomain shedding) and differential splicing of the IL-6R mRNA. Ectodomain shedding of the IL-6R is mainly mediated by A Disintegrin and Metalloproteinase domain (ADAM) proteases ADAM10 or ADAM17 [6,7]. Proteolytic cleavage of the IL-6R appears to be the predominant mechanism, as it is believed to account for 90% of the sIL-6R present in human serum.

The IL-6R single nucleotide polymorphism (SNP) variant rs2228145 is associated with reduced C-reactive protein expression and a reduced risk to develop coronary heart disease [8,9]. The IL-6R variant rs2228145

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leads to an amino acid substitution of aspartic acid to alanine (Ala) at amino-acid position 358 within the extracellular domain of the IL-6R (Asp358Ala) and is strongly associated with a two-fold increase in sIL-6R levels [10,11]. In Europe, the Asp358Ala mutation is rather common, as it has a minor allele frequency (MAF) of 30–40% [12]. This SNP was recently shown to result in increased generation of the differentially spliced soluble IL-6R [13], reduced cell surface expression of IL-6R [14] and impaired IL-6 responsiveness of target cells indicating that the IL-6R Asp358Ala variant is biologically active [15]. The amino acid exchange is directly located within the ADAM17 cleavage site between Gln357 and Asp358 of the common IL-6R variant (Ala358) [16], whereas the cleavage site for ADAM10 was suggested to be located elsewhere [17]. It was hypothesized that the IL-6R Asp358Ala variant has a higher proteolytic conversion rate than the common IL-6R variant, concomitantly reducing IL-6

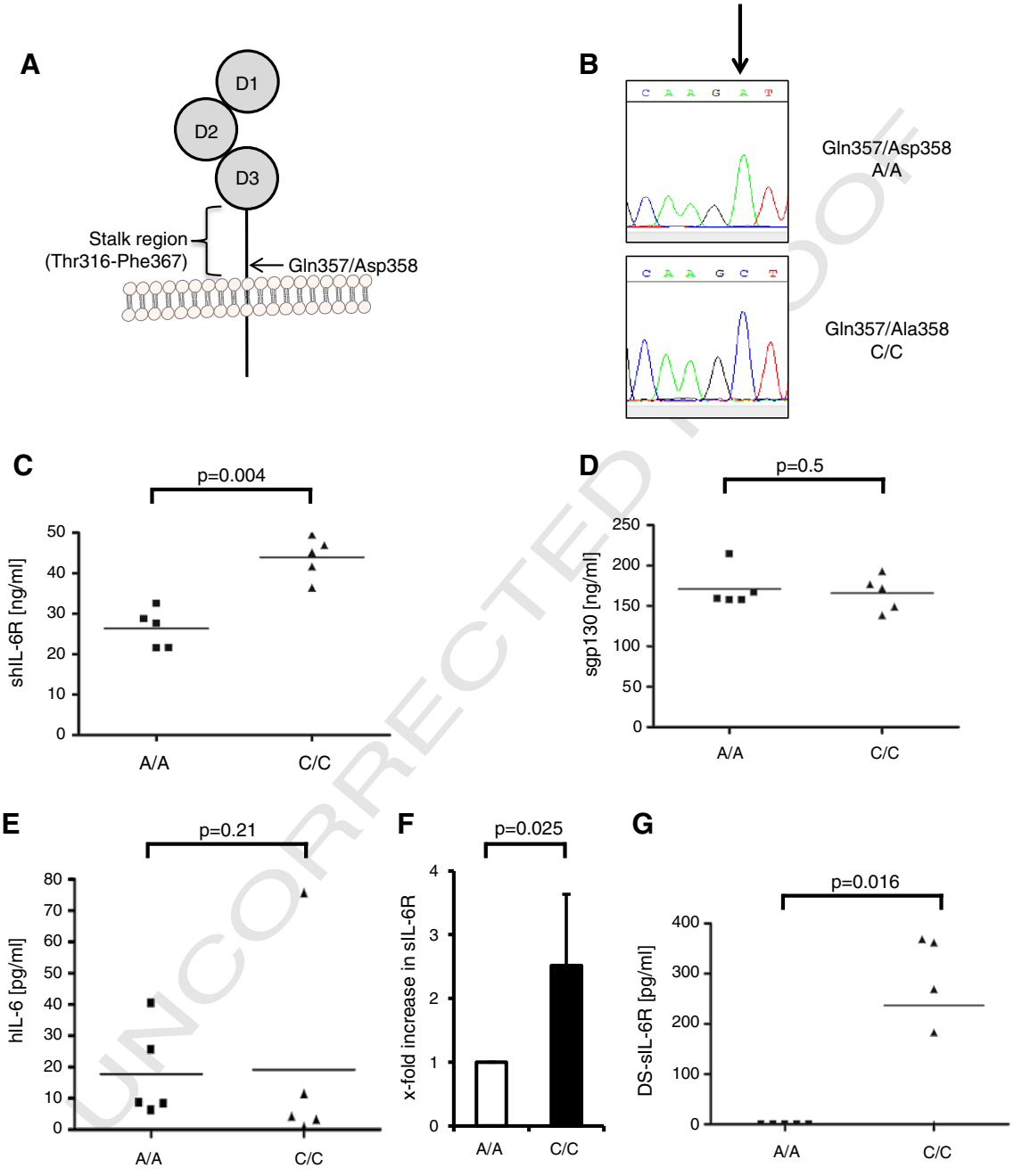

Fig. 1. The coding single nucleotide polymorphism (SNP) rs2228145 (Asp358Ala) is associated with increased soluble IL-6R levels in humans. A. Schematic representation of the human IL-6R consisting of an Ig-like domain (D1), the two domains containing the cytokine-binding module (CBM, D2 and D3), the stalk region, transmembrane and intracellular domain. The ADAM17 cleavage site between Gln357 and Asp358 is indicated with a black triangle. B. Chromatograms showing the SNP rs2228145 (Asp358Ala) in exon 9 of IL6R. Upper panel shows a wildtype situation (358Asp, A/A), whereas the lower panel shows a human homozygous for the mutation (358Ala, C/C). C. Full blood was taken from five healthy donors homozygous for the IL-6R SNP rs2228145 (referred to as C/C) and five healthy donors not carrying the SNP as controls (referred to as A/A) by venipuncture and serum isolated. Determination of sIL-6R levels within the serum was performed with an ELISA specific for hIL-6R. D. Serum levels of sgp130 were determined in the serum samples described under C with an ELISA specific for sgp130. E. Serum levels of IL-6 were determined in the serum samples described under C with an ELISA specific for human IL-6. F. PBMCs were isolated pair-wise from fresh blood of humans with the A/A or C/C IL-6R genotype (see Materials and methods for details). Equal amounts of PBMCs were incubated for 2 h, and the amount of sIL-6R in the supernatant of the PBMCs was quantified with an ELISA specific for the IL-6R. The data shown in this graph are the mean ± S.D. of five independent experiments. G. The serum samples of the 10 healthy donors described under panel C were analyzed with an ELISA that specifically recognizes only the differentially spliced hIL-6R, but not the sIL-6R generated by limited proteolysis. Samples below the detection limit (<31.25 pg/ml) are shown with a symbol directly on the x-axis. In graphs B, C, D and F, each symbol represents the mean of the ELISA measurement for the individual participants. The horizontal lines indicate the mean of each group. P-values of the statistical analysis are given above the respective figure.

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signaling in target cells due to lower membrane-bound IL-6R expression [15]. Importantly, all previously published point mutations and deletions surrounding the ADAM17-cleavage site in the IL-6R lead to decreased proteolytic conversion rates [16,17].

Here, we show that Asp358Ala confers higher constitutive and induced proteolytic conversion rates of the IL-6R mainly mediated by ADAM10 and ADAM17.

2. Materials and methods

2.1. Cells and reagents

Ba/F3-gp130 cells were obtained from Immunex (now part of Amgen Inc., Thousand Oaks, CA, USA [18]) and Ba/F3-gp130-hIL-6R cells described previously [19]. HEK293 and HeLa cells were obtained from DMSZ (Braunschweig, Germany). Murine embryonic fibroblasts deficient for ADAM10, ADAM17, or both have been described previously [20–22]. Parental Ba/F3-gp130 cells were cultured using 10 ng/ml recombinant hyper-IL-6 [23,24], and after transduction with the different IL-6R constructs with 10 ng/ml recombinant human IL-6 [25]. PMA and ionomycin were purchased from Sigma-Aldrich (Steinheim, Germany). The anti-hIL-6R mAb tocilizumab (RoACTEMRA) was kindly provided by Roche (Grenzach, Germany) and GI254023X and GW280264X by Glaxo Smith Kline (Stevenage, UK) [26].

2.2. Construction of the hIL-6R plasmids

pcDNA3.1 containing human IL-6R was previously described [7]. The point mutation hIL-6R-Asp358Ala was introduced using standard techniques.

2.3. Retroviral transduction and proliferation assays of Ba/F3-gp130 cells

All necessary IL-6R constructs were subcloned into the pMOWS vector. Ba/F3-gp130 cells were retrovirally transduced [27] and cytokine-dependent proliferation was determined as described previously [28].

2.4. Flow cytometry

IL-6R cell surface expression on transiently transfected HEK293 cells was stained with 1:100 diluted anti-hIL-6R 4–11 mAb [19] and a 1:100 dilution of FITC-conjugated anti-mouse mAb (Dianova, Hamburg, Germany) in FACS buffer (0.5% BSA in PBS). Cells were analyzed on a BD FACS Canto II (Becton-Dickinson, Heidelberg, Germany) as described previously [17].

2.5. IL-6R protein turnover assay

Ba/F3-gp130-hIL-6R and Ba/F3-gp130-hIL-6R-Asp358Ala cells were collected and pelletized at 5000 rpm for 1 min. Supernatants were discarded, and cells were suspended at a concentration of 1×10^6 cells/ml in ice cold PBS. Cells were washed twice in ice cold washing buffer (0.5% BSA/PBS) and pelletized (300 g, 4 °C, 5 min). Cells were afterwards incubated with anti-hIL-6R antibody on ice for 1 h. To allow internalization, cells were washed 3 times with washing buffer and incubated in DMEM with 10% FBS at 37 °C for the times indicated (0–120 min). After the incubation, cells were washed three times with washing buffer at 4 °C and subsequently incubated at 4 °C for 1 h with FITC-conjugated anti-mouse IgG. After additional washing, expression of receptors remaining at the cell surface was assayed as described above.

2.6. Immunofluorescence staining and confocal microscopy

HeLa cells were seeded onto glass coverslips in 6 well plates and transiently transfected with either pcDNA3.1-hIL-6R or pcDNA3.1-hIL-6R_Asp358Ala using TurboFect (Thermo Scientific, St. Leon-Rot, Germany). To determine transfection efficiency, cells were transfected with a control plasmid containing GFP. GFP fluorescence was determined microscopically and was detectable in 60–70% of the cells. 48 h after transfection, cells were fixed in 4% PFA for 20 min at room temperature. After washing with PBS, cells were blocked for 1 h in PBS with 1% BSA and 0.25% Triton X-100 for 1 h. Cells were stained with α-hIL-6R 4–11 mAb (diluted 1:500 in 1% BSA/PBS) at 4 °C over night. Afterwards, cells were washed three times with PBS and stained with Alexa Fluor 546 goat anti-mouse IgG (Invitrogen, Karlsruhe, Germany, diluted 1:500 in 1% BSA/PBS) for 1 h at room temperature. Coverslips were washed three times with PBS afterwards and mounted with ProLong Gold Antifade reagent containing DAPI (Invitrogen, Karlsruhe, Germany) onto microscopy slides. Analyses were performed with a Leica TCS SP2/AOBS microscope equipped with a HCX PL APO 63 × immersion objective.

2.7. Genotyping and isolation of human peripheral blood mononuclear cells (PBMCs)

Ethic approval for this study was obtained from the institutional review board of the Heinrich-Heine-University (study #3949). All participants gave written informed consent. Genomic DNA was isolated from buccal cells, part of the IL-6R amplified (5′: GAGGGGAAGGTTCCTTTG AG, 3′: CATGGCATGCTTTTTGTAGC) and genotype determined by sequencing. Afterwards, peripheral blood from healthy volunteers was collected by venipuncture. PBMCs were isolated using LSM 1077 Lymphocyte Separation Medium (PAA Laboratories, Pasching, Austria) analogous as described previously [19].

2.8. Shedding assays and ELISA

Shedding assays were performed as previously described [7], and the data analysis and calculation was done according to Baran et al. [17]. An ELISA specific for all hIL-6R variants [7,19] as well as an ELISA specific for the differentially spliced shIL-6R (DS-sIL-6R) were described previously [29]. The gp130 ELISA was from R&D Systems (Wiesbaden, Germany), and the IL-6 ELISA was purchased from ImmunoTools (Friesoythe, Germany).

2.9. Western blotting

Transiently transfected HEK293 and MEF cells were lysed in mild lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, complete protease inhibitor mixture tablets), and 50 μg was loaded per lane onto 10% SDS gels. Western blotting using anti-human IL-6R [4–11] and anti β-actin antibodies was performed as described previously [7,30].

2.10. Statistical analysis

Data are expressed as mean values ± standard deviation calculated from at least three independent experiments unless otherwise stated. Statistical analysis was performed using a one-tailed Mann–Whitney-*U* test. P-values are either given directly within the figures, or a P-value below 0.05 is indicated with an asterisk (*).

3. Results

3.1. Increased sIL-6R serum levels in homozygous carriers of the IL-6R SNP rs2228145 (Asp358Ala variant) were not caused by differential splicing of the IL-6R mRNA

The soluble IL-6R serum level is derived from differential splicing or limited proteolysis of the membrane-bound IL-6R, and the latter is believed to account for at least 90–99% of the total sIL-6R found in human blood. ADAM10 and ADAM17 are the major proteases responsible for IL-6R shedding, and the cleavage site of ADAM17 within the

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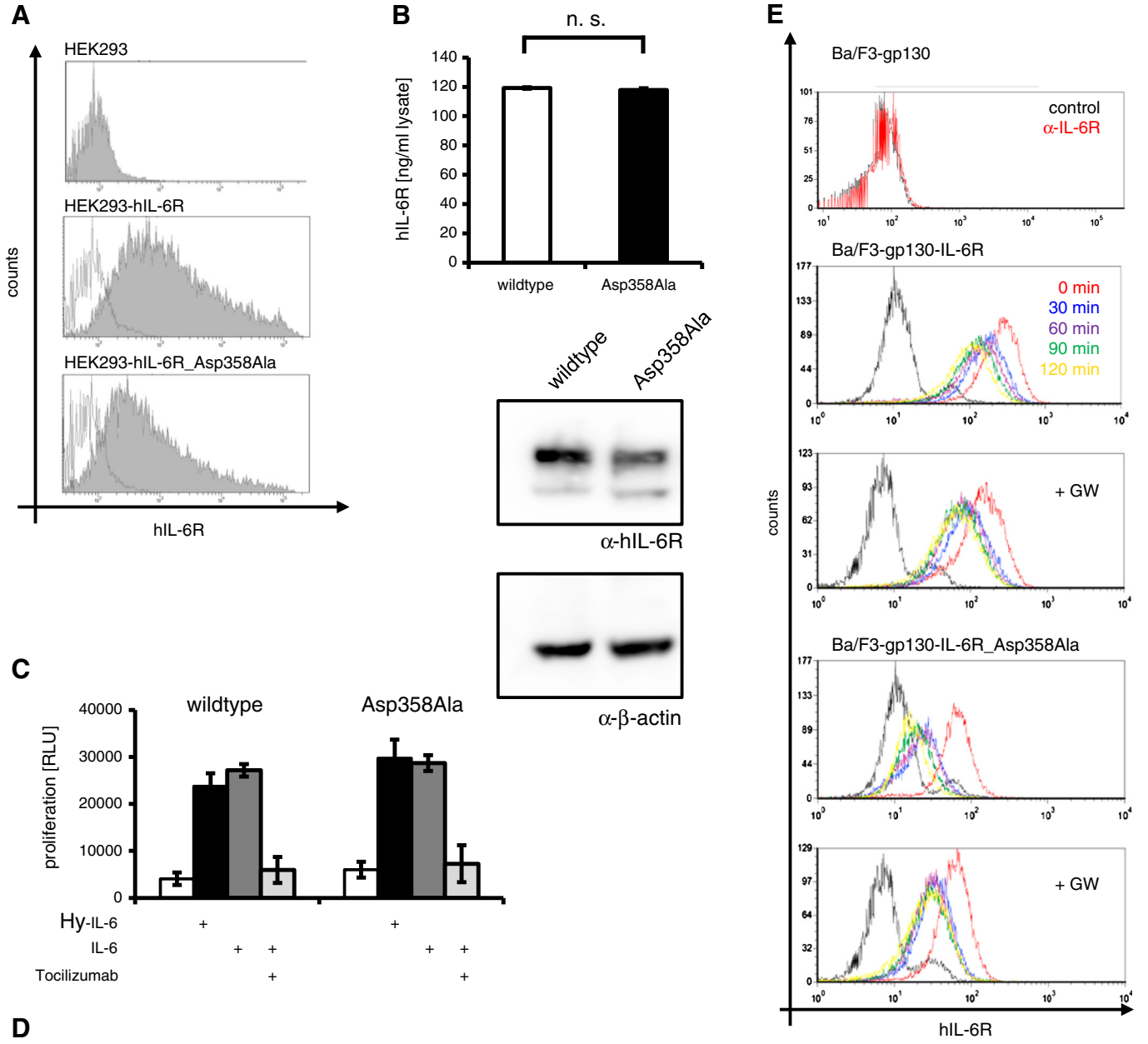
juxtamembrane stalk region of the IL-6R has been determined to be located between Gln357 and Asp358 ([31] and Fig. 1A). Interestingly, the single nucleotide polymorphism (SNP) rs2228145 resulted in an amino acid substitution within the ADAM17 cleavage site, as aspartic acid 358 is converted to an alanine (Asp358Ala, Fig. 1B). Cell surface expression of the IL-6R Asp358Ala variant is markedly reduced compared to the



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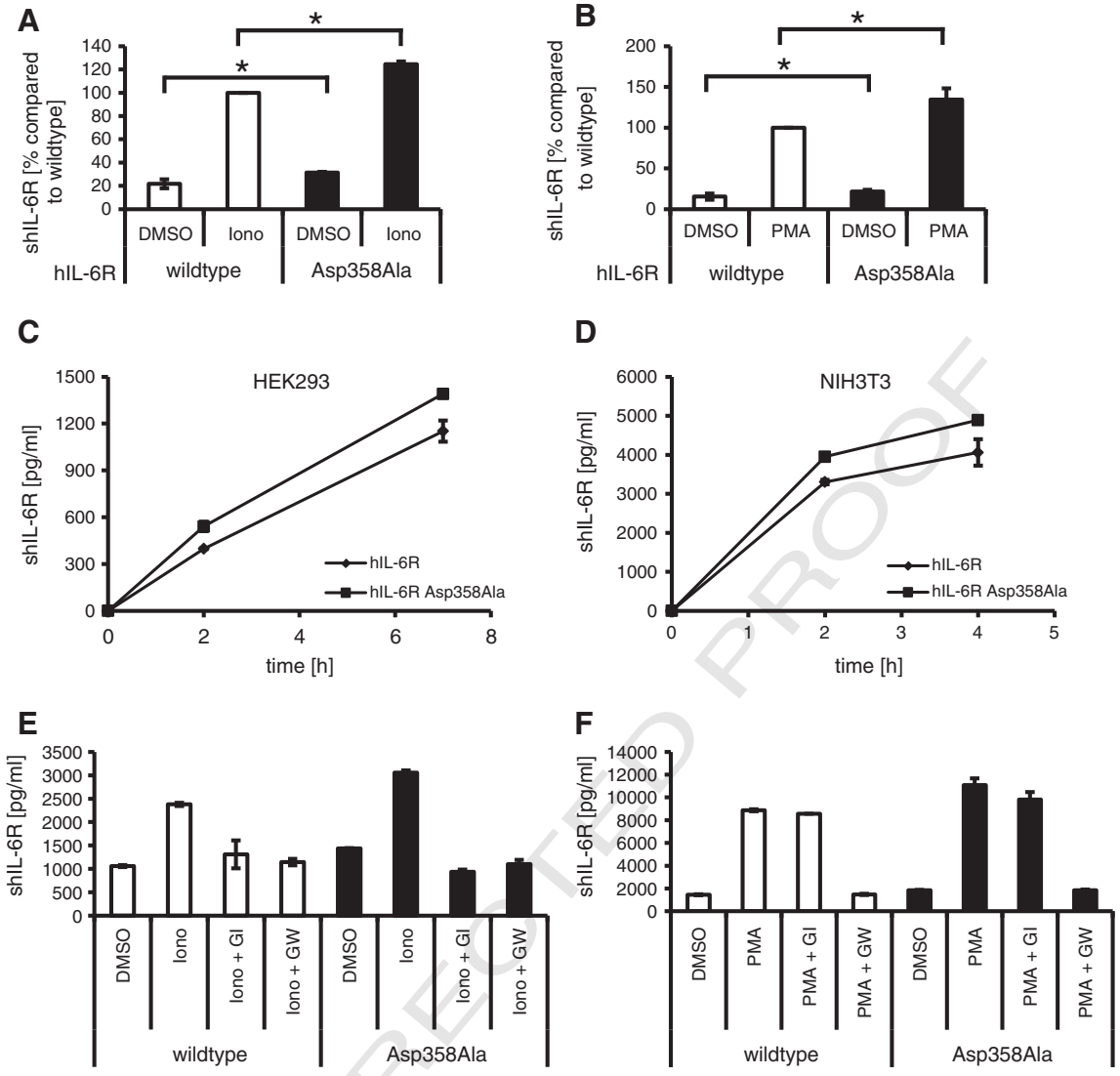

Fig. 3. Constitutive and stimulated shedding of the interleukin 6-receptor variant Asp358Ala is increased compared to the common IL-6R variant. A. HEK293 cells were transiently transfected with expression plasmids coding for IL-6R wildtype and Asp358Ala. Cells were equally distributed one day later onto 6 well-plates, and stimulated 24 h later for 60 min with 1 μM ionomycin (Iono) or treated with DMSO as negative control. The soluble IL-6R in the supernatant was quantified via ELISA. The amount of soluble wildtype IL-6R was set to 100%, and all other values were calculated accordingly. ELISA results show the mean ± S.D. of three independent experiments. B. The experiment was performed as described under panel A, except that cells were stimulated with 100 nM PMA for 120 min. C. HEK293 cells were transiently transfected with expression plasmids coding for IL-6R wildtype and Asp358Ala. Cells were equally distributed one day later onto 6 well-plates. The next day, medium was replaced and conditioned media collected at the time points indicated. The soluble IL-6R in the supernatant was quantified via ELISA. C. The experiment described under panel C was performed with NIH3T3 cells. E. The experiment was performed as described under panel A. Cells were pretreated for 30 min with the metalloprotease inhibitors GI (specific for ADAM10) or GW (specific for ADAM10 and ADAM17) where indicated. One representative experiment of three performed is shown. F. The experiment was performed as described under panel C, except that cells were stimulated with 100 nM PMA for 120 min. One representative experiment is shown.

common IL-6 variant on naïve CD4+ T-cells, CD4+ memory T cells, CD4+ regulatory T cells and monocytes, resulting in reduced IL-6 responsiveness of these cells [15]. Moreover, the IL-6R Asp358Ala variant has been shown to be associated with increased sIL-6R level in the serum of homozygous carriers [10,11]. To confirm this finding, 37 healthy age-matched (25–35 years) volunteers were genotyped for the occurrence of the IL-6R SNP rs2228145 (15 A/A (wild-type, common allele), 16 A/C (heterozygous), 6 C/C (rs2228145)), and sera were drawn from five volunteers carrying the common IL-6R (A/A) allele and five homozygous carriers of rs2228145 (C/C). Indeed, homozygosity for the Asp358Ala mutation was associated with significantly increased sIL-6R serum levels (A/A: 26.4 ± 4.8 ng/ml; C/C: 44.0 ± 5.1 ng/ml; p =

Fig. 2. The Asp358Ala IL-6R variant shows a reduced cell-surface expression and an increased protein turnover. A. HEK293 cells were transiently transfected with either IL-6R wildtype, IL-6R Asp358Ala or left untransfected. Cell surface expression of the IL-6R was determined via flow cytometry 48 h after transfection. B. The cells described under panel A were lysed, and the amount of IL-6R within the lysate determined via ELISA and Western blotting. One representative experiment of three performed is shown. n. s.: No significant difference. C. HeLa cells were transiently transfected with either IL-6R wildtype or IL-6R Asp358Ala. Cells were grown on cover slips, fixed and stained 48 h later. The DAPI stained nucleus is shown in blue, whereas the IL-6R staining is shown in red. Five different microscopic fields per IL-6R variant are shown. D. Equal amounts of Ba/F3-gp130-hIL-6R and Ba/F3-gp130-hIL-6R-Asp358Ala cells were stimulated with 10 ng/ml hyper-IL-6, 10 ng/ml IL-6, 10 ng/ml IL-6 plus 100 μg/ml tocilizumab or left untreated. Cell viability was assessed 48 h later. One representative experiment of three performed is shown. E. Equal amounts of Ba/F3-gp130-hIL-6R and Ba/F3-gp130-hIL-6R-Asp358Ala cells were stained with anti-IL-6R antibody, and the time-dependent protein turnover of the IL-6R (0–120 min) was determined as described in *Experimental procedures*. Cells were pretreated for 30 min with the ADAM10/17 metalloprotease inhibitor GW where indicated. As control, parental Ba/F3-gp130 cells were stained with the anti-IL-6R antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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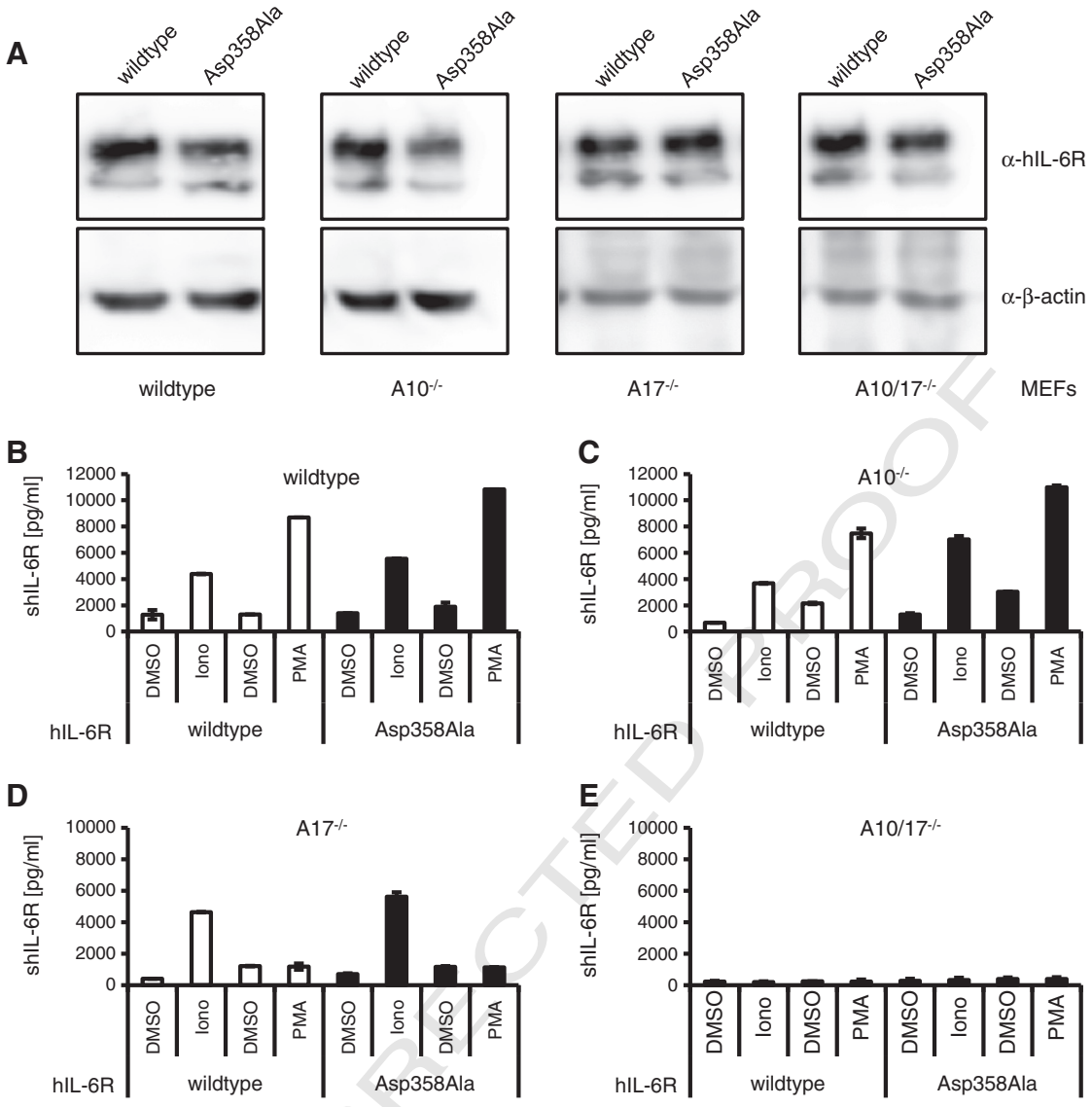

Q3 **Fig. 4.** Increased shedding of the Asp358Ala IL-6R variant in protease-deficient murine embryonic fibroblasts (MEFs). A. Wildtype, ADAM10$^{-/-}$, ADAM17$^{-/-}$ and ADAM10$^{-/-}$/17$^{-/-}$ MEFs were transiently transfected with expression plasmids coding for IL-6R wildtype and Asp358Ala. The level of IL-6R expression was determined by Western blotting, and β-actin served as internal loading control. B.–E. The indicated MEFs were stimulated for 60 min with 1 μM ionomycin (Iono) or 2 h with 100 nM PMA. DMSO was used as negative control for each stimulation (either 1 h or 2 h). Afterwards, sIL-6R in the cell supernatants was quantified via ELISA. In each panel, one out of three experiments with similar outcome is shown.

0.004; Fig. 1C). Importantly, the rs2228145 SNP solely influenced sIL-6R serum levels, since neither sgp130 (A/A: 170.9 ± 24.5 ng/ml; C/C: 165.9 ± 21.9 ng/ml: $p = 0.5$; Fig. 1D) nor IL-6 (A/A: 17.8 ± 14.8 pg/ml; C/C: 19.2 ± 31.9 pg/ml; $p = 0.21$; Fig. 1E) showed any statistical significant difference between the two groups investigated.

Moreover, peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples of the ten volunteers, homozygous either for the common or the IL-6R Asp358Ala allele. Blood was drawn on five different days from age and sex-matched pairs (one genotype of each on the same day), and cell culture supernatants were analyzed for sIL-6R by ELISA. The data were analyzed pairwise on a daily basis, to exclude qualitative differences between the preparations of the cells on different days and subsequently summarized as x-fold increase of sIL-6R using the sIL-6R levels from the daily prepared common IL-6R variant as basis. As shown in Fig. 1F, conditioned cell culture supernatants of PBMCs homozygous for the Asp358Ala IL-6R variant contained significantly more sIL-6R (2.5 ± 1.1-fold, $p = 0.025$) compared to homozygous carriers of the common IL-6R variant.

Stephens et al. have previously shown that rs228145 increases the differential splicing of the IL-6R [13]. Therefore, we analyzed if the increased sIL-6R level were due to increased level of differentially spliced sIL-6R (DS-sIL-6R). An ELISA specifically detecting the alternative C-terminus of DS-sIL-6R but not the proteolytically processed sIL-6R revealed that below 1% of the sIL-6R is generated by differential splicing (Fig. 1G), which is consistent with other studies [32,33]. We can, however, not exclude that the new C-terminus of the differentially spliced sIL-6R variant is further processed after secretion, which might lead to underestimation of the differentially spliced sIL-6R variants. Four out of five serum samples from healthy volunteers, homozygous for the C allele in rs2228145, had detectable DS-sIL-6R levels, whereas in all other serum samples, DS-sIL-6R was below the detection limit (<31.25 pg/ml) of the ELISA.

Even though our results confirmed the earlier report that the C/C genotype conferred increased production of spliced sIL-6R as compared to the A/A variant [13], they suggested that ecto-domain shedding but not differential splicing mechanism mainly

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contributes to the overall generation of the sIL-6R found in human plasma.

3.2. The IL-6R Asp358Ala variant has an increased cell surface release rate compared to the common IL-6R variant

Cell surface expression of the IL-6R Asp358Ala variant has been shown to be reduced compared to the common IL-6R variant [14,15] and human PBMCs release more sIL-6R (Fig. 1F), suggesting that ectodomain shedding is the main driving force behind the increased sIL-6R serum level. Interestingly, cell surface expression of the IL-6R Asp358Ala variant in transiently transfected HEK293 cells was also lower compared to the common IL-6R variant (Fig. 2A), even though cellular IL-6R expression was comparable (Fig. 2B). Both IL-6R variants were able to induce IL-6 dependent signaling in stable transduced Ba/F3-gp130 cells, which could be blocked by the monoclonal antibody tocilizumab (Fig. 2C). Using confocal microscopy, intense cell surface staining for the common IL-6R variant was detected (Fig. 2D, top panel), whereas the IL-6R Asp358Ala variant was only sparely detected on the cell surface (Fig. 2D, lower panel). Taken together, ectopic expression of the IL-6R Asp358Ala variant led to reduced cell-surface expression compared to the common IL-6R variant, which resembles the in vivo situation.

Next, we analyzed if an increased proteolytic turnover of the IL-6R Asp358Ala variant contributes to the diminished amount of membrane-bound IL-6R. Reduced overall cell surface expression of the Asp358Ala IL-6R variant was also detected in Ba/F3-gp130-IL-6R cells (Fig. 2E). Cell surface expressed IL-6R was labeled with hIL-6R antibodies on these cells. Labeling was conducted at 4 °C and non-bound anti-IL-6R antibodies were washed away. Cells were shifted back to 37 °C for 0, 30, 60, 90 and 120 min to allow IL-6R ectodomain shedding and internalization. Thereafter, the cells were stained with secondary FITC-conjugated anti-mouse IgG detection antibody at 4 °C to quantify the remaining cell surface expression of IL-6R by flow cytometry. During the pulse-chase experiment, time-dependent reduction of the cell surface IL-6R expression was faster for the IL-6R Asp358Ala variant compared to the common IL-6R variant (Fig. 2E). To differentiate between ectodomain shedding and receptor internalization, constitutive IL-6R shedding was blocked by the metalloproteinase inhibitor GW280264X (GW), which is selective for the main IL-6R sheddases ADAM10 and ADAM17. Inhibition of IL-6R shedding had almost no effect on the cell surface expression of the common IL-6R variant, suggesting that the reduction of IL-6R cell surface expression was mainly caused by receptor internalization (Fig. 2E, compare upper two panels). Interestingly, blockade of ADAM10 and ADAM17 decelerated the cell surface down-regulation of the IL-6R Asp358Ala variant almost to the rate observed for the common IL-6R variant (Fig. 2E, compare lower two panels). Our data suggest that mainly ectodomain shedding contributes to the accelerated IL-6R cell surface down-regulation of the IL-6R Asp358Ala variant and directly cause the increased sIL-6R levels.

3.3. The IL-6R Asp358Ala variant has an increased inducible proteolytic conversion rate

Cellular stimulation with the calcium ionophor ionomycin or the phorbolester PMA resulted in induced shedding of the IL-6R by ADAM10 and ADAM17, respectively [7]. Therefore, we transiently transfected HEK293 cells with cDNAs coding for either the common or the Asp358Ala IL-6R variant and stimulated shedding with either ionomycin or PMA.

We set the amount of sIL-6R after stimulation to 100% and calculated all other values according to this, which allows the comparison of the different IL-6R variants [17]. The amount of shed sIL-6R of the Asp358Ala variant was slightly but significantly increased compared to the common IL-6R variant after ionomycin or PMA treatment (125 ± 2.4%, $p < 0.05$, Fig. 3A; 134.5 ± 13.7%, $p < 0.05$, Fig. 3B, respectively). Interestingly, also constitutively shed sIL-6R of the Asp358Ala IL-6R variant was increased in HEK293 cells compared to the common IL-6R variant (18.7 ± 5.2% vs. 27.7 ± 5.7%, $p < 0.05$, Fig. 3A; 17.2 ± 4.9% vs. 25.3 ± 4.9%, $p < 0.05$, Fig. 3B). Time-course experiments with transiently transfected HEK293 (7 h) and NIH3T3 (4 h) confirmed the increased constitutive proteolytic sIL-6R release of the Asp358Ala variant (Fig. 3C and D).

To ensure that ionomycin and PMA selectively activated ADAM10 and ADAM17, we used the protease inhibitors GI254023X (GI), which is selective for ADAM10, and GW280264X (GW), which is selective for both ADAM10 and ADAM17. Again, stimulation with ionomycin or PMA induced more IL-6R shedding for the Asp358Ala variant, which

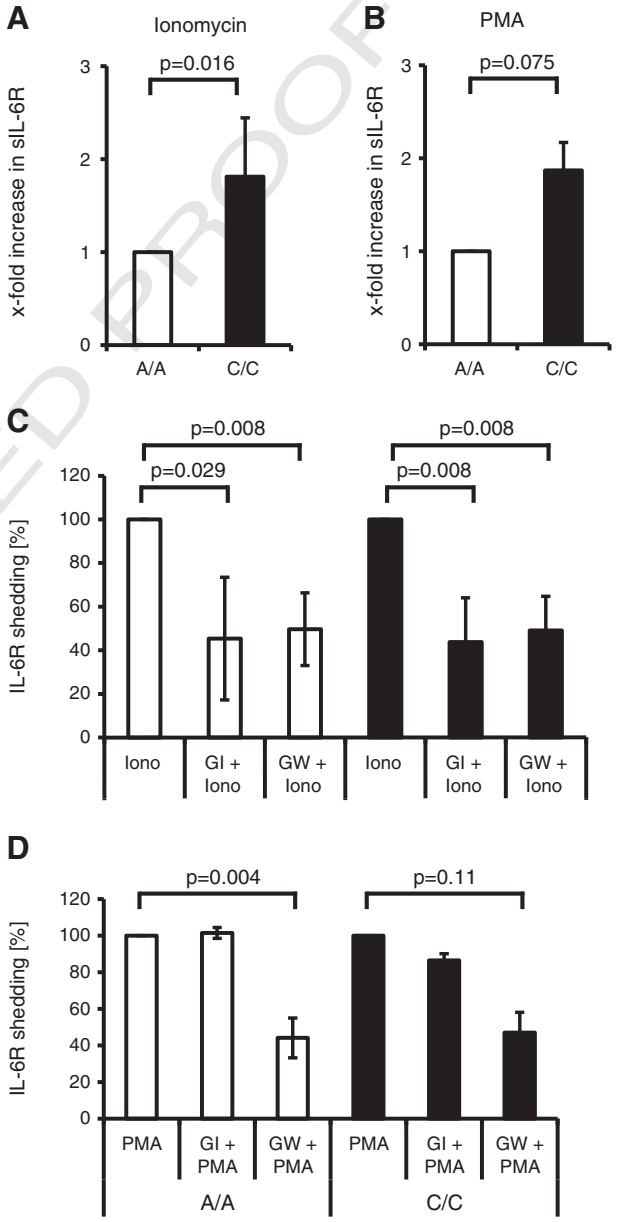

Fig. 5. Primary human peripheral blood mononuclear cells (PBMCs) from individuals homozygous for the rs2228145 (Asp358Ala) variant show higher ectodomain shedding of the IL-6R. PBMCs were isolated pair-wise from fresh blood of humans with the A/A or C/C IL-6R genotype (see Materials and methods for details). In all experiments, equal amounts of PBMCs were used, and the amount of sIL-6R in the supernatant of the PBMCs quantified with an ELISA specific for the IL-6R. Cells were stimulated as follows: A. 1 μM ionomycin (1 h), B. 100 nM PMA (2 h), C. 1 μM ionomycin plus either 3 μM GI or GW, D. 100 nM PMA plus either 3 μM GI or GW. Cells were pre-incubated with the inhibitors 30 min before addition of either PMA or ionomycin. P-values of the statistical analysis are given above the respective figure.

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was blocked by GI and GW or only GW, respectively (Fig. 3E and F). These results confirmed that ionomycin-induced ADAM10-mediated IL-6R shedding, whereas PMA selectively activated ADAM17 and that both proteases contribute to increased shedding of the Asp358Ala IL-6R variant.

3.4. ADAM10 and ADAM17 are responsible for increased proteolysis of the Asp358Ala IL-6R variant

Next, we used murine embryonic fibroblasts (MEFs) deficient for either ADAM10, ADAM17, or both proteases [7], to further analyze shedding of the Asp358Ala IL-6R variant. Overall cellular expression of the common and the Asp358Ala IL-6R variant was comparable or, if at all, minimally reduced for the Asp358Ala IL-6R in all MEFs tested (Fig. 4A). If the increased shedding of the IL-6R Asp358Ala IL-6R variant would be due to an increased protein biosynthesis than increased IL-6R expression of the Asp358Ala IL-6R variant would have been expected. Again constitutive and ionomycin- or PMA-induced shedding of the Asp358Ala IL-6R variant was slightly increased compared to the common IL-6R variant (Fig. 4B). ADAM10 deficient MEFs also released more sIL-6R after ionomycin- and PMA-stimulation (Fig. 4C). Although ADAM10 is lacking in these cells, we observed ionomycin-induced IL-6R shedding. As reported previously, compensatory shedding of human IL-6R was mediated by ADAM17 after ionomycin stimulation in ADAM10-deficient murine embryonic fibroblasts [7]. This phenomenon has also been described for other ADAM10/17 substrates [34]. As

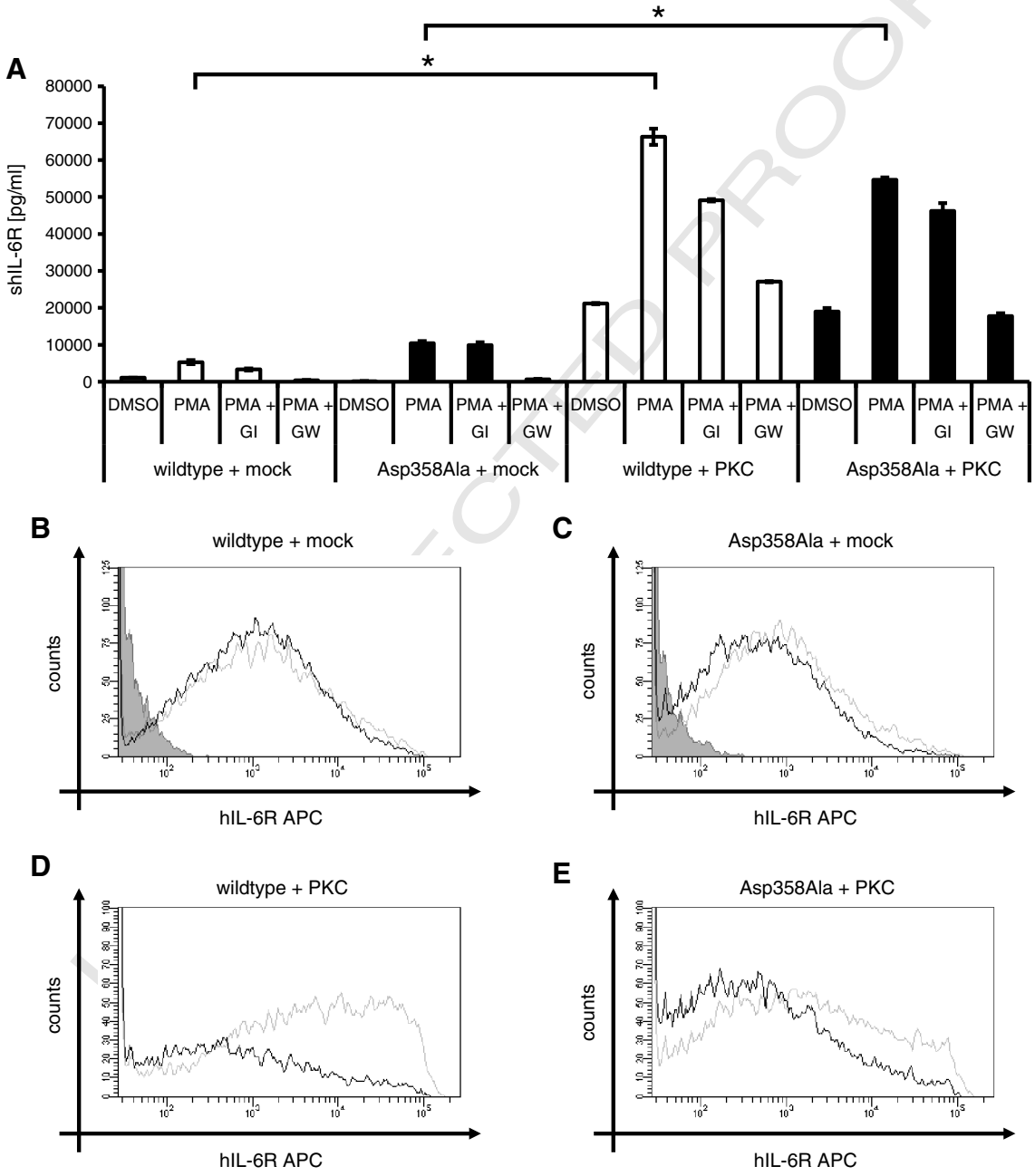

Fig. 6. Overexpression of PKC strongly enhances IL-6R proteolysis. A. HEK293 cells were transfected with either wildtype IL-6R or Asp358Ala IL-6R and either an expression plasmid encoding PKCα or a control plasmid. Cells were stimulated with PMA for 2 h. Where indicated, cells were pretreated with the ADAM inhibitors GI or GW 30 min before stimulation. The amount of sIL-6R within the supernatant was quantified via ELISA. One out of three experiments with similar outcome is shown. B–E. The transfected HEK293 cells of panel A were detached, washed and stained for cell-surface IL-6R expression as described under *Experimental procedures*. DMSO-treated cells are shown in light gray, PMA-stimulated cells in black. Filled gray histograms denote negative control to ensure no unspecific binding of the antibodies.

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expected, IL-6R shedding in ADAM17 deficient MEFs was not induced after PMA-treatment, since PMA solely activates ADAM17, which cannot be compensated by ADAM10. Ionomycin-induced shedding led to slightly more shed IL-6R in case of the Asp358Ala variant as compared to the common variant (Fig. 4D). In MEFs deficient for both ADAM10 and ADAM17 constitutive and PMA- and ionomycin-induced shedding IL-6R shedding was completely abrogated for both IL-6R variants (Fig. 4E). From these experiments we concluded that increased shedding of the Asp358Ala IL-6R variant was mediated by ADAM10 and ADAM17.

3.5. The Asp358Ala IL-6R variant has an increased inducible shedding susceptibility on PBMCs

To confirm our data in primary cells, we analyzed ectodomain shedding of the IL-6R Asp358Ala variant on human PBMCs. Unstimulated PBMCs from healthy volunteers had already shown a significantly increased sIL-6R release (2.5 ± 1.1-fold, $p = 0.025$) from PBMCs homozygous for the Asp358Ala IL-6R variant (Fig. 1F). Thus, PBMCs were used again from the ten volunteers, homozygous either for the common or the IL-6R Asp358Ala allele. PBMCs were either stimulated with ionomycin, PMA or left untreated to analyze induced IL-6R ectodomain shedding. Stimulation with ionomycin or PMA strongly induced IL-6R proteolysis of both IL-6 variants, shedding of the Asp358Ala IL-6R variant was 1.8 ± 0.6 ($p = 0.016$) and 1.9 ± 0.3-fold ($p = 0.075$) increased compared to common IL-6R variant, respectively (Fig. 5A, B). Ionomycin and PMA-induced shedding was significantly suppressed by co-incubation with GI and GW or GW alone, respectively (Fig. 5C, D), indicating that the sIL-6R was generated by ADAM10- and ADAM17-mediated ectodomain shedding and not by differential splicing.

3.6. Overexpression of protein kinase C strongly enhanced ADAM17-mediated IL-6R shedding and abolished the differential shedding of the IL-6R variants

PMA-induced ADAM17-mediated shedding of the IL6R is dependent on activation of the protein kinase C (PKCα) [35]. Co-transfection of cDNAs coding for PKCα and the common IL-6R variant led to massively increased constitutive and PMA-induced IL-6R shedding as compared to IL-6R transfected HEK293 cells ($p < 0.05$, Fig. 6A), demonstrating that the endogenous PKCα level is rate limiting for IL-6R shedding, which was boosted by PKCα co-expression. Interestingly, co-expression of IL-6R and PKCα abolished differences between the shedding of the common and the Asp358Ala IL-6R variant (Fig. 6A). Inhibition of ADAM17-mediated shedding by GW reduced PMA-induced shedding, but not below the level of constitutive shedding (Fig. 6A), suggesting that PKCα might induce a so-far not-identified IL-6R sheddase. It is, however, not known if this unknown protease is also responsible for constitutive shedding under non-PKCα-overexpressing conditions.

We verified these findings via flow cytometry. Whereas we observed only a very small reduction of cell-surface IL-6R when we stimulated HEK293 cells transfected with wildtype IL-6R (Fig. 6B), the cell-surface reduction of the Asp358Ala variant was more pronounced after PMA stimulation (Fig. 6C), thus reflecting the ELISA measurements (Fig. 6A). Interestingly, the majority of the IL-6R appeared to be still present on the cell surface. This suggests that the endogenous level of PKCα in HEK293 cells is not sufficient to induce full-blown ADAM17-mediated IL-6R shedding. In sharp contrast, PKC overexpression led to an increased cell-surface loss of both IL-6R variants (Fig. 6D and E), thereby confirming the results obtained via ELISA measurement (Fig. 6A). PKCα overexpression seemed not only to even the differences between the common and the Asp358Ala variant of the IL-6R, but also to lead to a preferential cleavage of the common variant (Fig. 6A, D, E). The reason behind this is currently unknown and warrants further investigation.

4. Discussion

Here, we provide evidence that the IL-6R SNP rs2228145 leads to increased constitutive and induced ectodomain shedding of the IL-6R by ADAM proteases. Overall steady state cell surface expression of Asp358Ala IL-6R variant is reduced compared to the common IL-6R variant. Paradoxically, despite reduced cell surface IL-6R expression, inducing ectodomain shedding by ionomycin or PMA still resulted in increased sIL-6R generation of the Asp358Ala IL-6R variant compared to the common IL-6R variant. This effect might be explained by incomplete PMA- and ionomycin-induced shedding of the IL-6R, which is not sufficient to release all cell surface IL-6R molecules. This notion is supported by our PKC co-expression experiments. Forcing ectodomain shedding of the IL-6R by over-expression of PKCα revealed the full potential of IL-6R ectodomain shedding. Even though PMA is the strongest inducer of IL-6R shedding described to date, co-expression of PKCα result in 3–4-fold increase of PMA-induced IL-6R shedding revealing the full potential of ADAM-mediated IL-6R shedding. Interestingly, under these conditions no differences in PMA-induced shedding of the common and the Asp358Ala IL-6R variants were observed.

Limited proteolysis of transmembrane proteins occurs usually in close proximity to the plasma membrane, and the shedding susceptibility of this is critically influenced by the amino acids surrounding the cleavage sites. ADAM17 cleaves the IL-6R between Gln357 and Asp358 within the so-called stalk region [16], which consists of 52 amino acids [17]. The stalk region is considered to function as a spacer to position the three extracellular domains of the IL-6R towards gp130. Until now, no structural data of the stalk region are available [36,37], but overall the stalk is considered to lack a domain structure. Interestingly, other non-naturally occurring point mutations within the IL-6R resulted in reduced shedding of the IL-6R [16]. Also small deletions within the stalk region reduced IL-6R shedding [17]. To date, no consensus sequence for ADAM10 or ADAM17 cleavage sites has been identified. However, our data show that ADAM17 might favor the cleavage after a small, non-charged amino acid like alanine instead of aspartic acid.

IL-6 is found in the blood at 1–5 pg/ml but increases to ng/ml concentrations during pathophysiology [1]. The serum concentration of the sIL-6R is about 20 ng/ml and of sgp130 is about 100–200 ng/ml [1]. The IL-6R SNP rs2228145 leads to a doubling of sIL-6R level in the blood [10,11], and this has been shown to be associated with reduced CRP level [8,9]. Two different mechanisms might be responsible for this, or may act in concert. Circulating IL-6 will bind to sIL-6R and subsequently to sgp130, which is the natural inhibitor of the IL-6 trans-signaling [1]. Thus, sIL-6R and sgp130 in the serum can act to buffer systemic activities of IL-6. Since sgp130 is available in excess, a doubling of sIL-6R would indeed increase this buffer capacity. However, thus far no experiments were conducted to proof this hypothesis. An increased proteolytic conversion of IL-6R due to the Asp358Ala SNP might therefore contribute to this increased buffer capacity of IL-6 and to overall reduced IL-6 activity [5,38]. Furthermore, the reduced membrane-bound IL-6R on hepatocytes of humans homozygous for the rs2228145 SNP might lead to reduced IL-6 classic-signaling and subsequently to reduced CRP production [15,39].

Taken together, our data strongly suggested that ectodomain shedding but not alternative splicing mainly contributes to the reduced cell surface IL-6R levels and increased sIL-6R serum levels found in individuals homozygous for the IL-6R rs2228145 SNP.

Conflict of interest statement

The authors have no conflict of interest to declare.

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