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Original Article

Tumor-infiltrating mast cells stimulate ICOS$^+$ regulatory T cells through an IL-33 and IL-2 axis to promote gastric cancer progression

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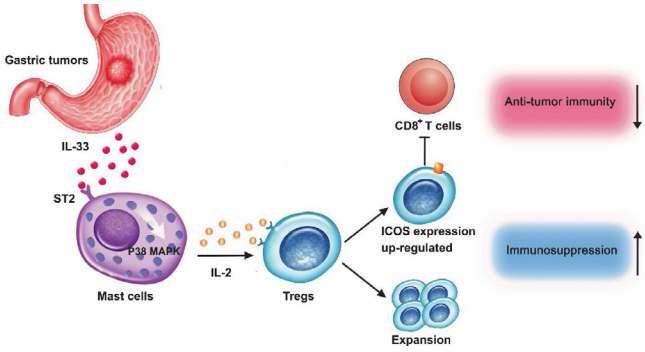
HIGHLIGHTS

- IL-33 is increased in gastric cancer and promotes tumor-associated mast cell survival by inhibiting its apoptosis.
- Tumor-derived IL-33 mediates Treg expansion by inducing mast cells to secrete IL-2.
- IL-2-induced Tregs display an activated and immunosuppressive phenotype.
- IL-2–expanded ICOS$^+$ Tregs exhibit increased inhibition of CD8$^+$ T cell proliferation and anti-tumor effector activity.
- Blockade of immunosuppressive ICOS$^+$ Tregs inhibits GC tumor growth and progression *in vivo*.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: In solid tumors, regulatory T cell (Treg) and mast cell perform different roles depending on the microenvironment. Nevertheless, mast cell and Treg-mediated interactions in gastric cancer (GC) are

Abbreviations: GC, gastric cancer; IL, interleukin; Foxp3, forkhead box P3; TTCS, tumor tissue culture supernatants; NTCS, non-tumor tissue culture supernatants; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TNF-α, tumor Necrosis Factor α; CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed cell death protein 1; ICOS, the inducible T cell costimulator; LAP, latency-associated peptide; IFN, interferon; PBMCs, peripheral blood mononuclear cells; hCBMCs, human umbilical cord blood-derived cultured mast cells.

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unclear, as are their regulation, function, and clinical significance.
Objective: The present study demonstrated the mechanism of tumor-infiltrating mast cells stimulating ICOS$^+$ regulatory T cells via the IL-33/IL-2 axis to promote the growth of gastric cancer.
Methods: Analyses of 98 patients with GC were conducted to examine mast cell counts, ICOS$^+$ Tregs, and the levels of IL-33 or IL-2. Isolated ICOS$^+$ Treg and CD8$^+$ T cell were stimulated, cultured and tested for their functional abilities *in vitro* and *in vivo*.
Results: GC patients exhibited a significantly more production of IL-33 in tumors. Mast cell stimulated by tumor-derived IL-33 exhibited a prolonged lifespan through IL-33 mediated inhibition of apoptosis. Moreover, mast cells stimulated by tumor-derived IL-33 secreted IL-2, which induced Treg expansion. These inducible Tregs displayed an activated immunosuppressive phenotype with positive expression for the inducible T cell co-stimulator (ICOS). In vitro, IL-2 from IL to 33-stimulated mast cells induced increased numbers of ICOS$^+$ Tregs with increased immunosuppressive activity against proliferation and effector function of CD8$^+$ T cell. *In vivo*, ICOS$^+$ Tregs were treated with anti-IL-2 neutralizing antibody followed by co-injection with CD8$^+$ T cells in GC mouse model, which showed an increased CD8$^+$ T cell infiltration and effector molecules production, meanwhile tumor growth and progression were inhibited. Besides, reduction in GC patient survival was associated with tumor-derived ICOS$^+$ Tregs.
Conclusion: Our results highlight a crosstalk between GC-infiltrating mast cells and ICOS$^+$ Tregs and provide a novel mechanism describing ICOS$^+$ Treg expansion and induction by an IL-33/mast cell/IL-2 signaling axis in GC, and also provide functional evidence that the modulation of this immunosuppressive pathway can attenuate GC-mediated immune tolerance.

Introduction

The global death toll from gastric cancer is estimated to reach 768,793 in 2020, with 1,089,103 new cases. Among all cancers, it has the sixth highest incidence and third highest death rate [1]. In some countries with low incomes and high rate of Helicobacter pylori infection, such as several Eastern Asian countries, deaths and morbidities associated with cancer have been significantly elevated by this disease [2]. Despite significant progress in diagnosis and treatment of GC [3], decreased Helicobacter pylori infection rates [4], improvements in food preservation and the promotion of gastrointestinal endoscopic screening, pathogenesis and regulatory mechanisms of GC remain largely unknown.

Gastric cancer development and prognosis are believed to be affected by its interaction with the immune system [5]. Clinical outcomes of GC patients are strongly influenced by adaptive immunity, according to most studies [6,7]. On the other hand, innate immune cell, for instance, residential mast cell is detected in GC little is known about their function in GC progression. A mast cell's innate role in allergic hypersensitivity type I is their most well-known function [8]. In tumor- microenvironment, mast cell can manipulate immunomodulatory effects [9] through reshaping tumor-microenvironment [10], promoting angiogenesis [11] and cross-talking with other immune cells [12]. Our previous study has shown that tumor-infiltrating mast cells could release cytokines to directly promote tumor progression [13]. As we know, tumors are associated with cytokines and the immune system. The cytokines are peptides produced by a wide variety of cell types, including immune system subpopulations. When they bind to appropriate receptors on membranes, they exert their effects as cellular mediators [14]. Mast cells can also interact with other stroma cells to indirectly affect tumor progression. An immunosuppressive subset of T cells known as Treg is found in human GC [15]. Some studies have shown evidence of a cross-talk between mast cell and Treg in allergic responses [16] and autoimmune disease [17]. However, the potential interactions between mast cells and Treg subsets and the underlying mechanisms have not been investigated for human GC.

There are several organ systems that express interleukin 33 (IL-33) of the IL-1 cytokine family, including the stomach [18]. This cytokine activates IL-1 receptor-like 1 (IL-1RL1) and IL-1 receptor accessory protein (IL-1RAcp), which constitute a heterologous receptor complex. [19]. In tumor-microenvironment, it has been found that mast cell and T helper (Th) 2 cell are involved in production of IL-1RL1, which is regulated by IL-33 [20]. Elsewhere, IL-2 is known to exert potent effect on T cell and NK cell reproduction and effector activity [21,22]. IL-2 from activated T cells is also known to be essential for Treg's Foxp3 expression and repressive function. However, whether IL-2 regulates Treg in GC with similar mechanisms requires further clarification.

Here, we researched the signaling mechanisms governing interactions between mast cells and Treg subsets in GC. Mast cells were found to secrete IL-2 in responding to GC-derived IL-33, which in turn promoted the expansion of immunosuppressive ICOS$^+$ Tregs, thereby contributing to GC progression.

Materials and Methods

Patients and specimens

Tissues comprising a gastric tumor (no necrosis), peritumoral tissues, and non-tumor tissues (a minimum distance of 5 cm should be kept from the tumor), and patients peripheral blood sample were all collected at the First Affiliated Hospital of Third Military Medical University) who had undergone surgical resection for GC. Autoimmune and infectious diseases, and multiple primary cancers were excluded, as was chemotherapy and radiation received before specimen collection. A TNM classification system (7th edition) was used to determine the clinical stage of tumors. Each subject gave written informed consent to participate in the study, which was approved by the Ethics Committee of the Southwest Hospital of Third Military Medical University. A total of 107 patients were initially enrolled, but 9 of them withdrew or terminated early from the follow-up due to personal preference. The Supplementary table 1 contains a list of all reagents used in this article.

Isolation of single cells from GC tissues

Hank's solution containing 1% FCS was used to wash fresh GC tissues three times and cut them into pieces. A MACS dissociator (Miltenyi Biotech, Germany) was used to mechanically separate samples in RPMI 1640 containing collagenase IV (1 mg/ml) (Gibco,

USA) and deoxyribonuclease I (10 mg/ml) (Sigma-Aldrich, USA). Cell suspensions were then incubated at 37 °C for 1 h under continuous rotation before being filtered with a 70 μm cell filter (BD Labware, USA) (cell viability>90%).

Preparation of TTCS and NTCS and TTCS-conditioned mast cell supernatants

By placing autologous tumor or non-tumor gastric tissues for 24 h in RPMI 1640, TTCS or NTCS were respectively prepared. The supernatant was collected. A 24-hour culture was performed on primary human umbilical cord blood mast cells (hCBMCs, 5×10^5/ml) in 50%TTCS or in 50%TTCS with neutralizing antibodies against human IL-33 (20 mg/ml) to obtain TTCS-conditioned mast cell supernatant (TTCS-hCBMCs sup). The supernatants were centrifuged, collected and frozen separately at −80 °C.

Mast cell stimulation

hCBMCs were stimulated with 50% TTCS with human IL-33 neutralizing antibody (20 μg/ml, Goat IgG) or an isotype control IgG (20 μg/ml) for 1 day, the supernatants were collected for IL-2 ELISA studies. A signaling pathway inhibition experiment was conducted by pretreatment with U0126 (MEK1/2 inhibitor), SB203580 (mitogen-activated protein kinase (MAPK) inhibitor), or SP600125 (c-Jun N-terminal kinase (JNK) inhibitor) in 5 μl (10 μM) for 1 h, followed by stimulation with 50% TTCS or human recombinant (hr) IL-33 (100 ng/ml) for 24 h and harvesting. In parallel to the inhibitor treatment, DMSO (5 μl) was used to control the inhibitors.

Treg cell induction assay

Isolating peripheral blood mononuclear cells (PBMCs) from healthy donors with ficoll density gradient centrifugation, as reported in the previous article [23]. For 5 days, Fluorescence-activated cell sorter (FACS) (BD, USA) sorted naive T cells ($CD4^+$-$CD45RA^+$) were cultured in 50% TTCS or 50% TTCS-hCBMCs sup plus neutralizing antibodies against human IL-33 (20 g/ml) or a control IgG (20 g/ml) respectively. Then, cells were harvested for intracellular cytokine staining. In addition, sorted naïve T cells were cultured with the supernatant of TTCS-stimulated hCBMCs (referred to as TTCS-hCBMCs sup) or the supernatant of IL-33-stimulated hCBMCs (referred to as IL-33-hCBMCs sup) with or without neutralizing antibodies against IL-2 (20 μg/ml, Rat IgG2a, κ) for 5 days. Finally, we stained intracellular cytokines in harvested cells using a FACSCanto II (BD Biosciences, USA).

In vitro Treg cell-$CD8^+$ T cell co-cultures

After sorting naïve T cells, they were cultured in TTCS-hCBMC supernatant for 5 days with or without neutralizing antibodies against IL-2 (20 ng/ml). Tregs were divided into $ICOS^+$ and $ICOS^-$ groups according to the expression of ICOS on $CD25^+Foxp3^+$Tregs. $ICOS^+$ and $ICOS^-$ Treg subsets were also sorted from GC tissues (FACSAria II, BD Biosciences, USA). The expression of Ki-67 in Treg subsets was detected by intracellular staining. Anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) antibodies were added to RPMI 1640 with 10% FCS and 2 × 105/well CFSE-labeled $CD8^+$ T cells (sorted by FACSAria II) from healthy donors were cultured. Sorted $ICOS^+$ and $ICOS^-$ Treg subsets were then added with $CD8^+$ T cells at 1:2 ratio. After a 5-day incubation, a CFSE fluorescence level and intracellular cytokine staining were measured by flow cytometry for $CD8^+$ T cells, and supernatants for ELISA analysis were collected.

Mast cell proliferation and apoptosis assays

TTCS and NTCS were collected as described above. hCBMCs were cultured with TTCS, NTCS, or TTCS with human IL-33 neutralizing antibody or a control IgG. Besides, mast cells were cultured with human recombinant (hr) IL-33 (100 ng/ml) during mast cell culture. As a measure of cell proliferation, CCK-8 (Dojindo, Japan) was used at 0, 12, 24 and 48 h. Annexin V Apoptosis Detection Kit (BD Biosciences, USA) or APO-Direct Apoptosis Detection Kit (Invitrogen, USA) was used for 72 h to detect apoptosis, the methods as reported in the previous article [24].

In vivo tumor inhibition assay

Third Military Medical University's Animal Ethical and Experimental Committee approved animal experiments. A total of 10^6 GC cells (SGC-7901 cells) were administrated subcutaneously to 5–7 weeks-old female NOD/SCID mice in 100 ml buffered saline. There were 4 groups, 5 mice in each group. This about 7 days to establish the GC mice model (Modeling was successful when a tumor the size of a needle tip was seen subcutaneously at the injection site in mice) and then the mice would undergo approximately three weeks of cell co-culture *in vivo*.

In cultured for 5 days, sorted naïve T cells were stimulated with TTCS-hCBMCs sup supplemented with neutralizing antibodies against IL-2 or a control IgG2a. On day 7 after tumor cell inoculation, 2×10^6 activated (2 μg/ml anti-CD3 and 1 μg/ml anti-CD28) polyclonal autologous $CD8^+$ T cells were co-cultured with or without sorted Treg subsets at a 2:1 ratio for 5 days, before being injected into the peritoneum (in 100 μl of buffered saline). Two independent observers measured tumor volumes (V) using the formula: $V = A \times B^2/2$ (A = axial diameter; B = rotational diameter) every 3 days with calipers fitted with vernier scales. ELISA, real-time PCR, and immunohistochemical staining of mouse tumors were conducted following their euthanasia. The flow cytometric analysis of mouse spleens was also performed on single cells dissociated from the spleen.

Statistical analysis

Results are expressed as mean ± SEM. Student's *t*-test was generally used to analyze the differences between two groups, but when the variances differed, the Mann-Whitney *U* test was used. The linear regression analysis was used to assess the correlation between different parameters. Survivorship was defined as the period between surgery and death, or surgery and the last observation for those who survived. Using Kaplan-Meier method, cumulative survival time was calculated and survival in months was assessed by log-rank test. In the study, SPSS statistical software (version 13.0) was used. A significance level of $P < 0.05$ was considered statistically significant.

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Animal Ethical and Experimental Committee of Third Military Medical University (2019YFC1302200). The experiments involving human samples were approved by the Ethics Committee of Southwest Hospital of Third Military Medical University (2018YFC1303300). The written informed consent was obtained from each subject.

Results

IL-33 is increased in gastric cancer tissues

To evaluate the potential role of IL-33 in human GC, we analyzed IL-33 levels in different tumor, peritumoral and non-tumor samples. According to our findings, GC tumors contain significantly higher expressions of both IL-33 mRNA (Fig. 1A) and protein (Fig. 1B) than peritumoral tissues and non-tumor tissues. Moreover, compared to Non-tumor culture supernatant (NTCS), IL-33 production in tumor-tissue culture supernatant (TTCS) was also significantly increased (Fig. 1C). IL-33 protein levels were similarly assessed by western blot (Fig. 1D) and immunohistochemical staining (Fig. 1E). Furthermore, immunohistochemistry staining showed that IL-33 was most likely derived from $CD326^+$ tumor cells in GC (Fig. 1F). Additionally, we found that sST2, IL-33 cell's surface receptor and an endogenous inhibitor of IL-33 signaling in a soluble form [25], was not changed in tumor tissues (Fig. 1G) or in TTCS (Fig. 1H) compared to peritumoral and non-tumor tissues or NTCS. This indicates that IL-33 bioactivity is not limited by sST2 in GC environments. We also detected other important members of the IL-1 family and similar observations were made when analyzing the mRNA (Supplementary Fig. 1A) and protein (Supplementary Fig. 1B) levels of IL-1β and IL-18 in different tissues. Altogether, there is an increase in IL-33 in the GC tissues of patients, as indicated by these findings.

IL-33 promotes tumor-associated mast cell survival by inhibiting its apoptosis

In several diseases, IL-33 induces mast cell to secret proinflammatory cytokines [2]. Therefore, we wondered whether IL-33 also modulated mast cell responses in GC environments. We first observed mast cell infiltration in tumor tissues (Fig. 2A), with expression ST2 (the IL-33 receptor) merged with tryptase staining on mast cells (Fig. 2B). Moreover, we observed IL-33 levels and mast cell infiltration in tumors were positively correlated (Fig. 2C), suggesting that mast cell might be targets of IL-33 within GC environment. To assess the effect of tumor-derived IL-33 on mast cell, we stimulated hCBMCs with TTCS and then assessed mast cell viability and survival rates. Using trypan blue staining, comparing hCBMCs exposed to TTCS with those exposed to NTCS from autologous GC patients, we found increased cell viability with TTCS (Fig. 2D). In comparison with hCBMCs exposed to NTCS, a delayed onset of apoptosis was observed by annexin V staining (Fig. 2E) and deoxyuridine triphosphate nucleotide analysis (Fig. 2F). To examine whether IL-33 might mediate these aforementioned effects on mast cells, we putted neutralizing antibodies against IL-33 in the hCBMCs/TTCS co-culture. Intriguingly, antibody blockade of IL-33 reversed TTCS-induced prolonged hCBMC viability and survival (Fig. 2D-F). Exogenous IL-33 addition was also demonstrated to prolong hCBMC viability (Supplementary Fig. 2B) by delaying hCBMC apoptosis (Supplementary Fig. 2C and D). These findings imply that tumor-derived IL-33 may function to increase mast cell survival through the inhibition of cell apoptosis.

Tumor-derived IL-33 mediates Treg expansion by inducing mast cells to secrete IL-2

IL-33 has been reported to contribute to Treg expansion in infection-associated sepsis [26] and allergic dermatitis [27]. To investigate whether analogous mechanisms may work in the GC microenvironment, we first stimulated $CD4^+CD45RA^+$ naïve T cells with IL-33. However, IL-33 alone had no effects on Treg expansion (Fig. 3A and Supplementary Fig. 3B). As IL-33 exerted effects on tumor-derived mast cells (Fig. 2), we postulated that IL-33 might still indirectly affect Treg expansion through mast cell stimulation. Thus, we stimulated naïve T cells with the supernatant from TTCS-stimulated hCBMCs and found that this significantly induced Treg expansion. The effect was attenuated following the addition of anti-IL-33 neutralizing antibodies (Fig. 3A). We also observed that supernatant from IL-33-stimulated hCBMCs significantly induced Treg expansion (Supplementary Fig. 3C). Moreover, signal pathway inhibition experiments showed that supernatant from IL-33-stimulated hCBMCs induced Treg expansion via the p38 MAPK pathway (Fig. 3C).

Next, we observed that IL-2 concentrations in the supernatant of TTCS-stimulated hCBMCs were significantly increased compared to supernatant from non-hCBMCs or supernatant from TTCS-stimulated hCBMCs treated with anti-IL-33 neutralizing antibodies (Fig. 3B). Within GC tumors, Treg infiltration and IL-2 production showed a strong positive correlation (Fig. 3E). To further determine whether supernatant-contained IL-2 from TTCS-stimulated mast cells contributed to Treg expansion, we added anti-IL-2 neutralizing antibodies to naïve T cells stimulated with the supernatant from TTCS-stimulated mast cells. Interestingly, antibody blockade of IL-2 efficiently attenuated supernatant-induced Treg expansion (Fig. 3F). Collectively, these results indicate that tumor-derived IL-33 can mediate Treg expansion by inducing mast cells to secrete IL-2.

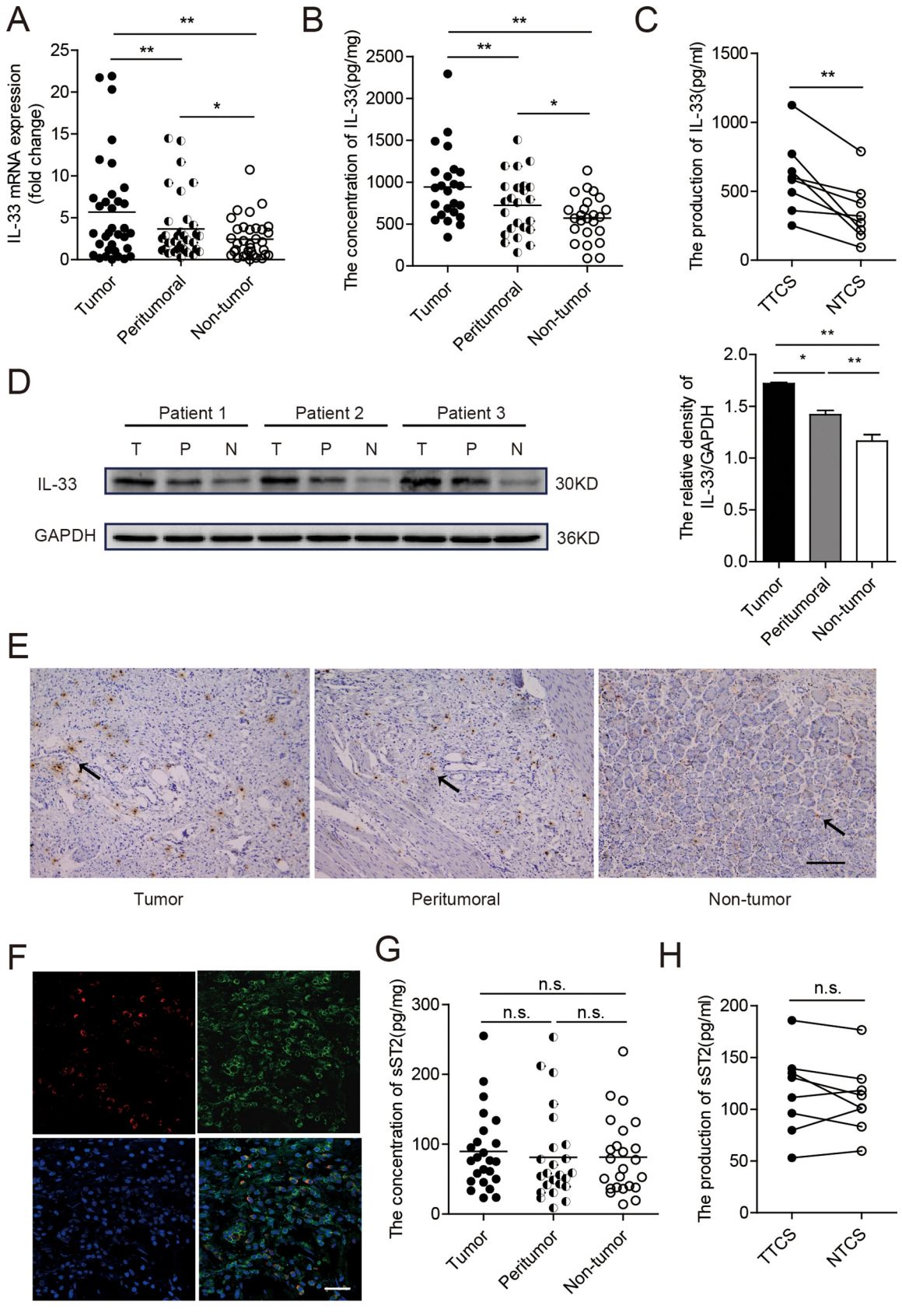
IL-2-induced Tregs display an activated and immunosuppressive phenotype

Phenotypic analysis of $CD4^+CD25^+Foxp3^+$ (G1 cell population) versus $CD4^+CD25^-Foxp3^-$ (G2 cell population) cells (Fig. 4A) induced by supernatant from TTCS-stimulated hCBMC containing control IgG2a or anti-IL-2 neutralizing antibody was conducted. The expression of classical Treg and T cell activation marker, including CD39, CD73, CTLA-4, LAP, ICOS, PD-1 and T cell activation and memory markers CD69, CD44 and CD103 were all assessed (Fig. 4B). Although Treg classical markers and T activation and memory markers (Helios, PD-1, CD39, CD44, LAP, GATA3, CTLA-4, ICOS, CD73, CD69 and CD103) were significantly overexpressed in the G1 cell population compared to the G2 cell population, ICOS expression on induced $Foxp3^+$ Tregs was notably increased in control IgG2a antibody containing cell cultures compared to those containing anti-IL-2 neutralizing antibody (Fig. 4B). As a costimulatory receptor, ICOS is a marker of T cell activation. In short, these results verify that Tregs induced by TTCS-stimulated hCBMC supernatant displayed an activated phenotype.

IL-2–expanded $ICOS^+$ Tregs exhibit increased inhibition of $CD8^+$ T cell proliferation and anti-tumor effector activity

Given the reported enhanced ability of $ICOS^+$ Treg to restrain T cell compared to $ICOS^-$ Tregs [28], we hypothesized that $ICOS^+$ Tregs induced by IL-2 from TTCS-stimulated hCBMCs may play an important part in immunosuppression. Purified $CD8^+$ T cells were co-cultured with $ICOS^+$ Tregs or $ICOS^-$ Tregs sorted from induced Tregs cultured in TTCS-stimulated hCBMC supernatant treated with either control IgG or anti-IL-2 neutralizing antibody respectively. Interestingly, $ICOS^+$ Tregs inhibited significantly more $CD8^+$ T cell IFN-γ, perforin and granzyme B production and proliferation compared to $ICOS^-$ Tregs (Fig. 5A and C and Supplementary Fig. 4B). $ICOS^+$ Tregs also displayed increased proliferative activity compared to $ICOS^-$ Tregs (Fig. 5B).

To confirm the inhibitive effects of tumor-infiltrating $ICOS^+$ Treg on $CD8^+$ T cell, we repeated this assay using $ICOS^+$ Tregs or $ICOS^-$


A
IL-33 mRNA expression (fold change)
Tumor
Peritumoral
Non-tumor
B
The concentration of IL-33(pg/mg)
Tumor
Peritumoral
Non-tumor
C
The production of IL-33(pg/ml)
TTCS
NTCS
The relative density of IL-33/GAPDH
Tumor
Peritumoral
Non-tumor
D
Patient 1
Patient 2
Patient 3
T P N T P N T P N
IL-33
30KD
GAPDH
36KD
E
Tumor
Peritumoral
Non-tumor
F
G
The concentration of sST2(pg/mg)
n.s.
Tumor
Peritumor
Non-tumor
H
The production of sST2(pg/ml)
n.s.
TTCS
NTCS

Tregs isolated from GC tumor tissue. Tumor-infiltrating ICOS$^+$ Tregs also significantly suppressed CD8$^+$ T cell IFN-γ, perforin and granzyme B production and proliferation compared to tumor-infiltrating ICOS$^-$ Tregs (Fig. 5D and F and Supplementary Fig. 4C). Tumor-infiltrating ICOS$^+$ Tregs also exhibited enhanced proliferative activity compared to ICOS$^-$ Tregs (Fig. 5E). The data obviously demonstrate that IL-2–expanded ICOS$^+$ Tregs exhibit enhanced suppression of CD8$^+$ T cell proliferation and anti-tumor effector activity.

Growth and progression of GC tumors are inhibited *in vivo* by blocking immunosuppressive ICOS$^+$ Tregs

To test the suppressive effects of ICOS$^+$ Tregs induced by TTCS-stimulated hCBMCs (iTregs) on CD8$^+$ T cell immunity *in vivo*, a GC mouse model (using NOD/SCID mice inoculating with SGC-7901) was established using iTregs that were treated with an anti-IL-2 neutralizing antibody or a control IgG followed by their co-injection with CD8$^+$ T cells. Tumor growth and progression were observed in mice without CD8$^+$ T cell transfusion and mice co-administered CD8$^+$ T cell and control IgG-treated iTregs (Fig. 6A and B). In agreement with *in vitro* observations, mice receiving CD8$^+$ T cells co-administered with anti-IL-2 neutralizing antibodies showed decreased tumor volumes and slower disease progression on day 19 (Fig. 6A and B). Mice co-administered CD8$^+$ T cells and anti-IL-2 neutralizing antibodies showed increased CD8$^+$ T cell infiltration in tumors (Fig. 6C) and increased IFN-γ, perforin and granzyme B production (Fig. 6D and E and Supplementary Fig. 5B). Moreover, increased IFN-γ producing CD8$^+$ T cell was observed in spleens of mice co-administered CD8$^+$ T cell and anti-IL-2 neutralizing antibody-treated iTregs (Fig. 6D and Supplementary Fig. 5A) compared with mice co-administered CD8$^+$ T cell and control IgG-treated iTregs. These findings suggest that IL-2 induced ICOS$^+$ Tregs from TTCS-stimulated hCBMCs suppress CD8$^+$ T cell immunity *in vivo* and thereby contribute to GC growth and progression. Finally, we evaluated the clinical relevance of the proportion of ICOS$^+$ Treg cells and the ICOS$^+$ Treg/CD8$^+$ T cell ratios in GC patients. Comparing patients with high versus low ICOS$^+$-Tregs percentage (or rates ratio of ICOS$^+$ Tregs / CD8$^+$ T cells), the 44-month overall survival rates were signifificantly lower for those within the higher percentage (or rates ratio of ICOS$^+$ Tregs / CD8$^+$ T cells) (Fig. 7A and B). Taken together, these findings suggest that increased intratumoral ICOS$^+$ Tregs are associated with tumor progression and poor survival of GC patients.

Discussion

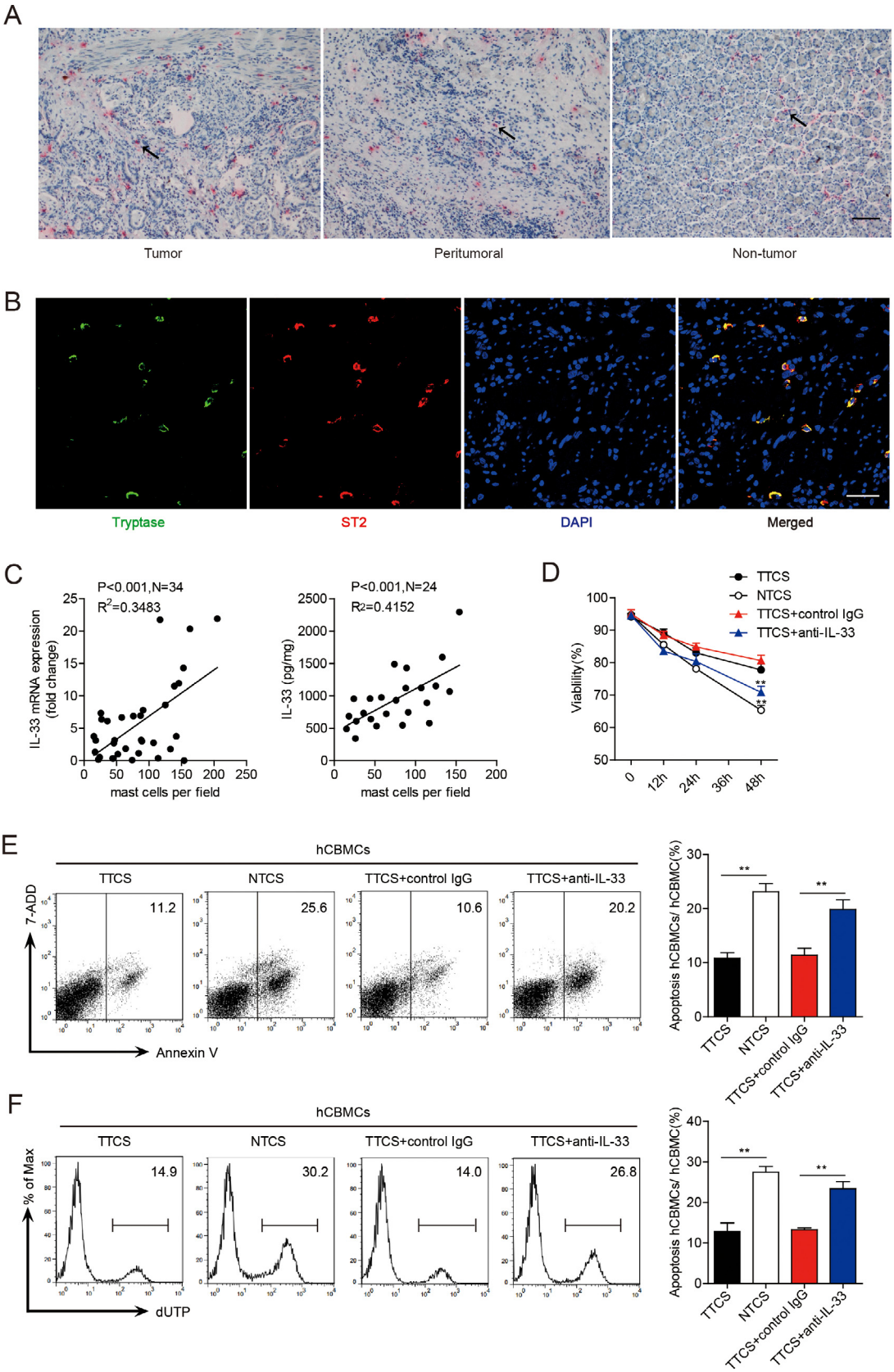
In an immunosuppressive tumor microenvironment, tumors can modulate immune cell to facilitate tumor progression [24]. Understanding precisely how immune cells can influence cancer progression has been a fundamental focus of cancer investigation. In recent decades, it has been extensively studied how tumor-mediated immunosuppression affects tumor growth [29]. Mast cells [30] and Tregs [31] are tumor-infiltrating immune cells within strong immunosuppressive effects. Here, we report that gastric cancer derived IL-33 can lead to local mast cell to secret IL-2 to promote Treg cell expansion and ICOS expression. Although tumor-infiltrating mast cells and Tregs have already been discovered in GC [32,33], to our knowledge, this is the first demonstration of a regulatory mechanism describing IL-33-induced mast cell to produce IL-2 to induce Treg expansion in GC. Our results uncover a novel mechanism for mast cell and Treg interaction and provide new proof for the pro-tumoral roles of mast cells and Tregs within the GC milieu.

The cytokine IL-33 is subordinate to IL-1 family and is involved in cancer growth and metastasis [34]. IL-33 overexpression is associated with hepatocellular carcinoma progression [35], and raised expression of IL-33 are also found in lung cancer [36] and head and neck squamous cell carcinoma [37]. In GC, the development and transfer of GC cells are influenced by IL-33 in a dose-dependent manner, according to previous studies [38]. A study reported IL-33 deficient *gp130*$^{F/F}$/IL-13$^{-/-}$ mice had reduced gastric tumor growth and fewer pro-tumorigenic myeloid cells [39]. Inflammation is a well-established hallmark of cancer development and progression and immune cells can be regulated by tumor-derived signals to promote these processes. However, relatively little is reported regarding the effects of IL-33 on immune cells in GC milieu. Here, we showed that IL-33 was mainly produced by GC cells, which exerted biological function via ST2 receptor. IL-33 binding allows membrane ST2 to interact with IL-1RAcP. The IL-33/ST2/IL1RAcP complex then activates MAPK signalling and NF-κB transcription factor via the MyD88 adapter, IL-1 receptor associated kinase 1 (IRAK1), IRAK4 and TNF receptor associated factor 6 (TRAF6). As a result, ST2-expressing immune cells are a key target of IL-33 signaling in tumor microenvironments. These cells include group 2 innate lymphoid cell (ILC2), Treg, Th1 cell, activated CD8$^+$ T cell and mast cell. IL-33 targets mast cells and contributes to the deterioration of allergic and inflammatory diseases. Soluble ST2 receptor (sST2) is an essential negative regulator of IL-33 activity [40]. We detected sST2 expression in GC tissue and non-tumor tissue and found low sST2 levels in both tissue sites. Previous studies related to IL-33 and mast cells mostly focused on IL-33 induced mast cell degranulation and cytokine secretion [41,42]. Here, we unexpectedly discovered that TTCS protected mast cells from spontaneous apoptosis and enhanced their proliferation via IL-33 signaling (Fig. 2D-F). We speculate that this may explain why mast cell infiltration of GC is positively correlated with IL-33 concentration (Fig. 2C).

Our previous studies have found that mast cells can exhibit a pro-tumor phenotype during the intermediate and advanced stages of GC, and that this is an independent and unfavorable prognostic factor for GC patients [13]. IL-33 induced mast cell activation can be dependent or independent of IgE/antigen–FcεRI signals, such as prostaglandins, histamine, chemokines, IL-1β, IL-6, IL-13 and TNF-α [43,44]. Our present study found significantly raised IL-2 production by mast cells following stimulation by GC derived-IL-33. Pre-treatment of mast cells with signaling pathway inhibitors identified the involvement of the MAPK pathway in IL-33-induced mast cell IL-2 production.

Inducing T cell death or suppressing T cell proliferation, Tregs are MHC class II restricted CD4$^+$ T cells [45]. NK cell-mediated anti-tumor activity can be suppressed by Tregs [46]. Antigen-

Fig. 1. There is increased IL-33 expression in gastric cancer tissues. (**A**) IL-33 mRNA expression was determined using real-time PCR in autologous tumor, peritumoral, and non-tumor tissue samples (n = 34). (**B** and **C**) IL-33 concentrations in autologous tumor, peritumoral and non-tumor tissues (n = 24) (**B**) or between autologous TTCS and NTCS (n = 8) (**C**) was analyzed by ELISA. (**D**) IL-33 production between autologous tumor (T), peritumoral (P) and non-tumor (N) tissues (3 pairs) were analyzed by western blot. (**E**) A representative immunohistochemical staining of tumor, peritumoral, and nontumor mast cells of GC patients. Scale bars: 100 μm. (**F**) Infiltrated CD326$^+$IL-33$^+$ cells were seen in tumor tissues of GC patients by immunofluorescence staining. Green corresponds to CD326, red to IL-33 and blue to DAPI-stained nuclei. Scale bars: 50 μm. (**G** and **H**) sST2 concentrations in autologous tumor, peritumoral and non-tumor tissues (n = 24) (**B**) or between autologous TTCS and NTCS (n = 8) (**C**) was analyzed by ELISA. The horizontal bars in panels **A**, **B** and **G** represent mean values. Each ring or dot in panels **A**, **B**, **C**, **G** and **H** represents 1 patient. *, $P < 0.05$; **, $P < 0.01$; n.s., $P > 0.05$ for groups connected by horizontal lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


A
Tumor
Peritumoral
Non-tumor
B
Tryptase
ST2
DAPI
Merged
C
P<0.001,N=34
R2=0.3483
IL-33 mRNA expression (fold change)
mast cells per field
P<0.001,N=24
R2=0.4152
IL-33 (pg/mg)
mast cells per field
D
TTCS
NTCS
TTCS+control IgG
TTCS+anti-IL-33
Viability(%)
0
12h
24h
36h
48h
E
hCBMCs
TTCS
NTCS
TTCS+control IgG
TTCS+anti-IL-33
7-ADD
11.2
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10.6
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Annexin V
Apoptosis hCBMCs/ hCBMC(%)
F
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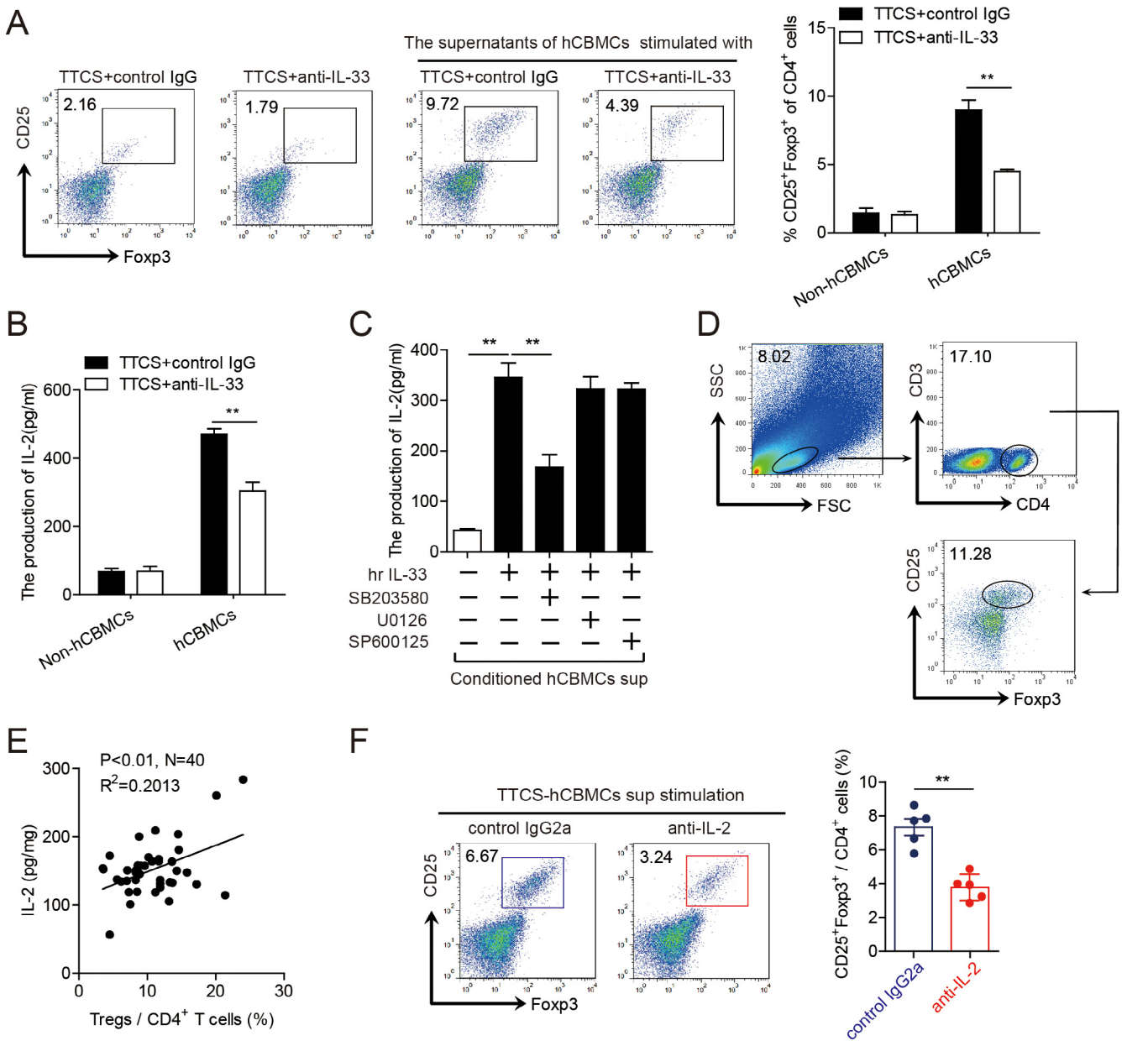

Fig. 3. Induced mast cell secretion of IL-2 by tumor-derived IL-33 mediates Treg expansion. (**A**) Naïve T cells ($CD4^+CD45RA^+$) were stimulated with different condition culture supernatants as described in Methods for 5 days, and the expansion of $CD25^+Foxp3^+$ Tregs in each group was compared (n = 5). (**B**) The concentration of IL-2 in different condition culture supernatants as described in Methods was analyzed (n = 5). (**C**) A comparison of the IL-2 production by hCBMCs exposed to hr IL-33 with and without signal transduction inhibitors was conducted (n = 5). (**D**) Dot plots of surface and intracellular molecule staining for $CD25^+Foxp3^+$ Tregs gating on $CD4^+$ T cells from tumor tissues of GC patients. (**E**) The correlations between IL and 2 concentration and $CD25^+Foxp3^+$ Tregs infiltrated in GC tissues were detected (n = 40). (**F**) Naïve T cells ($CD4^+CD45RA^+$) were stimulated with TTCS-hCBMCs sup with IL-2 neutralizing antibodies (20 μg/ml) or control IgG2a (20 μg/ml) for 5 days, and the expansion of $CD25^+Foxp3^+$ Tregs in each group was compared (n = 5). Each ring or dot in panel **E** represents 1 patient. *, $P < 0.05$, **, $P < 0.01$ for groups connected by horizontal lines. sup, supernatant.

Fig. 2. By promoting proliferation and inhibiting apoptosis, IL-33 increases the number of mast cells in tumor tissue. (**A**) Immunohistochemical analysis of mast cells positive for tryptase (red) in tumor, peritumoral, and non-tumor tissues from GC patients. Scale bars: 100 μm. (**B**) Representative immunofluorescence staining images showed $tryptase^+ST2^+$ mast cell infiltration interactions tumor tissues of GC patients. Green, tryptase; red, ST2; and blue, DAPI-stained nuclei. Scale bars: 50 μm. (**C**) Analyses were conducted on the correlation between mast cells and IL-33 production in GC tumors. Results were expressed as the number of mast cells per field and IL-33 mRNA expression (n = 34) or IL-33 concentration (n = 24) in tumor tissues of patients with GC. (**D-F**) Different culture supernatants were added to hCBMCs as described in Methods for the indicated time periods. Trypan blue staining was used to assess cell viability (**D**) (n = 5). The apoptosis of GC cells was analyzed by annexin V (**E**) and deoxyuridine triphosphate nucleotides (dUTP) (**F**) detection (n = 5). Each dot in panel **C** represents 1 patient. *, $P < 0.05$; **, $P < 0.01$ for groups connected by horizontal lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

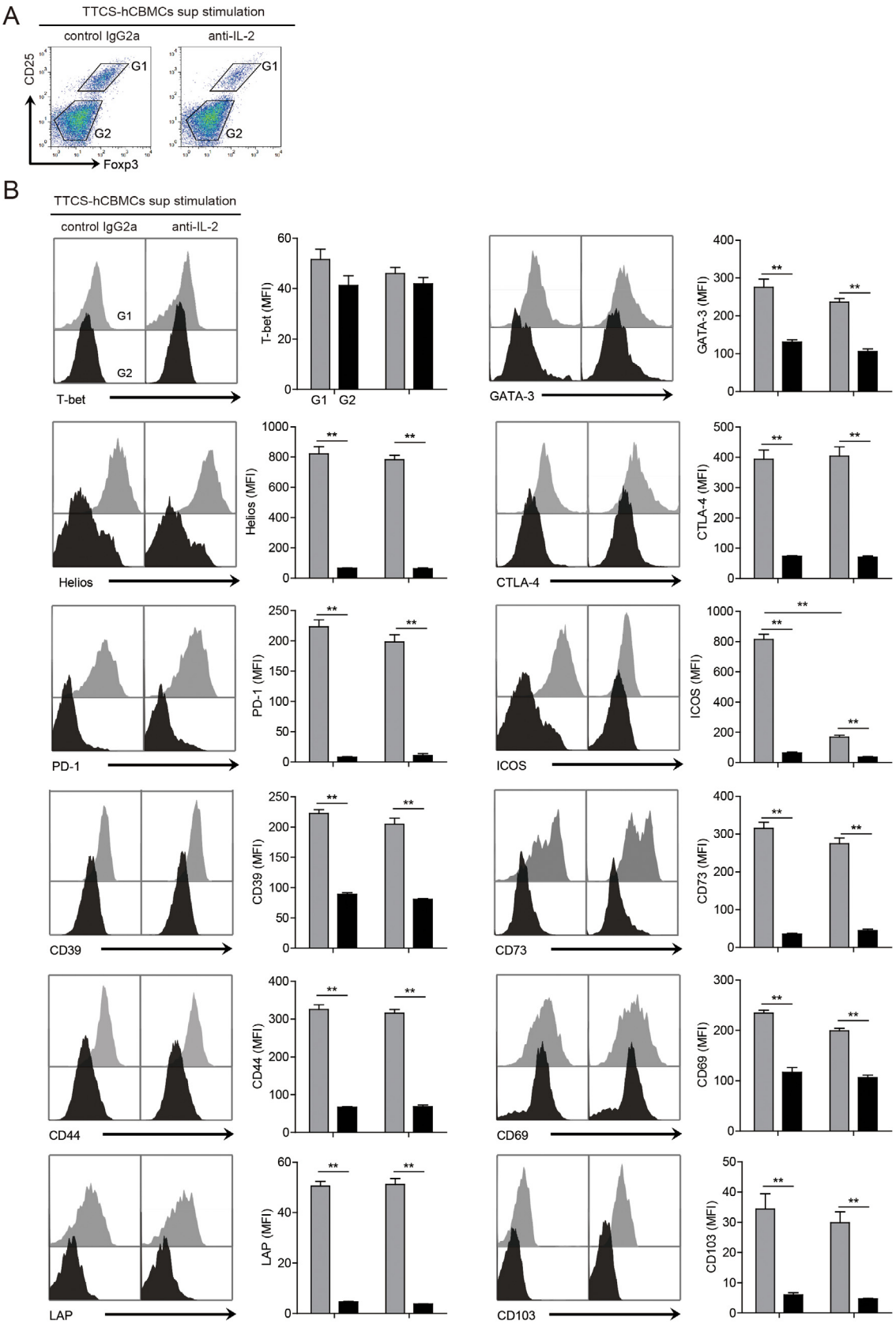

Fig. 4. Induced CD25$^+$Foxp3$^+$ Tregs express classical Treg markers and show an activated phenotype. Naïve T cells (CD4$^+$CD45RA$^+$) were stimulated with TTCS-hCBMCs supernatant with IL-2 neutralizing antibodies (20 μg/ml) or control IgG2a (20 μg/ml) for 5 days, and the cells were harvested and stained for flow cytometric analysis. (**A**) Gating strategy on CD4$^+$-gated cells and (**B**) expression of surface and intracellular markers on CD25$^+$Foxp3$^+$ (G1; gray; top) and CD25$^-$Foxp3$^-$ (G2; black; bottom), as indicated in (**A**). Bar graphs in (**B**) represent an average of the mean fluorescence intensity (MFI) for each marker (n = 3). **, $P < 0.01$ for groups connected by horizontal lines.

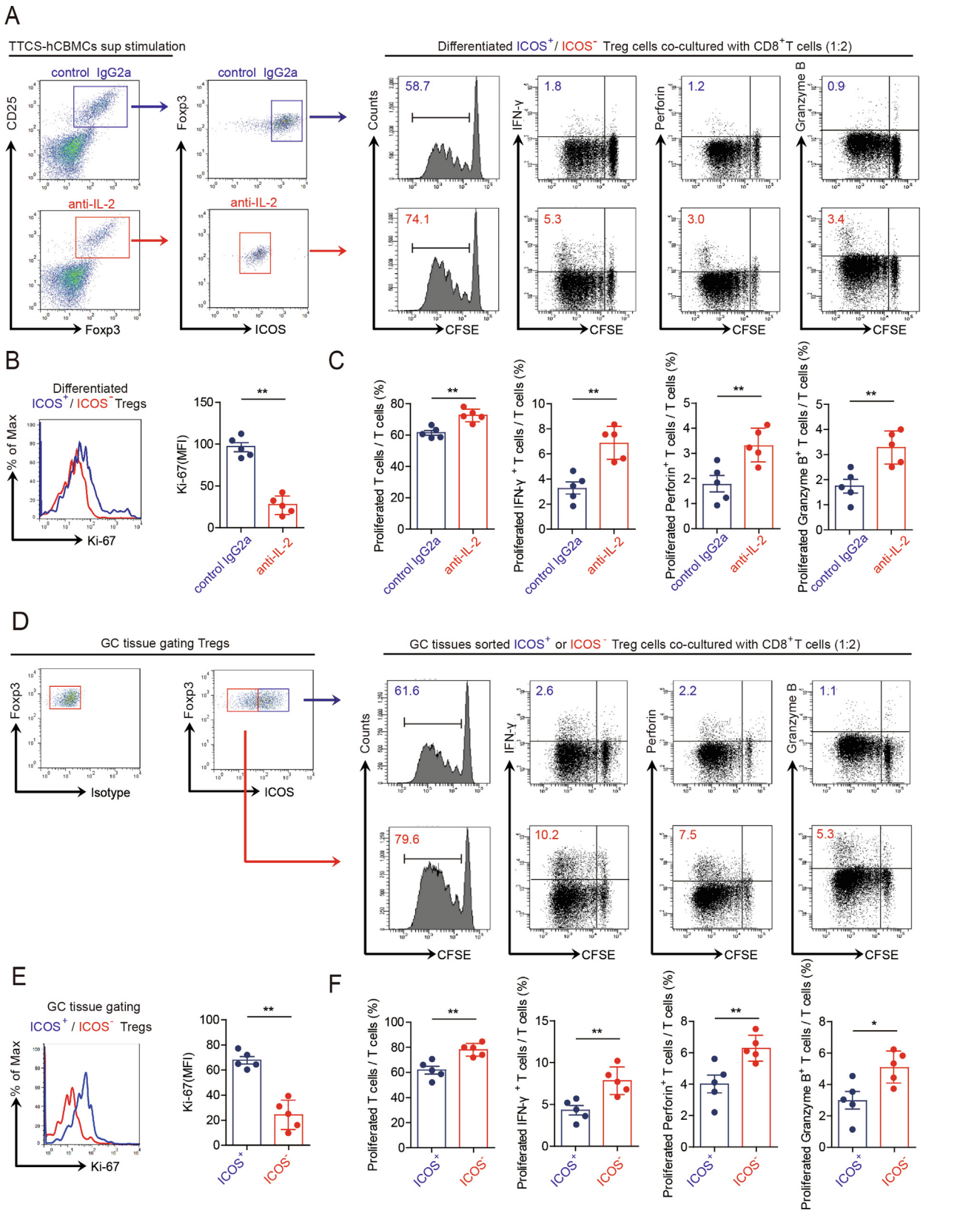

Fig. 5. IL-2–expanded ICOS$^+$ Tregs exhibit increased suppression of CD8$^+$ T cell proliferation and anti-tumor effector function. Naïve T cells (CD4$^+$CD45RA$^+$) were stimulated with TTCS-hCBMCs supernatant with IL-2 neutralizing antibodies (20 μg/ml) or control IgG2a (20 μg/ml) for 5 days. According to the expression of ICOS, the differentiated Tregs were divided into ICOS$^+$ Tregs or ICOS$^-$ Tregs subsets. (**A** and **C**) ICOS$^+$ Tregs and ICOS$^-$ Tregs subsets were sorted by FACS, and co-cultured with CD8$^+$ T cells for 5 days. Representative data (**A**) and statistical analysis (**C**) of CD8$^+$ T cell proliferation and intracellular cytokines production were shown (n = 5). (**B**) Representative data and statistical analysis of Ki-67 expression on ICOS$^+$ Tregs and ICOS$^-$ Tregs subsets respectively. (**D** and **F**) ICOS$^+$ Tregs and ICOS$^-$ Tregs subsets sorted from tumor tissues of GC patients, and co-cultured with CD8$^+$ T cells for 5 days. Representative data (**D**) and statistical analysis (**F**) of CD8$^+$ T cell proliferation and intracellular cytokines production were shown (n = 5). (**E**) Representative data and statistical analysis of Ki-67 expression on ICOS$^+$ Tregs and ICOS$^-$ Tregs subsets sorted from tumor tissues of GC patients. *, $P < 0.05$; **, $P < 0.01$ for groups connected by horizontal lines.

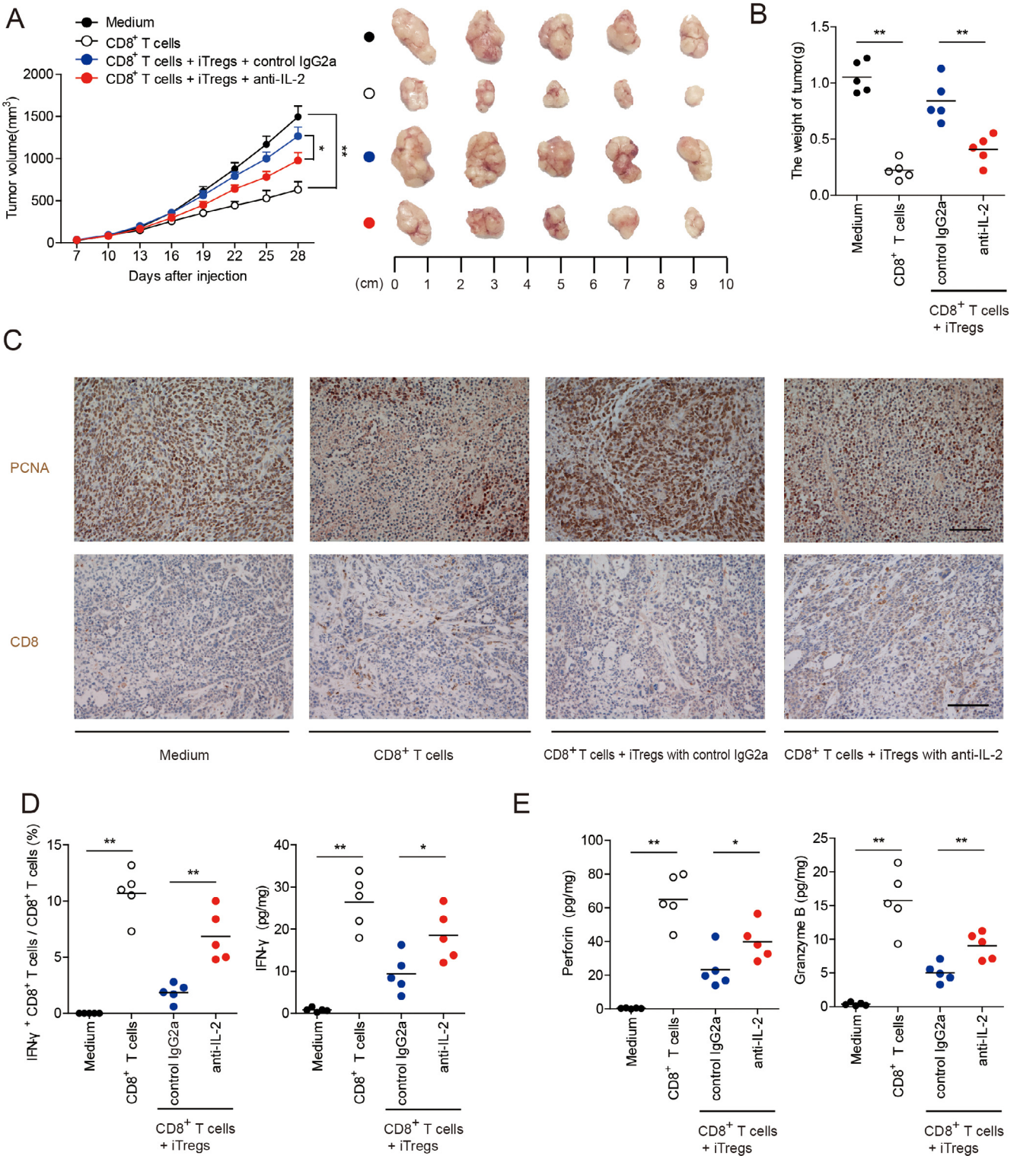

Fig. 6. *In vivo*, blocking immunosuppressive ICOS$^+$ Tregs inhibited tumor growth and progression of GC. (**A**) Mice were injected with human SGC-7901 cells, as described in Methods. Control animals () didn't receive any additional injections. Experiments involved injections of CD8$^+$ T cells () or CD8$^+$ T cells in combination with TTCS-hCBMCs supernatant with control IgG2a () or anti-IL-2 () induced Tregs (iTregs). In the illustrations, tumor volumes are represented for five mice in each group. The day of the tumor cell injection was counted as day 0, with the tumors being excised and photographed 28 days after injection. (**B**) The weights of tumors were compared. (**C**-**E**) Detection of proliferating cell nuclear antigen (PCNA) (brown) in tumors, infiltration of CD8$^+$ T cells (brown) (**C**), and production of IFN-γ (**D**), perforin and granzyme B (**E**) in tumors and IFN-γ-producing T cell response (**D**) in spleens of mice were compared (n = 5). Scale bars: 100 μm. The horizontal bars represent mean values. *$P < 0.05$; **, $P < 0.01$; n.s., $P > 0.05$ for groups connected by horizontal lines.

presenting cells (APCs) are evidently considerable for the regulation of T cell differentiation [47]. Although mast cells are non-professional APCs, the CD4$^+$ T cell response to infection was altered in mast cell-deficient mice, suggesting mast cells are capable of modulating T-cell responses as well [48,49]. Furthermore, mast cells could secrete IL-6 to promote Th17 differentiation leading

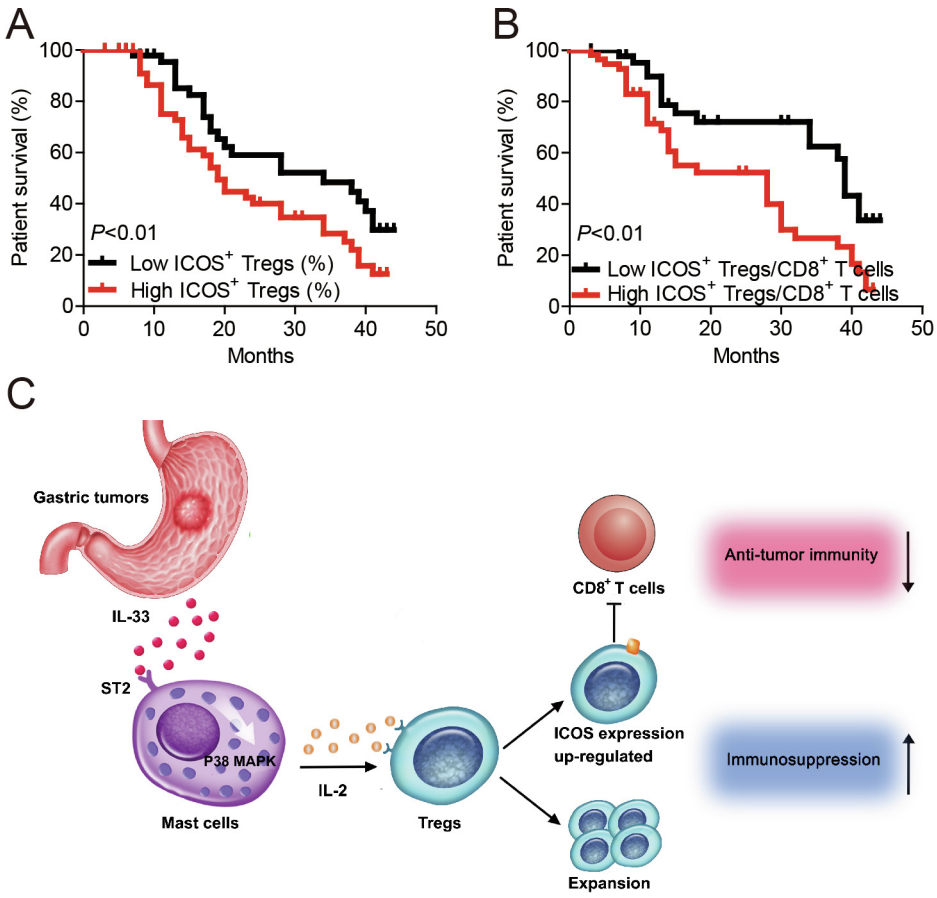

Fig. 7. The proportion of ICOS$^+$ Treg cells and the ICOS$^+$ Treg/CD8$^+$ T cell ratios in cancer patients are negatively correlated with patient survival. Here is a graphic depicting the GC, mast cell, and Treg cross-talk in the cancer microenvironment. (**A** and **B**) Kaplan-Meier plots for overall survival by median ICOS$^+$ Tregs percentage (63.5%) (**A**) or median ratio of ICOS$^+$ Tregs / CD8$^+$ T cells (5.28%) in GC (**B**). (**C**) By activating the p38 signaling pathway, GC-derived-IL-33 stimulates mast cells to release IL-2, increasing the expression of ICOS on Tregs. In GC, ICOS$^+$ Tregs inhibit CD8$^+$ T cell proliferation and antitumor immunity, facilitating cancer progression.

to increased inflammation and autoimmunity in diabetic mice [50]. However, little is known about the effects of mast cells and mast cell-derived IL-2 in the tumor microenvironment. In our study, we confirmed that mast cell-derived IL-2 is important for Treg differentiation, expansion and CD8$^+$ T cell suppression in the GC milieu.

Treg subsets with differing states of differentiation/activation can be discerned using various markers. Here, we found that Treg expansion was suppressed *in vitro* when mast cell derived-IL-2 was neutralized. It's known that CD25 is the receptor for IL-2, CD25$^+$ Foxp3$^+$ Treg may respond to IL-2 stimulation in comparison to the CD25$^-$ population of cells that do not have the IL-2 receptor. Several recent studies suggest that CD25 molecules on Treg cells and possibly other cells are capable of influencing T-cell homeostasis via IL-2 deprivation [51]. In addition, IL-2 neutralizing antibodies can be added to the *in vitro* culture system to deepen our understanding of IL-2 on T cell homeostasis. Phenotypic analysis of CD25$^+$Foxp3$^+$ compared with CD25$^-$Foxp3$^-$ cells cultured with TTCS-mast cell supernatant treated with control IgG2a or anti-IL-2 neutralizing antibody showed altered surface marker expression on CD25$^+$ Foxp3$^+$ Treg cells, including CTLA-4, PD-1, ICOS, CD39, CD73, LAP, CD103, CD44 and CD69. ICOS expression on CD25$^+$ Foxp3$^+$ cells were reduced following IL-2 blockade. CD28 family member ICOS is well known as a costimulatory molecule expressed on T cells after they have been activated. Treg ICOS expression marks an activated Treg phenotype with enhanced suppressive capacity [52]. In follicular lymphoma, follicular Tregs have been reported to exhibit high ICOS expression and strong inhibition of CD4$^+$ effector T cell activity [53]. Besides, ICOS$^+$ Tregs in GC and colorectal cancer (CRC) patients were closely associated with *H. pylori* in gastric epithelium and their prognosis, overall survival was longer in patients with low ICOS$^+$ Tregs than in those with high ICOS$^+$ Tregs, and patients with anti-*H. pylori* antibody (Hp-Ab) showed shorter recurrence-free survival than those without Hp-Ab, which suggested pre-operative *H. pylori* eradication has potential as a novel immunotherapy for GC and CRC patients [54]. Nonetheless, whether and how ICOS$^+$ Tregs could modulate anti-tumor CD8$^+$ cytotoxic T cells has not been entirely researched in GC. Using a combination of flow cytometry, cell sorting, cell co-cultures and neutralizing antibodies, our results show that mast cell-derived IL-2 can up-regulate ICOS expression in Tregs. Previous studies have shown that the way to create LAK cells is to use high doses of IL-2, which has shown toxicity. A recent study with innovative value shows effects of IL-2 (200 U/ml) with immune-enhancing effects on the expression of activating NKG2D, inhibitory CD158a and CD158b receptors on CD8$^+$ T, NKT-like and NK cell lymphocyte subsets originating from regional lymph nodes of melanoma patients *in vitro* [55]. Furthermore, Tregs were divided into ICOS$^+$ and ICOS$^-$ subsets and ICOS$^+$ Tregs displayed a superior proliferative and immunosuppressive ability compared to ICOS$^-$ Tregs.

Tumor-infiltrating lymphocytes (TILs) have been used as a biomarker of the host immune response to cancer and the level of TIL infiltration is closely related to cancer patient prognosis [33]. ICOS$^+$ Tregs were abundantly observed in the late stages of gastric cancer and exhibited the ability to produce IL-10, but not IFN-γ, TNF, or IL-17, which was closely related to plasmacytoid dendritic cells

(pDCs) and their expression of ICOS-L and TLR9 as well as *H. pylori* infection [56]. $CD8^+$ T cells are the vital component of TILs that play crucial anti-tumor role. In our study, we co-cultured $CD8^+$ T cells with Tregs *in vitro* and found that $ICOS^+$ Tregs greatly inhibited $CD8^+$ T cell proliferation and their anti-tumor effector functions. Importantly, using an *in vivo* GC model, we revealed that $ICOS^+$ Tregs inhibited $CD8^+$ T cell immunity and promoted GC development in an IL-2 dependent manner. Our results are consistent with those from Tu JF et al, in which $ICOS^+$ Tregs were identified as the major immunosuppressive cells in the liver carcinoma microenvironment [57].

The relationship between infiltrating $ICOS^+$ Tregs and GC patient outcomes is of clinical interest. Our results found that patients with higher proportions of $ICOS^+$ Tregs in their GC showed worse prognoses than those with lower $ICOS^+$ Treg proportions. In addition, patients with a higher $ICOS^+$ Tregs/$CD8^+$ T cell ratio in their GC exhibited a significantly increased risk of death than those with a lower ratio, and GC survival may be influenced by this factor independently. Together with other studies [58], our findings highlight that $ICOS^+$ Treg infiltration plays an indispensable role in GC tumor development and clinical prognosis through the suppression of anti-tumoral $CD8^+$ T cell proliferation and effector function, thereby leading to GC tumor progression.

Conclusions

Here, we reveal that GC-derived IL-33 induces mast cells to produce IL-2 through the P38 MAPK pathway, which further promotes the expansion of $ICOS^+$ Tregs. This leads to enhanced Treg immunosuppression and decreased anti-tumor $CD8^+$ T cell activity (Fig. 7C). Overall, it provides new insight into how these mechanisms work, through which mast cells and Treg subset communicate to regulate the GC microenvironment in favor of tumor progression. Our discoveries could help the development of new immunomodulatory strategies for improved GC diagnosis, treatment and patient prognosis.

Compliance with Ethics Requirements

Each participant signed an informed consent form before participating in the study, which was approved by the ethics committee of the Southwest Hospital of Third Military Medical University.

CRediT authorship contribution statement

Yipin Lv: Conceptualization, Methodology, Investigation, Writing – original draft, Validation, Formal analysis, Visualization, Supervision, Project administration, Funding acquisition. **Wenqing Tian:** Validation, Formal analysis, Visualization, Supervision, Project administration. **Yongsheng Teng:** Resources, Data curation. **Pan Wang:** Resources, Data curation. **Yongliang Zhao:** Resources, Data curation. **Zhengyan Li:** Resources, Data curation, Funding acquisition. **Shanhong Tang:** Funding acquisition. **Weisan Chen:** Resources, Writing – review & editing. **Rui Xie:** Supervision, Project administration, Resources, Writing – review & editing. **Muhan Lü:** Supervision, Project administration, Resources, Writing – review & editing. **Yuan Zhuang:** Conceptualization, Methodology, Investigation, Writing – original draft, Validation, Formal analysis, Visualization, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2023.04.013.

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