IL-23 selectively promotes the metastasis of colorectal carcinoma cells with impaired Socs3 expression via the STAT5 pathway

Le Zhang, Jun Li, Li Li*, Jie Zhang, Xiaodong Wang1, Chuanhua Yang1, Yanyan Li, Feng Lan and Ping Lin*

Division of Geriatrics, Center for Medical Stem Cell Biology, State Key Laboratory of Biotherapy and 1Gastrointestinal Surgery Center, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, People’s Republic of China

*To whom correspondence should be addressed. Division of Geriatrics, Center for Medical Stem Cell Biology, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, 1 Keyuan 4 Road, Gaoping Avenue, Hi-tech Development, Chengdu, Sichuan 610041, People’s Republic of China. Tel: +86 28 85164016; Fax: +86 28 85582944; Email: linping8@yahoo.com

Interleukin-23 (IL-23) is a conventional proinflammatory IL-12-like cytokine signaling (Socs) molecules, respectively, serve as agonists and antagonists of IL-23-associated inflammation. However, it remains unknown whether IL-23 directly affects CRC metastasis. In this study, we measured the metastasis of several human CRC cell lines stimulated by IL-23 in vitro and in vivo. Interestingly, the prometastatic effect of IL-23 was observed only in SW-620 cells. IL-23-associated migration and invasion was mediated by the phosphorylation of STAT5. The expression of Socs3 in SW-620 was impaired by IL-23 via DNA methylation and DNA methyltransferase-1 (DNMT1)-dependent way. The DNMT1-associated regulation was not observed in the other three cells. Socs3 was further confirmed to inhibit the prometastatic function of IL-23 both in vitro and in vivo. We analyzed the clinical correlation between the level of IL-23 in tumors and the metastasis of CRC and found that higher IL-23 levels along with lower Socs3 in CRC tissues accounted for more metastatic cases. In conclusion, it was demonstrated that IL-23, assisted by STAT5, might only promote the metastasis of CRC cells with deficient Socs3 expression in which IL-23-induced DNMT1 was involved. It was elucidated that Socs3 seemed to be one of the important factors that mediate the selectivity of IL-23. Taken together, these discoveries give rise to new insights into the role of IL-23 in cancer biology and provide additional preclinical data regarding IL-23-associated therapy for CRC.

Introduction

Colorectal carcinoma (CRC) is one of the most fatal neoplastic diseases worldwide. It has been reported as being relevant to some inflammatory bowel diseases, such as Crohn’s disease and ulcerative colitis (1–3), and many inflammatory factors are involved in the progression of CRC (4). Interleukin-23 (IL-23), which is a general proinflammatory factor, has been shown to participate in the malignant transformation of the colorectal epithelium, based on our own observation (5). We further demonstrated the existence of the IL-23 receptor (IL-23R) on the surface of CRC cells, which provided the premise that IL-23 acts directly on these cells (5). IL-23 has a unique subunit, IL-23p19, and a subunit that is shared with IL-12, IL-12p40 (6); however, it performs differently from IL-12. It has been demonstrated that, apart from its IL-12-like immune-enhancing functions (7), IL-23 enhanced autoimmune and inflammation-associated diseases via the Th17 axis (8). In addition, the procarcinogenic role of IL-23 has also been investigated extensively (8,9). Various signaling pathway are involved in IL-23-associated signaling transduction. The signal transducer and activator of transcription 5 (STAT5), which is one of the major downstream signaling transducers of the IL-23 signal, serves as a consequential intermediary in IL-23-associated inflammation (10). However, negative regulating mechanisms of IL-23-signaling transduction are discovered as well. As an important member of the superfamily of suppressors of cytokine signaling (Socs), Socs3 may block the phosphorylation of STAT3 and STAT5 by inhibiting the Janus kinase 2 (JAK2) directly, subsequently crippling the STAT signal (11). As a result, it is widely accepted that Socs3 is an effective feedback regulator of the IL-23 STAT signaling pathway. It is indicated that the expression of Socs3 in ulcerative colitis-related CRC is effectively regulated by IL-6 via Socs3 gene methylation. DNA methyltransferase-1 (DNMT1) is verified to play a crucial role in this process (12).

Recently, it was also shown that IL-23 is associated with the metastasis of cancer, as IL-23 promoted the metastasis of hepatocellular carcinoma via matrix metalloproteinase 9 (13). Furthermore, an anti-IL-23p19 monoclonal antibody effectively suppressed the metastasis of lung cancer in a mouse model, which also indicated the prometastasis effect of IL-23 (14). In conclusion, IL-23 is a potential metastasis stimulus. However, whether IL-23 promotes the metastasis of CRC and, if so, its underlying mechanism, remains unclear.

It is undisputed that the IL-23-signaling pathway is related to the progression of CRC; however, the direct link between IL-23 and metastatic CRC remains a mystery. In fact, our own data have demonstrated that low doses of IL-23 promote the proliferation of lung cancer cells (15). Nevertheless, whether IL-23 affects cellular migration warrants clarification. Here, to determine the direct effect of IL-23 on the migration of CRC cells, we explored the migration and invasion of CRC cells treated with recombinant human IL-23 and its underlying mechanism both in vitro and in vivo, and further analyzed the clinical correlation between tissue IL-23 levels and the metastatic condition of CRC.

Materials and methods

Patients and samples

Human CRC samples were dissected from 96 CRC patients in Gastrointestinal Surgery Center of West China Hospital. The diagnosis of all samples was confirmed by pathologist. The tissues were snap-frozen in liquid nitrogen immediately after dissection and stored in liquid nitrogen until further assessment by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry. The study was approved by the local Ethical Committee on Human Experimentation of West China Hospital, Chengdu, and informed written consent was acquired from all patients. Then all collected samples were eligible for experimental purpose. And the information about pathological types of all samples had been listed in Supplementary Table 1, available at Carcinogenesis Online.

Cells, reagents and transfection

SW-480, SW-620, HT29 and HCT-116 cell lines were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum. pEFLAG-Socs3 and pEFLAG-vector plasmids (both were kindly provided by Prof. D.Hilton from the Walter and Eliza Hall Institute of Medical Research, the University of Melbourne) were expanded in competent Escherichia coli and extracted with Axyprep™ plasmid miniprep kit (Axygen, Union City, CA). Small interfering RNA (siRNA) for Socs3 and negative control were purchased (Genepharma, Shanghai, China). And the respective sequences: siRNA for Socs3: 5'-GAC CCA GTC TGG GAC CAA G-3'; siRNA for negative control: 5'-UCU UUC GAA CGU GUC AGC UTT-3'. Transfection was mediated by TurboFect Transfection Reagent (Thermo Fisher Scientific, Waltham, MA). Cells had been precultured in serum-free DMEM for 2 h before transfection. Then 8 µg plasmid or 10 nmol siRNA was introduced into the cell using 6 µl transfection reagent according to the manufacturer’s instruction. Human recombinant IL-23, anti-IL-23p19 neutralizing antibody (B-Z23) (ab84471)
IL-23 promotes the metastasis of CRC cells

and Jak inhibitors AG490 were, respectively, purchased from R&D Systems (Minneapolis, MN), Abcam (Cambridge, UK) and Cayman chemical (Ann Arbor, MI). 1 µg/ml anti-IL-23p19 neutralizing antibody was used to block the endogenous IL-23 in vitro culture system, which was absolutely sufficient to block the effect of 10 ng/ml IL-23 according to the manufacturer’s instruction.

**Wound-healing assay and transwell assay**

Before wound-healing or transwell assay were conducted, the cells of each group were transfected with or without Socs3-specific plasmid or siRNA as the way described above. Wound-healing assay was carried out by scratching down the single cell layer with tips. The images of the scratch area were recorded by three random spots at 0 and 24 h. The migrating distance from the wound edge was measured using a standard size field for each image. The mean migrating distances of three spots were calculated according to the scaleplate. Wound-healing assays were performed in triplicates and all data were statistically processed.

Transwell assays for estimating the migration and invasion were conducted using 24-well Millicell Hanging Cell Culture Insert 8.0 µm PET (for migration; Merck Millipore, Darmstadt, Germany) and Cell invasion Assay (for invasion; Millipore, Billerica, MA). Cells were resuspended in serum-free DMEM and prepared in upper chamber, whereas complete DMEM with 10% fetal bovine serum. Twenty-four hours later, the migratory or invasive cells on the basolateral side of the chamber were fixed for formaldehyde for 10 min and stained with crystal violet (Merck Millipore, Darmstadt, Germany) for another 15 min, then the images were captured. The 33% acetic acid was utilized to dissolve the crystal violet for further optical density value reading by the ELISA plate reader (Model 550; Bio-Rad, Hercules, CA) at 570 nm.

**MTT assay**

Cells proliferation was evaluated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All cells were plated at density of 5 × 10^4 cells per well in 96-well plates treated with IL-23. Twenty-four hours later, MTT assay was conducted. Finally, the optical density was determined at 570nm using the ELISA plate reader (Model 550; Bio-Rad). At least three independent experiments were ensured.

**Quantitative real-time PCR**

Total RNA was extracted from cells with TAKARA kit (Takara, Dalian, China), according to the manufacturer’s protocol. One microgram total RNA was subjected to reverse transcription with a PrimeScript RT MMLV reagent kit (Takara) according to the manufacturer’s instructions. PCR reactions were performed with SYBR Premix Ex Taq II kit (Takara), and PCR protocol consisted of one cycle at 95°C for 10 s followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s. Gene-specific primers used to determine the relative expression levels of Socs3, DNMT-1 and β-actin were as follows: human Socs3, forward: 5'-ATG GTG ACC ACC AGC AAG TT-3' and reverse: 5'-ACT GAG CAG CAG GTT CGC C-3'; human DNMT-1, forward: 5'-AGG CCG TAG AGT GGG ATT GGG TTT-3' and reverse: 5'-TGAG GTC ACC TCT GAA ATG CTT-3'; human β-actin, forward: 5'-CTG GAA CGG TGA AGG TCA-3' and reverse: 5'-AAG GGA CCT TCT GTA ACA ATG CA-3'. The expression of β-actin was utilized to normalize for transcription. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence exceeded the fixed threshold. All samples were performed in triplicates.

**Western blotting**

Samples were lysed by General Protein Extraction Reagent (Biotek, Beijing, China). Thirty-five micrograms of proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred electrophoretically to a 0.45 µm polyvinyl difluoride membranes. The following anti-human antibodies were used: anti-p53 monoclonal antibody (Abcam, Darmstadt, Germany) and rabbit anti-p53 polyclonal antibody (Zhongshanjinqiao, Beijing, China). The diluents of anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were 1:1000. The membranes were developed using Immobilonenhanced Western Chemiluminescent HRP Substrate (Millipore). Protein level was quantified by scanning blots on a Gel Doc EZ imager (Bio-Rad) and analyzed with Quantity One 1D image analysis software 4.40 (Bio-Rad).

**Methylation-specific PCR**

DNA Total DNA was isolated using trizol reagent and bisulfite modified using the EZ DNA methylationTM kit (ZYMO Research, Irvine, CA). Methylated DNA and unmethylated DNA (Millipore) were acquired and used as a positive and negative control, respectively. Methylation-specific PCR was performed on bisulfite-converted genomic DNA. The PCR was performed in a 25 μl volume containing 40 ng bisulfite-modified DNA, 2x Master Mix (Biotek, Beijeing, China) and 10 pmol specific primer mix (forward and reverse primers). Three regions of SOCS3 promoter were targeted and each primer mix were as follows: primer set 1: methylated forward: 5'-GGT TCG TGT TCG TAC TGA GTT AGT C-3' and reverse: 5'-TAA ATT CCA CAA AAA AAC CTT CG. PCR-3′ (annealing at 55°C), unmethylated forward: 5'-TTT TGG TCG TCT TGG AGT TAG TTA GTT G-3' and reverse: 5'-TAA ATT CCA CAA AAA AAC CTT CAC-3′ (annealing at 56°C); primer set 2: methylated forward: 5'-GGGA GAT TTT AGG TTT TCG GAA TAT TTC-3' and reverse: 5'-CCT CCC AGA AAA CTA CCT AAA CAC CGG-3' (annealing at 56°C); unmethylated forward: 5'-GTT GGA GAT TTT AGG TTT TTG GAA TAT TTC-3' and reverse: 5'-AAA CCC CCA AAA GTA CCT AAA CAC CA-3′ (annealing at 56°C); primer set 3: methylated forward: 5'-GGT TGA TTC GTA GGG TTT TTC-3' and reverse: 5'-CAG CTA AAA AAC CTC CGG AT-3' (annealing at 55°C); unmethylated forward: 5'-GTT TGA TTT GTA GGG TTT TTG-3' and reverse: 5'-CAA CCT AAA AAA CCT CCC AAT-3′ (annealing at 55°C). Protocol consisted of one cycle at 95°C for 3 min followed by 40 cycles at 95°C for 15 s and at respective annealing temperature for 30 s. The PCR products were visualized on a 1.5% agarose gel using ethidium bromide and ultraviolet illumination.

**Demethylation treatment**

5-aza-2-deoxycytidine (5-Aza-dC; Sigma, St Louis, MO) was used to revert the effect of DNMT-1 and added to a final concentration of 10 μM. Three days (72h) after 5-Aza-dC treatment, the cells were stimulated by IL-23 (100 ng/ml) and harvested for quantitative real-time PCR and western blot.

**In vivo assay for migration and invasion**

BALB/c male nude mice, used at 6–8 weeks, were bred and maintained under standard housing conditions in the animal facility of Sichuan University. All experiments in this study for 15 min at 4°C were performed in accordance with nation’s relevant laws and animal welfare requirements. To construct in vivo model, lentiviral vectors (pGLV-H1-GFP+Puro) were designed to establish the stably IL-23 expressing SW-180 and SW-620 cell lines according to the manufacturer’s instruction (GenePharma, Shanghai, China). These modified cells were further transfected with Socs3 plasmid or siRNA-Socs3 described above. 1 × 10^6 cells for each mouse were injected into the circulation via tail vein. 5 μg/g (per gram of the mouse weight) anti-IL-23p19 neutralizing antibody was given intraperitoneally in IL-23 neutralizing groups 1 day after the injection of tumor cells. Ten mice were randomly assigned to each group. All mice were killed 2 weeks later. Metastases were microscopically checked and counted. According to quite a few previous reports, only the metastases which were large enough to form a colony in target site (>30 cells in one colony which were reliable and countable metastases) were counted, for only this part of metastases had biological and clinical significance. For each mouse, the whole lung was embeded and the qualified metastases on 10 random slices were counted. Counting of lung metastases was statistically processed.

**Enzyme-linked immunosorbent assay**

Protein was extracted by tissue homogenate method in which the normal saline with protease inhibitor cocktail (Roche, Mannheim, Germany) was used. All protein samples for further analysis by ELISA have been standardized to same total protein concentration. The IL-23 level of tumor tissues and CRC cell lines culture supernatant were analyzed by commercial ELISA kits (R&D Systems), according to the manufacturer’s protocol. The color reaction was measured as OD450 units on the ELISA plate reader (Model 550; Bio-Rad). The concentration of cytokines was determined by a standard curve that was obtained using the kit’s standards.

**Immunofluorescence and immunohistochemistry**

Cells were plated onto sterile round microscope slides in 6-well plates and used at 70% confluence at 4°C. After three washes, cells were blocked with 5% house serum for 1h at 37°C followed by incubation with rabbit-anti-human IL-23r antibody (dilution: 1:100; Boaoen, Beijing, China) and rabbit-anti-human IL-12Rβ1 antibody (1:100 dilution; Boaoen) at 4°C for overnight. After several washes, cells were incubated with an FITC-conjugated (for IL-23r) or Alexa 594-conjugated (for IL-12Rβ1) secondary antibodies (1:100 dilution; Zhongshanjinqiao) for 1h. 4,6-diamidino-2-phenylindole (5 μg/ml) (Invitrogen, Eugene, OR) was used to stain nuclei. Rabbit isotype IgG (1:300) was designed to be the corresponding native control. The positively stained tumor cells were assessed in 40 fields selected randomly, at a final magnification of ×400.

All CRC tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Then the embedded samples in paraffin were sliced with 4-μm thickness, and were pretreated with hydrogen peroxide. The slides were incubated with 3,3-diaminobenzidine (0.05% in Tris-HCl buffer containing 0.01% Hydrogen Peroxide) for 10 min. After several washes with PBS, the slides were incubated with the 1:100 dilution of rabbit anti-IL-23 antibody overnight at 4°C. Subsequently, the tissues were incubated with horseradish peroxidase-labeled secondary anti-rabbit immunoglobulin...
Diaminobenzidine was used as a substrate chromogen, and slides were counterstained with hematoxylin. Rabbit isotype IgG (1:300) was designed to be the corresponding negative control. The positively stained tumor cells were assessed in 40 fields selected randomly, at a final magnification of ×400. The expression of IL-23, Socs3 and pSTAT5 was scored by proportion and intensity, according to Allred’s procedure. The proportion score represented the estimated proportion of tumor cells staining positive was as follows: 0 (none), 1 (<1/100), 2 (1/100–1/10), 3 (1/10–1/3), 4 (1/3–2) and 5 (>2/3). Any brown cytoplasmic or nuclear staining in CRC cells was counted toward the proportion score. The intensity score represented the average intensity of the positive cells was as follows: 0 (none), 1 (weak), 2 (intermediate) and 3 (strong). The total score was calculated by summation of proportion and intensity scores. Tumors with scores <3 were identified as negative staining or otherwise positive.

Statistical analysis

Statistical analysis was performed with SPSS 13.0 (SPSS, Chicago, IL). The results of real-time PCR were treated by rank-sum test. One-way analysis of variance test was utilized to process the result of MTT assay, ELISA, wound-healing assay, transwell assay and in vivo lung metastases counting. Chi-square test was used to analyze the relationship between IL-23 level and metastasis in clinical CRC cases. Fisher’s exact probabilities test was used to analyze the relationship between Socs3 expression and metastasis in IL-23-highest subgroup of CRC cases. A correlation analysis was used to analyze the relationship between the expression of IL-23 and Socs3 messengers RNA expression. The equality of variances was assured by Levene’s test. P values <0.05 were considered as statistically significant. *Indicated that the P values were <0.05 and >0.01, whereas **Indicated P values <0.01.

Results

IL-23 selectively promoted the migration and invasion of SW-620 cells, rather than SW-480, HT29 and HCT-116 cells

For the association between IL-23 and CRC metastasis remained unclear, we preferred to determine whether IL-23 was able to promote the migration and invasion of CRC cells. Human recombinant IL-23 was used to trigger the cells to detect its direct influence. It was initially indicated that IL-23R complex (IL-23r and IL-12Rβ1) was expressed on SW-480, SW-620, HT29 and HCT-116 cells (Supplementary Figure 1A, available at Carcinogenesis Online). It has suggested that all cell lines were possibly affected by the direct action of IL-23. Thus, they were set as the representative CRC cells for this study. Considering the effect on proliferation of tumor cell treated with IL-23, MTT assay was used to estimate the possibility that the proliferation of the cells might disturb the results of the migration and invasion assay. It was indicated that the proliferation of these CRC cells was hardly affected by any dose of IL-23 (Supplementary Figures 1B, 2E and 3A, available at Carcinogenesis Online). The migratory and invasive ability of CRC cell lines stimulated with different doses of IL-23 was estimated in vitro. Interestingly, although all cell lines expressed IL-23R (Supplementary Figure 1A, available at Carcinogenesis Online), only SW-620 cells acquired enhanced movement and invasion in the presence of 40 and 100 ng/ml of IL-23, as assessed in the transwell assay (Figure 1A and B). Furthermore, the wound-healing assay confirmed the accelerated migration of SW-620 cells triggered by high doses of IL-23 (Supplementary Figure 1C and D, available at Carcinogenesis Online). In contrast, SW-480, HT29 and HCT-116 cells were unaffected after IL-23 administration (Figure 1A and B). These results indicated the preferentially and dose-dependently promigratory and proinvasive effect of IL-23 in vitro.

It was widely accepted that mesenchymal transition of carcinoma was an important process to initiate metastasis. To further assess whether IL-23 was linked to mesenchymal transition, metastasis-associated mesenchymal molecular expression pattern was estimated. Although 40 ng/ml IL-23 was sufficient to promote the metastasis of SW-620, only 100 ng/ml IL-23 induced SW-620 to present detectable mesenchymal pattern. The expression of mesenchymal markers, Neuronal-Cadherin (N-Cadherin) and Vimentin, was enhanced, whereas the expression of epithelial marker, Epithelial-Cadherin (E-Cadherin) was largely attenuated (Supplementary Figure 1E, available at Carcinogenesis Online). It was indicated that mesenchymal transition required higher level of IL-23. Nevertheless, the morphology of SW-620 stimulated by IL-23 remained unchanged (data not shown).

High level of IL-23 in CRC tissue has been widely reported. Various subsets of inflammatory cells were indicated to contribute to this high IL-23 microenvironment including tumor-associated macrophage and fibroblast. However, we observed that CRC cells were able to secrete IL-23 in vitro as well (Supplementary Figure 2A, available at Carcinogenesis Online). The autocrine loop of IL-23 was potential to play a role in IL-23-associated metastasis. In order to address whether an autocrine mechanism existed, we utilized anti-IL-23p19 neutralizing antibody (IL-23 nAb) to block the action of CRC-derived endogenous IL-23. However, neutralization of the endogenous IL-23 had no effect on both migration and invasion of all CRC cell lines (Supplementary Figure 2B and C, available at Carcinogenesis Online). Indeed, for as much as 10 ng/ml exogenous IL-23 could barely affect the migration and invasion of CRC cells in vitro (Figure 1A and B), a relatively lower level of IL-23 produced by CRC themselves was incapable either.

IL-23 induced the phosphorylation of STAT5 in SW-620 cells, which mediated the acceleration of cellular migration and invasion

STAT3 and STAT5 were considered to be critical to the IL-23-signaling pathway. To decipher the deeper signaling pathway that was involved in the IL-23-mediated enhancement of cellular migration and invasion, two major mediators of the IL-23-signaling pathway, STAT3 and STAT5, were rationally checked. Surprisingly, phosphorylation of STAT3, the most conventional second messenger of IL-23 signaling, was unable to be induced in four CRC cell lines. The phosphorylation of STAT3 was almost undetectable in SW-620, SW-480 and HT29 (Figure 1C). Conversely, the activation of STAT5 in SW-620 cells was effectively induced by IL-23; however, no similar effect was observed in SW-480, HT29 and HCT-116 cells (Figure 1C). To clarify the role of STAT5 in this process, AG490, a conventional Jak2 inhibitor, was used to block the activation of STAT5. Attenuation of the phosphorylation of STAT5 by AG490 dramatically interrupted the IL-23-induced migration and invasion of SW-620 cells (Figure 1D). In conclusion, the phosphorylation of STAT5 might play an important role in the mediation of the IL-23-enhanced migration of SW-620 cells.

Socs3 expression was divergently regulated by IL-23 in different CRC cell lines via DNA methylation and DNMT-1-dependent pathway

The dual effect of IL-23 on the cellular migration and invasion of these four cell lineages drove us to elucidate the intrinsic mechanism underlying these observations. To analyze the dissimilarity, we considered that the negative regulatory mechanism of the IL-23/STAT signaling pathway was involved in this process. Socs3, which acts as an efficient inhibitor of the STAT pathway, might be related to the signaling pathway was involved in this process. Socs3, which acts as an efficient inhibitor of the STAT pathway, might be related to the IL-23-associated metastasis. We estimated the level of expression of Socs3 in the presence of different concentrations of IL-23. Although it was barely affected by 10 ng/ml of IL-23, the expression of Socs3 was bidirectionally modulated by higher doses of (40 and 100 ng/ml) IL-23 in different cell lines. In this situation, Socs3 expression was increased in SW-480, HT29 and HCT-116 cells and reduced in SW-620 cells, as assessed using quantitative real-time PCR (Figure 2A, left lane) and western blotting (Figure 2B).

Socs3 was conventionally induced by IL signal. However, the induced expression of Socs3 was observed in SW-480, HT29 and HCT-116 cells. On the contrary, Socs3 expression was inhibited by IL-23 in SW-620. To illustrate the contradiction, the methylation of Socs3 gene was taken into consideration. Initially, the expression of DNMT-1, a conventional DNA methyltransferase, was measured after IL-23 treatment. Interestingly, the expression of DNMT-1 was only elevated in SW-620 triggered by IL-23 rather than in SW-480, HT29 and HCT-116 (Figure 2A, right lane, 2B). Additionally, the methylation status of Socs3 gene in four CRC cell lines triggered by IL-23 was detected using methylation-specific PCR. As the Figure 2C shown, the methylation of three potential methylation targets within
IL-23 promotes the metastasis of CRC cells

Socs3 gene was enhanced by IL-23 only in SW-620. Moreover, in Figure 2D, it was displayed that Socs3 expression of SW-620 was not suppressed by IL-23 in the presence of general methylation inhibitor, 5-aza-2-deoxycytidine. It was implied that IL-23 promoted the expression of DNMT-1 only in SW-620 which could further induce the methylation of Socs3 gene. These results confirmed that the methylation of Socs3 induced by IL-23 was associated with the loss of Socs3 in SW-620. The distinct expression pattern of Socs3 induced
by IL-23 led us to believe that Socs3 seemed to be one of the specific molecules regulating the IL-23-induced migration and invasion in different CRC cells.

**Socs3 was a negative regulator of the cellular migration and invasion induced by IL-23**

The different expression patterns of Socs3 in the four cell lines and its underlying mechanism were confirmed. As a result, it was important to clarify whether this difference resulted in divergent responses to IL-23 in different CRC cells. For this purpose, a Socs3 expression plasmid or an siRNA for Socs3 were, respectively, transfected into SW-620 and SW-480, HT-29, HCT-116 cells to evoke or revoke Socs3 expression after stimulation with IL-23. Concordant with our hypothesis, the IL-23-induced migration and invasion were both canceled by the overexpression of Socs3 in SW-620 cells (Socs3-OE; Figure 3A, Supplementary Figure 3B, available at Carcinogenesis Online). In contrast, SW-480, HT29 and HCT-116 cells triggered by IL-23 exhibited superior migration and invasion after the inhibition of Socs3 expression by an siRNA for Socs3 (si-Socs3; Figure 3B, Supplementary Figure 3D, available at Carcinogenesis Online). It was shown that Socs3 might play a critical role in IL-23-induced migration and invasion in vitro: if Socs3 was not induced, cellular migration and invasion were
IL-23 promotes the metastasis of CRC cells

Enhanced by IL-23, as observed in wild-type SW-620 and the other three cell lines with defective Socs3 expression. Otherwise, the induction of the expression of Socs3 might be sufficient to attenuate the promigration and proinvasion effect of IL-23, as observed in SW-620 cells that overexpressed Socs3 and the other wild-type cell lines.

Furthermore, the phosphorylation of STAT5 in SW-620 cells was not increased in the presence of a high level of Socs3 expression (Figure 4A). In contrast, the production of pSTAT5 was elevated by IL-23 in SW-480, HT29 and HCT-116 cells after inhibition of Socs3 expression (Figure 4A). Similarly, the impairment of the expression of pSTAT5 by AG490 effectively canceled the IL-23-induced migration.

Fig. 3. IL-23 induced migration and invasion of SW-620 cells that overexpressed Socs3 (Socs3-OE) and that of the other cells with Socs3 knockdown (si-Socs3). (A) Transwell assays indicating that IL-23 did not promote the migration and invasion of SW-620 cells that overexpressed Socs3 (Socs3-OE) compared with the vector control. Quantitative absorbance measurement of the crystal violet released by migratory (upper portion) and invasive cells (lower portion) at 570 nm was performed in triplicates and statistical analysis was shown in the diagram. The absorbance represented the amount of migratory and invasive cells. **P< 0.01.

(B) Transwell assays indicating that IL-23 facilitated the migration and invasion of SW-480, HT29 and HCT-116 cells with impaired Socs3 expression (si-Socs3). Quantitative absorbance measurement of the crystal violet released by migratory (left portion) and invasive (right portion) cells at 570 nm was performed in triplicates and statistical analysis was shown in the diagram. The absorbance represented the amount of migratory and invasive cells. **P< 0.01.
and invasion of Socs3-defective SW-480, HT29 and HCT-116 cells (Figure 4B). Hence, these results indicated that the phosphorylation of STAT5 might also act as an intermediary for the IL-23-induced migration and invasion when Socs3 had been impaired. In summary, the effect of IL-23 on the migration and invasion of CRC cells might be restricted by Socs3 in vitro.

**IL-23-enhanced metastasis of CRC cells was regulated by Socs3 in vivo**

In order to clarify the prometastatic effect of IL-23 on SW-620 and SW-480 in vivo, lung metastasis model was constructed via tail vein injection in nude mice system. IL-23 stably expressing SW-620 and SW-480 cell lines were established to create the microenvironment of high IL-23 level in vivo. More metastases of SW-620 stimulated with IL-23 (IL-23-OE) were observed compared with control (IL-23, leftmost lane). The metastasis of SW-480 triggered by IL-23 (IL-23-OE) remained unchanged in vivo (Figure 5A and C, second lane from the right). It was further proved that Socs3 also played a critical role in IL-23-induced metastasis in vivo. High level of IL-23 failed to increase the metastasis of SW-620 with sufficient Socs3 when overexpression of Socs3 blocked the prometastatic effect of IL-23 on SW-620 (Figure 5A and C, second lane from the left). Meanwhile, interference of Socs3 facilitated the metastasis of SW-480 stimulated by IL-23 (Figure 5A and C, rightmost lane). All the metastases were confirmed at histological level (Figure 5B). Consequently, IL-23 and Socs3 might involve in the regulation of the metastasis of CRC cell in vivo.
IL-23 promotes the metastasis of CRC cells

To further address the effect of IL-23 on metastasis in vivo, anti-IL-23 neutralizing antibody was used. Though having no statistically significance, the trend that treatment of anti-IL-23 antibody reduced the spontaneous metastasis of wild-type SW-620 in vivo was observed (Supplementary Figure 4A and B, left lane C, available at Carcinogenesis Online). More metastases were observed in IL-23 overexpression SW-480 cell group only when Socs3 had been attenuated (IL-23-OE, rightmost lane lower portion). Overexpression of Socs3 effectively canceled the IL-23-promoted metastasis of SW-620 (second lane from the left). More metastases were observed in IL-23 overexpression SW-480 cell group only when Socs3 had been attenuated (IL-23-OE, rightmost lane lower portion). (B) The lung metastases of the SW-620 (left lane) and SW-480 (right lane) were confirmed using hematoxylin and eosin staining. (C) The number of qualified metastases (a metastatic colony containing at least 30 cells) of each mouse was microscopically counted and the average number of metastases of each group was shown. **P < 0.01.

A high level of IL-23 was associated with the metastasis of the CRC with defective Socs3 expression

To estimate the correlation between IL-23 and metastasis from a clinical perspective, 96 tumor tissues from different CRC patients were collected, including 48 primary and 48 metastatic cases. First of all, we found that most of the CRC cells, same as tumor-associated fibroblasts and inflammatory cells, were able to produce IL-23 (Supplementary Figure 4D, available at Carcinogenesis Online). Relative low production of local IL-23 and low efficiency of the antibody treatment might contribute to an undetectable therapeutic effect of the neutralizing antibody in SW-620 wild-type group. However, neutralization of the IL-23 could attenuate the metastasis of SW-620 manipulated with high level of IL-23 (IL-23-OE) to some extent (Supplementary Figure 4A and B, right lane C, available at Carcinogenesis Online). Treatment with anti-IL-23 antibody seemed to be a potential way to inhibit IL-23-induced SW-620 metastasis in our model.
We designated the level of 4.2 ng/mg IL-23, 2-fold of mean IL-23 concentration of normal colorectal tissues, as the inclusion criteria of IL-23\textsuperscript{high} subgroup of CRC. Consequently, we found that 54.5% CRC cases in IL-23\textsuperscript{high} subgroup underwent metastasis, whereas in IL-23\textsuperscript{low} subgroup 31.6% cases did. However, this difference did not have statistical significance according to chi-square test (Figure 6A). It seemed that relative high level of IL-23 could not result in more metastasis. However, if the effect of Socs3 was taken into consideration, it was uncovered that 90.6% Socs3-negative cases in IL-23\textsuperscript{high} subgroup underwent metastasis which was much higher than Socs3-positive cases (Figure 6B). It might underscore the potential prometastatic role of IL-23 only in the absence of Socs3. It was demonstrated that the high dose of IL-23 might only be associated with the metastatic progress in CRC patients with defective Socs3 expression.

Furthermore, within the primary group, a positive correlation between IL-23 and Socs3 messenger RNA expression was observed, which paralleled the in vitro data (Figure 6C, left lane). Meanwhile, negative correlation between IL-23 and Socs3 messenger RNA expression was observed in the metastatic group (Figure 6C, right lane). It was illustrated that IL-23 might also modulate the expression of Socs3 via other covert and intrinsic factors.

To clarify the potential role of STAT5 in the metastasis of CRC, we investigated the pSTAT5 expression in all CRC cases. Indeed, we observed that more pSTAT5-positive cases underwent metastasis compared with negative cases (Supplementary Figure 4E, available at Carcinogenesis Online). pSTAT5 seemed to involve in the progression of CRC as well.

![Fig. 6. Clinical correlation between tissue IL-23 levels and the metastatic condition of CRC. (A) IL-23 levels in CRC tissues were measured using ELISA. Cases which had IL-23 >4.2 ng/mg were designated to IL-23\textsuperscript{high} subgroup, whereas the others were assigned to IL-23\textsuperscript{low} subgroup. Metastatic rate of each subgroup was calculated and no statistic significance was observed; χ² test was used to analyze the statistic significance. (B) Metastatic rate of Socs3-positive [Socs3(+)] or negative [Socs3(-)] cases within IL-23\textsuperscript{high} subgroup. Cases with negative Socs3 expression have much higher metastatic rate. Fisher’s exact probabilities test was used to analyze the statistic significance. ** P < 0.01. (C) Correlation analysis was used to analyze the correlation between IL-23 concentration and Socs3 messenger RNA relative expression of CRC in primary (left) and metastatic (right) cases.](https://academic.oup.com/carcin/article/35/6/1330/450214)
Discussion

IL-23 has wide-ranging influence on inflammation and autoimmune diseases. We reported previously that human CRC cells express IL-23R on their surface and permit IL-23 to act directly on colorectal tumor cells. Indeed, IL-23 was reported to participate in the progression and metastasis of CRC both in vitro and in vivo (16,17). Here, in accordance with previous investigations (16,17), we further discovered that IL-23 might selectively induce the migration and invasion of CRC cells in which Socs3 expression was synchronously shut off in vitro. Moreover, in these cells, the IL-23-induced phosphorylation of STAT5 was the key mediator of the promotion of migration and invasion. Conversely, IL-23 might no longer facilitate the metastasis of CRC cells in which the Socs3-associated feedback system was potent, as observed in the SW-480, HT29 and HCT-116 cell lines. In addition, it was indicated that IL-23-induced DNMT-1 and Socs3 gene methylation might play an important role in regulating Socs3 expression which further affected metastasis. In conclusion, IL-23 failed to promote the migration and invasion of CRC cells if Socs3 expression was sufficient. However, when the Socs3-associated feedback inhibition was repressed by DNA methylation, the potential of IL-23 to enhance the movement of CRC cells might emerge immediately. In fact, the level of IL-23 was positively correlated with the metastasis of CRC patients in whom the expression of Socs3 was impaired. Taken together, these data might suggest a preferentially prometastatic role of IL-23 in CRC progression.

The role of IL-23 in canceration remains highly controversial. It was indicated previously that IL-23 inhibits malignance via the enhancement of antitumor immunity and the promotion of apoptosis (18–20), while inducing tumor-associated inflammation and angiogenesis to promote cancer progression (8,9). To clarify the exact underlying mechanisms of the multiple effects of IL-23 on malignant progression, we proposed another new potential answer to this question, which involved the IL-associated feedback regulation mechanism. In this study, we found the regulating role of Socs3 in IL-23-enhanced metastasis. It might establish a novel potential model to explain the contradictory effect of IL-23 on cancer development. According to our previous data, we also observed that the proliferation of lung cancer cells was either promoted or inhibited at different doses of IL-23, respectively (15). Nonetheless, unlike lung cancer cells, the in vitro proliferation of CRC cell lines was exempted from the effect of IL-23. There must be some intrinsic differences between lung cancer and intestinal cancer. However, according to our CRC lung metastasis model, interestingly, extremely large metastases were observed after high IL-23 stimulation. The quantification of the metastases was conventionally sufficient to indicate the metastatic capacity of tumor cells. Nevertheless, more rapid growth of tumor cells usually led to the large sizes of the lesions. Therefore, the pro-proliferative function of IL-23 in vitro could not be ruled out in addition to its prometastatic role.

Tumor-associated fibroblasts and inflammatory cells were reported to be the major sources of local IL-23 in tumor microenvironment. However, we found that CRC cells themselves might produce certain level of IL-23 both in vitro and in vivo. Considering the possible expression of IL-23R on CRC cells, an autocrine loop probably existed. However, we discovered that blocking the CRC-derived IL-23 could not effectively inhibit the migration and invasion of CRC in vitro. Nonetheless, in line with the observation of Michele’s group (14), treatment of anti-IL-23p19 neutralizing antibody could reduce the metastasis of CRC cells in vivo to some extent. Indeed, tumor-associated inflammatory niche was too complicated to distinguish the CRC-derived and inflammatory cells-derived IL-23 in vivo. Both might involve in this process. More effort needed to be paid to address the underlying mechanism and hypothesized autocrine loop.

As a conventional downstream messenger of the IL-23 signal, STAT3 was considered a potential mediator of tumor migration (21,22). However, the IL-23-induced activation of STAT3 in our cell line models was undetectable. This excluded the possibility that STAT3 mediates IL-23-induced migration in our case. However, from a clinical perspective, the role of STAT3 might not be overshadowed, as Zizi-Sermpetzoglou’s research group observed the pSTAT3 expression and nuclear localization in most human CRC samples (23). Thus, the interpretation of the exact influence of STAT3 on IL-23-associated metastasis warrants deeper investigation. Regarding STAT5, the situation seemed to be more complex. In contrast to our discovery, the Sultan group found that STAT5 was a favorable prognosis factor for mammary carcinoma that effectively inhibited the metastasis of tumor cells in a mouse model (24). To explain this contradiction, it is rational to think about the essential differences between mammary and CRC. In fact, in prostate carcinoma, STAT5b was indicated to promote migration, which was in keeping with our findings (21). Additional factors may be implicated in this process; these should be identified in future studies.

Surprisingly, we observed that the effect of IL-23 on Socs3 expression varied in different cells. IL-23 induced Socs3 expression in accordance with its conventional role and reduced the expression of Socs3 via another mechanism. It was shown previously that promoter methylation led to the attenuation of Socs3 expression in various kinds of tumors (25,26). In fact, some growth and inflammatory factors, such as insulin-like growth factor-I and tumor necrosis factor-α, were reported to change the status of methylation of the Socs3 promoter and further inhibit Socs3 expression (27).

The Li group also observed that IL-6 induced the DNMT1 gene and facilitated the methylation of Socs3 in the dysplastic epithelium of patients with ulcerative colitis (12). According to our own data, it was proved that IL-23 also modified the methylation of the Socs3 promoter and blocked the expression of Socs3, which further promoted the migration and invasion of SW-620 cells. Regarding intrinsic Socs3, this molecule was reported to impair tumor migration by enhancing cellular adhesion (28,29). The direct link between Socs3 and the downstream metastasis-associated pathway needs to be uncovered.

In conclusion, we showed that the promalignant role of IL-23-associated inflammation seemed to be complicated and restricted. Negative regulators, such as Socs3, might limit the action of inflammatory mediators. Only both the presence of proinflammatory factors and the absence of their respective inhibitors may lead to a synergistic effect on metastasis. Together with the malignancy of carcinomas, the increase in IL-23-associated proinflammatory factors and the attenuation of negative regulators may worsen the prognosis of cancer. This represents one of the procarcinogenic mechanisms that may become a novel target for cancer treatment. Finally, the combination of anti-inflammatory strategies with the recovery of intrinsic anti-inflammatory factors may bring about a promising era of anticancer therapy.

Supplementary material

Supplementary Table 1 and Figures I–4 can be found at http://carcin.oxfordjournals.org/

Funding


Acknowledgements

The authors would like to thank Prof. D.Hilton from the Walter and Eliza Hall Institute of Medical Research, the University of Melbourne for kindly providing pEF-Flag-Socs3 plasmid to strongly support this study.

Conflict of Interest Statement: None declared.
References

6. Oppmann, B. et al. (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity, 13, 715–725.

Received August, 11, 2013; revised January 10, 2014; accepted January 15, 2014.