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IL-33/ST2 pathway contributes to metastasis of human colorectal cancer

Xuejian Liu [a], Leilei Zhu [b], Xin Lu [b], Hairong Bian [b], Xia Wu [a], Wenchuan Yang [a], Qingliang Qin [b,*]

[a] Department of Oncology, The People's Hospital of Linyi Economic and Technological Development Zone, Linyi 276000, China
[b] Department of Radiotherapy, East Hospital, Tongji University School of Medicine, Shanghai 200120, China

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ABSTRACT

Interleukin-33 (IL-33) was recently implicated in cancer pathogenesis. However, the possible effect of IL-33 on tumor progression of colorectal cancer (CRC), which is one of the most commonly diagnosed and lethal cancers worldwide, was still unclear. Here we evaluated the potential role of IL-33/ST2 pathway in metastasis of human CRC. We found an elevated expression of IL-33 and ST2 in tumor tissues of CRC patients. Higher expressions of IL-33 and ST2 were observed in poor-differentiated human CRC cells. Of note, IL-33 stimulation promoted the invasion of human CRC cells in a dose dependent manner. Enhanced IL-33/ST2 signaling promoted CRC metastasis, while attenuated IL-33/ST2 signaling decreased CRC metastasis. In consistent, enforced IL-33 expression in human CRC cells enhanced their growth, metastasis and reduced the survival time in nude mice, while decreased IL-33 expression in human CRC cells inhibited their growth, metastasis and prolonged the survival time in nude mice. Finally, we observed an increased expression of IL-6, CXCR4, MMP2 and MMP9 in response to IL-33/ST2 signaling in human CRC cells, which were crucial for the enhanced metastasis by IL-33 stimulation. Collectively, our findings demonstrated that IL-33/ST2 pathway could contribute to the metastasis of human CRC, which could enlarge the understanding of CRC pathogenesis and provide clues for developing new CRC therapeutics.

1. Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed and lethal cancers worldwide [1]. Currently, surgical resection is a major treatment and highly effective for localized CRC [2]. However, up to 30–40% of patients can develop recurrence even within the first few years after initial surgery [2]. Within CRC patients, about 60% have liver metastasis [3]. The CRC patient's 5-year post-surgical survival chances could fall from 90% to 10% or even less after metastasis [4]. Thus, identification of critical effectors involved in CRC metastasis was essential and might ultimately aid the clinical treatment of CRC patients.

Interleukin-33 (IL-33), a member of the IL-1 family of cytokines, could bind to Toll-interleukin 1 (IL-1) receptor (TIR) domain-containing receptor ST2 and induce NF-kB and MAPK activation [5,6]. Accumulating data showed that IL-33/ST2 signaling was implicated in cancer growth and metastasis [5]. As such, IL-33/ST2 axis could promote breast cancer growth and metastases by facilitating intratumoral accumulation of immunosuppressive and innate lymphoid cells [7]. High level of serum IL-33 was reported to be a diagnostic and prognostic marker of non-small cell lung cancer and hepatocellular carcinoma [8,9]. Of interest, IL-33 blockade reduced mucositis and enabled prolonged irinotecan (CPT-11) treatment of ectopic CT26 colon carcinoma in mice, suggesting that IL-33/ST2 pathway was participated in CRC pathogenesis [10]. However, whether IL-33/ST2 pathway was involved in the metastasis of CRC remains undefined.

In this study, we evaluated the potential role of IL-33/ST2 pathway in human CRC metastasis. Our findings demonstrated an important role of IL-33/ST2 interaction in metastasis of human CRC cells, which could enlarge the understanding of CRC pathogenesis and facilitate the development of novel CRC therapeutics.

2. Materials and methods

2.1. Patients

The Ethics Committee of Tongji University approved the study with patients. Fifty-two CRC patients were enrolled and given written informed consent. Pathology reports confirmed the diagnosis and provided the differentiation state of tumor cells. Subjects with autoimmune diseases or infections were excluded.

* Corresponding author at: Department of Radiotherapy, East Hospital, Tongji University School of Medicine, 150 Jimo Road, Pudong New Area, Shanghai 200120, China. Fax: +86 21 58798999.
E-mail address: qlqintj@126.com (Q. Qin).

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2.2. Reagents and cell culture

The human CRC cells were isolated from surgical tumor tissues by the Cancer Cell Isolation Kit (Panomics) according to the manufacturer's instructions. SW620 cells were obtained from ATCC and maintained in our lab. Cells were cultured at 37 °C under 5% CO_2 in complete RPMI 1640 medium (GIBCO) containing 10% heat-inactivated fetal bovine serum supplemented with 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin sulfate. For metastasis, CRC cells (2×10^5/ml) were stimulated with recombinant IL-33 protein (Biologend) for 48 h and detected for their invasion. Human full-length IL-33 and ST2 expression vectors were purchased from Sino Biological Inc. Human IL-33 shRNA vector was purchased from Santa Cruz Biotechnology. Human ST2 shRNA vector was purchased from Invivogen. Neutralizing antibody for human ST2, IL-6 and CXCR4 were purchased from R&D Systems. Transfection of CRC cells was achieved using the Amaxa Nucleofector Kit or according to the manual's instructions. MMP inhibitor GM6001 was purchased from Merck Millipore.

2.3. Real-time PCR

Quantitative Real-time RT-PCR was performed as previously described [11,12]. All the primers and probes were obtained from Applied Biosystems. Total RNA was extracted using TRIzol reagent. cDNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa). Quantitative RT-PCR (qRT-PCR) analyses were carried out in duplicate to detect mRNA expression using SYBR Premix Ex Taq (TaKaRa), and β-actin was used as an internal control.

2.4. Invasive assay

The invasion assay was performed using BD Biocoat Matrigel Invasion Chamber assay (8 μm, BD Bioscience) as previously described [13]. Briefly, the Matrigel inserts were rehydrated and 5×10^4 of testing cells were resuspended in 0.5 mL of serum-free media and then seeded onto the upper chamber of Matrigel-coated filters. In the lower chambers, 0.75 mL of complete medium was added as a chemoattractant. The whole chamber was placed in one well of a 24-well plate, and cells were cultured in routine conditions. After 24 h, the cells on the upper side of the chamber were scraped, and the ones on the lower side of the chamber were fixed by methanol, stained with hematoxylin, and invaded cells were counted under the microscope. Five predetermined fields were counted for each membrane, and the mean values from three independent experiments in duplicates were used. Data are expressed as the percentage of invasion through the Matrigel Matrix and membrane relative to the migration through the control membrane according to the manufacturer's manual.

2.5. Flow cytometry

The protein expression levels of IL-33 and ST2 in primary CRC cells were analyzed with flow cytometry. Briefly, 5×10^5 cells were stained with FITC-conjugated anti-human IL-33 antibody (Sino Biological Inc.) or PE-conjugated anti-human ST2 antibody (MBL International) after fixation and permeabilization (BD), and analyzed on a FACSCalibur flow cytometer (BD). Appropriate isotype controls were used and all data were analyzed with FlowJo software (Tree Star).

2.6. In vivo experiments

Approval for mouse experiments was obtained from the Institutional Animal Care and Use Committee of Tongji University. BALB/c nude mice of 6–8 weeks old were purchased from Shanghai SLAC laboratory Animal Co. Ltd. and housed under specific pathogen-free conditions. In brief, 3×10^6 SW620 cells were suspended in 0.2 ml PBS and injected subcutaneously into the nude mice. Tumors volumes were measured every 5 days following tumor challenge using vernier calipers and were presented as the means ± SD. Thirty days after challenge, metastasis index to lung was graded as 1–4 and calculated as previously described [13]. Specifically, each metastasis less than 0.5 mm in diameter was graded as 1, between 0.5 and 1 mm as Grade 2, between 1.0 and 2 mm as Grade 3, and >2 mm as Grade 4. All the grade scores were then added to determine the metastatic index for a given animal, and the mean index was then calculated for a given control or experimental group of animals.

2.7. Statistical analyses

Statistical evaluation was performed using *T* tests or two-way analysis of variance (ANOVA) using the program PRISM 6.0 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered as statistical significant.

3. Results

3.1. Elevated expression of IL-33 and ST2 in tumor cells of CRC patients

To evaluate the potential role of IL-33/ST2 pathway in CRC metastasis, we detected the expression of IL-33 and ST2 in tumor tissues and adjacent tissues of CRC patients. As shown in Fig. 1A and B, the expression of IL-33 and ST2 was significantly higher in tumor tissues than that in adjacent tissues ($P < 0.05$). Of note, the expression levels of IL-33 and ST2 in tumor tissues were associated with the clinical stages of CRC patients (Fig. 1C and D, $P < 0.05$). When primary CRC cells were isolated from the surgical tissues and detected for their mRNA expressions of IL-33 and ST2, we found that IL-33 and ST2 mRNA expression levels in poor-differentiated CRC cells were significantly higher than that in medium- and well-differentiated CRC cells (Fig. 1E and F, $P < 0.05$). We then detected the protein levels of IL-33 and ST2 in primary CRC cells and found similar results (Fig. 1G and H, $P < 0.05$). These results suggested an involvement of IL-33/ST2 pathway in CRC metastasis.

3.2. Enhanced IL-33/ST2 pathway promoted the metastasis of human CRC

When human primary CRC cells were treated with an increasing dose of IL-33, a dose dependent manner of enhanced CRC invasion was observed (Fig. 2A, $P < 0.05$). An increased expression of ST2 of primary CRC cells was achieved by the transfection of ST2 expression vector (Fig. 2B, $P < 0.05$). Of important, enforced ST2 expression on primary CRC cells further promoted their invasion induced by IL-33 stimulation (Fig. 2C, $P < 0.05$). In consistent, transfection with IL-33 expression vector also resulted in enhanced invasion of primary CRC cells (Fig. 2D and E, $P < 0.05$). To detect this phenomenon in vivo, nude mice were challenged with SW620 cells that were stably transfected with IL-33 expression vector. We found that enforced IL-33 expression could significantly promote the tumor growth and metastasis of SW620 cells in nude mice (Fig. 2F and G, $P < 0.05$). Further, enhanced IL-33 expression in SW620 cells decreased the survival time of tumor bearing nude mice (Fig. 2H, $P < 0.05$).

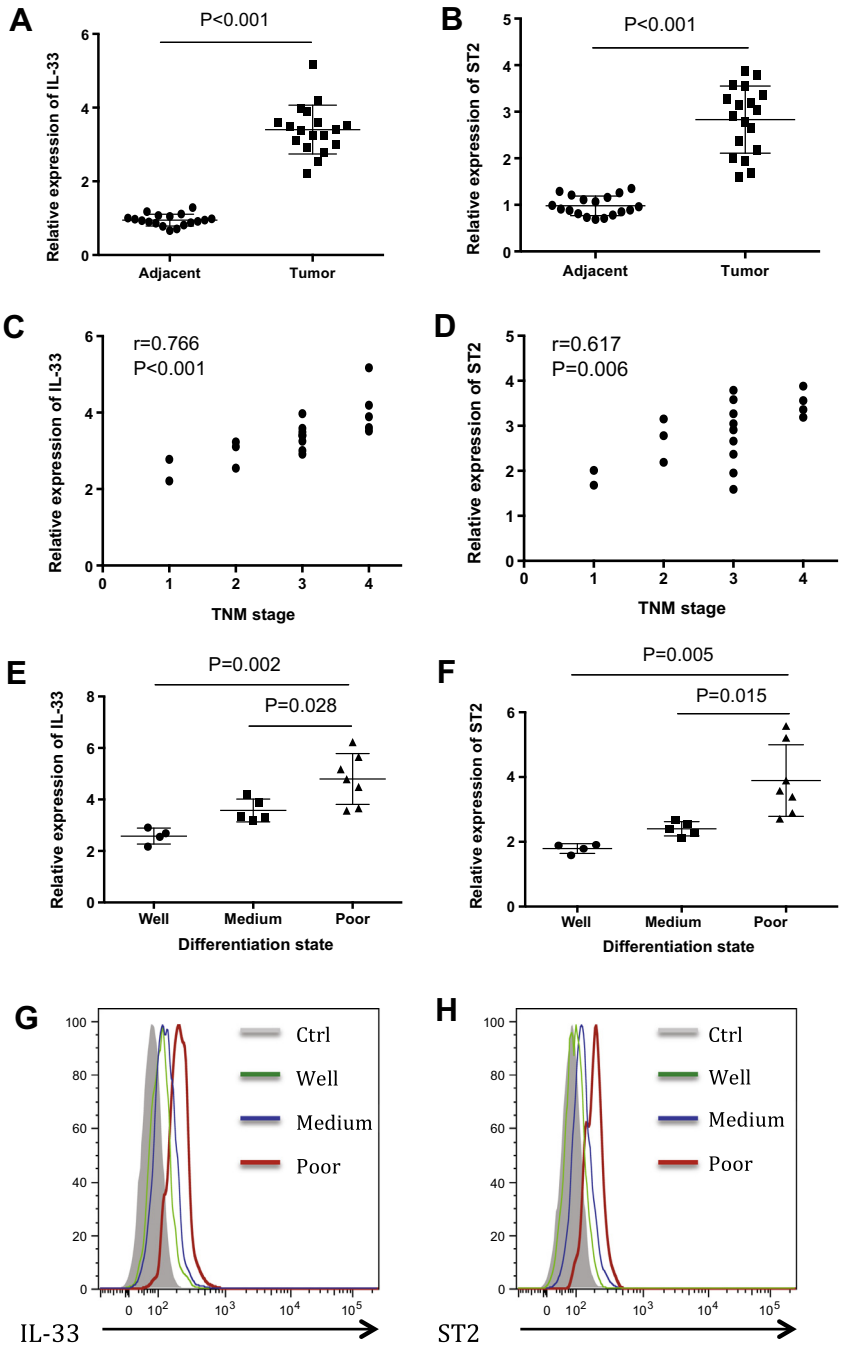

Fig. 1. IL-33 and ST2 expression in tumor cells of CRC patients. (A and B) The expressions of IL-33 and ST2 in tumor tissues and adjacent tissues of CRC patients ($n = 18$) were detected using q-PCR. (C and D) The correlation of IL-33 and ST2 expression levels with the TNM stage of CRC patients ($n = 18$) was analyzed. Each dot represented the results from one patient. (E and F) The mRNA expression levels of IL-33 and ST2 were analyzed for their correlation with the differentiation state of primary CRC cells isolated from surgical tissues ($n = 16$). Each dot represented the results from one patient. (G and H) The protein expression levels of IL-33 and ST2 in different differentiation state of primary CRC cells were analyzed using flow cytometry.

3.3. Blockade of IL-33/ST2 pathway inhibited the metastasis of human CRC

We observed that ST2 neutralization abrogated the enhanced CRC metastasis by IL-33 stimulation in vitro (Fig. 3A, $P < 0.05$). We then down-regulated ST2 expression in primary CRC cells by transfection with ST2 shRNA (Fig. 3B, $P < 0.05$). We found that decreased ST2 expression effectively inhibited the enhanced CRC invasion by IL-33 stimulation (Fig. 3C, $P < 0.05$). In consistent, transfection with IL-33 shRNA reduced the invasion of primary CRC cells (Fig. 3D and E, $P < 0.05$). When nude mice were challenged with SW620 cells that were stably transfected with IL-33 shRNA, we found that IL-33 shRNA significantly inhibited the tumor progression of SW620 cells and prolonged the survival time of nude mice (Fig. 3F–H, $P < 0.05$).

3.4. IL-33/ST2 pathway modulated expressions of IL-6, CXCR4, MMP2 and MMP9 in human CRC

To further explore the possible mechanisms involved in enhanced CRC metastasis by IL-33/ST2 pathway, we determined the expression of IL-6, CXCR4, MMP2 and MMP9 in primary CRC

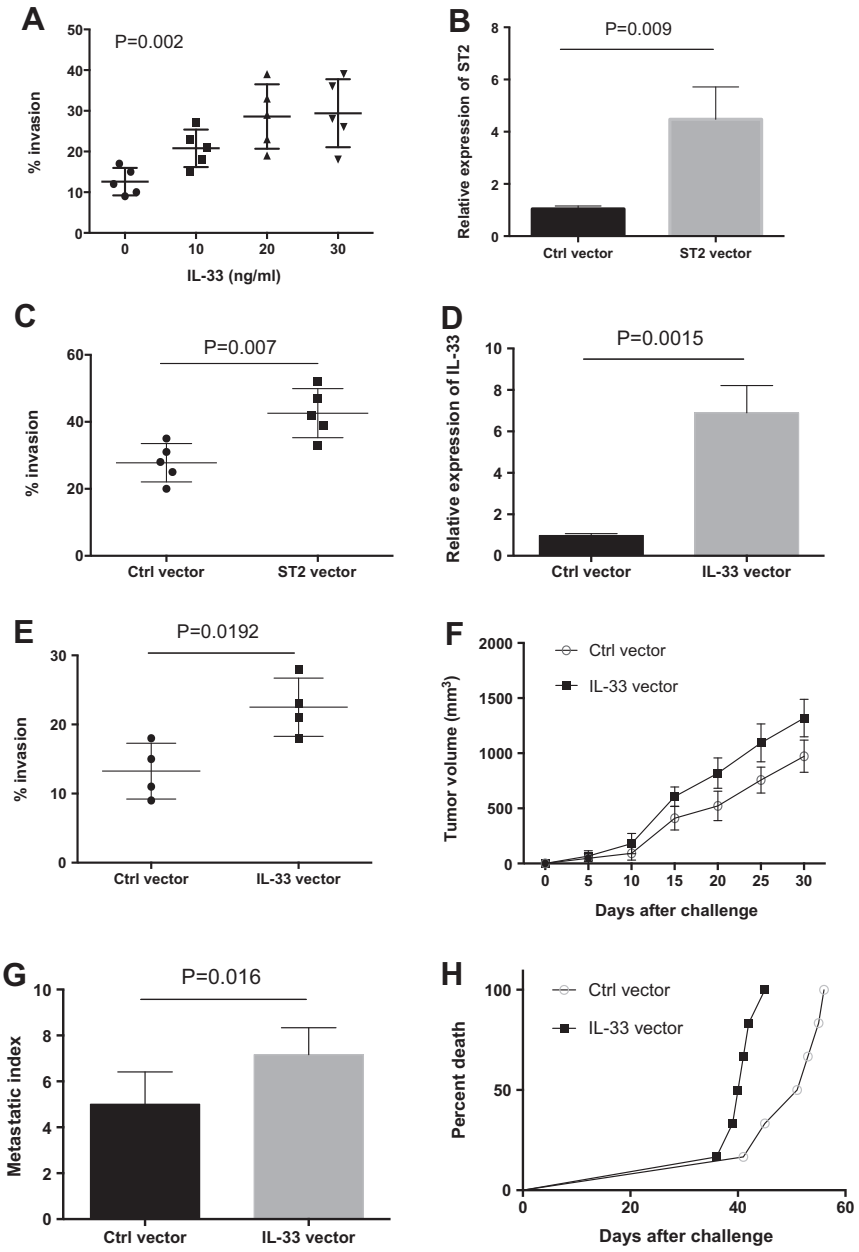

Fig. 2. Enhanced IL-33/ST2 signaling promoted CRC metastasis. (A) Human CRC cells isolated from surgical tissues ($n = 5$) were stimulated with the indicated dose of IL-33 for 48 h and then assayed for their invasion. (B) Human primary CRC cells were transfected with ST2 expression vector or the control vector for 12 h. (C) Human primary CRC cells ($n = 5$) were transfected with ST2 expression vector or the control vector respectively, and then treated with IL-33 (20 ng/ml) for 48 h. (D) Human primary CRC cells were transfected with IL-33 expression vector or the control vector for 12 h. (E) Human primary CRC cells ($n = 4$) were transfected with IL-33 expression vector or the control vector respectively, and then detected for their invasion. Each dot represented the results from one patient. (F–H) Groups of nude mice ($n = 6$) were challenged with SW620 cells that were stably transfected with IL-33 expression vector or the control, and detected for their tumor growth, metastasis index and survival time. Each bar represented the means (±SD) from six mice per group.

cells as they have been implicated in CRC metastasis. IL-33 stimulation of human CRC cells up-regulated the expression of IL-6, CXCR4, MMP2 and MMP9 (Fig. 4A, $P < 0.05$). We found an increased expression of IL-6, CXCR4, MMP2 and MMP9 in CRC cells in response to enhanced IL-33/ST2 signaling (Fig. 4B, $P < 0.05$). In consistent, decreased expressions of IL-6, CXCR4, MMP2 and MMP9 in CRC cells were observed in response to attenuated IL-33/ST2 signaling (Fig. 4C, $P < 0.05$). Of important, blockade of IL-6 and CXCR4 with specific neutralizing antibodies, as well as of MMP2 and MMP9 with MMP inhibitor GM6001, could effectively alleviate the enhanced metastasis of primary CRC cells induced by IL-33 stimulation (Fig. 4D–F, $P < 0.05$).

4. Discussion

Cancers develop in complex tissue environments in which they depend on sustained growth, invasion and metastasis that represents the end products of a multistep cell-biological process involves dissemination of cancer cells to anatomically distant organ sites and their subsequent adaptation to foreign tissue microenvironments [14,15]. The underlying mechanisms for cancer metastasis including CRC metastasis are still far from clear. Recently, accumulating data suggested that IL-33 might be involved in development of CRC [10,16]. Here we showed that enhanced IL-33/ST2 signaling could promote the metastasis of

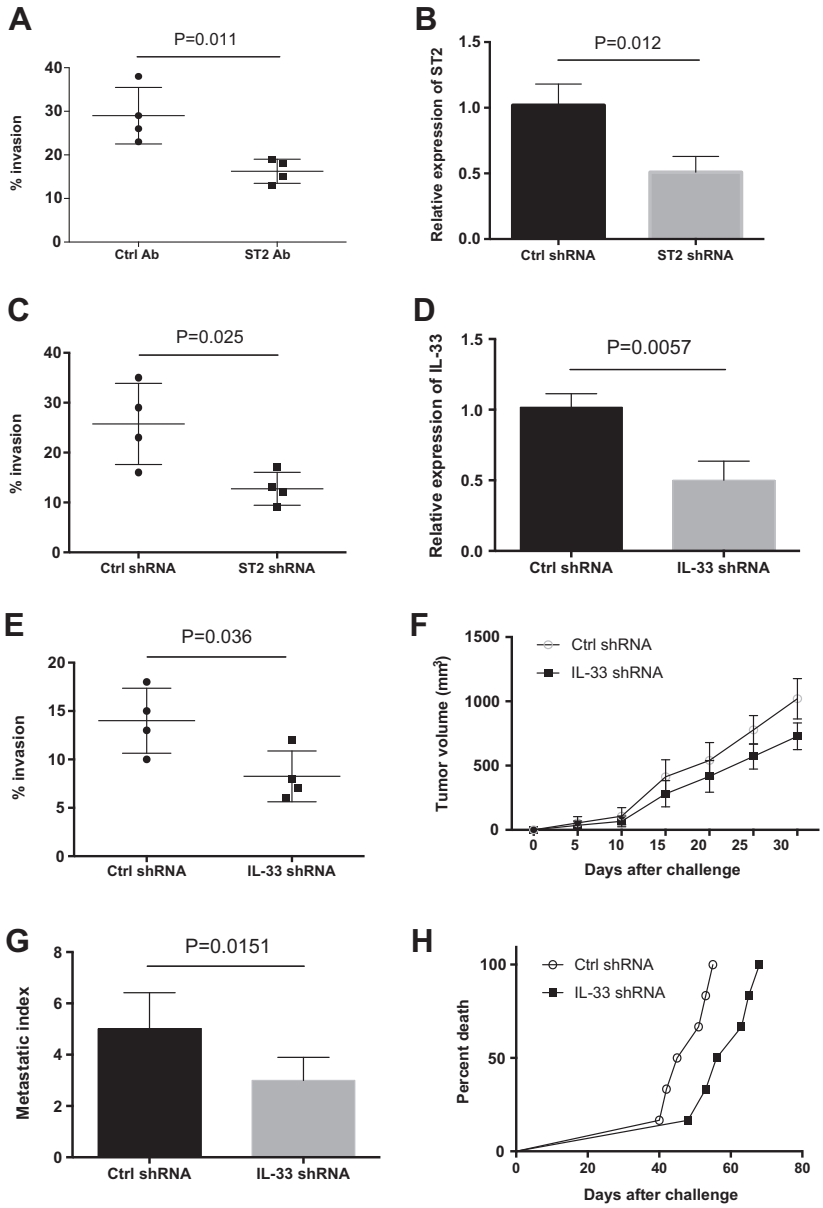

Fig. 3. Attenuated IL-33/ST2 signaling decreased CRC metastasis. (A) Human primary CRC cells ($n = 4$) were stimulated with IL-33 (20 ng/ml) in the presence of ST2 neutralizing antibody (10 μg/ml) or the control antibody for 48 h. (B) Human primary CRC cells were transfected with ST2 shRNA vector or the control vector for 12 h. (C) Human primary CRC cells ($n = 4$) were transfected with ST2 shRNA vector or the control vector respectively, and then treated with IL-33 (20 ng/ml) for 48 h. (D) Human primary CRC cells were transfected with IL-33 shRNA or the control for 12 h. (E) Human primary CRC cells ($n = 4$) were transfected with IL-33 shRNA or the control respectively, and then detected for their invasion. Each dot represented the results from one patient. (F–H) Groups of nude mice ($n = 6$) were challenged with SW620 cells that were stably transfected with IL-33 shRNA or the control, and detected for their tumor growth, metastasis index and survival time. Each bar represented the means (±SD) from six mice per group.

human CRC, while blockade of IL-33/ST2 signaling resulted in attenuated metastasis of human CRC. These findings could enlarge the understanding of CRC pathogenesis and provide clues for developing new CRC therapeutics.

In this study, we found an elevated expression of IL-33 and ST2 in tumor tissues in CRC patients. Further, we revealed the higher expression of IL-33 and ST2 in poo-differentiated human CRC cells. IL-33 stimulation resulted in enhanced metastasis of human CRC cells in a dose dependent manner. These data strongly demonstrated that IL-33/ST2 signaling could directly promote the metastasis of human CRC. In line with our results, accumulating data have reported the elevated expression of IL-33 in tumor tissues [17–21]. Besides, we found that increased IL-33 expression could enhance tumor progression of SW620 cells, while decreased IL-33 expression inhibited their tumor progression in nude mice. We found that transfection of SW620 cells with IL-33 expression vector resulted in higher level of secreted IL-33 in their culture supernatant, and transfection with IL-33 shRNA induced a lower secreted IL-33 level (data not shown). Thus, we speculated that secreted IL-33 from CRC cells could contribute to their growth and metastasis in nude mice. In consistent, we also found an elevated serum IL-33 in CRC patients (data not shown). However, the precise mechanisms underlying the secretion of IL-33 from CRC cell and whether the nuclear IL-33 in CRC cells could affect their biological behavior undoubtedly deserved further studies.

In the past decades, many important effectors involved in cancer metastasis have been explored. Of interest, recent study showed that stimulation with IL-6 could contribute to migration

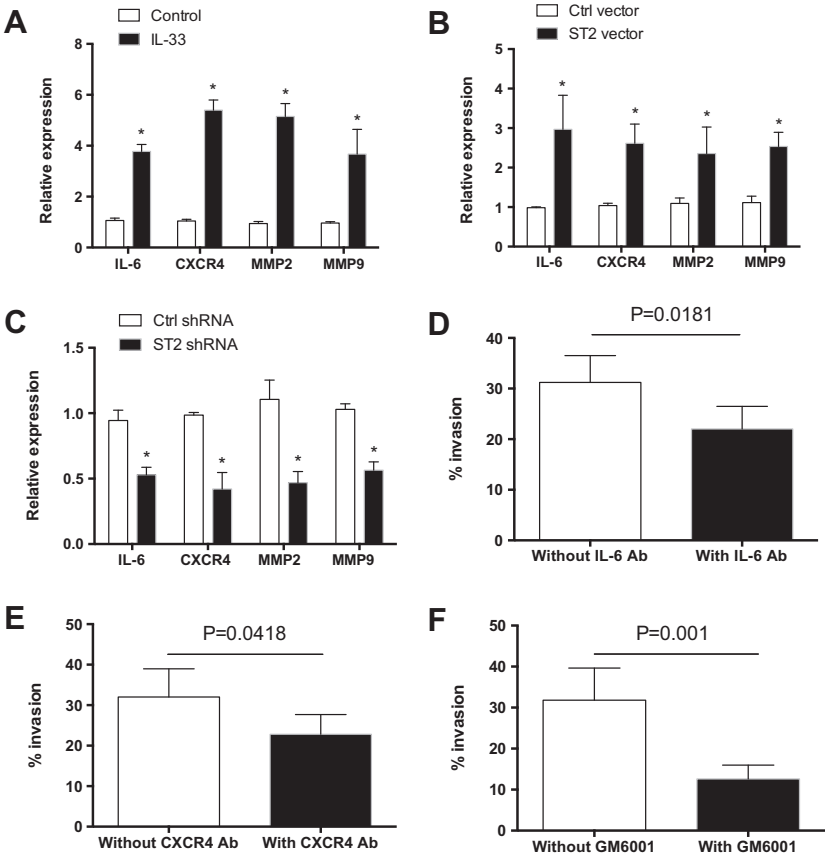

Fig. 4. IL-33/ST2 signaling regulated the expression of IL-6, CXCR4, MMP2 and MMP9 in human CRC cells. (A) Human primary CRC cells ($n = 4$) were treated with or without IL-33 (20 ng/ml) for 12 h and then assayed for their expression of IL-6, CXCR4, MMP2 and MMP9. (B) Human primary CRC cells ($n = 4$) were transfected with ST2 expression vector and treated with IL-33 (20 ng/ml) for 12 h. (C) Human primary CRC cells ($n = 4$) were transfected with ST2 shRNA vector and treated with IL-33 (20 ng/ml) for 12 h. (D–F) Human primary CRC cells ($n = 5$) were treated with IL-33 (20 ng/ml) in the presence or absence of IL-6 neutralizing antibody (2 μg/ml), CXCR4 neutralizing antibody (5 μg/ml) or GM6001 (10 μM) for 48 h, respectively, and assayed for their invasive potentials. Each bar represented the means (±SD) from three independent experiments performed in duplicate. $^{*}P < 0.05$.

and invasion of human CRC cells [22]. CXCR4 was reported to play an important role in the metastasis of CRC [23–27]. MMP2 and MMP9 were well-acknowledged crucial factors involved in CRC metastasis [28–30]. In the present study, we found that IL-33/ST2 signaling could modulate expressions of IL-6, CXCR4, MMP2 and MMP9 in human CRC. Abrogation of IL-6, CXCR4 and MMPs could inhibit the enhanced CRC metastasis by IL-33 stimulation. These data might, at least in part, account for the crucial effect of IL-33/ST2 pathway on metastasis of human CRC.

To our knowledge, this is the first report that demonstrated an important role of IL-33/ST2 interaction in metastasis of human CRC, which could further understanding of CRC pathogenesis and development of new therapeutics.

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The authors declared none potential financial conflict of interest.

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