# TSU68 Prevents Liver Metastasis of Colon Cancer Xenografts by Modulating the Premetastatic Niche

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## Abstract

**The aim of this study was to investigate the inhibitory effect of TSU68 [(*Z*)-5-[(1,2-dihydro-2-oxo-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-propanoic acid; SU6668], an inhibitor of vascular endothelial growth factor receptor 2, platelet-derived growth factor receptor β, and fibroblast growth factor receptor 1 (FGFR1), on colon cancer liver metastasis, and to test the hypothesis that TSU68 modulates the microenvironment in the liver before the formation of metastasis. First, we implanted the highly metastatic human colon cancer TK-4 orthotopically into the cecal walls of nude mice, followed by twice-daily administration of TSU68 (400 mg/kg/d) or vehicle. Five weeks of treatment with TSU68 significantly inhibited liver metastasis compared with the control group ($P < 0.001$). Next, we analyzed the gene expression profile in premetastatic liver using microarrays. Microarray and quantitative reverse transcription-PCR analysis showed that mRNA levels for the chemokine *CXCL1* were significantly increased in tumor-bearing mice compared with non–tumor-bearing mice. Moreover, *CXCL1* expression was significantly decreased by TSU68 treatment. *CXCR2* expression was detected predominantly on tumor cells in orthotopic tumors compared with ectopic tumors. The number of migrating neutrophils in premetastatic liver was significantly decreased in the TSU68-treated group ($P < 0.001$). The amount of interleukin-12 (IL-12) p40 in the portal vein was significantly decreased by TSU68 ($P = 0.02$). Blockade of both CXCR2 and IL-12 p40 with a neutralizing antibody significantly inhibited liver metastasis. These results suggest that the CXCL1/CXCR2 axis is important in cancer metastasis and that TSU68 may modulate the premetastatic niche in the target organ through suppression of the inflammatory response, which might be an alternative mechanism used by antiangiogenic agents.** [Cancer Res 2008;68(23):9754–62]

## Introduction

The idea of targeting angiogenesis to treat tumors was first proposed more than 30 years ago (1). Since then, several approaches to block or disrupt tumor angiogenesis have been explored. Finally, in 2004, it was shown that the addition of bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF)-A, to conventional chemotherapy prolonged survival in patients with metastatic colorectal cancer compared with chemotherapy alone in a randomized, controlled phase III clinical trial (2). The original concept of antiangiogenesis was the inhibition of outgrowth of new blood vessels (3); however, it became more obvious that bevacizumab may affect the vasculature through various mechanisms: It (*a*) causes regression of the tumor vasculature, (*b*) normalizes the tumor vasculature, (*c*) inhibits the formation of new blood vessels, and (*d*) prevents recruitment of progenitor cells from the bone marrow (3–6). Understanding of these detailed mechanisms provided the rationale for combination therapy using antiangiogenic agents and cytotoxic chemotherapy (4, 7). Nowadays, various other kinds of antiangiogenic agents, such as anti-VEGF receptor antibodies (8), soluble VEGF receptors (9), and VEGF receptor tyrosine kinase inhibitors (10), have been developed. In particular, receptor tyrosine kinase inhibitors are small, synthetic, selective molecules that have favorable toxicity profiles, do not induce an immune response, and have an ability to inhibit multiple receptors simultaneously (11).

TSU68 [(*Z*)-5-[(1,2-dihydro-2-oxo-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-propanoic acid; SU6668] is a potent antiangiogenic agent that targets multiple tyrosine kinase receptors for VEGFR2, platelet-derived growth factor receptor β (PDGFRβ), and FGFR1 (12). Therefore, TSU68 can target not only endothelial cells (via VEGFR2, FGFR1, and PDGFRβ) but also tumor cells (via FGFR1 and PDGFRβ) and surrounding stromal cells (via PDGFRβ on pericytes or FGFR1 on fibroblasts; ref. 13). The strong potency of TSU68 for preventing colon cancer liver metastasis has been shown previously in some preclinical models (13–15); however, the detailed mechanisms by which TSU68 suppresses tumor metastases remain unclear.

Cancer metastasis depends on the interaction of tumor cells with the microenvironment at the site of metastasis (16). Recently, extensive studies have suggested that chemokines produced in metastatic organs may play a major role in mediating tumor metastasis (17–20). Moreover, tumor-associated cells, such as macrophages and hematopoietic bone marrow progenitors, prepare the "niche" in premetastatic organs and thus enhance metastasis (21–23). From these recent observations, we speculated that TSU68 might modulate the microenvironment in the liver before the formation of metastasis.

In this study, we showed the effect of TSU68 in preventing colon cancer liver metastasis using an orthotopic implantation model. Furthermore, to elucidate the possible role of this agent in modulating the premetastatic microenvironment in the liver, we analyzed the gene expression profile of liver in the premetastatic phase using GeneChip microarrays.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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## Materials and Methods

### Animals and Tumor Models

The following experiments were done according to the guidelines of the Committee on Experimental Animals of Hamamatsu University School of Medicine. TSU68 was kindly provided by Taiho Pharmaceutical Co. TK-4 is a solid tumor tissue strain with strong liver metastatic potential, which was derived from a human colon carcinoma obtained from a surgically resected specimen in our institution. TK-001, used as another model of colon carcinoma, has no liver metastasis ability. TK-4 was implanted orthotopically into cecal walls by suturing 120-mg tumor pieces with 6-0 Polysorb (Tyco Healthcare) or transplanting them s.c. into the right axillary regions of 6-wk-old male BALB/c nu/nu mice (Clea Japan), as previously described (24–27).

**Treatment protocol 1.** The animals were treated with TSU68 (400 mg/kg/d, twice daily, p.o.; $n = 19$) or vehicle ($n = 19$) from 7 d after orthotopic implantation. We defined the day on which treatment was initiated as day 0 (Supplementary Fig. S1*A*). After 5 wk of drug administration, mice were sacrificed and tumors were removed and weighed. Metastatic foci on the liver surface were counted macroscopically and were confirmed microscopically, as reported previously (28, 29).

**Treatment protocol 2.** Six-week-old mice received TK-4 tumor implantation orthotopically as described above or a sham operation in which the cecum was stitched with 6-0 Polysorb, without tumor tissue. From 1 wk after the operation, treatment with TSU68 or vehicle was initialized as in protocol 1 (Supplementary Fig. S1*A*). At days 4 and 7, mice were sacrificed and samples were removed. Livers were extracted from the left lateral lobe because metastasis is often observed in this area. All samples were fixed in formalin or treated with RNAlater (Applied Biosystems) for further analyses.

### Immunohistochemistry

For immunohistochemical analysis of Ki67, CD34, CXCL1, and CK20, formalin-fixed, paraffin-embedded sections (thickness, 4 μm) were used. For Mac-1 (CD11b) and NIMP R-14 staining, frozen sections were used. After irradiation in a hydrated autoclaving device at 121°C for 15 min in 10 mmol/L sodium citrate buffer (pH 6) to remove the masking effect of formalin fixation, the slides were rinsed with PBS and incubated with 0.3% $H_2O_2$ in absolute methanol for 10 min to quench endogenous peroxidase activity. After another washing step with PBS, antimouse CD34 antibody (1:50; Abcam), antihuman Ki67 antibody (1:50; DAKO), antimouse CXCL1 antibody (1:50; Abcam), antimouse NIMP-R14 antibody (1:100; Abcam), antimouse Mac-1 antibody (1:100; BD Pharmingen), or antihuman CK20 (1:50; DAKO) was applied. Immunocomplexes were detected with biotin-conjugated secondary antibodies, streptavidin-conjugated horseradish peroxidase, and diaminobenzidine, using Histofine (Nichirei). The sections were lightly counterstained with hematoxylin and mounted with a permanent mounting medium. TIFF images were obtained with a digital microscope BZ-8000 (Keyence) and converted into gray-scale images with Adobe Photoshop 7.0.1 (Adobe Systems, Inc.) for the extraction of stained areas. For microvessel density quantitation, the size of the CD34-positive area was determined by use of Scion Image software (Scion).[4] Tumor cell density was evaluated by calculating the size of the CK20-positive area per high-power field (HPF) using the same methods. For the evaluation of CXCL1 and CD11b, the number of positive cells in 10 HPFs was counted under a light microscope at ×400 magnification. For the evaluation of Ki67 labeling index, the ratio between the numbers of Ki67-positive cells and all tumor cells was calculated in 10 HPFs at ×400 magnification.

### RNA Extraction

Total RNA was isolated from mouse livers or cecal tumors using an Isogen kit (Wako), following purification with an RNeasy mini kit (Qiagen).

### Microarray Analyses

Biotin-labeled complementary RNA was synthesized from 5 μg of total RNA using the One-Cycle cDNA Synthesis Kit and the GeneChip IVT Labeling Kit (Affymetrix) according to the manufacturer's instructions. The labeled complementary RNA samples were hybridized to the complete Affymetrix mouse genome 430A 2.0 GeneChip set (Affymetrix). Signal intensities were detected using an Affymetrix GeneChip scanner and analyzed using GeneSpring GX 7.3.1 (Agilent). Three chips per group were hybridized, and the mean value was used for data comparison. Changes in gene expression of >2-fold were defined as significant.

### Quantitative Reverse Transcription-PCR Analysis

Total RNA was subjected to reverse transcription with random hexanucleotide primers and SuperScript Reverse Transcriptase II (Invitrogen). The resulting cDNA was subjected to real-time PCR using the Rotor-Gene 3000 System (Corbett Research) and a QuantiTect SYBR Green PCR kit (Qiagen; ref. 30). The primer sequences were as follows: 5′-GCTGGGATTCACCTCAAGAA-3′ and 5′-TCTCCGTTACTTGGGGACAC-3′ for mouse *CXCL1;* 5′-GCCTTCCGTGTTCCTACCC-3′ and 5′-TGCCTGCTTCACCACCTTC-3′ for mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); 5′-CTGCATCAGTGACGGTAAAC-3′ and 5′-CCTTTGGGCTGTTGTGCTTA-3′ for mouse stromal cell–derived factor 1 (*SDF-1*); 5′-ACTCCAATAACAGCAGGTCAC-3′ and 5′-TCAAAGCTGTCACTCTCCATG-3′ for human *CXCR2;* and 5′-GGGCTGCTTTTAACTCTGGT-3′ and 5′-TGATTTTGGAGGGATCTCGC-3′ for human *GAPDH*. Data were normalized to *GAPDH* mRNA levels.

### Reverse Transcription-PCR

cDNA was synthesized as described above, and human *CXCR2* cDNA was amplified by PCR using the oligonucleotide primer pair 5′-ACTCCAATAACAGCAGGTCAC-3′ and 5′-TCAAAGCTGTCACTCTCCATG-3′. PCR products were electrophoresed through 3.0% agarose gels containing ethidium bromide.

### Quantitative Analysis of Cytokines in Blood

Mice were treated in the same fashion described in protocol 2. Seven days after the initial treatment with TSU68, mice were anesthetized with ether and a midline incision was made in the upper abdomen. The portal vein was carefully exposed. After cutting off the vessel, the blood flowing out was suctioned and collected. Peripheral blood was obtained by pulling out the eye ball. Blood samples were applied to a Bioplex Cytokine Assay System (Bio-Rad) according to the manufacturer's instructions.

### Treatment with Neutralizing Antibodies against Human CXCR2 and Mouse Interleukin-12 p40 Subunit

Seven days after tumor implantation, an antihuman CXCR2 neutralizing antibody (48311 clone; R&D Systems) was administrated i.p. at a dose of 20 μg in 200 μL of PBS on alternate days ($n = 10$). The CXCR2 neutralizing antibody has been shown to inhibit CXCR2 effectively when used at 20 μg per mouse (31). Antihuman IgG (20 μg in 200 μL of PBS; R&D Systems) was used as a control ($n = 7$). After 5 wk of treatment, mice were killed and the livers collected for the evaluation of liver metastases. The antimouse interleukin (IL)-12 p40 neutralizing antibody (C17.8 clone; R&D Systems) was administrated i.p. at a total dose of 500 μg (100 μg in 200 μL of PBS twice a week, total of five times; $n = 9$). Antimouse IgG (100 μg in 200 μL of PBS; R&D Systems) was used as a control ($n = 6$). Five weeks after the initial treatment, mice were killed and the livers collected for the evaluation of liver metastases and CXCL1 expression.

### Statistical Analysis

Data are presented as mean ± SE. The statistical significance of differences was assessed with the Student $t$ test. $P < 0.05$ was considered statistically significant.

## Results

**Antitumor and antimetastatic effects of TSU68 (protocol 1).** To determine the antimetastatic and antitumor effects of TSU68, the colon cancer xenograft TK-4 was orthotopically transplanted

[4] http://www.scioncorp.com/

into the cecal walls of nude mice, and mice were treated with TSU68 (200 mg/kg/d) or vehicle alone as a control. After 5 weeks of treatment, the number of liver metastases was counted macroscopically and primary tumors were resected. As shown in Fig. 1*A*, no visible liver metastasis was observed in TSU-treated mice, whereas between one and four foci were seen (average, 1.28) in control animals (Fig. 1*A*). Immunohistochemical analysis confirmed that the liver metastases observed in control mice were positive for human specific epithelial marker CK20 (Fig. 1*B*) and thus those originated in transplanted human colon tumor. These results suggest an antimetastatic effect of TSU68 on colon cancer xenografts. The tumor cell density evaluated by human CK20 staining (Fig. 1*C*) was significantly lower in the TSU-treated group ($0.06 \pm 0.01$ mm$^2$) compared with the control group ($0.15 \pm 0.01$ mm$^2$; Fig. 1*D*). Immunohistochemistry for Ki67 and CD34 revealed a significant decrease in tumor cell proliferation and microvessel density, respectively, at the primary site in the TSU-treated group (Fig. 1*C* and *D*). Furthermore, the percentage of apoptotic cells in the primary tumor was greater in the TSU-treated group than in the control group (data not shown). These data suggest an antitumor effect of TSU68 on colon cancer xenografts.

**Gene expression changes in premetastatic livers (protocol 2).** Recently, some reports have emphasized the significance of microenvironmental changes in the metastatic site in advance of metastatic development (21, 22). In the present study, it also seems that TSU68 might alter the microenvironment in liver during the premetastatic phase. To examine this possibility, we sought to determine the premetastatic phase when micrometastasis has not occurred in the liver but the primary tumor is growing. Two weeks after tumor implantation, when the primary tumor had already begun to grow, no micrometastasis was observed in mouse livers (Supplementary Fig. S1*B*). Therefore, we defined day 0 to day 7 (0–2 weeks after tumor implantation) as the premetastatic phase (Supplementary Fig. S1*B*).

To address the genetic mechanisms by which TSU68 alters the microenvironment in liver during the premetastatic phase, we performed DNA microarray analyses. First, we established four different groups of mice: non–tumor-bearing and treated with vehicle alone (NT-Co), non–tumor-bearing and treated with TSU68 (NT-TSU), tumor-bearing and treated with vehicle (T-Co), and tumor-bearing and treated with TSU68 (T-TSU; Fig. 2*A*). Non–tumor-bearing mice were given a sham operation without tumor implantation, as described in Materials and Methods. The treatment was initialized 7 days after implantation in the same fashion as protocol 1. At day 7, mouse livers in all groups were resected and gene expression profiles were analyzed using microarrays. We extracted 1,947 genes that were increased in the T-Co group compared with the N-Co group and 700 genes that

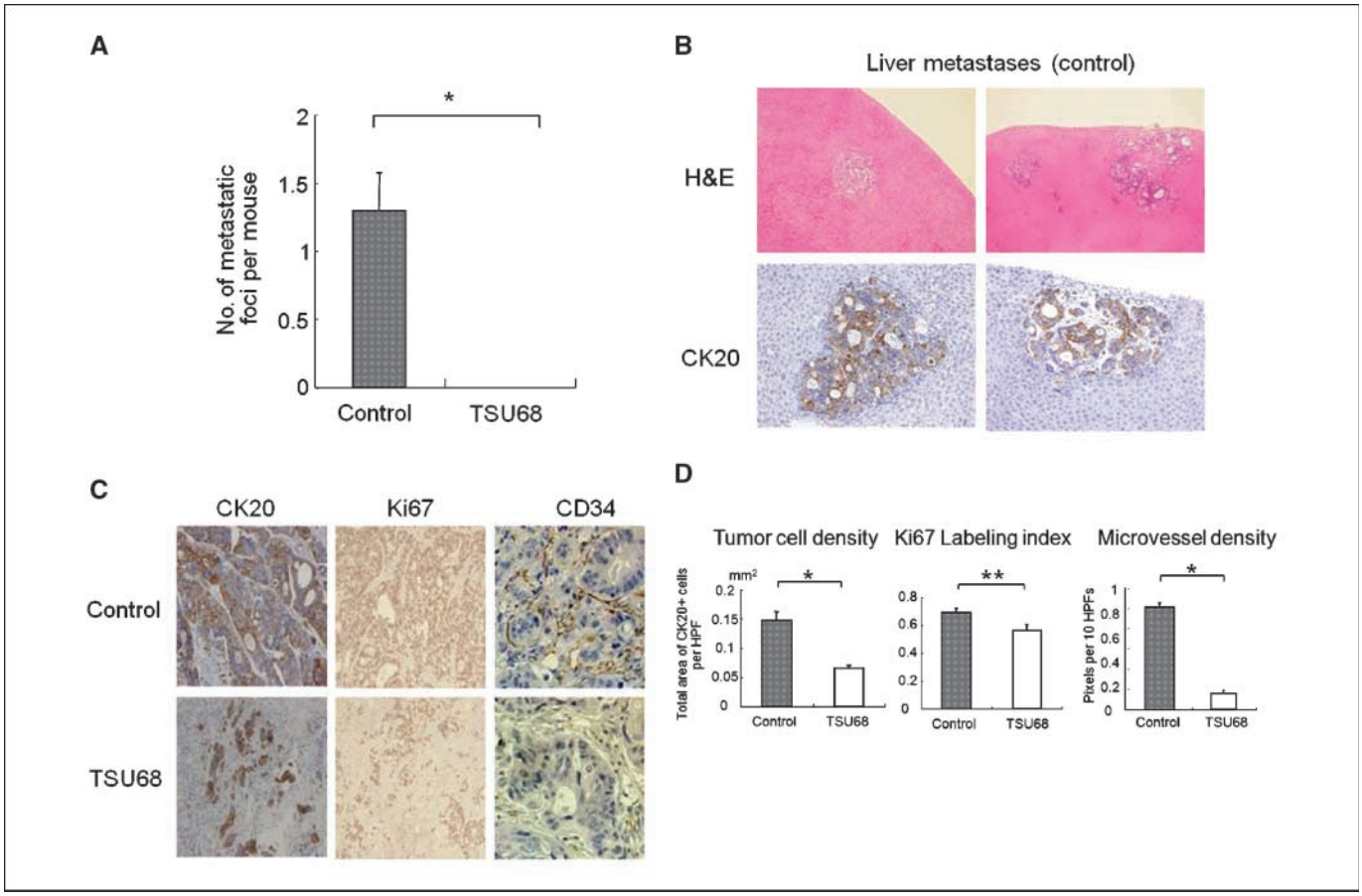


**Figure 1.** Protocol 1. The effect of TSU68 on primary tumors and in preventing liver metastasis. *A,* numbers of liver metastases in control ($n = 19$) and TSU68-treated mice ($n = 19$). *B,* histologic examination of liver metastases with H&E staining and CK20 immunostaining used as a human-specific epithelial marker. *C,* histologic examination of primary tumors with CK20 staining (×100 magnification), Ki67 immunostaining (proliferating tumor cells; ×100 magnification), and CD34 immunostaining (endothelial cells; ×400 magnification). *D,* quantitative evaluation of tumor cell density, Ki67 labeling index, and microvessel density in control and TSU68-treated mice. *Bars,* SE. *, $P < 0.01$; **, $P < 0.05$.

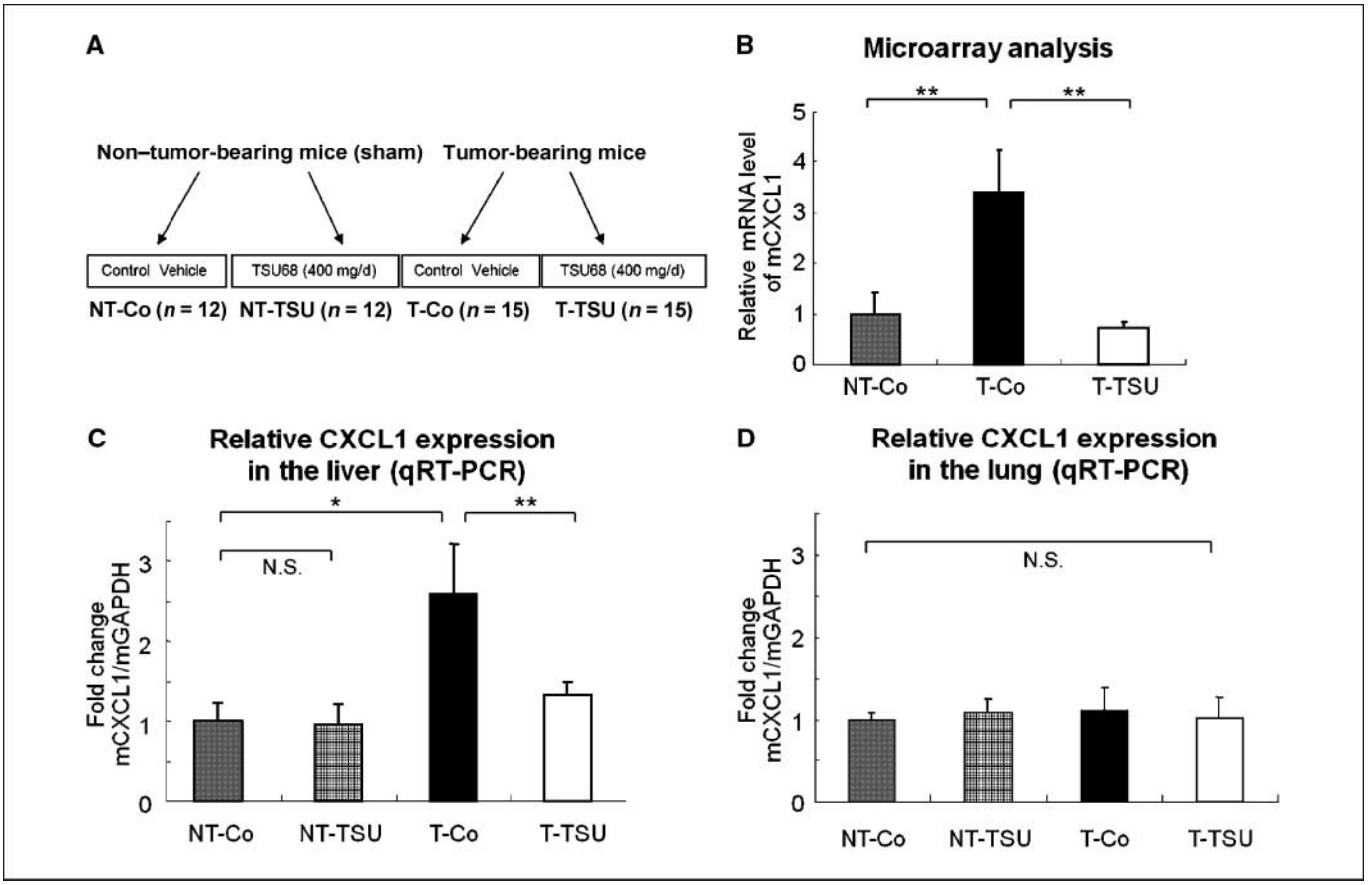


**Figure 2.** Protocol 2. *A,* schematic representation of the four groups of mice. Mice were divided into tumor-bearing and non–tumor-bearing groups, with a control group and TSU68-treated group for each type. *B,* relative mRNA levels of mouse CXCL1 (*mCXCL1*) obtained from microarray analysis. *C* and *D,* quantitative RT-PCR (*qRT-PCR*) analysis of mouse CXCL1 during the premetastatic phase in liver (*C*) and lung (*D*). Data are normalized to internal mouse GAPDH (*mGAPDH*). *Bars,* SE. *, $P < 0.01$; **, $P < 0.05$. *N.S.,* not significant ($P > 0.05$).

were decreased by TSU68 treatment. We further extracted 44 genes that showed a >2-fold difference in expression level (Table 1). Among these genes, we focused on *CXCL1*, one of the CXC chemokines, the expression level of which was decreased by about a quarter by TSU68 treatment (Fig. 2*B*), because a number of reports have shown that chemokines are closely related to cancer metastasis (17, 32, 33). In microarray analysis, *CXCL1* mRNA expression was 4-fold increased in the liver of T-Co mice compared with that of NT-Co mice, and this was suppressed by TSU68 treatment. *CXCL1* mRNA expression was confirmed by quantitative reverse transcription-PCR (RT-PCR), and it was consistent with the microarray data (Fig. 2*C*). The significant increase of *CXCL1* mRNA in the liver was also observed at day 4 (Supplementary Fig. S2*A*). The inhibitory effect of TSU68 was not significant, but there was a tendency toward inhibition at this time point. To determine whether these changes could be observed in other models, TK001 strain, which is also a solid tissue strain of human colon carcinoma with no ability of liver metastasis, was used. In the TK001 model, *CXCL1* mRNA was significantly increased in the liver of T-Co mice compared with that of NT-Co (6-fold) at day 7, but TSU68 failed to suppress this induction (Supplementary Fig. S2*B*). Interestingly, *CXCL1* mRNA expression was not affected in non–tumor-bearing mice by TSU68 treatment, regardless of tumor type or time point (Fig. 2*C*; Supplementary Fig. S2*A* and *B*). Moreover, no significant change of *CXCL1* mRNA was observed in the lung during the same phase (Fig. 2*D*; Supplementary Fig. S3*A* and *B*). To address the mechanisms underlying *CXCL1* induction in the liver, CXCL1 protein expression was evaluated. The immunohistochemical analysis revealed that CXCL1-positive cells recruited into the premetastatic liver were significantly increased in tumor-bearing mice, and this was not observed when treated by TSU68 (Fig. 3*A* and *B*). These data suggest that up-regulation of *CXCL1* observed specifically in the liver resulted from recruitment of CXCL1-positive cells that can be inhibited by TSU68 treatment. We also assessed mRNA expression of *SDF-1*, which is known as an important chemokine involved in the formation of the premetastatic niche (17–19). However, no significant changes were observed among the four groups in this study (Supplementary Fig. S3*C*).

**Migration of neutrophils into premetastatic liver.** The primary function of CXCL1 is the chemoattraction and activation of specific leucocytes expressing its receptor CXCR2 and the subsequent induction of various immunoinflammatory responses (32). To evaluate the neutrophil migration to the liver and the effect of TSU68, we examined immunohistochemical staining of neutrophil-specific marker NIMP-R14. The number of migrating neutrophils in the liver was increased in T-Co mice compared with NT-Co, and this was suppressed by TSU68 treatment (Fig. 3*C* and *D*). In addition, CD11b staining revealed that the number of macrophages recruited to the liver was also increased in T-Co mice compared with NT-Co mice, and this was suppressed by TSU68 treatment (Supplementary Fig. S4*A* and *B*). These data suggest that the cecal tumors recruit neutrophils as well as macrophages to the liver, and

**Table 1.** Gene list showing significant increases and decreases in gene expression levels in tumor-bearing mice following TSU68 treatment

| Fold change (T-Co > T-TSU) | NT-Co < T-Co > T-TSU |
|---|---|
| 0.111 | flavin-containing monooxygenase 3 |
| 0.191 | DNA-damage-inducible transcript 4 |
| 0.198 | squalene epoxidase |
| 0.23 | insulin-like growth factor binding protein 1 |
| 0.23 | chemokine (C-X-C motif) ligand 1 |
| 0.276 | transducer of ERBB2, 2 |
| 0.285 | early growth response 1 |
| 0.294 | S100 calcium binding protein A8 (calgranulin A) |
| 0.305 | metallothionein 1 |
| 0.321 | nicotinamide *N*-methyltransferase |
| 0.324 | lipocalin 2 |
| 0.326 | cytochrome *P*450, family 39, subfamily A, polypeptide 1 |
| 0.351 | peroxisome proliferator activated receptor, γ, coactivator 1α |
| 0.356 | chemokine (C-C motif) ligand 6 |
| 0.359 | S100 calcium binding protein A9 (calgranulin B) |
| 0.369 | complement component 1, q subcomponent, α polypeptide |
| 0.378 | Jun-B oncogene |
| 0.393 | orosomucoid 3 |
| 0.393 | DNase IIα |
| 0.396 | transformation related protein 53 inducible nuclear protein 1 |
| 0.398 | serum amyloid A2 |
| 0.413 | extracellular link domain containing 1 |
| 0.415 | metallothionein 1 |
| 0.42 | cysteine sulfinic acid decarboxylase |
| 0.424 | cytochrome *P*450, family 2, subfamily B, polypeptide 10 |
| 0.427 | orosomucoid 2 |
| 0.43 | glutamate oxaloacetate transaminase 1, soluble |
| 0.437 | RIKEN cDNA 1810015C04 gene |
| 0.444 | serine dehydratase-like |
| 0.448 | cytochrome *P*450, family 2, subfamily B, polypeptide 10 |
| 0.452 | chemokine (C-C motif) ligand 6 |
| 0.454 | cytotoxic T lymphocyte–associated protein 2α |
| 0.455 | CD9 antigen |
| 0.462 | UDP-Gal:βGlcNAcβ-1,3-galactosyltransferase, polypeptide 1 |
| 0.466 | serum amyloid A1 |
| 0.467 | apolipoprotein A-IV |
| 0.472 | tumor differentially expressed 1 |
| 0.473 | gap junction membrane channel protein α1 |
| 0.479 | transformation related protein 53 inducible nuclear protein 1 |
| 0.48 | serine (or cysteine) peptidase inhibitor, clade A member 3M |
| 0.482 | Jun oncogene |
| 0.486 | cytochrome *P*450, family 2, subfamily B, polypeptide 10 |
| 0.487 | lipopolysaccharide binding protein |
| 0.494 | nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor, ζ |

NOTE: These data were sorted by fold change between T-Co and T-TSU shown at the left.

TSU68 inhibits this recruitment. Furthermore, the CXCL1 protein level in peripheral blood was significantly increased in tumor-bearing mice (Supplementary Fig. S5*A* and *B*), suggesting that CXCL1 in the bloodstream may be one of the factors that mobilize neutrophils from bone marrow.

**Expression of the *CXCR2* gene in primary tumors.** CXCL1 has been shown to bind its receptor CXCR2 with high affinity (34). CXCR2 on tumor cells is known to contribute to their growth, survival, and motility (33). Thus, we confirmed the expression of *CXCR2* mRNA in primary cecal tumors by RT-PCR. Expression of *CXCR2* mRNA was observed in 8 of 14 (57.1%) samples from primary cecal tumors (Fig. 4*A*). Interestingly, when TK-4 was implanted into the subcutaneous space, its expression was detected in only 1 of the 14 samples (7.1%; Fig. 4*A*). These results indicate that the tumors are more likely to express *CXCR2* mRNA detectable by RT-PCR when implanted into orthotopic sites compared with ectopic sites. The expression level of *CXCR2* mRNA in each tumor was quantified by quantitative RT-PCR, and as expected, it was significantly increased in the cecal tumors compared with the subcutaneous tumors (Fig. 4*B*).

**Blocking CXCR2 with a neutralizing antibody.** To clarify the importance of the CXCL1/CXCR2 axis in the formation of metastasis, we inhibited human CXCR2 on tumor cells with a neutralizing antibody. After 5 weeks of treatment, we observed a significant decrease in liver metastasis compared with the human control IgG (Supplementary Fig. S6*A* and *B*). These data indicated that the CXCL1/CXCR2 axis is important in the regulation of colon cancer liver metastasis.

**Quantitative determination of cytokines in the portal vein.** Blood from the cecum is carried to the liver through the portal vein. Therefore, we speculated that some cytokines secreted by primary tumors might flow to the liver via the portal vein, resulting in an enhancement of metastasis, and that TSU might suppress this process. To assess this hypothesis, we quantified cytokines in the portal vein using a Multiplex Suspension Array System. Among the 32 cytokines we analyzed, only the IL-12 p40 subunit was significantly increased in the T-Co group, and this increase was suppressed by TSU68 treatment (Fig. 5*A* and *B*). These results suggest that the IL-12 p40 subunit secreted by primary tumors might be involved in the process of liver metastasis, and that this process could be impaired by TSU68 treatment.

**Blocking the IL-12 p40 subunit with a neutralizing antibody.** Because IL-12 p40 has been shown to abrogate the antitumor activity of IL-23, resulting in an enhanced tumorigenesis (35), and induce the activation of nuclear factor-κB (36), which plays a central role in the *CXCL1* induction (37), blocking of p40 would be considered to suppress tumor metastasis. To address this possibility, we used an antimouse IL-12 p40 neutralizing antibody in the TK-4 model. As expected, significant inhibition of liver metastasis was observed following IL-12 p40 antibody treatment compared with control IgG treatment (Supplementary Fig. S6*C* and *D*). These data suggest that IL-12 p40 plays an important role in the development of liver metastasis.

## Discussion

The pathogenesis of cancer metastasis depends on the multiple favorable interactions of metastatic cells with host homeostatic mechanisms (16). In 1889, Paget described the concept of "seed" (tumor cell) and "soil" (specific organ) for the nonrandom metastasis of breast cancer to specific organs (38). This theory

states that different organs provide growth conditions optimized for specific cancers, and the outcome of metastasis depends on multiple interactions of metastatic cells with homeostatic mechanisms, which the tumor cells can usurp (16). In our experiment, CXCR2, which contributes to the growth, survival, and motility of tumor cells, was more frequently expressed in tumors growing in the cecum than in those growing in the subcutaneous space (Fig. 4*A* and *B*). Greene and colleagues (39) reported that tumors growing at orthotopic sites exhibit higher levels of epidermal growth factor receptor, basic fibroblast growth factor, IL-8, type IV collagenase, and the multidrug resistance (*mdr-1*) gene than those growing in the subcutaneous space; this change in expression would affect tumor growth, invasion, and angiogenesis. These observations indicate that cancer cells can exhibit an innate malignant phenotype when they grow in an optimal microenvironment, in which they can express various metastasis-related factors including CXCR2. It is also suggested that orthotopic implantation is mandatory for the assessment of cancer metastasis.

In 44 genes extracted from microarray data, which were increased in mice in the tumor-bearing group and decreased in those in the TSU68-treated group (Table 1), some of them are metabolism-related genes, such as flavin-containing monooxygenase 3 or squalene epoxidase. The expression changes of those genes might be the effect of drug metabolism because TSU68 is metabolized in the liver. Therefore, these genes were excluded for further analysis. We also excluded genes that have not been reported to be involved in cancer progression and metastasis. Among the remaining chemokines, chemoattractants, and oncogenes, the chemokine *CXCL1* showed the most marked fold change following TSU68 treatment, so we focused on this factor for further analysis.

CXCL1 is known to play a major role in inflammation, wound healing, angiogenesis, and tumorigenesis (40). It showed an ~3-fold increase at day 7 in the livers of TK-4-bearing mice, in concert with *CXCR2* expression in primary cecal tumors. We also showed significant inhibition of liver metastasis by a CXCR2 neutralizing antibody (Supplementary Fig. S6*A* and *B*). These observations strongly suggest that the CXCL1/CXCR2 axis plays an important role in cancer progression and metastasis. Moreover, we also observed the up-regulation of *S100A8* and *S100A9,* which are known to act as strong chemoattractants for monocytes and neutrophils (41), in premetastatic liver (Table 1). Hiratsuka and colleagues showed that VEGF-A, transforming growth factor β, and tumor necrosis factor α, which are released from primary

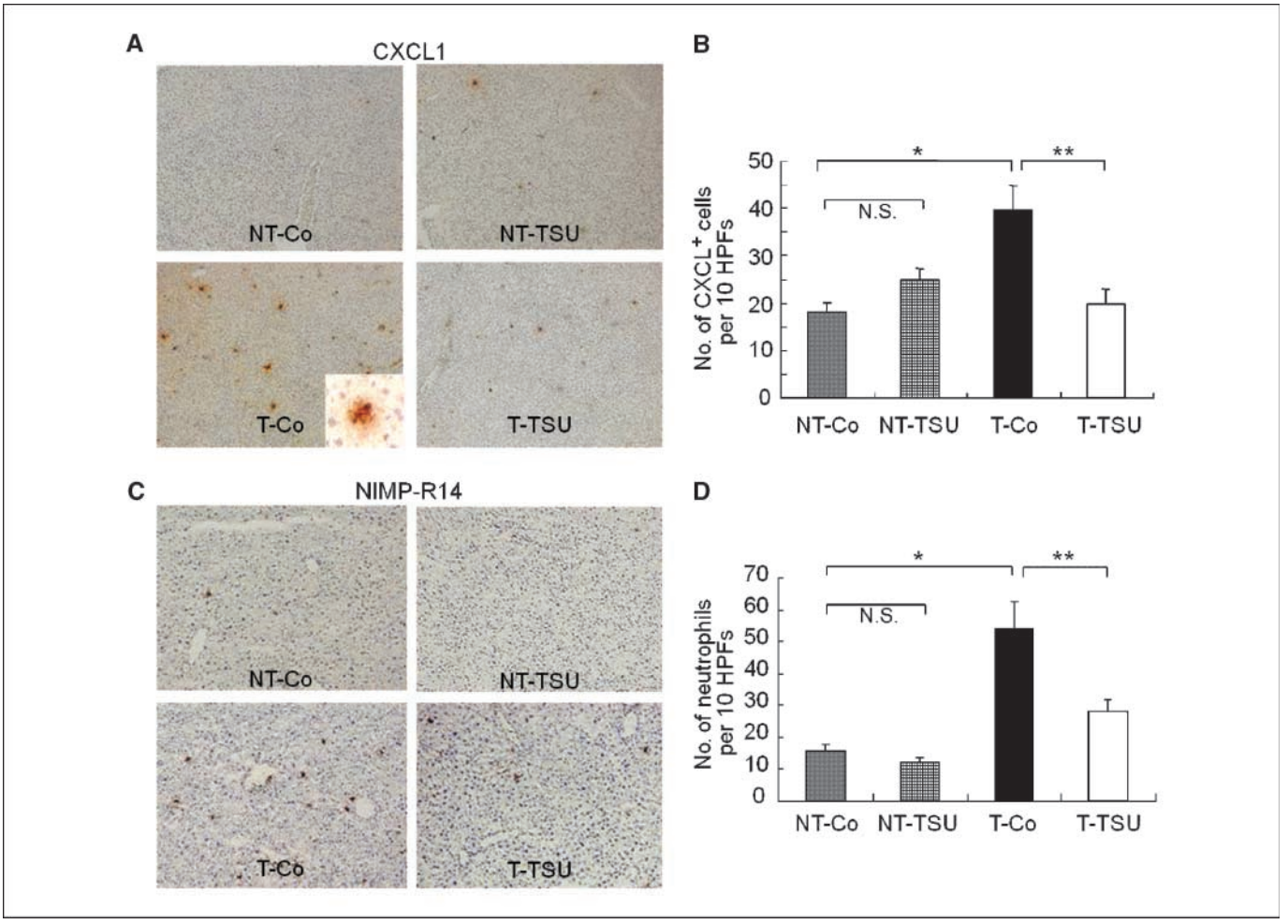


**Figure 3.** *A,* immunohistochemical staining of mouse CXCL1 in premetastatic liver (magnification, ×100). *Inset,* high magnification (×400). *B,* quantitative analysis of CXCL1-positive cells per 10 HPFs in premetastatic liver. *C,* immunohistochemical staining of mNIMP-R14 as a marker of neutrophils (magnification, ×100). *D,* quantitative evaluation of migrated neutrophils. *Columns,* mean number of neutrophils per 10 HPFs; *bars,* SE. *, $P < 0.01$; **, $P < 0.05$. *N.S.*, not significant ($P > 0.05$).

tumor cells, induce the expression of *S100A8* and *S100A9* in lung endothelium and myeloid cells, thereby facilitating the homing of tumor cells to the premetastatic sites within the lung parenchyma. Their results suggest that tumor metastasis may be dependent on a selective induction of organ-specific chemoattractants, which can be induced by specific soluble factors released from the primary tumors. In the present study, we observed an elevated level of IL-12 p40 subunit in the portal veins of tumor-bearing mice (Fig. 5*A* and *B*) and the antimetastatic effect of antimouse IL-12 p40 neutralizing antibody (Supplementary Fig. S6*C* and *D*). IL-12 p40 is known to act as an antagonist of IL-23 and abrogates IL-23–mediated antitumor effects (35). It is also reported that IL-12 p40 can induce the activation of nuclear factor-κB (36), which plays a central role in the induction of *CXCL1* (37). These data support our hypothesis that IL-12 p40 may play an important role in the development of metastasis by inducing CXCL1. In the present study, however, we could not confirm the suppression of *CXCL1* in the liver by IL-12 p40 neutralization (data not shown). Although further verifications are needed, treatments targeting IL-12 p40 may have potential for the prevention of liver metastasis.

Moreover, it is also suggested from recent observations that other soluble factors might be involved in the establishment of the premetastatic niche. In a previous study, we confirmed that TK-4 expresses VEGF-A, cyclooxygenase (COX)-1, COX-2 (24), and the active form of matrix metalloproteinase-2 (42). COX-2 expression was significantly up-regulated in the orthotopically implanted tumors and was positively correlated with the number of liver metastases (24). Kaplan and colleagues (23) have shown that VEGF and placental growth factor derived from tumor cells up-regulate fibronectin in resident fibroblasts, which results in the activation of VEGFR1-positive hematopoietic bone marrow progenitor cells in premetastatic organs, providing a permissive niche for incoming tumor cells. Taken together, these findings suggest that various tumor-derived soluble factors provide a premetastatic niche in target organs to facilitate the homing of cancer cells, which is vital for the development of cancer metastasis.

We have also shown that TSU68 significantly suppresses CXCL1 expression in the premetastatic liver. TSU68 also decreased *S100A8* and *A9* expression (Table 1). The reduction in the levels of these chemokines/chemoattractants may lead to a suppression of the homing of CXCR2-expressing neutrophils and tumor cells to the liver. Fidler (16) emphasized that therapy for metastasis should be targeted not only against tumor cells but also against the host microenvironment that contributes to and supports the progressive growth and survival of metastatic cancer cells. Our results may indicate that antiangiogenic agents can modulate the microenvironment in target organs through suppression of the inflammatory response, leading to marked inhibition of liver metastasis, which may be an alternative mechanism of action used by these agents.

The mechanism by which TSU68 suppresses CXCL1 expression in the liver remains to be elucidated; however, it can be explained by several hypotheses. First, the number of tumor cells and endothelial cells that can produce various cytokines might be reduced by TSU68 treatment. The marked decrease in tumor cell density and microvessel density in the primary tumors observed in this study (Fig. 1*C* and *D*) might reflect such a reduction. A second possibility is that TSU68 might reduce the influx of these cytokines into drainage vessels. Jain and colleagues (7, 43, 44) have argued that anti-VEGF therapies can reduce interstitial fluid pressure and decrease the shedding of metastatic cells and growth factors out of tumors through a vascular normalization mechanism. Indeed, in our preliminary data, TSU68 could induce tumor vessel normalization and reduce interstitial fluid pressure (data not shown). In addition, we have shown that the protein level of the

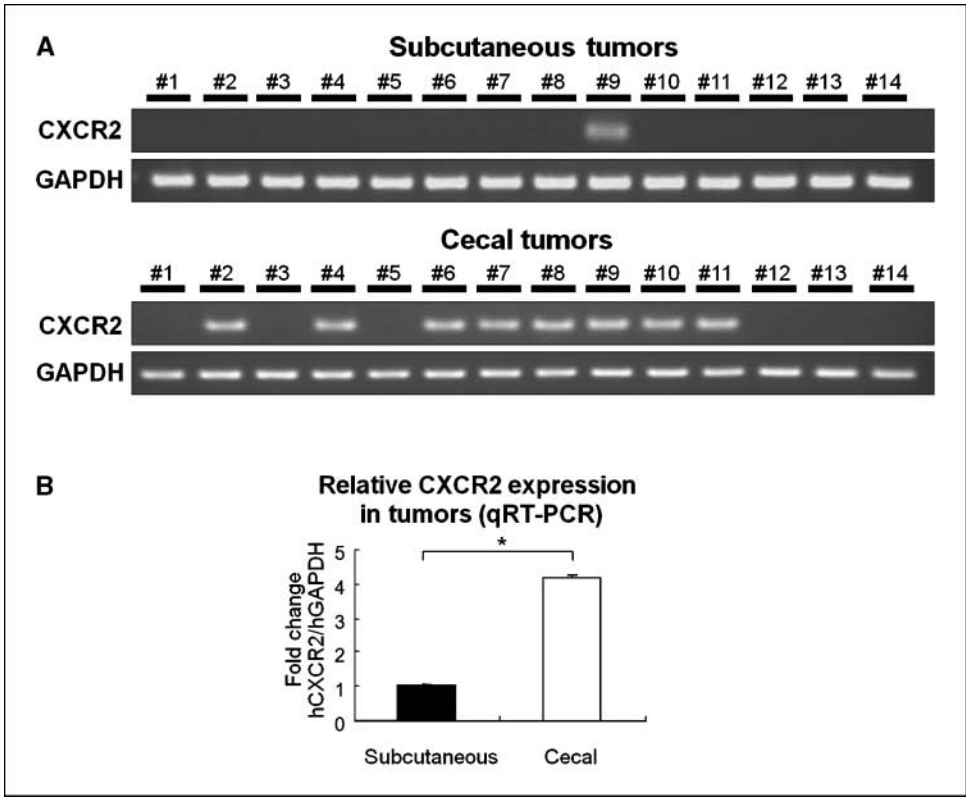


**Figure 4.** *A,* expression of human CXCR2 mRNA in tumors growing in ectopic sites (subcutaneous; *top*) and orthotopic sites (cecum; *bottom*), which were analyzed by RT-PCR. Expression of human CXCR2 mRNA was observed in 8 of 14 (57.1%) samples from the cecal tumors, whereas its expression was detected in only 1 of 14 (7.1%) samples from the tumors growing in the subcutaneous space. *B,* quantitative RT-PCR analysis of human CXCR2 in each tumor. Data are normalized to internal human GAPDH. *Bars,* SE. *, $P < 0.01$.

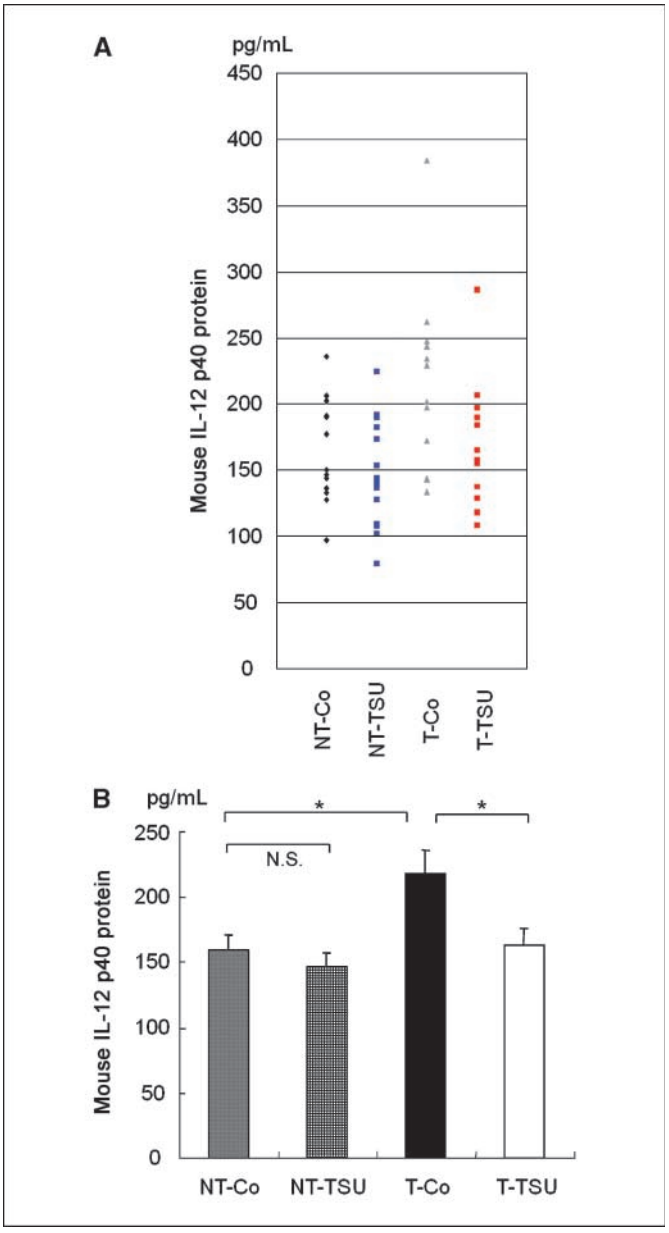


**Figure 5.** The amount of IL-12 p40 subunit protein in the portal vein was elevated in tumor-bearing mice and decreased in TSU68-treated mice. Data are shown in a scatter plot view (*A*) and as a bar graph (*B*). *Bars,* SE. *, $P < 0.05$. *N.S.*, not significant ($P > 0.05$).

IL-12 p40 subunit in portal veins was decreased by TSU68 (Fig. 5*A* and *B*). These results suggest that TSU68 might suppress the influx of various cytokines into the drainage vessels by means of the vascular normalization mechanism. Third, TSU68 might directly target the bone marrow–derived endothelial progenitor cells recruited into premetastatic liver, resulting in a decrease in their production of CXCL1. Gao and colleagues have reported that endothelial progenitor cells are involved in the angiogenic switch in lung metastasis (45). They mentioned that the efficacy of antiangiogenic inhibitors in patients with metastatic tumors may be a consequence of directly targeting bone marrow–derived endothelial progenitor cells as well as the nascent tumor vasculature. In their report, *CXCL1* expression was up-regulated ~2.5-fold in tumor-recruited bone marrow–derived endothelial progenitor cells. It is necessary to address the role of bone marrow–derived endothelial progenitor cells in liver metastasis and whether TSU68 could target bone marrow–derived endothelial progenitor cells directly.

In summary, we have shown that the CXCL1/CXCR2 axis has an important role in colon cancer liver metastasis and that TSU68 significantly inhibits CXCL1 expression in the premetastatic liver, leading to a marked inhibition in liver metastasis. These results suggest that antiangiogenic agents can modulate the premetastatic niche in target organs. Interruption of these interactions between the tumor cells and the host environment by antiangiogenic agents can lead to the inhibition or regression of cancer metastasis. These results provide a new rationale for the use of antiangiogenic agents to treat cancer metastasis and may lead to development of new strategies aimed at targeting premetastatic niches.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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