Metastatic cells can escape the pro-apoptotic effects of TNF-α through increased autocrine IL-6/STAT3 signalling.

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Running Title: Rescue from TNF-α–induced apoptosis by IL-6

Key Words: metastasis, inflammation, apoptosis, IL-6, IGF

∞ This study was supported by grant MOP-81201 from the Canadian Institute for Health Research.

# Supported by a doctoral fellowship from the Research Institute of the McGill University Health Center.

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ABSTRACT

The liver is a common site for cancer metastases where the entrance of tumor cells has been shown to trigger a rapid inflammatory response. In considering how an inflammatory response may affect metastatic colonization in this setting, we hypothesized that tumor cells may acquire resistance to the pro-apoptotic and tumoricidal effects of TNF-α, a cytokine that is elevated in a pro-inflammatory tissue microenvironment. In this study, we investigated molecular mechanisms by which such resistance may emerge by using tumor cells in which the overexpression of the type I insulin-like growth factor receptor (IGF-IR) enhanced the inflammatory and metastatic capacity of poorly metastatic cells in the liver. Mechanistic investigations in vitro revealed that IGF-IR overexpression increased cell survival in the presence of high levels of TNF-α, in a manner associated with increased autocrine production of IL-6. In turn, tumor cell-derived IL-6 induced gp130 and IL-6R–dependent activation of STAT3, leading to reduced caspase-3 activation and apoptosis. We found that cell death resistance was dose-dependent with increasing IGF-I levels. Additionally, RNAi-mediated knockdown of either IL-6 or gp130 that established a blockade to autocrine STAT3 induction was sufficient to abolish the pro-survival effect of TNF-α and to inhibit liver metastasis. Taken together, our findings define an IGF-IR-mediated mechanism of cancer cell survival that is critical for metastatic colonization of the liver.
INTRODUCTION

Cancer metastasis remains the main cause of cancer-related mortality. In order to colonize a distant site, cancer cells must complete several rate limiting steps, including detachment from the primary site and invasion, intravasation, survival in the circulation, extravasation and finally, survival and growth in the target organ (1-3). Once in the target organ, cancer cells can induce a proinflammatory microenvironment involving host leukocytes and monocytes that reside in the affected tissue or are recruited in response to signals elaborated by the tumor cells. These host cells can further promote the development of secondary tumors by releasing cytokines and chemokines such as tumor necrosis factor-α (TNF-α), IL-1 and IL-6 that promote tumor cell migration, tumor survival and the induction of endothelial cell adhesion receptors that mediate tumor cell arrest and trans-endothelial migration (4-6).

TNF-α is a pleiotropic, pro-inflammatory cytokine that can have multiple and diametrically opposing effects on tumor cell survival and malignant progression. While at high concentrations, it can cause hemorrhagic necrosis, induce apoptosis and stimulate anti-tumor immunity, it can also, under the appropriate conditions, promote tumor initiation and progression (7, 8). TNF-α is produced as a cell membrane-associated 26-kDa pro-protein that can be cleaved by matrix metalloproteinases (MMPs) to release a soluble 17-kDa form (reviewed in (9)). TNF-α is active as a self-assembling, non-covalently associated trimer that binds to 2 cell surface receptors; the p55/p60 TNFR1 and the p75/p80 TNFR2. Binding to TNFR1 triggers the recruitment of the adaptor TNFR-associated death domain (TRADD) that serves as a platform to assemble alternative signalling complexes. This can lead to activation of IKK/IκBα/NF-κB
signalling and upregulation of NF-κB target genes such as *IL-6* or the recruitment of Fas-associated death domain-containing protein (FADD) and caspase 8, leading to activation and cleavage of the effector caspase-3 and apoptosis (10, 11).

*IL-6* belongs to a family of cytokines that signal through receptor complexes containing the signal-transducing protein gp130 (12). It can play diverse roles both as a regulator of the acute inflammatory response and as a growth and survival factor. Binding of *IL-6* to its cellular receptor *IL-6R*α (CD126) triggers the recruitment of gp130 (CD130) and results in Janus kinase (JAK) activation and phosphorylation of transcription factors of the signal transducer and activator of transcription (STAT) family, particularly STAT3. Phosphorylated STAT3 dimers translocate to the nucleus where direct DNA binding leads to transcriptional activation of various genes, including oncogenes such as c-Myc, cell survival genes such as Bcl-X<sub>L</sub>, cell cycle regulators such as cyclin D1, cytokines such as *IL-6*, and molecular modulators of angiogenesis and metastasis such as MMP-2, MMP-9, HIF-1α, and VEGF (13, 14). Activation of an autocrine regulatory loop whereby *IL-6* activates STAT3 and STAT3 in turn, upregulates *IL-6* transcription was shown to maintain cell resistance to apoptosis and contribute to tumor progression (15). *IL-6* can also bind to gp130 via an extracellular soluble form of *IL-6R*α (sIL-6R) resulting in activation of gp130/JAK/STAT3 signaling and enhancement of the effects of *IL-6* (16).

The receptor for the type 1 insulin-like growth factor (IGF-IR) has also been identified as a survival factor for cancer cells and implicated in cellular transformation, malignant progression and the acquisition of an invasive/metastatic phenotype (17). Ligand-induced activation of the intrinsic IGF-IR tyrosine kinase can initiate several signalling pathways including the Ras/Raf/ERK pathway implicated in receptor-mediated
mitogenesis and transformation and the PI3-Kinase (PI3-K)/Akt pathway implicated in the transmission of cell survival signals. Crosstalk with integrins and with the JAK/STAT pathway has also been described (17-19). Upregulated expression of IGF-IR has been documented in many types of cancers and it has been recognized as a therapeutic target in the treatment of malignant disease (17, 20, 21).

Previously, we have shown that lung carcinoma M-27 cells ectopically expressing human IGF-IR acquired a liver-metastasizing potential that correlated with increased cell survival in the liver (22). We have also shown that metastatic lung and colon carcinoma cells that invade the liver initiate a rapid host inflammatory response that entails increased production of cytokines TNF-α and IL-1 by activated Kupffer cells and results in increased tumor cell adhesion to local microvascular endothelial cells, transmigration and metastasis. This ability to induce a rapid inflammatory response was tumor type-specific, correlated with the liver-metastasizing potential of the cells and was also noted in cells with ectopic IGF-IR expression (6, 23, 24). These results imply that metastatic tumor cells acquire mechanisms of resistance to the pro-apoptotic effects of TNF-α produced in the microenvironment of the target organs in order to survive and give rise to new metastatic foci.

Based on these findings, we hypothesized that IGF-IR overexpression plays a role in the acquisition of resistance to the pro-apoptotic effects of TNF-α by shifting TNFR1-induced signals from pro- to anti-apoptotic. The objective of the present study was therefore to identify molecular mechanism(s) that contribute to the acquisition of TNF-α resistance in highly metastatic tumor cells and investigate the role that IGF-IR plays in this process.
MATERIALS AND METHODS

Cells. Sublines M-27 and M-27IGFIR were generated in our laboratory. Their origin, properties, metastatic phenotypes, and culture conditions have been described in detail previously (22, 25). Murine colon carcinoma MC-38 cells that are highly metastatic to the liver in an IGF-I dependent manner (26) were a kind gift from Dr. Shoshana Yakar (Mount Sinai Hospital, NY, NY). These cell lines have been tested and authenticated as per the McGill University Animal Care committee and the McGill University Biohazard committee guidelines.

Antibodies and reagents. The polyclonal rabbit antibodies to p65, gp130, IL-6Rα (M-20), USF-2, and STAT3 (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies to Akt, p-Akt (Ser473) and p-STAT3 (D3A7, Tyr705) were from Cell Signal Technology (Beverly, MA). The monoclonal mouse anti-β-actin antibody was from Sigma Chemical Co. (St. Louis, MO). The anti-Rat Cathepsin B antibody was a generous gift from Dr. John S Mort (The Shriner’s Hospital for children, Montreal, Quebec, Canada). The antibody to Caspase-3 (#9662) (Cell signal, Beverly, MA) was a generous gift from Dr. Teruko Taketo (Department of Urology, McGill University, Quebec, Canada). The anti-murine IL-6 antibody (MAB406), a generous gift from Centocor Co. (Horsham, PA), the goat antibody to mouse IL-6 Rα and normal rat IgG1 were from R&D (Minneapolis, MN). The recombinant human IGF-I was from United States Biological (Swampscott, MA). The recombinant TNF-α and goat serum IgG were from Invitrogen (Carlsbad, CA) and JSH-23 was from Calbiochem, a brand of EMD Biosciences, Inc. (San Diego, CA). The inhibitors LY294002 and PD98059 were from Calbiochem (San Diego, CA).
RT-PCR. RNA was extracted using the TRIzol reagent (Life Technologies, ON, Canada) and RT-PCR was performed using the M-MLV reverse transcriptase and Taq DNA polymerase (both from Invitrogen, Carlsbad, CA) as we previously described (27). Primer sequences are listed in Supplementary Table 1.

Quantitative Real-time PCR (qRT-PCR). RNA extraction and reverse transcription were performed as described above. qRT-PCR was performed essentially as we previously described (27).

Western blotting. Cell fractionation and nuclear extraction were performed as described in detail elsewhere (28). Whole cell lysate or subcellular fraction proteins were separated by PAGE using 10% SDS gels and Western blotting was performed as we previously described (29). The primary antibody dilutions are shown in Supplementary Table 2 and the secondary antibodies were used at a dilution of 1:10,000.

Apoptosis assay. Apoptosis was measured using an ELISA kit for detection of mono-oligonucleosomes (Roche Diagnostics, Mannheim, Germany), as per the manufacturer’s instructions and 50,000 cells per analysis.

ELISA. Tumor cell conditioned media were harvested, concentrated 100X using Centrifugal Filter Units (Milipore, Billerica, MA) and IL-6 concentrations quantified using the mouse IL-6 DuoSet ELISA Development System (R&D Systems, Minneapolis, MN).

Gene silencing by shRNA. Tumor cells cultured in 6-well plates (3x10^5 cells per well) were transfected with the pRS plasmid (Origene, Rockville, MD) expressing gp130 or IL-6 shRNA sequences or a scrambled sequence as a control, using serum-free media containing Lipofectamine. Four μg/ml puromycin were added 48 hr later and drug-
resistant cells cloned by the limiting dilution method. Clones with a stable reduction in the respective mRNA levels were selected for further study.

**Experimental metastasis assay.** Experimental liver metastases were generated by the intrasplenic/portal injections, as we previously described (22, 30). Metastases visible on the surface of the liver were enumerated and sized without prior fixation. All the procedures were approved by the McGill University Animal Care committee.

**Statistical analysis.** The student’s t test was used for analysis of *in vitro* data and the non-parametric Mann-Whitney test for analysis of metastasis data.
RESULTS

Increased resistance to TNF-α-induced apoptosis in liver-metastasizing tumor cells.

Previously we have shown that highly metastatic lung and colon carcinoma cells that invade the liver initiate a rapid host inflammatory response that entails increased production of the cytokine TNF-α and results in increased tumor cell adhesion, transmigration and metastasis (6, 23). This prompted us to investigate whether the ability of metastatic cells to survive in this local inflammatory microenvironment involved an altered response to TNF-α. To this end, we used Lewis lung carcinoma subline M-27 cells that acquired a liver-metastasizing potential following ectopic expression of the human IGF-I receptor (M-27IGFIR cells) (22, 31). We compared the response of these, and wild type, poorly metastatic M-27 cells to treatment with increasing concentrations of TNF-α by measuring apoptosis using a detection system for mono-oligonucleosomes. We observed that M-27IGFIR cells had an altered response, particularly at high TNF-α concentrations. While at the lower concentration of 10 ng/ml, TNF-α in serum-free medium could partially protect both cell types from apoptosis (50% reduction), at the higher concentrations of 25-50 ng/ml, M-27IGFIR, but not M-27 cells were significantly protected (> 85% reduction at 50 ng/ml) from cell death (Fig 1A), suggesting that under these conditions, TNF-α activated distinct signalling pathways in these cells. TNFR activation can lead to the induction of caspase-3-mediated cell apoptosis when death domain signalling predominates. We therefore analyzed caspase-3 expression and activation levels in these cells under basal conditions and in response to TNF-α. We found that the metastatic M-27IGFIR cells expressed reduced caspase-3 mRNA and protein levels relative to M-27 cells and these levels declined even further when the cells were
treated with TNF-α (Fig 1 B &C). When the tumor cells were treated with TNF-α, an increase in cleaved caspase-3 levels could be detected by Western blotting in M-27, but not in M-27IGFIR cells (Fig 1D), even when the protein load for the latter cells was doubled to adjust for the relative decrease in total caspase-3 levels (Supplementary Fig 1). This difference in detectable cleaved caspase-3 levels was consistent with the reduction in apoptosis seen in M-27IGFIR cells.

**Increased IL-6/STAT3 signalling in metastatic M-27IGFIR cells.**

Caspase-3 production is negatively regulated by activated STAT3. We therefore next analyzed the expression levels and activation status of STAT3 in these cells. Quantitative RT-PCR and Western blotting (Fig 2 A&B) revealed that basal STAT3 expression levels in M-27IGFIR cells were actually lower than those in non-metastatic M-27 cells. However, activated STAT3 (p-STAT3) levels were markedly increased (4 fold) in M-27IGFIR cells (Fig 2B). Furthermore, treatment of M-27IGFIR, but not M-27 cells with TNF-α for 24 hr increased p-STAT3 levels and a further increase of 10-50% was seen when IGF-I was also added (Fig 2C&D). STAT3 activation was also observed when M-27IGFIR cells were stimulated with TNF-α (with or without IGF-I) for a shorter interval of 4 hr (Supplementary Fig 2). This indicated that upon cell treatment with TNF-α, a signal transduction mechanism upstream of STAT3 was activated and suggested that in these cells, there was an increased production of a STAT3-activating cytokine under basal conditions that could be further augmented by TNF-α.

IL-6 is a potent activator of JAK/STAT signalling and in both M-27 and M-27IGFIR cells, treatment with exogenous IL-6 led to STAT-3 activation (Supplementary Fig 3). When we measured IL-6 mRNA expression levels and compared secreted levels in the
conditioned media by ELISA, we found that basal IL-6 mRNA levels were 2-4 fold higher in M-27<sup>IGFIR</sup> cells, as shown in Fig 3 (A and B) and this corresponded to an increase in protein production levels, as shown in Fig 3D (extreme left bars). Furthermore, when IL-6 production was measured following cell treatment with TNF-α, a marked difference was observed in the response of these tumor cells. Namely, while in M-27 cells, IL-6 mRNA and protein levels remained virtually unchanged, in M-27<sup>IGFIR</sup> cells, they were highly induced, with mRNA levels increasing 7.5-9.5 fold, as measured by qRT-PCR (Fig 3C) and protein production increasing up to 60-fold, as measured by ELISA (Fig 3D). Similarly, in murine colorectal carcinoma MC-38 cells that are highly metastatic to the liver in an IGF-I-dependent manner (26), IL-6 production was markedly increased (60 fold) in response to treatment with TNF-α (Fig 3E). Furthermore, in both cell types, the increase in IL-6 production was TNF-α dose-dependent and could be further augmented when the cells were treated simultaneously with TNF-α and IGF-I (Fig 3, C-F), confirming that signals generated by ligand-activated IGF-IR could enhance transcriptional activation of IL-6. The TNF-α-inducible increase in IL-6 production was mediate mainly via NF-κB activation because pre-treatment of M-27<sup>IGFIR</sup> cells with JSH23, a cell-permeable diamino compound that selectively blocks nuclear translocation of p65 (Rel-A) (Supplementary Fig 4), diminished the increase in IL-6 expression due to TNF-α in the presence or absence of IGF-I (Fig 3G and 3H). In these experiments, IL-6 concentrations in the conditioned media of stimulated cells were within a range (17-252 pg/ml) that was confirmed to activate STAT3 in these cells (see supplementary Fig 5).
The Soluble IL-6 receptor α (sIL-6Rα) acts as an agonist to activate the gp130/JAK/STAT3 pathway. It was therefore of interest to analyze whether TNF-α treatment affected its production by the tumor cells. When conditioned media obtained from M-27 and M-27IGFIR cells before and after TNF-α treatment were analyzed for the presence of sIL-6Rα using Western blotting, we found that M-27IGFIR produced a higher level of the soluble receptor. However this level did not increase following TNF-α treatment (supplementary Fig 6), suggesting that the effect of TNF-α on STAT3 activation was not due to changes in the release of sIL-6R, although its presence per se may have contributed to enhanced STAT3 activation in these cells.

IGF-I does not directly activate STAT3 signalling in M-27IGFIR cells. The IGF-IR has previously been implicated in STAT3 signalling (32). To assess its role in STAT3 activation in our cells, M-27IGFIR cells were stimulated directly with IGF-I and the effect on STAT3 phosphorylation analyzed by Western blotting. Treatment of the cells with IGF-I for up to 2 hr failed to increase phospho-STAT3 levels (Fig 4A) under conditions that triggered signalling, as evidenced by rapid Akt phosphorylation (Fig 4B), suggesting that IGF-IR signalling could not directly activate STAT3 in these cells and that its major effect was to augment TNF-α/NF-κB signalling and thereby IL-6 production. Stimulation of the cells with IGF-I for 24 hr also failed to increase p-STAT3 levels (Supplementary fig 7), suggesting that the presence of TNF-α was required to stimulate IL-6 production and autocrine STAT3 activation in these cells. Moreover, STAT3 activation in response to TNF-α and IGF-I could be inhibited by a neutralizing antibody to IL-6Rα (Fig 4C), providing direct evidence for the involvement of IL-6 in STAT3 activation by these ligands. To identify the signal transduction pathway downstream of
IGF-IR involved in the enhancement of TNF-α/NF-κB-induced IL-6 transcription, we used the chemical inhibitors LY294002 and PD98059 to block PI3-K and ERK signalling, respectively. Inhibition of PI3-K signalling completely abolished the incremental increase in IL-6 production due to IGF-IR activation, while also reducing (2 fold) the effect of TNF-α alone. Inhibition of ERK signalling also reduced the increase in IL-6 production due to IGF-I, but to a lesser extent (64%, Fig 4D). The results implicate both pathways in IGF-I-mediated enhancement of IL-6 synthesis, although PI3-K/Akt signalling appears to play a more critical role.

**Silencing of gp130 or IL-6 abolishes the anti-apoptotic effect of TNF-α.** The protein gp130 is an IL-6 co-receptor required for transmitting IL-6-derived signals. We confirmed that M-27^IGFIR cells express both gp130 and IL-6Rα (Supplementary Fig 8A). To determine the role of gp130 in maintaining STAT3 activity and the pro-survival effect of TNF-α in these cells, we generated an M-27^IGFIR cell line with a stable knockdown of gp130 using short hairpin RNA (shRNA, see Supplementary Fig 8B and 8C). In these cells, the transcriptional activity of STAT3 was markedly reduced as demonstrated by significant downregulation of the STAT3 target genes - IL-6 (> 99% reduction) and MMP-2 (90% reduction) and a concomitant upregulation (10 fold increase) in the expression of caspase-3 (Fig 5A), confirming that gp130 signalling was essential for STAT3 activity in these cells. When the effect of stable knockdown of gp130 on the ability of TNF-α to rescue the tumor cells from apoptosis was analyzed, we found that a knockdown of gp130 expression significantly decreased the ability of TNF-α to rescue M-27^IGFIR cells from apoptosis and also reversed the anti-apoptotic effect seen when the cells were co-treated with TNF-α and IGF-I (Fig 5B). Similarly, tumor cells in which IL-
production in response to TNF-α was blocked by shRNA-mediated silencing of IL-6 (Supplementary Fig 9A and 9B), completely lost the survival advantage in the presence of high TNF-α concentrations (Fig 5B) and could not be protected by the addition of IGF-I. Together, these results indicated that IL-6/gp130 signalling was essential for the pro-survival effect of TNF-α on these cells.

**Loss of metastatic potential in cells with reduced autocrine IL-6-mediated survival signalling.** Finally, to assess the effect of IL-6 inhibition on tumor cell potential to colonize the liver, we used M-27IGFIR and colon carcinoma MC-38 cells in which IL-6 expression was stably silenced by shRNA (Supplementary Figures 9 and 10), as well as treated tumor cells with a neutralizing anti-IL-6 antibody prior to injection. As already shown above, the pro-survival effect of TNF-α (with or without IGF-I) was abolished in M-27IGFIR cells stably transfected with IL-6 shRNA (Fig 5B). Similarly, in colon carcinoma MC-38 cells with silenced IL-6 expression that resulted in reduced STAT3 activation levels (Fig 6A), TNF-α-mediated apoptosis levels were significantly increased (Fig 6B). Moreover, when M-27IGFIR cells were treated with a neutralizing antibody to murine IL-6 (but not an isotype–matched control), prior to treatment with 50 ng/ml TNF-α, the protective effect of TNF-α was completely abolished and apoptosis levels were restored to those seen with untreated cells (Fig 6C), clearly identifying IL-6/gp130/STAT signalling as crucial to the pro-survival effect of TNF-α in the different tumor cell types. When M-27IGFIR or MC-38 cells with stable knockdown of IL-6 were then inoculated into syngeneic C57Bl/6 mice via the intrasplenic/portal route to assess their liver-colonizing potential, a significant reduction in the number (and size) of hepatic metastases, relative to controls was found in both groups of mice (Fig 7A-E), and this was also observed in
mice injected with M-27IGFIR cells pre-treated with a neutralizing, anti-IL-6 antibody (Fig 7F). Taken together, these results show that activation of autocrine IL-6/gp130/STAT3 survival signalling in response to TNF-α was essential for liver metastasis of these carcinoma cells.
DISCUSSION

Our results show that in the presence of high TNF-α-concentrations, highly metastatic tumor cells could be rescued from apoptosis by activating autocrine IL-6/gp130/STAT3 pro-survival signalling and implicate the IGF-IR axis in shifting the balance from cell death towards IL-6 induction and cell survival. Our finding that TNF-α can rescue cells from cell death under conditions of serum-deprivation may be particularly relevant to cell fate during the early stages of liver metastasis when the tumor cells may encounter the combined insult of ischemia/reperfusion due to sinusoidal vessel occlusion (33) and a surge in the local production of TNF-α, as we and others have previously shown (23) (reviewed in (34)). Our results are in agreement with other studies that identified IL-6 as a survival factor in the liver both for primary hepatocellular carcinoma (35) and for liver metastases (36). However, while in those studies, IL-6 was liver-derived and acting in a paracrine fashion, our results show that in the presence of inflammatory stimuli, tumor cells can activate this survival mechanism in an autocrine fashion and thereby escape the pro-apoptotic effects of the microenvironment. Of note, we did not observe a significant increase in circulating IL-6 levels (as measured by ELISA) in mice injected with MC-38 and M-27^{IGFIR} cells as compared to non-injected controls (levels variable and ranging from ≤5-150 pg/ml in both groups). This suggests that in the present tumor models, tumor–derived IL-6 did not have a measurable effect on systemic IL-6 levels and also that the contribution of paracrine IL-6 to STAT3 activation and tumor cell survival may have been secondary to the effect of autocrine IL-6 production and signaling. Taken together with findings by others and our own previous data that implicated TNF-α in the induction of endothelial cell adhesion receptors and thereby in tumor trans-endothelial
migration and extravasation (6, 23, 37), the present study identifies a dual role for this cytokine in liver metastasis namely, as a modifier of the microenvironment on one hand and a survival factor for the tumor cells on the other.

We observed that at lower concentration (10ng/ml), TNF-α also had an anti-apoptotic effect on M-27 cells that was lost at higher concentrations. This is consistent with observations in other systems where TNF-α could be shown to mediate opposing effects at different concentrations (7). While the mechanism mediating the anti- and pro-apoptotic effects of TNF-α in these cells remain to be fully elucidated, we have observed that following treatment of both M-27 and M-27<sup>IGFIR</sup> cells with 10 ng/ml TNF-α, the NF-κB p65/RelA protein is activated and translocates to the nucleus (unpublished observation), where it can directly activate transcription of anti-apoptotic factors. However, we did not observe a significant upregulation of IL-6 expression or increased STAT3 activation in M-27 cells at any of the TNF-α concentrations used.

In our M-27/M-27<sup>IGFIR</sup> model, the upregulation of IL-6 production in response to TNF-α depended on increased IGF-IR expression levels in the tumor cells. We observed that although ligand-induced activation of IGF-IR did not, in itself, significantly increase IL-6 production and STAT3 activation, the activated receptor could act synergistically with TNF-α to increase IL-6 levels. This effect was RelA/p65 dependent because it was inhibited when p65 translocation was blocked. It was also abolished when PI3-K signalling was inhibited; suggesting that IGF-IR/PI3-K and NF-κB signalling converged to enhance IL-6 production in these cells. This synergistic effect was not unique to the M-27<sup>IGFIR</sup> cells as it was also observed in the liver-metastasizing Lewis lung carcinoma variant H-59 (results not shown) and murine colon carcinoma MC-38 cells (Fig 3E) that
express high IGF-IR levels and can also induce an acute inflammatory response in the liver (38, 39), suggesting that this survival mechanism is a broader phenomenon for liver-metastasizing cells.

While the crosstalk mechanism(s) between the IGF and TNF-α axes in our cells remain(s) to be fully characterized, two potential interactions are likely candidates. IL-6 gene expression is activated by transcription factors NF-κB, AP-1 and C/ERB and IGF-IR was identified as an upstream activator of AP-1 depended transcription (40, 41). It is possible, therefore, that the synergistic effect of IGF-IR and TNF-α is due to the concomitant activation of the two cis-acting transcription elements (NF-κB and AP-1) in the IL-6 promoter. Alternatively, IGF-IR may increase NF-κB signalling more directly, through the PI3-K pathway. Indeed, TNF-α was identified as a direct activator of Akt phosphorylation in premalignant keratinocytes (42) and NF-κB was shown to be activated downstream of the PI3-K/Akt pathway in prostate epithelial cells (43). This is consistent with our finding that the IGF-I effect in our model was PI3-K dependent and suggests that IGF-IR may act directly on the NF-κB pathway via PI3-K/Akt signalling to enhance IL-6 production in response to TNF-α.

In this context, it is noteworthy that low levels of IL-6 and p-STAT3 could be detected in M-27$^{IGFIR}$ (but not M-27) cells under basal conditions, even without stimulation of the cells by exogenous IGF-I. This may be due to serum IGF-I and insulin present in the culture medium that maintain a basal level of IGF-IR/PI3-K/Akt and ERK signalling in these cells, or to an autocrine effect of the low levels of IGF-II (but not IGF-I) produced by these cells (unpublished observation). Moreover, IGF-IIR expression in these cells was found to be reduced 2.5 fold relative to M-27 cells (unpublished gene array data).
Because IGF-IIR does not activate intracellular signalling and acts as a “sink” to reduce ligand bioavailability (44), a reduction in its levels in the presence of tumor-derived IGF-II could result in low level autocrine IGF-IR activation and signalling that are further enhanced in the presence of exogenous ligand. Autocrine IL-6/STAT3 signalling in these cells may also be enhanced by increased production of sIL-6R that acts as an agonist to activate gp130. While the regulatory link between increased IGF-IR expression and the increased production of sIL-6R in our cells is presently unknown, we have previously shown that M-27 and M-27\textsuperscript{IGFIR} cells express distinct MMP repertoires (27, 29, 45). It is possible therefore, that cleavage of the membrane-bound IL-6R by a proteinase preferentially expressed or activated in M-27\textsuperscript{IGFIR} cells is involved. Our data indicate however, that TNF-\(\alpha\) did not directly regulate sIL-6R production levels, suggesting that its major role in STAT3 activation was mediated through the upregulation of IL-6.

Crosstalk between the IGF axis and the IL-6/STAT3 survival pathway has been documented in other models. The evidence for this association is particularly strong for multiple myeloma cells where co-signalling by the IGF-IR and IL-6R were shown to be critical for survival and growth (46, 47). Conversely in T-cells, the neutralization of IL-6 could partially abrogate the anti-apoptotic effect of IGF-I (48), suggesting that IGF-IR and IL-6 signalling were interdependent in these cells. To our knowledge, however, our results are the first to document a synergistic effect of IGF-IR signalling in the induction of autocrine IL-6/STAT3 signalling by TNF-\(\alpha\). Moreover, the data suggest that the ability of the IGF axis to regulate IL-6 production and STAT3 signalling may be a function of the NF-\(\kappa B\) activation status in the cells and therefore provide insight into the crosstalk between the IGF axis and NF-\(\kappa B\) signalling.
In some studies, IGF-IR was identified as a direct activator of JAK/STAT signalling (49). However, STAT3 activation was not observed in M-27\textsuperscript{IGFIR} cells stimulated with IGF-I for up to 24 hr and was blocked in the presence of an antibody to IL-6R\(\alpha\), while in MC-38 cells, the silencing of IL-6 reduced p-STAT 3 levels. These data suggest that in the present cells, STAT3 activation was IL-6-dependent.

The receptor gp130 is an essential component of the IL-6 receptor complex and is required for the activation of JAK/STAT3 signalling (50). We show here that stable silencing of gp130 or IL-6 in different metastatic tumor cells abolished the anti-apoptotic effect of TNF-\(\alpha\) and that inhibition of IL-6 blocked hepatic metastasis. These data are further evidence that the pro-survival effect of TNF-\(\alpha\) depended on IL-6/gp130/STAT3 signalling in these cells. In this respect, our findings are in line with other studies that identified IL-6 and STAT3 as progression factors and potential prognostic predictors and therapeutic targets in several malignancies, including hepatocellular (51), colon (52), pancreatic (53) and gastric (54) carcinomas that either arise in, or are commonly metastatic to the liver.

Among the genes that are transcriptionally regulated by STAT3 are mediators of cell death and survival (e.g. caspase-3 and Bcl-X\(_L\)), cell cycle progression factors (Cyclin D1), cytokines and chemokines (e.g. IL-6, CCL5) and mediators of migration (Cten), invasion (MMP-2, MMP-9) and angiogenesis (HIF-1\(\alpha\), VEGF) (55) that can all contribute to tumor progression and the crosstalk between inflammation and carcinogenesis. We observed that inhibition of STAT3 phosphorylation by gp130 silencing significantly decreased IL-6 and MMP-2 gene expression while also increasing caspase-3 expression levels. MMP-2 was identified as a critical mediator of liver
metastasis (56), and we have previously shown that MMP-2 is an IGF-IR/PI3-K regulated gene in M-27IGFIR cells (45, 57). The present results suggest that the constitutive increase in IL-6/STAT3 activity in these cells (even in the absence of TNF-α stimulation) may also contribute to the increased expression of MMP-2. In addition, the increase in caspase-3 expression in gp130-silenced cells confirms that the pro-survival effects seen in the presence of TNF-α were indeed linked to the STAT3-mediated reduction in caspase-3 levels.

Collectively, these results provide new insight into the multifaceted role that the IGF axis can play in providing survival and growth cues to cancer cells in the pro-inflammatory microenvironment of the liver. They also identify additional candidate molecules in the search for anti-metastatic, therapeutic targets.

**Acknowledgments**

This project was supported by grant MOP-81201 from the Canadian Institute for Health Research. Shun Li was the recipient of a doctoral fellowship from the Research Institute of the McGill University Health Center. The authors thank Ms. Boram Ham for help in preparation of the manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1. IGF-IR expression increases the anti-apoptotic effect of TNF-α. (A) Cells in serum-free RPMI medium were treated (or not) with the indicated concentrations of TNF-α for 48 hr, lysed and the cytoplasmic mono-oligonucleosomes analyzed using ELISA. The results are expressed as apoptosis (means and SD) relative to the respective control untreated cells, based on three separate experiments. (B) Constitutive caspase-3 expression levels were analyzed by qRT-PCR. Shown are means and SD of three separate analyses normalized to GAPDH and relative to M-27 cells that were assigned a value of 1. (C) Cells were treated (or not) with 10 ng/ml TNF-α for 48 hr and caspase-3 protein levels in total cell lysates analyzed by Western blotting. Shown are representative results of three analyses performed. (D) Cells were treated with 50 ng/ml TNF-α for the indicated time interval, lysed and the intact and cleaved forms of caspase-3 detected by Western blotting after loading 200 μg total protein per lane. Shown are representative results of two analyses performed. *- p<0.05. **- p<0.01.

Figure 2. Increased STAT3 activation in metastatic M-27IGFIR cells. Expression levels of STAT3 (A) were analyzed by qRT-PCR. Results are based on three separate analyses and expressed as the mean ratios to GAPDH (±SD), relative to M-27 cells assigned a value of 1. Western blot analyses (B&C) were performed on total cell lysates Results in (B) are of a representative experiment (n=3). Results in (C) are of a representative experiment (n=4) performed with cells treated (or not) for 24 hr with 50 ng/ml TNF-α, with or without 10 ng/ml IGF-I. Results of densitometry based on 4 different experiments and expressed as means (and SD) of the ratios of p-STAT3:STAT3.
in treated relative to untreated M-27IGFIR cells are shown in (D). p = 0.04 for TNF-α treated vs untreated cells and p=0.07 for TNF-α +IGF-I vs TNF-α-treated cells.

Figure 3. Increased constitutive and TNF-α-induced IL-6 production in liver-metastatic tumor cells. RT-PCR (A) and qRT-PCR (B, C, E and G) were performed on total RNA extracted from the indicated cells. Shown in (A) are representative results of 3 experiments and in (B and C) means and SD (n=3) expressed as the ratios of IL-6:GAPDH relative to M-27 cells that were assigned a value of 1. Results in (E) are expressed as means and SD (n=3) of the ratios of IL-6:GAPDH relative to untreated MC-38 cells. Treatment with 50 μg/ml JSH23 (in G) was for 30 min prior to stimulation with 10 ng/ml TNF-α for 10 min and the results are expressed as means and SD (n=3) of the ratios of IL-6:GAPDH relative to untreated M-27IGFIR cells. ELISAs (D, F and H) were performed on conditioned media harvested from the indicated cells that were treated (or not) for 48 hr with the indicated concentration of TNF-α (or 10 ng/ml in (D) and (H)), 10 ng/ml IGF-I, or both, without (D and F) or with (H) 100 μg/ml JSH23. The results (in D) are expressed as the concentration of IL-6 (in pg/ml) under each experimental condition and in (F and H) as fold change relative to untreated M-27IGFIR cells that were assigned a value of 1 (n=3). *-p<0.05. **-p<0.01 for the indicated comparison groups.

Figure 4. IGF-IR signalling enhances TNF-α – mediated IL-6 production and IL-6R activation but does not directly activate STAT3. M-27IGFIR cells were serum starved overnight, stimulated with 10 ng/ml IGF-I for the indicated durations, lysed and Western blotting performed on total cell lysates. Results of a representative experiment (n=3) are shown (A). Cells cultured in serum-containing medium were used as a positive control (Ctl). Shown in (B) are results of a Western blot performed on the same cell lysates but
probed with an antibody to p-Akt (as an indicator of IGF-IR activation). Cells were also incubated for 24 hr with (or without) 50 ng/ml TNF-α and 10 ng/ml IGF-I, in the presence of 2.0 μg/ml of a neutralizing goat anti-IL-6Rα antibody or non-immune goat IgG, as a control. Shown in (C) are the results of a representative Western blot (n=3) performed as above. Results of an ELISA performed on conditioned media harvested from M-27IGFIR cells treated (or not) with 50 ng/ml TNF-α, with or without 10 ng/ml IGF-I, and in the presence or absence of 20 μM of the indicated inhibitors are shown in (D). They are expressed as the means and SD of the changes in IL-6 concentration (n=3), relative to non-treated M-27IGFIR cells assigned a value of 1. *- p<0.05, **- p<0.01 as compared to cells stimulated in the same manner but without the inhibitors.

**Figure 5. Silencing of gp130 abolishes the transcriptional activity of STAT3 and the anti-apoptotic effect of TNF-α.** qRT-PCR (A) was performed on total RNA extracted from M-27IGFIR cells stably transfected with gp130 shRNA (Supplementary Fig 6C- sh1), or a scrambled sequence (Ctl). Shown are the means and SD of three experiments expressed as fold change relative to non-transfected cells. Apoptosis (B) was measured using the Cell Death Detection ELISA (Roche) following treatment of the specified M-27IGFIR cells with serum-free RPMI containing (or not) 50 ng/ml TNF-α, with or without 10 ng/ml IGF-I, as indicated, for 48 hr. *- p<0.05 (student’s t-test) for the indicated comparison groups. **- p<0.01 (student’s t-test) as compared to control cells (Ctl) treated in the same manner.

**Figure 6. Loss of survival advantage in cells with reduced IL-6 expression or function.** STAT3 activation (A) was measured in non-transfected MC-38 cells (WT) and cells stably expressing IL-6 shRNA (IL-6sh) or a scrambled sequence (Ctl) (see
Supplementary Fig 8). All cells were treated (or not) with 50 ng/ml TNF-α for 24 hr and Western Blotting performed on total cell lysates. Apoptosis (B) was measured following treatment of the above cells with serum-free RPMI containing (or not) 50 ng/ml TNF-α. Cells (10^5 per condition) were lysed and cytoplasmic mono-oligonucleosomes were analysed using the Cell Death detection ELISA kit (Roche). Results shown are based on a representative experiment performed in triplicates. They are expressed as mean O.D. values (405 nm) relative to the respective untreated cells that were assigned a value of 1.

M-27IGFIR cells in (C) were treated with an anti-mouse IL-6 antibody or normal rat IgG as control, both at a concentration of 0.5 μg/ml and then with 50 ng/ml TNF-α. Apoptosis was analyzed as in (B) and the results, based on three separate experiments are expressed relative to cells treated with TNF-α only. *- p<0.05, **- p<0.01 (student’s t-test) for the comparison groups indicated.

**Figure 7. Gene silencing or blockade of IL-6 reduce the liver-metastasizing potential of tumor cells.** Liver metastases were enumerated 20 days after the intrasplenic/portal inoculation of 2x10^5 M-27IGFIR cells (A and B) or 5X10^4 MC-38 cells (C -E) stably expressing IL-6 shRNA (IL-6sh) or mock-transfected (Ctl). In a separate experiment (F), M-27IGFIR were pretreated for 1 hr with 50 μg/ml of an anti-mIL-6 antibody, or vehicle as control, prior to the injection. Metastases were enumerated (and sized) before fixation of the liver and the livers then fixed in 10% neutral buffered formalin for the preparation of paraffin blocks. Shown are the numbers of metastases in individual livers and means (horizontal bar) per group in a representative experiment of 2 performed with M-27IGFIR (A, B and F) and 2 with 2 different clonal sublines of MC-38 with IL-6 silencing (C-E). Numbers on the top indicate the incidence of hepatic metastases/group and below the
mean nodule size. Representative livers from one of these experiments are shown in (B) and (D), and representative H&E stained sections of FFPE livers from an experiment with MC-38 cells showing (top) a metastasis corresponding to the mean nodule size in this group are shown in (E). Mag (in (E)) : images on left – 50X; image in inset on right (same metastases as shown on the left) – 400X. *-p<0.05, **-p<0.01 in comparison to control group as determined by the Mann-Whitney test. L-liver, T-tumor.
**A**

M-27 M-27IGF-IR M-27Mock

IL-6

GAPDH

**B**

IL-6 mRNA transcripts (fold change)

M-27 M-27IGFIR M-27Mock

**C**

IL-6 mRNA transcripts (fold change)

- M-27
- M-27IGFIR

**D**

IL-6 concentration (pg/ml)

- M-27
- M-27IGFIR

**E**

MC-38

IL-6 mRNA transcripts (fold change)

MC-38

**F**

M-27IGFIR

IL-6 concentration (fold change)

- + + +
- + + +

**G**

M-27IGFIR

IL-6 mRNA transcripts (fold change)

- + + +
- + + +

**H**

M-27IGFIR

IL-6 concentration (fold change)

- + + +
- + + +

Li et al., Fig. 3
Li et al., Fig. 4
A

![](chart)

B

![](chart)

Li et al., Fig. 5
A

<table>
<thead>
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<th></th>
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<th>IL6sh</th>
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<td>+</td>
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**Apoptosis** to control (p-STAT3)

**B**

**MC-38**

Apoptosis relative to control (%)

TNF-α : - + - +

Ctl IL-6sh

**C**

**M-27IGFIR**

Apoptosis relative to control (%)

TNF-α : - + + +

IgG1 IL-6 Ab

Li et al., Fig. 6
Metastatic cells can escape the pro-apoptotic effects of TNF-α through increased autocrine IL-6/STAT3 signalling

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Cancer Res  Published OnlineFirst December 22, 2011.

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Access the most recent version of this article at:
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