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

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Interleukin-23 (IL-23) is a conventional proinflammatory IL-12-related cytokine (1) that plays a key role in the pathogenesis of many inflammatory diseases (2). IL-23 is a heterodimeric cytokine consisting of a unique p19 subunit produced by activated T cells, and a common p40 subunit released by dendritic cells and macrophages (3). IL-23 acts on IL-17-producing T cells, natural killer (NK) cells and γδ T cells to stimulate their proliferation and production of TNF-α, IL-17 and IL-22 (4). IL-23 is involved in the pathogenesis of many inflammatory diseases such as rheumatoid arthritis, psoriasis, Crohn’s disease and type 1 diabetes (5). However, the role of IL-23 in colorectal carcinoma (CRC) remains unclear.

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. Gene transfection 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)

Abbreviations: CRC, colorectal carcinoma; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; IL-, interleukin; IL-23R, IL-23 receptor; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA; Socs, suppressors of cytokine signaling; STAT, signal transducer and activator of transcription.

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and Jak2 inhibitor 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 at three random spots at 0 and 24 h. The migration distance of 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; 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 was prepared in lower chamber for facilitating the migration and invasion. Twenty-four hours later, the migratory or invasive cells on the basolateral side of the chamber were fixed by 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.

MIT assay

Cells proliferation was evaluated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-di-phenyltetrazolium 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 PrimeScriptTM RT Reagent kit (Takara) according to the manufacturer’s instructions. PCR reactions were performed with SYBR Premix Ex Taq II (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 45 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 GTC ACC CAC AGC AGT TTG-3‘ and reverse: 5‘-ACT GAG CAG CAC GAG TGG C-3‘; human DNMT-1, forward: 5‘-GGG CGG TAG AGT GGG AAT GG-3‘ and reverse: 5‘-TCA TCT TGT GAG TTC GGA-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-bodies were used: rabbit polyclonal antibody:anti-Socs3, pSTAT5, STAT5 (1:350) (Cell signaling technology, Boston, MA), DNMT1 (1:500) (Abcam, Cambridge, UK) and E-Cadherin (1:200), Vimentin (1:200) and N-Cadherin (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibody;β-actin (1:1000) (Zhongshan jinqiao, Beijing, China). The diluents of anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were 1:8000. The membrane was developed using Immobilon™ Western Chemiluminescent IP 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 4.4.0 (Bio-Rad).

Methylation-specific PCR

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-reated genomic DNA. The PCR was performed in a 25 μl volume containing 40ng bisulfite-modified DNA, 2x Master Mix (Biotek, Beijing, China) and 10 pmol specific primer mix (forward and reverse primers). Three regions of Socs3 promoter were targeted and each primer mix were as follow: primer set 1: methylated forward: 5’-GTT TCG TTG TCG TAG TAA GGT C-3‘ and reverse: 5‘-TAA AAT CCA CAA AAA AAT CCTG G-3‘; primer set 2: unmethylated forward: 5‘-TTT TGG TTG AGT TGG TAG TTA GTT G-3‘ and reverse: 5‘-TAA AAT CCA CAA AAA AAT CCTC C-3‘ (annealing at 56°C); primer set 2: methylated forward: 5‘-GGA GAT TTT AGG TTT CCG GAA TAT TTC-3‘ and reverse: 5‘-CCC CCG AAA CTA CCT AAA CGC CG-3‘ (annealing at 56°C); unmethylated forward: 5‘-GTT GGA GAT TTT AGG TTG GAA TAT TTC-3‘ and reverse: 5‘-AAA CCC CCA AAA GTA CCT AAA AAC CAC-3‘ (annealing at 56°C); primer set 3: methylated forward: 5‘-GTT GGA TTC TTA GGG TTG GGA TTC-3‘ and reverse: 5‘-GAC CTA AAA AAC CTC CGC AT-3‘ (annealing at 55°C); unmethylated forward: 5‘-GTT GGA TTC TTA GGG TTG GGA TTC-3‘ and reverse: 5‘-CAA CCT AAA AAC CTC CGC AT-3‘ (annealing at 55°C). Protocol consisted of one cycle at 95°C for 3 min followed by 50 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 (100ng/ 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 were performed in accordance with national 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 overexpressing 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 embedded 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 via 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 for immunofluorescence 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-12R1β 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-12R1β) secondary antibodies (1:100 dilution; Zhongshan jinqiao) for 1h. 4',6-diamidino-2-phenylindole (5 μg/ml) (Invitrogen, Eugene, OR) was used to stain nuclei. Rabbit isotype IgG (1:100) 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 x400.

All CRC tissue samples were fixed in 10% neutral-buffer formalin and embedded in paraffin. Then the embedded samples in paraffin were sliced and the sections were pretreated with 3% H2O2. The sections were blocked with rabbit-anti-human IL-23, pSTAT5 and Socs3 antibody (1:100; Santa Cruz Biotechnology) overnight at 4°C. Subsequently, the tissues were incubated with horseradish peroxidase-labeled secondary anti-rabbit immunoglobulin...
(1:800 dilution; Zhongshanjinqiao). 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 (1/3–2/3) 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

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ng/ml of IL-23, as

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 Socs3 expression and metastasis in IL-23 subgroup of CRC cases. A correlation analysis was used to analyze the relationship between the expression of IL-23 and Socs3 messenger 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-12R) 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 promigrative 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 STAT5 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 cells 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 IL-23-associated migration. 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 methylation inducer, 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
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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 rather than the other three cell lines. M denoted the methylated DNA, whereas U denoted the respective unmethylated DNA. Synthesized methylated or unmethylated DNA were used as positive and negative control, respectively. (D) Quantitative real-time PCR (left lane) and western blot (right lane) showing that 100 ng/ml of IL-23 did not inhibited Socs3 expression anymore when Socs3 gene methylation had been prohibited by 5-aza-2-deoxycytidine (DAC) compared with control. *0.01 < P < 0.05, **P < 0.01.

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### Fig. 2. Socs3 expression and its regulation in CRC cells stimulated with different doses of IL-23. (A) Quantitative real-time PCR showing that 40 and 100 ng/ml of IL-23 inhibited the expression of Socs3 in SW-620 cells, whereas they promoted the expression of Socs3 in SW-480, HT29 and HCT-116 cells. IL-23 could induce the expression of DNMT-1 in SW-620 rather than in the other three cell lines. IL-23 at 10 ng/ml did not yield any changes. *0.01 < P < 0.05, **P < 0.01. (B) Western blot showing the Socs3 and DNMT-1 expression in CRC cell lines. (C) Methylation-specific PCR showing that IL-23 increased the methylation of Socs3 gene in SW-620 rather than the other three cell lines. M denoted the methylated DNA, whereas U denoted the respective unmethylated DNA. Synthesized methylated or unmethylated DNA were used as positive and negative control, respectively. (D) Quantitative real-time PCR (left lane) and western blot (right lane) showing that 100 ng/ml of IL-23 did not inhibited Socs3 expression anymore when Socs3 gene methylation had been prohibited by 5-aza-2-deoxycytidine (DAC) compared with control. *0.01 < P < 0.05, **P < 0.01.
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 three 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.
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. In line with the observation in vitro, more metastases of SW-620 stimulated with IL-23 (IL-23-OE) were observed compared with control (Figure 5A and C, 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.
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To further address the effect of IL-23 on metastasis in vivo, anti-IL-23 neutralizing antibody was used. Though having no statistical 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). Treatment with anti-IL-23 antibody seemed to be a potential way to inhibit IL-23-induced SW-620 metastasis in our model.

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. 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). It was indicated that CRC cells themselves might act as a source of the local IL-23 in the tumor microenvironment.

![Fig. 5. Metastasis of SW-620 and SW-480 stimulated by IL-23 in vivo.](https://academic.oup.com/carcin/article-abstract/35/6/1330/450214)

(A) Lung metastases of SW-620 (left lane) and SW-480 (right lane) were indicated. More metastases were observed in IL-23 overexpressing SW-620 group (IL-23-OE, leftmost 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.
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 statistic 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 \textit{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 \textit{Carcinogenesis} Online). pSTAT5 seemed to involve in the progress of CRC as well.

\textbf{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; $\chi^{2}$ 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.
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 vivo 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 positive 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 prohibit 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 hypothesised 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, STAT3b 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-1 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 carcinoma, 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 1–4 can be found at http://carcin.oxfordjournals.org/

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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.

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