Tocilizumab unmasks a stage-dependent interleukin-6 component in statin-induced apoptosis of metastatic melanoma cells

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The interleukin (IL)-6 inhibits the growth of early-stage melanoma cells, but not metastatic cells. Metastatic melanoma cells are susceptible to statin-induced apoptosis, but this is not clear for early-stage melanoma cells. This study aimed to investigate the IL-6 susceptibility of melanoma cells from different stages in the presence of simvastatin to overcome loss of growth arrest. ELISA was used to detect secreted IL-6 in human melanoma cells. The effects of IL-6 were measured by western blots for STAT3 and Bcl-2 family proteins. Apoptosis and proliferation were measured by caspase 3 activity, Annexin V staining, cell cycle analysis, and a wound-healing assay. Human metastatic melanoma cells A375 and 518A2 secrete high amounts of IL-6, in contrast to early-stage WM35 cells. Canonical IL-6 signaling is intact in these cells, documented by transient phosphorylation of STAT3. Although WM35 cells are highly resistant to simvastatin-induced apoptosis, coadministration with IL-6 enhanced the susceptibility to undergo apoptosis. This proapoptotic effect of IL-6 might be explained by a downregulation of Bcl-X$_L$, observed only in WM35 cells. Furthermore, the IL-6 receptor blocking antibody tocilizumab was coadministered and unmasked an

IL-6-sensitive proportion in the simvastatin-induced caspase 3 activity of metastatic melanoma cells. These results confirm that simvastatin facilitates apoptosis in combination with IL-6. Although endogenous IL-6 secretion is sufficient in metastatic melanoma cells, exogenously added IL-6 is needed for WM35 cells. This effect may explain the failure of simvastatin to reduce melanoma incidence in clinical trials and meta-analyses. *Melanoma Res* 25:284–294 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

The principle of wide local excision of the primary melanoma as first described by William Norris in 1857 remains the gold standard of therapy for localized melanoma to date [1]. In contrast, the metastatic disease represents a therapeutic challenge with a poor prognosis, although new therapeutic approaches have emerged [1,2].

Serum level of interleukin (IL)-6 is a prognostic factor in melanoma [3–5]. High levels of IL-6 are predictive for reduced overall survival in metastatic melanoma patients and a high tumor burden [3,4]. IL-6 is part of the IL-6-like cytokine family, which includes IL-11, leukocyte inhibitory factor, oncostatin M, ciliary neurotrophic factor, and cardiotrophin 1 [6]. The cytokine IL-6 represents a key player in inflammatory processes. In melanoma, IL-6 acts like a double-edged sword. Growth of untransformed melanocytes and early-stage melanoma is inhibited, whereas metastatic melanoma cells are

mostly resistant to the antiproliferative effects of IL-6 [6]. It has been shown that almost 50% of metastatic melanoma express IL-6 mRNA and secrete IL-6, which enables proliferation in the absence of growth factors [7,8].

Inhibitors of the 3-hydroxy-3-methylglutaryl-coenzym A (HMG-CoA) reductase, usually referred to as statins, inhibit the rate-limiting step of the mevalonate pathway [9]. Mevalonate is a precursor of several major products including ubiquinone, dolichol, geranylgeranylpyrophosphate, and farnesylpyrophosphate [10]. Although statins are well tolerated, in micromolar concentrations, statins have been found to exert multiple pleiotropic effects. Thus, in-vitro application of a statin impacts the mitochondrial respiratory chain, protein glycosylation, and post-transcriptional lipid modification of proteins, in particular, small G proteins [11]. In various tumor cell lines, statins induce apoptosis, in particular in melanoma cells, which has led to a discussion to use statins in anticancer regimens [12–16]. In-vitro studies on murine and human melanoma cells have shown that lovastatin used in combination with doxorubicin potentiates

antitumor effects, which may be explained by compartmentalization of doxorubicin into the nucleus because of the inhibition of the ATP-binding cassette transporter P-glycoprotein (ABCB1) by statins [17,18]. The sensitivity of melanoma cells toward statins was further corroborated by enhanced apoptosis, reduced invasion, and metastasis [16,19]. Interestingly, on comparison of various melanoma cell lines, different sensitivities for lovastatin have emerged [20]. However, the reasons for these different sensitivities are yet not fully understood.

We have therefore asked the question whether the different susceptibilities toward statin-induced apoptosis are related to the action of IL-6? By comparison of human early-stage WM35 melanoma cells with metastatic 518A2 and A375 cells, the effects of IL-6 were investigated in the presence of the statin simvastatin. The WM35 cells were sensitive to IL-6-induced apoptosis in particular in the presence of simvastatin. Metastatic melanoma cells were insensitive to IL-6, but potently killed by simvastatin. The combination with tocilizumab, an IL-6-neutralizing antibody, unmasked an endogenous IL-6-dependent induction of apoptosis by simvastatin. The clinical implications of these findings are discussed.

Materials and methods
Cell culture and protein preparation
The human metastatic melanoma cell line A375 was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and the 518A2 cells were obtained from Dr Edgar Selzer (Department of Radiation Oncology, Medical University of Vienna, Austria). The human primary melanoma cell line WM35 was obtained from Dr Meenhard Herlyn (Wistar Institute, Philadelphia, Pennsylvania, USA). Human melanoma cell lines A375 and 518A2 were grown in Dulbecco's modified Eagle medium (DMEM)-high glucose (Invitrogen; Paisley, Scotland, UK) complemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. WM35 cells were grown in DMEM/Ham's F12 medium supplemented with 20 mmol/l HEPES (pH 7.4), 2% FCS, 20 mmol/l L-glutamine, and 1% penicillin/streptomycin. Cells were kept at 37°C under a 5% CO_2 humidified atmosphere.

The human melanoma cells were exposed to various compounds as indicated in the figure legends, and thereafter lysed as described previously [13]. For detection of phosphorylated proteins, cells were lysed with IP-buffer (25 mmol/l tris–HCl, pH 7.5, 150 mmol/l NaCl, 10 mmol/l EDTA, 0.1% Tween-20, 0.5% NP-40, 10 mmol/l β-glycerophosphate, 10 μg/ml aprotinin, 100 μmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mmol/l NaF, and 1 mmol/l Na_3VO). The protein fractions were rapidly frozen and stored at − 80°C.

Western blot analyses
Protein samples from cell lysis (cytosolic fraction) were used for western blot analysis and separated on a SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose membranes (Whatman, Dassel, Germany) and exposed to primary antibodies against cyclin D1 (M-20), Bcl-X_L (L-19), Bak (G-23), and Bax (N-20) from Santa Cruz (Santa Cruz, California, USA) and phospho-STAT3 (9131), STAT3 (9132) and Bcl-2 (2872) from Cell Signalling Technology (Danvers, Massachusetts, USA). The α-tubulin was used as a loading control (B 5-1-2; Sigma, St Louis, Missouri, USA). Appropriate horseradish peroxidase-conjugated secondary antibodies were used to detect the proteins of interest using the ECL Plus detection system (GE Healthcare, Bucks, UK). The intensity of the protein bands was determined using ImageJ software (*http://rsbweb.nih.gov/ij*) and expressed relative to the intensity of the α-tubulin band.

Interleukin-6 detection by ELISA
Equal amounts of cells (15 000) were seeded and kept in serum-free medium to prevent IL-6 contamination by FCS. Human IL-6 ELISA Development Kit (Peprotech, Rocky Hill, New Jersey, USA) and IL-6 (human) EIA kit (Enzo Life Sciences, Farmingdale, New York, USA) were used to detect IL-6 in the medium of melanoma cells. Experiments were conducted according to the manufacturer's protocol. Recombinant human IL-6 (Peprotech) was used as a standard.

Apoptosis detection
Apoptosis was measured in melanoma cells using double staining with Annexin V/propidium iodate using the apoptosis kit from Bender MedSystems (Vienna, Austria) according to the instruction manual. Double staining was analyzed by FACS as described previously [13]. Alternatively, caspase assays were carried out as described previously [15,21].

Cell cycle analysis
Melanoma cells were synchronized in serum-free medium overnight and treated with drugs for 48 h as indicated in the figure legends. Trypsinized cells were washed and fixed with 70% ethanol at 4°C for 1 h, washed, and incubated with 60 μg/ml RNase A in PBS (30 min, 37°C). Following staining with propidium iodide (5 μg/ml; 30 min, 4°C), samples were analyzed on a FACScan (Becton Dickinson, San Jose, California, USA). The cell cycle phases were analyzed using Cyflogic software (Version 1.2.1; Cyflogic, Turku, Finland).

Wound-healing assay
Melanoma cells were grown to 90% confluence as a monolayer in six-well plates. Subsequently, a wound was scratched with a 200 μl plastic pipette tip across the bottom of each well. Thereafter, cells were washed twice with PBS and treated for up to 32 h with the drugs indicated in the figure legends. Four representative spots were labeled and four pictures per scratch were taken at these orientation points. Up to four wells per condition were analyzed at the indicated time points. The

percentage of cell-free surface was calculated using the TScratch software tool (CSElab, ETH Zürich, Switzerland).

Miscellaneous procedures

The protein concentration was determined by a Bradford protein assay using bovine serum albumin as a standard [22]. Experiments were conducted at least three times in duplicate. Data are expressed as mean ± SD if not otherwise stated. The concentration − response curves were subjected to nonlinear least-squares regression with the Hill equation using the Sigmaplot software (Jandl, Erkrath, Germany). Statistical analysis was carried out using Student's t-test or for multiple comparisons with analysis of variance and post-hoc Bonferroni correction. A value of P less than 0.05 was considered statistically significant.

Results

Interleukin-6 is secreted by melanoma cells

Secretion of IL-6 has long been confirmed for murine and human melanoma cells [7,8]. However, high levels of IL-6 have been found in late-stage melanoma correlated with a poor prognosis [23,24]. We have therefore compared and characterized human early-stage WM35 melanoma cells with metastatic 518A2 and A375 cells. The metastatic melanoma cell lines secrete high amounts of IL-6 (121.4 ± 39.9 pg/ml A375 cells; 540 ± 169 pg/ml 518A2 cells) (Fig. 1). Conversely, WM35 cells secreted significantly less IL-6 (27.9 ± 6.9 pg/ml). This latter concentration is very close to that published by Molnar et al. [23] for these cells.

The activated IL-6 receptor is mirrored by STAT3 posphorylation at Tyr705, which results in dimerization, nuclear translocation, and DNA binding [25,26]. The IL-6-mediated activation of STAT3 in all three cell lines is intact (Fig. 1b). However, the strongest increase in STAT3 posphorylation is predominantely observed in WM35 cells. Conversely, in metastatic melanoma cells A375 and 518A2, the phosphorylation pattern was transient, with a clear peak after 60 min and a second peak in A375 cells after 120 min.

These results were further corroborated by analyses of the cell cycle, which showed a significant stimulation of the G_2 phase in A375 and 518A2 cells by IL-6 (Fig. 2). Conversely, WM35 cells accumulate in the G_0/G_1 phase, reflecting a reduction of cell cycle progression in the presence of IL-6 (Fig. 2c).

WM35 melanoma cells are resistant to simvastatin-induced apoptosis

Metastatic melanoma cells are sensitive to statin-induced apoptosis [13]. We have therefore also investigated WM35 cells from the radial growth phase of melanoma for simvastatin-induced apoptosis by FACS analysis using Annexin V and propidium iodide double staining (Fig. 3). Only concentrations exceeding 10 μmol/l

Fig. 1
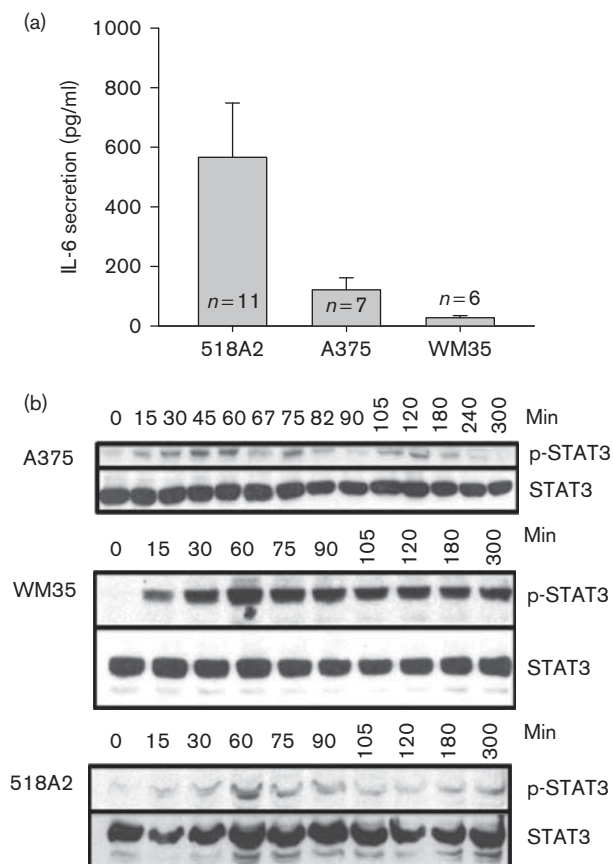
Interleukin (IL)-6 production and signaling in human melanoma cells of different leasional stages. (a) Human melanoma cells 518A2, A375, and WM35 were cultured in serum-free medium for 48 h and analyzed for IL-6 levels with ELISA. Bars and errors indicate the mean ± SD. (b) Serum starved human melanoma cells 518A2, A375, and WM35 were exposed to 20 ng/ml IL-6 for the indicated time points. Western blots show phosphorylated STAT3 (p-STAT3), which is compared with total STAT3 as a loading control.

simvastatin were capable of significantly inducing apoptosis in WM35 cells, whereas this effect has already been observed at 1 μmol/l simvastatin in 518A2 and A375 cells. However, in WM35 cells, coincubation with IL-6 shifted the concentration–response curve significantly to the left and no effect was observed in 518A2 and A375 cells (Fig. 3b and d).

The executer caspase 3 mirrors the point of no return in the apoptotic machinery. For comparison, simvastatin-induced caspase 3 activation was evaluated in all three melanoma cell lines to corroborate apoptosis by another method (Fig. 4a).

The corresponding EC_{50} values for A375 and 518A2 cells were 0.55 ± 0.09 and 0.47 ± 0.65 μmol/l (n = 3). Again, WM35 cells were 25-fold less sensitive to simvastatin-induced apoptosis, with an EC_{50} value of 14.2 ± 3.89 μmol/l

Fig. 2
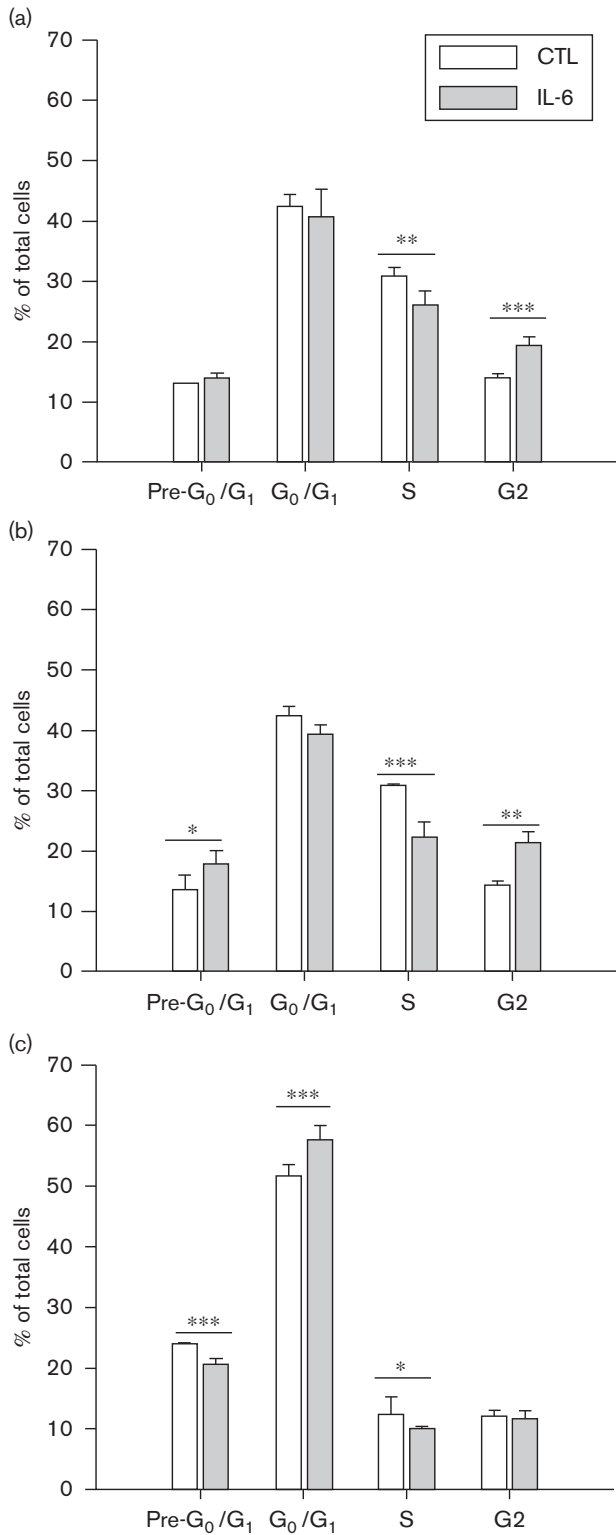
Cell cycle analysis of interleukin (IL)-6-treated human melanoma cells. Melanoma cells (a) 518A2, (b) A375, and (c) WM35 were incubated with IL-6 (20 ng/ml) under serum-free conditions for 48 h and compared with controls (CTL). For cell cycle analyses, cells were stained with propidium iodide and analyzed by FACS. Normalized data represent the mean ± SD ($n = 6$). Statistical significance versus control: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

($n = 3$), confirming the previous data (Fig. 3). Caspase 3 activity was not stimulated by IL-6 alone; however, in WM35, an insignificant increase was observed (Fig. 4). The combination of simvastatin with IL-6 exerted no effect in metastatic melanoma cells in comparison with simvastatin alone. In contrast, similar to the above-described positivity for Annexin V and propidium iodide double staining, a significant increase in caspase 3 activation was observed for this drug combination in WM35 cells (Fig. 4d).

We have previously shown that the proapoptotic effects of statins are triggered by translocation of the Bcl-2 homology (BH) domain member Bax into the mitochondrial membrane [15]. As the proapoptotic Bcl-2 members are counterbalanced by binding to antiapoptotic Bcl-2 members, we investigated these proteins in the absence and presence of IL-6 (Fig. 5). In the metastatic melanoma cells, the levels of proapoptotic Bak and Bax and antiapoptotic Bcl-2 and Bcl-X_L showed different responses upon IL-6 treatment. Bcl-X_L was clearly decreased in WM35 cells in the presence of IL-6. In addition, the amount of Bcl-2 was decreased, whereas the level of proapototic proteins remained unchanged (Fig. 5).

Effect of tocilizumab

To investigate the role of IL-6 in the proapoptotic effect of simvastatin, we used the inhibitory IL-6 receptor antibody, tocilizumab [27]. The addition of 50 µg/ml tocilizumab *per se* exerted no significant effect on melanoma cells in terms of cell cycle regulation or viability. Under control conditions, 64.8 ± 3.2, 62.1 ± 8.1, and 55.4 ± 9.3% of 518A2, A374, and WM35 cells were in G_0/G_1 phase, whereas 16.7 ± 5.0, 17.1 ± 3.5, and 18.1 ± 3.7% were in the S-phase, respectively. Under tocilizumab incubation, 67.8 ± 6.6, 62.5 ± 17.8, and 55.5 ± 9.6% of 518A2, A374, and WM35 cells were in the G_0/G_1 phase and 14.3 ± 4.2, 16.7 ± 5.4, and 18.7 ± 3.6% were in the S-phase, respectively.

These findings are further supported by the observation that tocilizumab exerted no effect on caspase 3 activation in all three melanoma cell lines, which was also the case for the corresponding human IgG1 isoform (Fig. 6). Interestingly, a significant stimulation of caspase 3 was detected for the combination of simvastatin and IgG1 in WM35 cells versus control (Fig. 6c). Compared with simvastatin treatment alone, the combination of simvastatin plus IgG1 was insignificant. However, the simvastatin-induced caspase 3 activation was significantly reduced by tocilizumab in metastatic melanoma cells A375 and 518A2 (Fig. 6a and b). These cells were therefore used in scratch assays to elucidate a functional effect of tocilizumab-induced abrogation of simvastatin-induced apoptosis. Again, the human IgG1 conctrol exerted no effect on wound closure or simvastatin-induced inhibition (Fig. 7a and c). Obviously,

Fig. 3
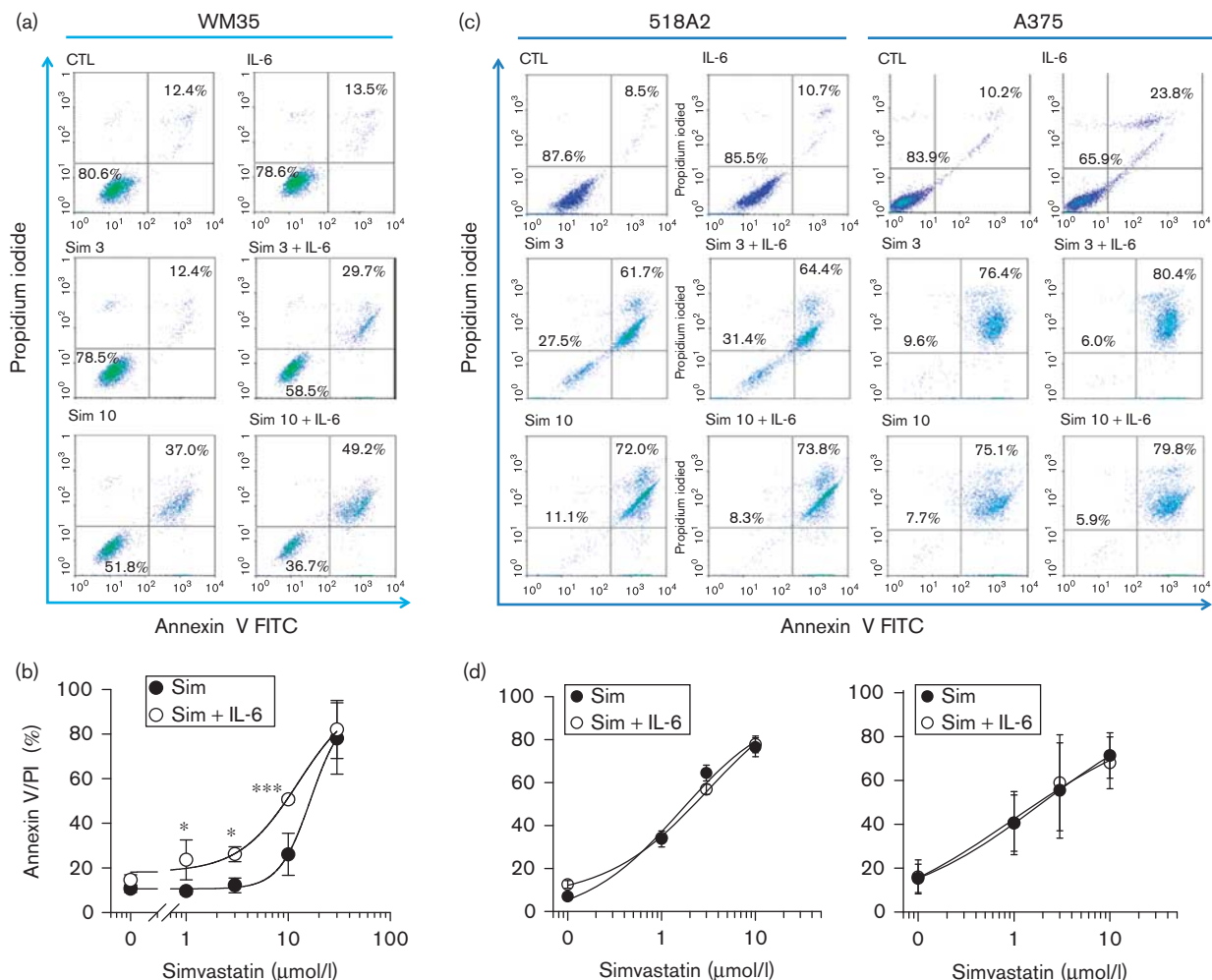
Interleukin (IL)-6 sensitizes WM35 melanoma cells for simvastatin-induced apoptosis, but not 518A2 and A375 cells. WM35 (a, b), 518A2, and A375 (c, d) cells were incubated in the absence (CTL) and presence of IL-6 (20 ng/ml) and the indicated concentrations of simvastatin (Sim) for 48 h. The cells were double stained with propidium iodide and Annexin V for quantification by FACS analysis in the upper panels (a, c). Three such experiments were collected for concentration–response curves (b, d). Statistical significance versus the corresponding simvastatin concentration: *$P < 0.05$; ***$P < 0.001$; $n = 3$.

proliferation in A375 and 518A2 cells was significantly prevented by simvastatin (Fig. 7). Although tocilizumab *per se* had no significant effect, coapplication with simvastatin resulted in accelerated reduction of the cell-free area, indicating accelerated proliferation. Hence, the latter observation confirmed abrogation of simvastatin-induced apoptosis by tocilizumab, which uncovered the involvement of proapoptotic IL-6 action in metastatic melanoma cells.

Taken together, these data show that simvastatin effectively triggers apoptosis in metastatic melanoma cells making use of the endogenous IL-6. The IL-6-neutralizing antibody tocilizumab unmasks this IL-6 component of the simvastatin-induced apoptosis, which is currently not understood at the molecular level. Conversely, in early-

stage melanoma cells, exogenous IL-6 is needed to enhance simvastatin-induced apoptosis.

Discussion
Interleukin-6 and melanoma
The cytokine IL-6 acts as a growth inhibitor in early-stage melanoma, which is reflected in this study by WM35 cells from the initial radial growth phase. In human metastatic melanoma cells A375 and 518A2, IL-6 acts as a growth factor. This dualistic action of IL-6 has long been known, but has not been fully understood at the molecular level [28–30].

Early studies showed that in WM35 cells, IL-6 leads to growth inhibition by upregulation of p21 and subsequent cell cycle arrest [31]. This observation is confirmed in this

Fig. 4
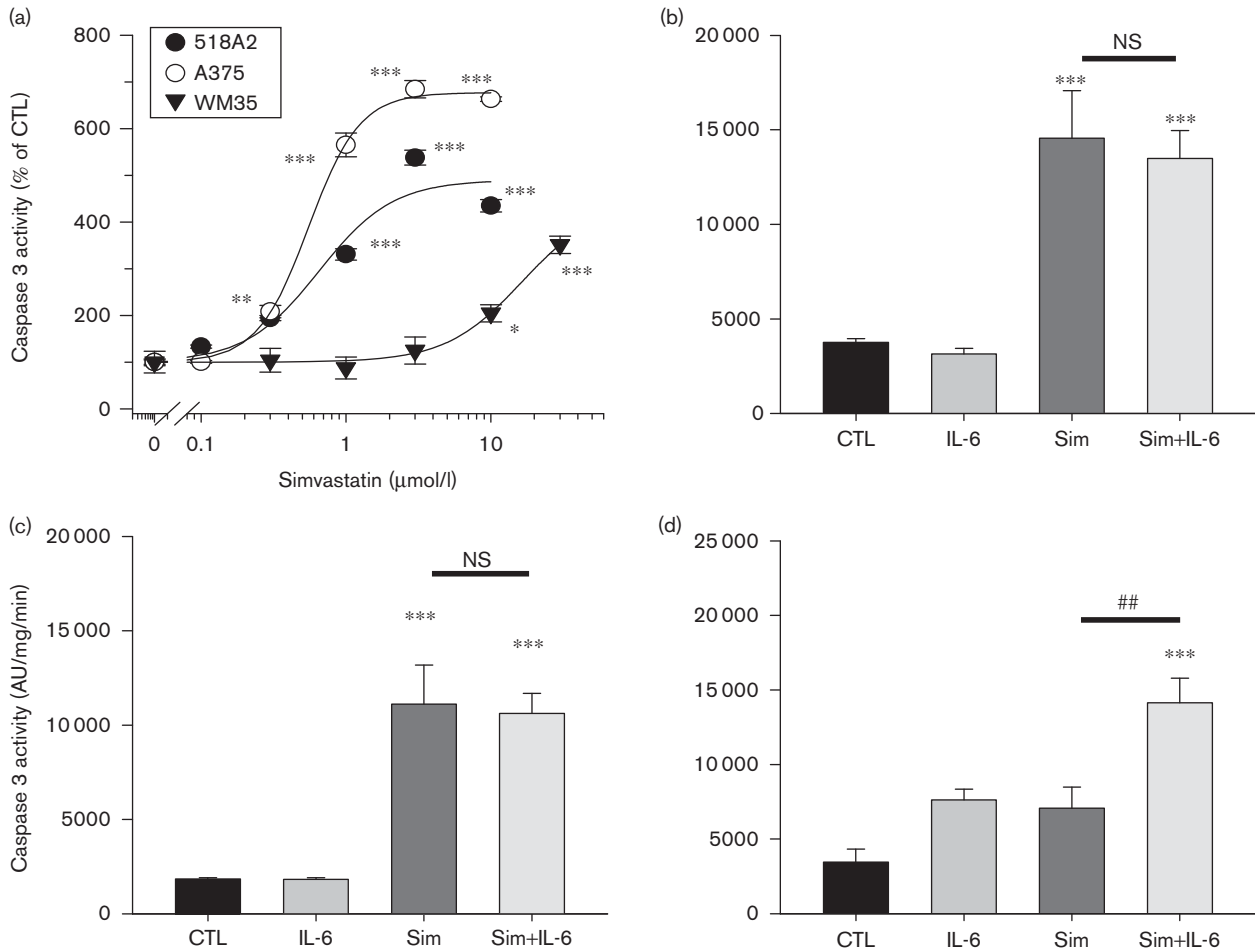
Simvastatin-induced caspase 3 activation in human melanoma cells. (a) The 518A2, A375, and WM35 cells were treated with the indicated concentrations of simvastatin for 48 h, lysed, and subjected to assessment of caspase 3 activity. Melanoma cells (b) 518A2, (c) A375, and (d) WM35 were treated for 48 h in the absence (CTL) and presence of 10 µmol/l simvastatin (Sim) and/or 20 ng/ml interleukin (IL)-6. Data points or bars represent the mean ± SEM ($n = 3$). Statistical significance versus control: **$P < 0.01$; ***$P < 0.001$, or versus simvastatin treatment: ###$P < 0.001$; NS, not significant.

study by a significant accumulation of WM35 cells in the G_0/G_1 phase and a reduction of the S-phase (Fig. 2). Transformation of WM35 cells by retroviral insertional mutagenesis was used to convert these cells into an aggressive phenotype [32]. Interestingly, these tumorigenic variants of the parental WM35 cells lost the responsiveness to IL-6-induced cell cycle arrest.

The IL-6-induced growth inhibition was not observed in late-stage melanoma cells, that is IL-6 shifted metastatic A375 and 518A2 cells into the G_2 phase (Fig. 2). These data are further corroborated by the fact that the latter cells also secrete high amounts of IL-6, which reflect roughly 5–15-fold higher levels compared with WM35 cells (Fig. 1). It is noteworthy that the concentration of IL-6 in the medium of WM35 cells is close to the upper limit for healthy individuals (20 pg/ml, 95th percentile in healthy individuals) [33,34].

Similar to IL-6, the melanoma cell lines investigated also showed a stage-dependent difference in sensitivity toward simvastatin-induced apoptosis (Figs 3 and 4a). Interestingly, WM35 cells were less sensitive to simvastatin-induced apoptosis compared with metastatic melanoma cells. The calculated EC_{50} values were 25-fold higher for WM35 cells. However, the coadministration of IL-6 enhanced the apoptotic effects of simvastatin, although IL-6 *per se* exerted no direct apoptotic effect (Figs 3 and 4d). The antiapoptotic Bcl-2 family members are often upregulated in melanoma cells, whereas the protein levels of proapoptotic proteins (Bax, Bak) were not changed [35]. We found a significant reduction of Bcl-X_L in IL-6-treated WM35 cells (Fig. 5). This observation provides a possible explanation for the facilitation of apoptosis triggered by coadministration with simvastatin (Figs 3 and 4d). An additional argument is provided by our recent observation that simvastatin triggers the synthesis of the

Fig. 5
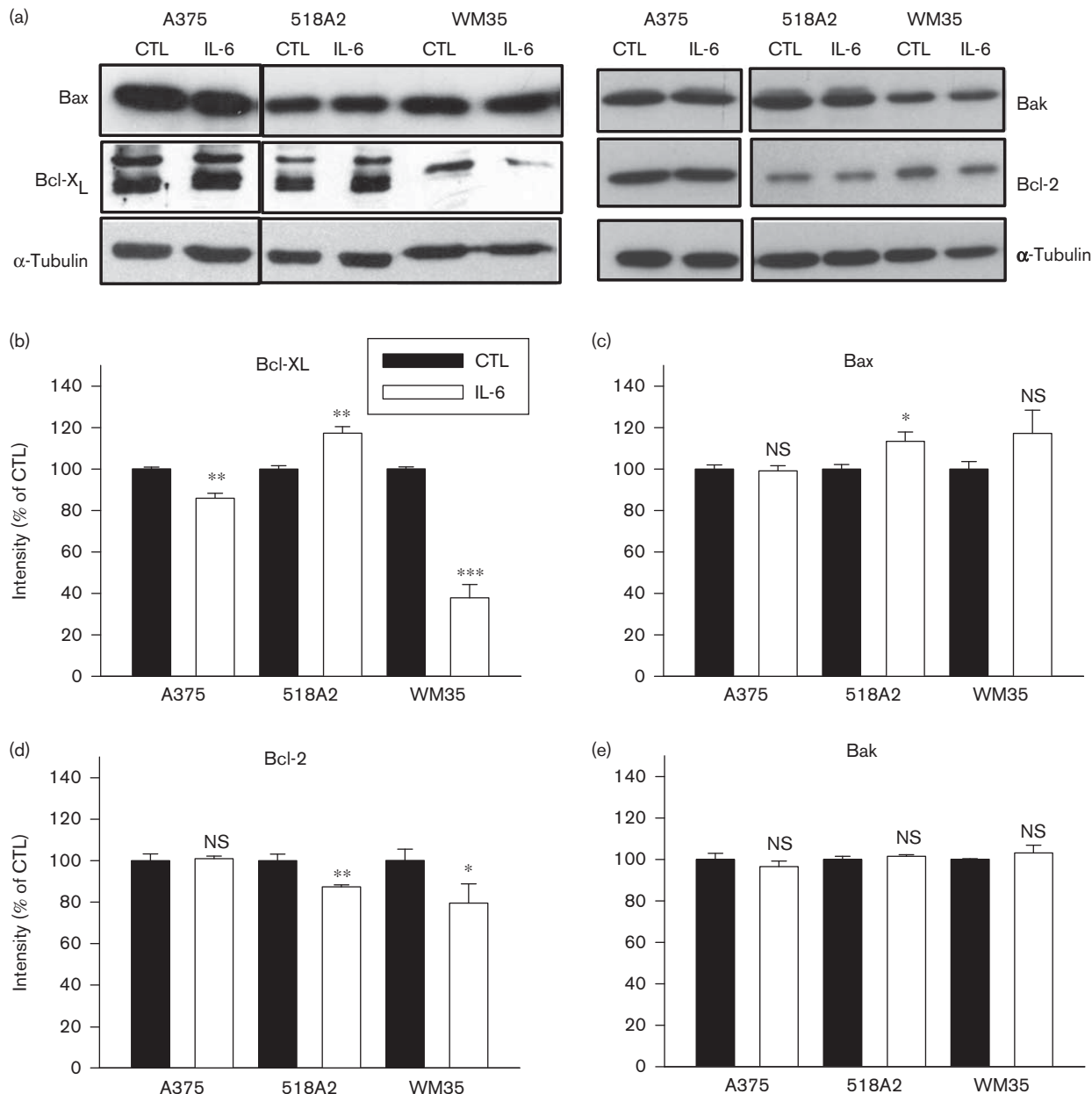
Interleukin (IL)-6 downregulates the Bcl-2 family member, Bcl-X$_L$ in WM35 melanoma cells. Melanoma cells A375, 518A2, and WM35 were kept under serum-free conditions in the absence [control (CTL)] and presence of 20 ng/ml IL-6 for 48 h. (a) Bcl-2 family members, Bcl-X$_L$, Bax, Bcl-2, and Bak were visualized using α-tubulin as a loading control. (b–e) Three independent experiments were analyzed for intensity and presented as mean ± SEM ($n = 3$). Statistical significance versus control: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.

prostaglandin, 15d-PGJ$_2$, which is an endogenous ligand of the peroxisome proliferator-activated receptors γ (PPARγ) [36]. Moreover, the fatty acid-binding protein 5, the lipid chaperon for 15d-PGJ$_2$, is strongly upregulated in simvastatin-treated A375 and 518A2 cells and activates the PPARγ ligand. Further investigations have to clarify whether simvastatin-induced activation of PPARγ downstream targets such as NFκB might affect IL-6 signaling.

Statins and melanoma
The fact that statins are so well tolerated when taken over long periods of time make it feasible now to analyze a possible anticancer action of statins in melanoma. In particular, the Breslow thickness has been found to be reduced in a statin-treated group [37]. Clinical studies and meta-analyses are available that confirmed a positive outcome and reduced the incidence of melanoma for statin

Fig. 6
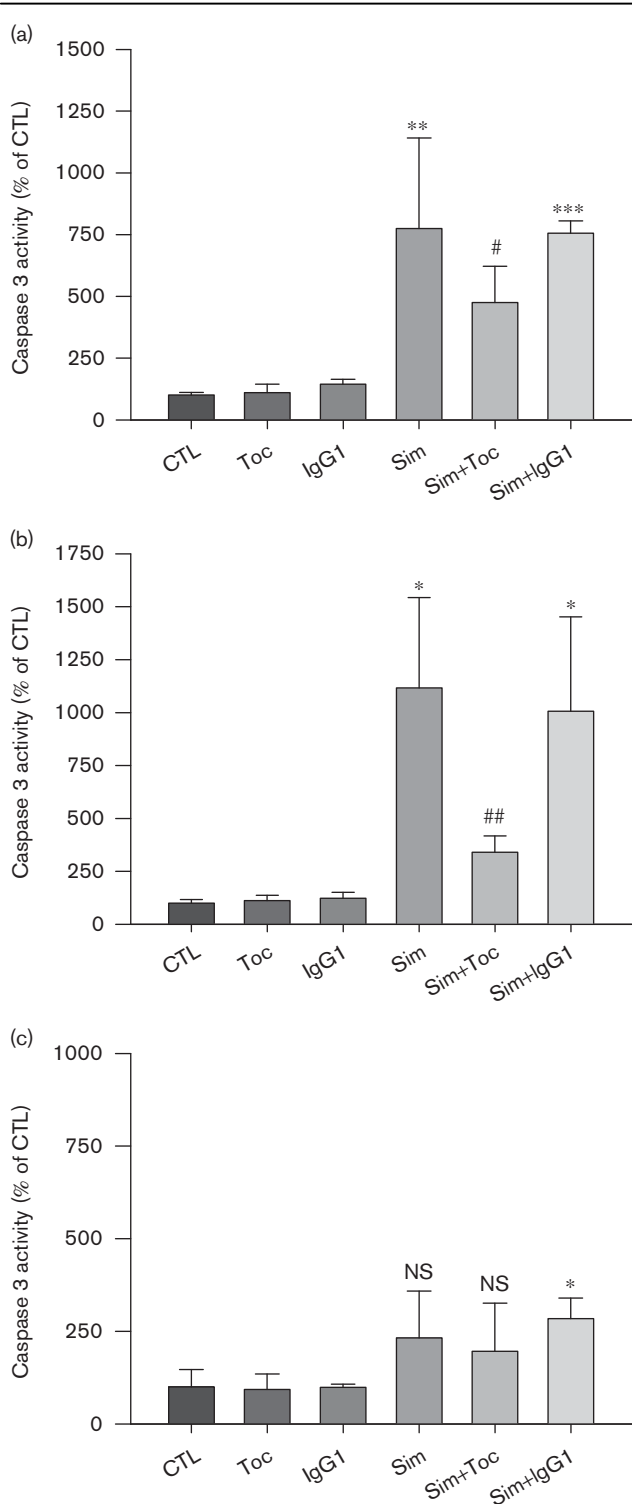
Tocilizumab unmasks an interleukin (IL)-6 contribution in simvastatin-induced caspase 3 activation. Melanoma cells (a) 518A2, (b) A375, and (c) WM35 were incubated in the absence [control (CTL)] and presence of 10 μmol/l simvastatin (Sim), human IgG1 (50 μg/ml), and/or tocilizumab (Toc; 50 μg/ml) for 48 h and the corresponding cell lysates used for caspase 3 assay ($n = 8$–34). Statistical significance versus control: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant, or versus simvastatin treatment: #$P < 0.05$; ##$P < 0.005$.

takers, with an estimated risk for melanoma incidence of 0.79 (95% confidence interval = 0.66–0.96) [37–39]. Besides these protective effects of statins, there are also reports and analyses that provide no significant evidence for a statin action in melanoma, even after correction for age, sex, or the individual statin used [40–42]. An explanation for these heterogeneous data could be the trial design *per se*. Statins are administered to patients with a cardiovascular risk and studies aim to confirm improved cardiovascular outcomes, but not the effect of statins on melanoma. Second, the follow-up periods of 5 years might be too short to evaluate or elucidate an anticancer effect. In part, these considerations have been addressed by Jacobs *et al.* [38] in their Cancer Prevention Study II Nutrition Cohort, which was carried out from 1997 to 2007 in 133 255 participants. The incidence of the 10 most common cancers and the overall cancer incidence in statin takers were investigated and no association nor increase in overall cancer incidence was found. However, a decreased risk of developing melanoma was observed (relative risk 0.70, 95% confidence interval 0.55–0.88, $P = 0.02$).

Interleukin-6 antagonism with tocilizumab

Tocilizumab is an approved specific humanized monoclonal antibody against the IL-6 receptor, competing for the binding site of IL-6 [43,44]. This therapeutic concept is used in the treatment of chronic inflammatory diseases such as rheumatoid arthritis and Castleman's disease [44,45]. We used tocilizumab to block IL-6 signaling of the endogenously secreted IL-6 to unmask an effect of this cytokine. Tocilizumab *per se* had neither an effect on the cell cycle progression nor on pre-G_0/G_1 accumulation of fragmented DNA independent of the cell line used (data not shown). Importantly, the IL-6/phospho-STAT3 axis is intact in WM35, A375, and 518A2 cells (Fig. 1b). Interestingly, in combination with simvastatin, tocilizumab unmasked a proapoptotic activity of IL-6 in metastatic A375 and 518A2 cells (Fig. 6), which is also reflected by improved wound healing of such treated cells (Fig. 7). These effects are considered to be specific as the control for the antibody isoform (human IgG1) had no effect on basal or simvastatin-induced caspase 3 activation (Fig. 6) or wound healing (Fig. 7a and c).

In the absence of simvastatin, IL-6 alone significantly enhanced cell cycle progression in metastatic melanoma cells (Fig. 2). In WM35 cells, exogenous IL-6 augments the G_0/G_1 phase and exerts a contrary effect (Fig. 2). This observation might be of clinical relevance as case reports have been published that tocilizumab, when administered to patients with rheumatoid arthritis, led to a rapid progression of early melanoma in two cases [46,47]. It is worth mentioning that, similar to melanoma, IL-6 plays a dualistic role in stage-dependent growth regulation of breast cancer [27]. Indeed, during a clinical trial with tocilizumab in patients with rheumatoid arthritis, one case of breast cancer emerged [48]. Thus, further clinical studies are needed to

Fig. 7
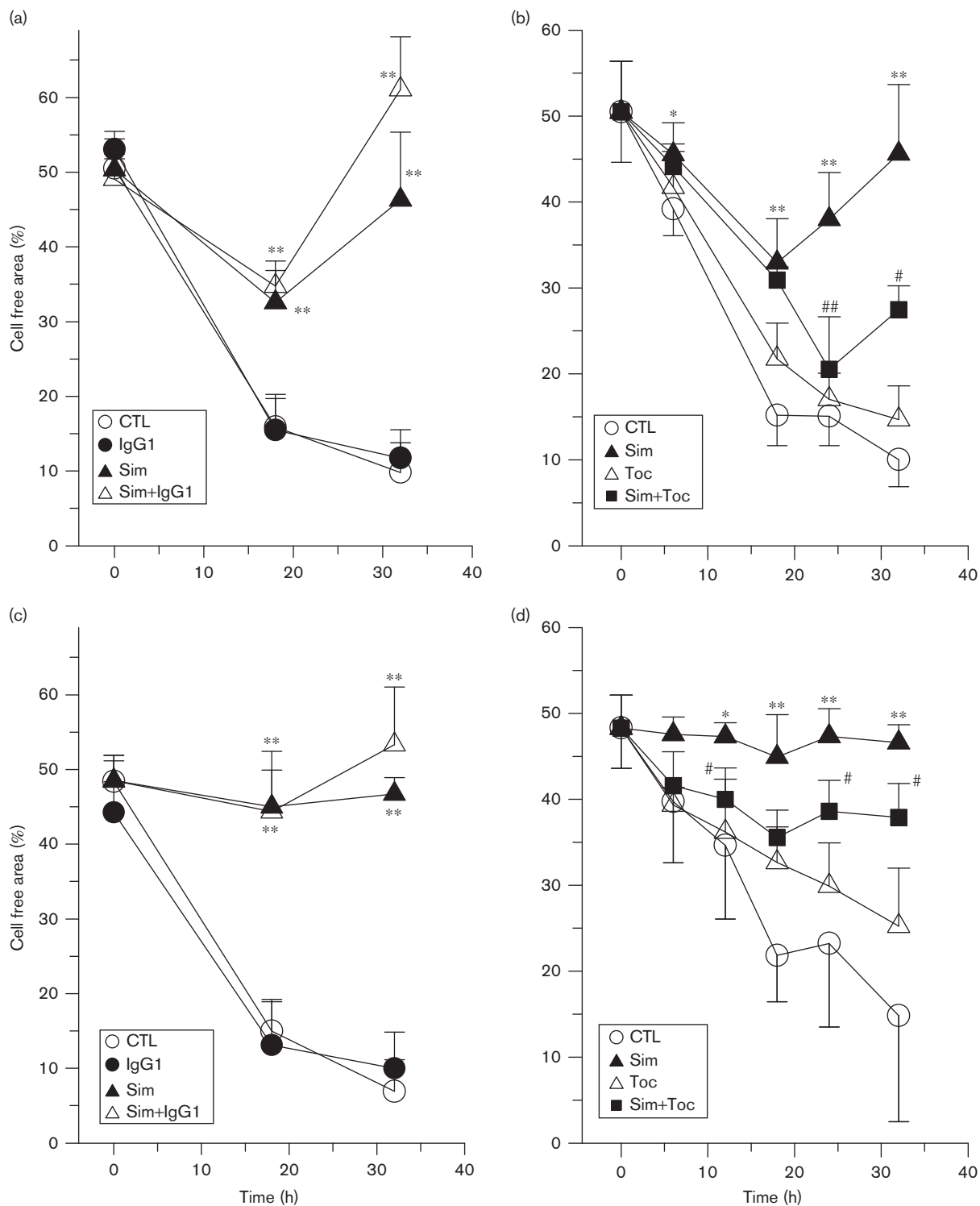
Tocilizumab abrogates simvastatin-induced inhibition of migration in scratch assays. The 518A2 (a, b) and A375 (c, d) cells were prepared for scratch assay and then treated in the absence and presence of 1 µmol/l simvastatin (Sim), 50 µg/ml human IgG1, 50 µg/ml tocilizumab (Toc), or a combination. Data points represent the mean ± SD ($n = 3$–6). Statistical significance versus control: *$P < 0.01$; **$P < 0.001$, or versus simvastatin treatment: #$P < 0.01$; ##$P < 0.001$.

exclude safety concerns related to tocilizumab-triggered melanoma development or progression.

In our study, we highlight an additional explanation for a possible mechanism behind tocilizumab facilitation of melanoma incidence. Exogenous IL-6 decreased the amount of the antiapoptotic protein Bcl-X_L in early-stage WM35 cells. Thus, blocking the IL-6 axis with tocilizumab could decrease the sensitivity of melanoma cells to apoptotic stimuli and this may lead to enhanced tumor progression. To prove such a hypothesis, further investigations are needed.

In metastatic melanoma cells A375 and 518A2, tocilizumab had no effect on proliferation nor apoptosis. One possible explanation for this is that advanced melanoma cells produce high IL-6 levels to communicate with the microenvironment [49]. For example, a recent study showed that treatment of melanoma with the specific BRAF inhibitor vemurafenib led to a decrease of IL-6 in the tumor microenvironment, which enhanced the infiltration with CD8^{+} cytotoxic T cells [50].

To our knowledge, this is the first investigation to show stage-dependent effects of tocilizumab on human melanoma cells, which implicates that tocilizumab-related safety concerns might be considered and investigated in melanoma models. Such an approach might also shed new light on the molecular switch, which transforms IL-6 from a growth-inhibitory cytokine to a driver of proliferation and growth of metastatic melanoma cells [51,52].

Acknowledgements

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Conflicts of interest
There are no conflicts of interest.

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