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

Christoph Minichsdorfer\textsuperscript{a,b}, Christine Wasinger\textsuperscript{b}, Evelyn Sieczkowski\textsuperscript{c}, Bihter Atil\textsuperscript{b} and Martin Hohenegger\textsuperscript{b}

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\textsubscript{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.

Keywords: apoptosis, HMG-CoA reductase inhibitors, interleukin-6, melanoma, simvastatin, tocilizumab

*\textsuperscript{a}Department of Oncology, Medicine I, \textsuperscript{b}Center for Physiology and Pharmacology, Comprehensive Cancer Center, Institute of Pharmacology and \textsuperscript{c}Department of Neurology, Medical University of Vienna, Vienna, Austria*

Correspondence to Martin Hohenegger, MD, Center for Physiology and Pharmacology, Comprehensive Cancer Center, Institute of Pharmacology, Medical University of Vienna, Waehringerstrasse 13A, A-1090 Vienna, Austria

Tel: + 43 1 40160 31358; fax: + 43 1 40160 931300; e-mail: martin.hohenegger@meduniwien.ac.at

Received 15 September 2014 Accepted 30 April 2015

**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-methylglutarlyl-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% CO2 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 Na3VO4). 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), Bel-XL (L-19), Bak (G-23), and Bax (N-20) from Santa Cruz (Santa Cruz, California, USA) and phospho-STAT3 (9131), STAT3 (9132) and Bel-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 Cytlogic software (Version 1.2.1; Cytlogic, 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 phosphorylation 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 phosphorylation is predominantly 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 G2 phase in A375 and 518A2 cells by IL-6 (Fig. 2). Conversely, WM35 cells accumulate in the G0/G1 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 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 executor 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 EC50 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 EC50 value of 14.2±3.89 μmol/l.
 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 anti-apoptotic 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-XL showed different responses upon IL-6 treatment. Bcl-XL 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 proapoptotic 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 G0/G1 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 G0/G1 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 control exerted no effect on wound closure or simvastatin-induced inhibition (Fig. 7a and c). Obviously,
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 unmaskes 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
study by a significant accumulation of WM35 cells in the G0/G1 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 G2 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 EC50 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-XL 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
prostaglandin, 15d-PGJ2, 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-PGJ2, 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
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-G0/G1 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 G0/G1 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...
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 Bel-XL 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 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
This work was supported by Herzfeldersche Familienstiftung and the Austrian Science Funds (P-22385) to M.H.

C.M., C.W., E.S., and B.A. conducted the experiments; C.M., C.W., and M.H. contributed toward data analyses and art work. Design, conception, and writing were performed by C.M. and M.H. All authors approved the final version of the manuscript.

Conflicts of interest
There are no conflicts of interest.

References
1 Tuong W, Cheng LS, Armstrong AW. Melanoma: epidemiology, diagnosis, treatment, and outcomes. Dermatol Clin 2012; 30:113–124. ix.
2 Batus M, Waheed S, Ruby C, Petersen L, Bines SD, Kaufman HL. Optimal management of metastatic melanoma: current strategies and future directions. Am J Clin Dermatol 2013; 14:179–194.
3 Mouawad R, Benhammouda A, Rie O, Antoine EC, Borel C, Wei M, et al. Endogenous interleukin-6 levels in patients with metastatic malignant melanoma: correlation with tumor burden. Clin Cancer Res 1996; 2:1405–1409.
4 Soubrane C, Rie O, Meric JB, Khayat D, Mouawad R. Pretreatment serum interleukin-6 concentration as a prognostic factor of overall survival in metastatic malignant melanoma patients treated with biochemotherapy: a retrospective study. Melanoma Res 2005; 15:199–204.
5 Hoierberg L, Basthoft L, Johansen JS, Christensen UJ, Gehl J, Schmidt H. Serum interleukin-6 as a prognostic marker in patients with metastatic melanoma. Melanoma Res 2012; 22:287–293.
6 Lázár-Molnár É, Hegyesi H, Toth S, Falus A. Autocrine and paracrine regulation by cytokines and growth factors in melanoma. Cytokine 2000; 12:547–554.
7 Colombo MP, Maccalli C, Mattei S, Melani G, Radrizanni M, Parmani G. Expression of cytokine genes, including IL-6, in human malignant melanoma cell lines. Melanoma Res 1992; 2:181–189.
8 Sun WH, Kreisle RA, Phillips AW, Esteller WB. In vivo and in vitro characteristics of interleukin-6-transfected B16 melanoma cells. Cancer Res 1992; 52:5412–5415.
9 Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990; 343:425–430.
10 Thumher M, Gruenbacher G, Nussbaumer O. Regulation of mevalonate metabolism in cancer and immune cells. Biochim Biophys Acta 2013; 1831:1009–1015.
11 Gazzero P, Proto MC, Gangemi G, Maftitano AM, Giaglia E, Pisanti S, et al. Pharmacological actions of statins: a critical appraisal in the management of cancer. Pharmacol Rev 2012; 64:102–146.
12 Demierre MF, Higgins PD, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. Nat Rev Cancer 2005; 5:930–942.
13 Minichsdorfer C, Hohenegger M. Double impact on IL-6 susceptibility of melanoma cells
14 Sieczkowski E, Lehrer C, Ambros PF, Hohenegger M. Double impact on p-glycoprotein by statins enhances doxorubicin cytotoxicity in human neuroblastoma cells. Int J Cancer 2010; 126:2025–2035.
15 Werner M, Sacher J, Hohenegger M. Mutual amplification of apoptosis by statin-induced mitochondrial stress and doxorubicin toxicity in human rhabdomyosarcoma cells. Br J Pharmacol 2004; 143:715–724.
16 Pich C, Teiti I, Rochaix P, Mariamé B, Couderc B, Favre G, Tíkín-Mariamé AF. Statins reduce melanoma development and metastasis through MICA overexpression. Front Immunol 2013; 4:62.
17 Feletszko W, Nylanczuk I, Oliszewska D, Jalil A, Grzela T, Lasek W, et al. Lovastatin potentiates antitumor activity of doxorubicin in murine melanoma via an apoptosis-dependent mechanism. Int J Cancer 2002; 100:111–118.
18 Werner M, Atil B, Sieczkowski E, Chiba P, Hohenegger M. Simvastatin-induced compartmentalisation of doxorubicin sharpens up nuclear topoisomerase II inhibition in human rhabdomyosarcoma cells. Naunyn Schmiedebergs Arch Pharmacol 2013; 386:605–617.
19 Colisson EA, Kleer C, Wu M, De A, Gambhir SS, Merajver SD, Kolodney MS. Atorvastatin prevents RhöC isoprenylation, invasion, and metastasis in human melanoma cells. Mol Cancer Ther 2003; 2:941–948.
20 Shellenman YG, Ribbe D, Miller L, Gendall J, Vanbuskirk K, Kelly D, et al. Lovastatin-induced apoptosis in human melanoma cell lines. Melanoma Res 2005; 15:83–89.
21 Sacher J, Weigl L, Werner M, Szegedi C, Hohenegger M. Delineation of myotoxicity induced by 3-hydroxy-3-methylglutaryl CoA reductase inhibitors in human skeletal muscle cells. J Pharmacol Exp Ther 2005; 314:1032–1041.
22 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–254.
23 Moinár EL, Hegyesi H, Toth S, Darvas Z, László V, Szalai C, Falus A. Biosynthesis of interleukin-6, an autocrine growth factor for melanoma, is regulated by melanoma-derived histamine. Am J Clin Dermatol 1995; 1997; 1995–1997.
24 Oh JW, Katz A, Harroch S, Eisenbach L, Revel M, Chebath J. Unmasking by soluble IL-6 receptor of IL-6 effect on metastatic melanoma: growth inhibition and differentiation of B16-F10.9 tumor cells. Oncoogy 1997; 15:569–577.
25 Zhong Z, Wen Z, Danell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 1994; 264:95–98.
26 Wen Z, Zhong Z, Danell JE Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995; 82:241–250.
27 Yao X, Huang J, Zhong H, Shen N, Faggioni R, Fung M, Yao Y. Targeting interleukin-6 in inflammatory autoimmune diseases and cancers. Pharmacol Ther 2014; 141:125–139.
28 Lu C, Kerbel RS. Interleukin-6 undergoes transition from paracrine growth factor to autocrine stimulator during human melanoma progression. J Cell Biol 1993; 120:1281–1288.
29 Lu C, Vickers MF, Kerbel RS. Interleukin 6: a fibroblast-derived growth inhibitor of human melanoma cells from early but not advanced stages of tumor progression. Proc Natl Acad Sci USA 1992; 89:9215–9219.
30 Hoeborg B, Bastholt L, Schmidt H, Hoeborg L, Bastholt L, Schmidt H, et al. Interleukin-6 and melanoma. Melanoma Res 2012; 22:327–333.
31 Flarenes VA, Lu C, Bhattacharya N, Rak J, Sheehan C, Slingerland JM, Kerbel RS. Interleukin-6 dependent induction of the cyclin dependent kinase inhibitor p21WAF1/CIP1 is lost during progression of human malignant melanoma. Oncogene 1999; 18:1023–1032.
32 Ban MR, Rak J, Adachi D, Wiltshire R, Trent JM, Kerbel RS, Ben-David Y. Multiple features of advanced melanoma recapitulated in tumorigenic variants of early stage (radial growth phase) human melanoma cell lines: evidence for a dominant phenotype. Cancer Res 1996; 56:3075–3086.
33 Tartoe E, Donvi T, Mosseri V, Deneux L, Mathiot C, Brailly H, et al. Serum interleukin 6 and C-reactive protein levels correlate with resistance to IL-2 therapy and poor survival in melanoma patients. Br J Cancer 1994; 69:911–913.
34 Knudsen LS, Christensen IJ, Lottenburger T, Svendsen MN, Nielsen HJ, Nielsen L, et al. Pre-analytical and biological variability in circulating interleukin 6 in healthy subjects and patients with rheumatoid arthritis. Biomarkers 2008; 13:59–78.
35 Piettenberg A, Ballaun C, Putter J, Mildner M, Strunk D, Weninger W, Tschaner E. Human melanocytes and melanoma cells constitutively express the Bcl-2 proto-oncogene in situ and in cell culture. Am J Pathol 1995; 146:651–659.
36 Wasinger C, Künzl M, Minichsdorfer C, Hohenegger M. Autocrine secretion of 15d-PGJ2 mediates simvastatin-induced apoptotic burst in human metastatic melanoma cells. Br J Pharmacol 2014; 171:5708–5727.
37 Koomen ER, Joosse A, Herings RM, Casparie MK, Hohenegger M. Interleukin-6 receptor inhibition with tocilizumab reduces disease activity in rheumatoid arthritis with inadequate response to disease-modifying antirheumatic drugs: the tocilizumab in combination with traditional disease-modifying antirheumatic drug therapy study. Arthritis Rheum 2008; 58:2968–2980.
38 Finet A, Amini-Adle M, Balme B, Colson F, Thomas L. Nodular progression of lentigo malignant melanoma during a treatment with tocilizumab: cause or coincidence? Clin Rheumatol 2013; 32:277–280.
39 Jagtap D, Rosenberg CA, Martin LW, Pettinger M, Khandekar J, Lane D, et al. Prospective analysis of association between use of statins and melanoma risk in the Women's Health Initiative. Cancer 2012; 118:5124–5131.
40 Bonovas S, Nikolopoulos G, Filippouli K, Peponi E, Bagos P, Sitaras NM. Can statin therapy reduce the risk of melanoma? A meta-analysis of randomized controlled trials. Eur J Epidemiol 2010; 25:29–35.
41 Freeman SR, Drake AL, Heilig LF, Graber M, McNealy K, Schilling LM, Delavalle RP. Statins, fibrates, and melanoma risk: a systematic review and meta-analysis. J Natl Cancer Inst 2006; 98:1538–1548.
42 Yamanaka H, Tanaka Y, Inoue E, Hoshi D, Momohara S, Hanami K, et al. Antitumor effect of humanized anti-interleukin-6 receptor antibody (tocilizumab) on glioma cell proliferation. Laboratory investigation. J Neurosurg 2009; 111:219–225.
43 Jagtap D, Rosenberg CA, Martin LW, Pettinger M, Khandekar J, Lane D, et al. Prospective analysis of association between use of statins and melanoma risk in the Women’s Health Initiative. Cancer 2012; 118:5124–5131.
44 Bonovas S, Nikolopoulos G, Filippouli K, Peponi E, Bagos P, Sitaras NM. Can statin therapy reduce the risk of melanoma? A meta-analysis of randomized controlled trials. Eur J Epidemiol 2010; 25:29–35.
45 Nishimoto N. Clinical studies in patients with Castleman’s disease, Crohn’s disease, and rheumatoid arthritis in Japan. Clin Rev Allergy Immunol 2005; 28:221–230.
46 Finet A, Amini-Adle M, Balme B, Colson F, Thomas L. Nodular progression of lentigo malignant melanoma during a treatment with tocilizumab: cause or coincidence? Clin Rheumatol 2013; 32:277–280.
47 Bonny M, Buysse V, Suys E. Rapidly progressive malignant melanoma in a patient treated with tocilizumab. J Am Acad Dermatol 2012; 67:e78–e79.
48 Yamanaka H, Tanaka Y, Inoue E, Hoshi D, Momohara S, Hanami K, et al. Efficacy and tolerability of tocilizumab in rheumatoid arthritis patients seen in daily clinical practice in Japan: results from a retrospective study (REACTION study). Mod Rheumatol 2011; 21:122–133.
49 Fisher DT, Appenheimer MM, Evans SS. The two faces of IL-6 in the tumor microenvironment. Semin Immunol 2014; 26:38–47.
50 Frederick DT, Piris A, Cogdill AP, Cooper ZA, Lezcano C, Ferrone CR, et al. BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. Clin Cancer Res 2013; 19:1225–1231.
51 Lu C, Rak JW, Kobayashi H, Kerbel RS. Increased resistance to oncostatin M-induced growth inhibition of human melanoma cell lines derived from advanced-stage lesions. Cancer Res 1993; 53:2708–2711.
52 Rak JW, Hegemann Jr, Lu C, Kerbel RS. Progressive loss of sensitivity to endothelium-derived growth inhibitors expressed by human melanoma cells during disease progression. J Cell Physiol 1994; 159:245–255.