Virchows Archiv (2019) 475:579–586
https://doi.org/10.1007/s00428-019-02579-9

ORIGINAL ARTICLE

IL-33 in the tumor microenvironment is associated with the accumulation of FoxP3-positive regulatory T cells in human esophageal carcinomas

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Received: 1 March 2019 / Revised: 7 April 2019 / Accepted: 18 April 2019 / Published online: 6 May 2019

Abstract

Forkhead box p3 (Foxp3$^+$) regulatory T cells (Tregs) are abundant in the tumor microenvironment where they dampen functions of host anti-tumor immunity and promote cancer progression. Cytokine signaling is essential for the generation and function maintenance of Tregs in patients with cancers. Recent in vitro and in vivo studies have described that interleukin (IL)-33 plays a critical role in regulating the expansion and function of Tregs. However, the regulatory role of IL-33 in Treg recruitment within the microenvironment of human esophageal squamous cell carcinoma (ESCC) to date is poorly understood. In this study, we have therefore characterized the expression of IL-33 by immunohistochemistry (IHC) and double immunofluorescence staining and analyzed its relationship with FoxP3$^+$ Treg accumulation in the microenvironment in 80 patients with ESCC. IHC observation revealed a high expression level of IL-33 in both ESCC mass and stroma, which paralleled to a high density of FoxP3$^+$ Tregs accumulated in the same compartments. Statistical analysis showed that the scores for cell densities of tumor- and stroma-expressing IL-33 were significantly correlated with the scores for density of FoxP3$^+$ Tregs in the tumor stroma. Further immunofluorescence images demonstrated that IL-33 functional receptor, ST2, was preferentially expressed in FoxP3$^+$ Tregs, suggesting a possible effecting pathway for IL-33. In addition, cyclooxygenase-2, one of the important immunosuppressive factors, was highly illustrated in FoxP3$^+$ Tregs. We have therefore concluded that microenvironmental-expressing IL-33 is associated with the recruitment of Tregs in human ESCCs.

Keywords Interleukin-33 · ST2 · Regulatory T cells · Microenvironment · Esophageal carcinoma

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Abbreviations

ESCC	Esophageal squamous cell carcinoma
IL	Interleukin
Treg	Regulatory T cell
FoxP3	Forkhead box p3
IHC	Immunohistochemistry
FITC	Fluorescein isothiocyanate
PGE2	Prostaglandin E2
TGF-β	Transforming growth factor-beta
COX-2	Cyclooxygenase-2
SEM	Mean of standard error
TNM	Tumor/node/metastasis

Introduction

Esophageal cancer is one of the commonest malignancies, being the eighth leading cause of cancer-related deaths in the world [9]. Esophageal squamous cell carcinoma (ESCC) is the

commonest histological subtype for esophageal cancers in Asia. Our location (Henan Province, Central China) has an extremely high incidence of ESCC and ESCC remains one of the deadliest cancers, with age-standardized incidence rates for both sexes exceeding 39.46/100,000/year and annual age-adjusted mortality rates of up to 27.24/100,000/year [14]. Due to its extremely aggressive nature, the systemic metastasis is often observed at diagnosis and the prognosis of ESCC becomes very poor [14]. To improve the clinical outcome, attempts to understand the controlling mechanisms of tumor invasion and metastasis have been made [30, 38]. A growing body of scientific evidence indicates that the growth, invasion, and metastasis of human cancers are not only determined by the cancer cells, but also by the interaction between cancer cells and the microenvironment [20, 25, 35]. In supporting this hypothesis, previous studies have revealed that tumor tissue is typically infiltrated by high densities of immune cells from the premalignant stage to malignant stage [6, 11, 28]. However, despite high density of immune cells observed in the tumor microenvironment, most tumors still progress invasively once they are established. One of the potential mechanistic explanations for such phenomenon is that host immunity in most patients with cancers does not develop a satisfactory anti-tumor immune response, and the function of immune cells in the tumor microenvironment may be inhibited by immunosuppressive factors produced by tumor cells or/and surrounding stromal cells [2, 16, 17, 31, 35].

The regulatory T cells (Tregs), formerly known as suppressor T cells, are a subpopulation of T cells that have been shown to be critical for the maintenance of immunologic tolerance and homeostasis [26]. Tregs have been proposed to dampen functions of host anti-tumor immunity and thus promote cancer progression [21, 34]. They may mediate powerful suppression of effector T cells via diverse mechanisms, produce immunosuppressive cytokines, notably transforming growth factor-beta (TGF-β) as well as prostaglandin E2 (PGE2) and adenosine, and are resistant to apoptosis or chemotherapies [8, 39]. It is now appreciated that generation and function of Tregs are regulated by factors from the tumor microenvironment [33, 37]. For example, interleukin (IL)-2/IL-2R pathway and TGF-β and cyclooxygenase-2 (COX-2)-PGE2 pathway are important for the activation of Tregs within the tumor microenvironment [37].

Studies performed over the past several years have revealed that the generation and function maintenance of Tregs are regulated by a vast and intricate network of cytokines [33]; therefore, investigating the role of diverse cytokines in Treg biology may carry the potential therapeutic significance in the context of human cancers [33]. IL-33, a novel IL-1 superfamily cytokine, is abundantly expressed in endothelial cells, epithelial cells, and fibroblast-like cells [1, 3–5]. IL-33 and its main receptor, ST2, form a functional axis that contributes to the development of chronic inflammation, immune tolerance, and tumorigenesis [1, 5]. Tregs are important for the establishment and maintenance of immunosuppressive and immune tolerance in patients with cancers [8, 39]. Thus, much interest has now focused on the modulation of Treg generation and function by factors released from the tumor microenvironment [33]. Indeed, recent progression has uncovered important roles of IL-33 in the stimulation of Treg expansion and function [12, 13, 18, 27]. However, in the light of the importance of IL-33 in controlling Treg expansion and function status, surprisingly in most previous studies having investigated human cancers, the involvement of IL-33 in the regulation of Treg recruitment in the ESCC microenvironment to date is poorly understood.

We have therefore evaluated the potential role of tumor- and stroma-expressing IL-33 in modulating Treg accumulation in the human ESCC microenvironment.

Materials and methods

Patients and tissue blocks

Eighty cases of surgical resected ESCC and 20 cases of non-tumor esophageal tissues were randomly retrieved from the paraffin tissues bank at the Department of Pathology, the Second Affiliated Hospital of Zhengzhou University between 2000 and 2010. The mean age at treatment for ESCCs was 60.38 years (range 32~79 years); male/female, 52/28; TNM stage I/II/III, 4/56/20; and node involvement positive/negative, 23/57. Twenty non-tumor esophageal tissues taken from far distance from esophageal cancer served as controls (mean age 54.35 years, range 27~72 years); male/female, 13/7. No patient received preoperative radiotherapy and/or chemotherapy. Histological diagnoses for all the biopsies were reviewed at the Department of Pathology, the Second Affiliated Hospital of Zhengzhou University. Use of human materials was approved by the Local Ethic Committee of the Second Affiliated Hospital, Zhengzhou University, and written concerns were obtained from all the participants.

Immunohistochemistry

Immunohistochemistry (IHC) for IL-33 and forkhead box P3 (FoxP3, to label Tregs) was performed with a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions and our published methods [3, 7, 11]. The following primary antibodies were used: goat anti-IL-33 polyclonal antibody (working dilution 1:100; R&D systems, Minneapolis, MN, USA) and mouse anti-FoxP3 polyclonal antibody (working dilution 1:100, Abcam, Cambridge, UK). Antibodies were incubated at 4 °C overnight. 3-Amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as chromogen,

and slides were slightly counterstained with Mayer's hematoxylin.

Double immunofluorescence staining

Here, to define whether Tregs in the tumor stroma express IL-33 receptor ST2, double immunofluorescence staining with ST2 (rabbit polyclonal, working dilution 1:100; Thermo Fisher Scientific, USA)/FoxP3 antibodies was conducted according to the protocol described in our previous publication [2, 6, 7]. FoxP3 immunoreactivity (IR) was developed with Texas red–conjugated secondary antibody and ST2 IR with fluorescein isothiocyanate (FITC)–conjugated secondary antibody (both from Jackson ImmunoReach Lab., West Grove, PA, USA).

To further examine the possible mechanisms by which Tregs induce immunosuppression in the ESCC microenvironment, we have analyzed the producing potential of COX-2, which is a well-known tumorigenesis promoter and immunosuppressive factor, by Tregs. Double immunofluorescence with COX-2 (rabbit polyclonal, working dilution 1:400, Cayman Chemical, Ann Arbor, MI, USA)/FoxP3 antibodies was used to evaluate the potential role of FoxP3$^+$ Tregs in producing COX-2 within the ESCC microenvironment. COX-2 IR was developed with fluorescein (FITC)-conjugated and FoxP3 IR was with Cy3-conjugated secondary antibodies (Jackson ImmunoReach Lab., West Grove, PA, USA). Nuclear counterstaining was not applied and the stained slides were observed and photographed with a confocal microscope (LSM-700, Carl Zeiss, Jena, Germany) under × 200 mediate-power fields (MPF). Negative controls were performed with (1) primary antibodies were substituted with the isotype-matched control antibodies and (2) the cross-reactivity was examined by crossing different secondary antibodies.

Multicolor immunofluorescence staining

Since the regulatory effect of IL-33 on ST2-expressing Treg cells has been reported [27], we have therefore evaluated the potential autocrine loop of IL-33/ST2 in Tregs within the ESCC microenvironment. Multicolor immunofluorescence staining with IL-33/ST2/FoxP3 antibodies was performed in 10 selected ESCC specimens. IL-33 IR was developed with Texas red–conjugated secondary antibody, ST2 IR with FITC-conjugated secondary antibody, and FoxP3 IR with Brilliant Violet™ Dye 480–conjugated secondary antibody (Jackson ImmunoReach Lab., West Grove, PA, USA).

Morphometric evaluation

All the stained slides were evaluated under light microscopy and the density of IL-33$^+$ cells was quantitatively graded in both ESCC mass and stroma respectively. The density of IL-33$^+$ cells was graded in three well-orientated high-power fields (× 400) with abundant distribution as follows: (0), < 30% of total cell mass; (1), 30~50% of total cell mass; (2), 50~70% of total cell mass; (3), > 70% of total cell mass. The densities of FoxP3$^+$ Tregs in the stroma and ESCC mass were quantified in at least three optical high-power fields (× 400) with abundant distribution respectively. The average values of positive cells per slide were used for statistical analysis.

Statistical analysis

The data were presented as the mean ± SEM (standard error of the mean) unless otherwise stated. *P* values were evaluated by the Mann-Whitney test and Kruskal-Wallis test. The correlation between densities of ESCC mass- and stroma-expressing IL-33$^+$ cells and the density of FoxP3$^+$ Tregs was analyzed with non-parametric correlation Spearman's *R* coefficient analysis. P values < 0.05 were considered statistically significant.

Results

Immunohistochemistry of IL-33$^+$ cells and FoxP3$^+$ Treg distribution patterns in the tumor microenvironment of ESCC

In the adjacent non-tumor sections, the immunoreactivity of IL-33 was primarily observed in the lamina propria cells and in the deep layer of squamous epithelial cells (Fig. 1a). In the ESCC sections, many IL-33$^+$ cells were observed in both the tumor stroma (arrowhead pointed in Fig. 1c) and the ESCC tumor mass (Fig. 1b).

In adjacent non-tumor sections, low density of FoxP3$^+$ Tregs could be observed in both squamous epithelium and stromal cells (Fig. 1c). In the ESCC sections, increased density of FoxP3$^+$ Tregs was observed in both stroma (arrowhead pointed in Fig. 1d) and ESCC mass (arrow pointed in Fig. 1d).

Further semi-quantitative data of IL-33$^+$ cells and FoxP3$^+$ Tregs confirmed IHC observations and showed that grading scores of IL-33$^+$ cells and FoxP3$^+$ Tregs in both the ESCC epithelium and tumor stroma were significantly higher than those in the non-tumor tissue (see Fig. 2a–d).

Spearman's *R* coefficient analysis of density correlation between IL-33$^+$ cells and FoxP3$^+$ Tregs in the ESCC microenvironment

In the ESCC stroma, the densities of IL-33$^+$ ESCC cells and IL-33$^+$ stromal cells were correlated with the densities of FoxP3$^+$ Tregs ($P < 0.01$ or 0.05 respectively, see Table 1). However, such a correlation was not found in the ESCC epithelium (see Table 1).

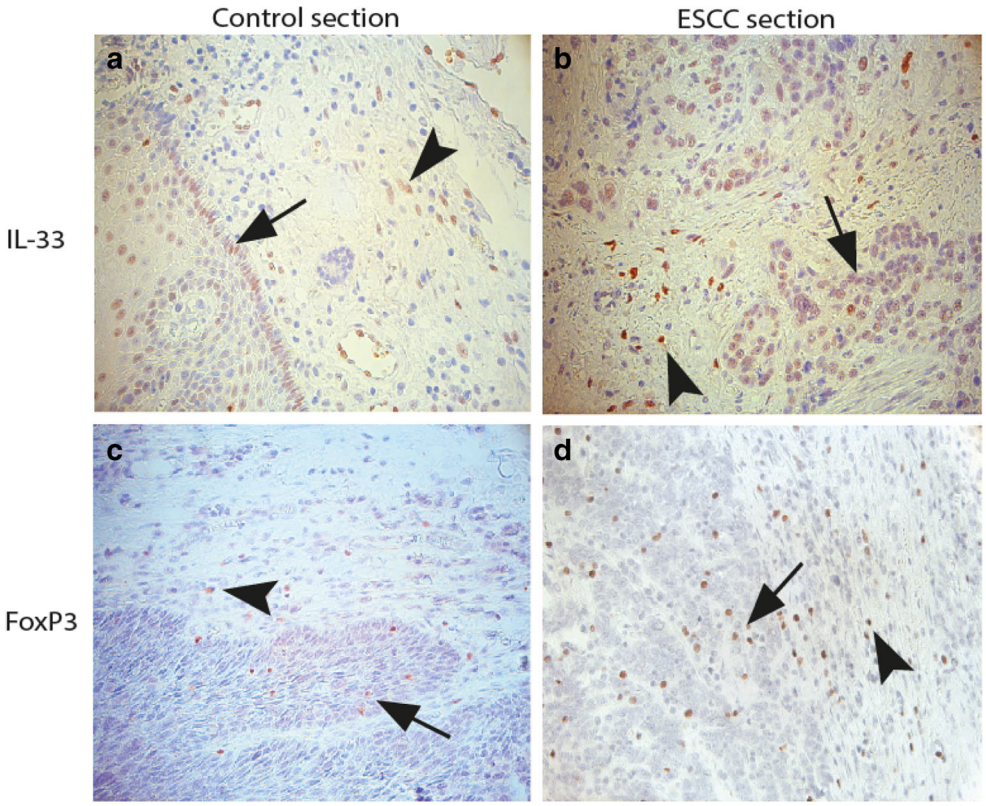

Fig. 1 Immunohistochemical examination of IL-33 and FoxP3$^+$ Tregs in the ESSC microenvironment. In the lamina propria of representative non-tumor esophageal section (**a**), IL-33 immunoreactivity (IR) was observed in squamous epithelium particularly in the deep layer (*black* arrow pointed in (**a**)) and stromal cells (*black* arrowhead pointed in (**a**)). In the stroma of representative ESCC section (**b**), IL-33 IR was observed in both ESCC cells (*black* arrow pointed in (**b**)) and stromal cells (*black* arrowhead pointed in (**b**)). In the representative non-tumor esophageal section (**c**), some FoxP3$^+$ Tregs could be observed in both the lamina propria (*black* arrow pointed in (**c**)) and epithelium (*black* arrowhead pointed in (**c**)). Increased density of FoxP3+ Tregs was highly enriched in both the ESCC epithelium (*black* arrow pointed in (**d**)) and ESCC stroma (*black* arrowhead pointed in (**d**)) (**a**–**d** IHC, counterstained with hematoxylin, original magnification × 400)

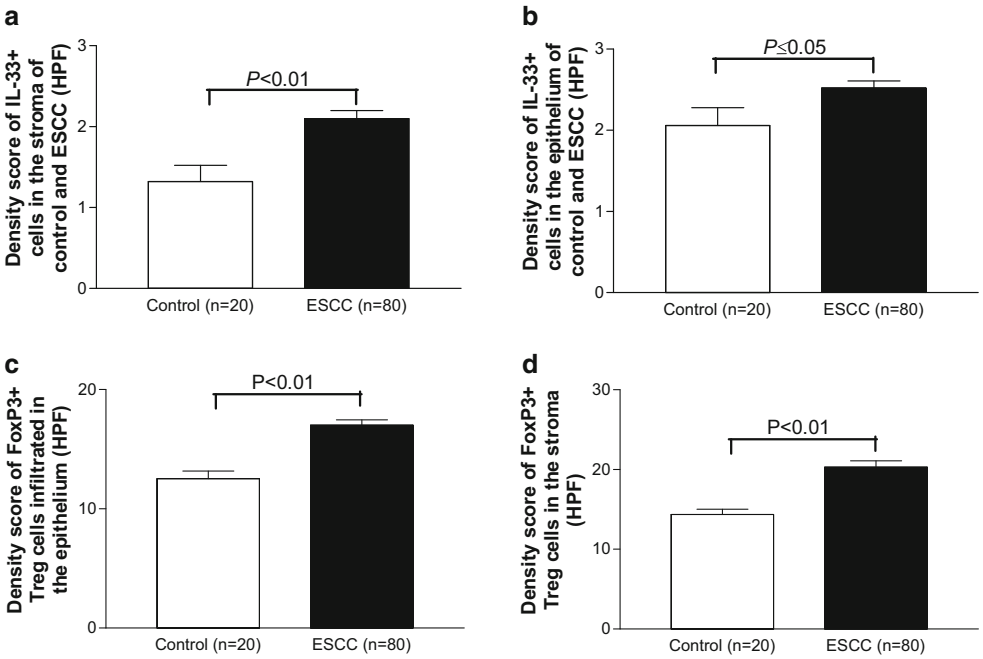

Fig. 2 Semi-quantitative density score results showed that the density scores of IL-33$^+$ cells and FoxP3$^+$ Tregs in both the ESCC epithelium and tumor stroma. Semi-quantitative density score results revealed that the densities of IL-33$^+$ cells in the ESCC mass and stroma (*black* bars in (**a**, **b**)) were higher than that in the controls (*white* bars in (**a**, **b**)). Similarly, densities of FoxP3$^+$ Tregs in both the ESCC epithelium and stroma (*black* bars in (**c**, **d**)) were also higher than those in the controls (*white* bars in (**e**, **h**)) (HPF, high powder field; *P* values were from Mann-Whitney tests)

Table 1 Spearman's *R* coefficient analysis of density correlation between IL-33$^+$ cells and FoxP3$^+$ Tregs in the ESCC microenvironment

	IL-33$^+$ ESCC cells	IL-33$^+$ stroma cells
FoxP3$^+$ cells in ESCC epithelium		
r	0.080	0.1112
P	0.4759	0.3262
FoxP3$^+$ cells in ESCC stroma		
r	0.4962	0.2497
P	< 0.001	0.0255

ST2, the functional receptor for IL-33, is highly expressed in FoxP3$^+$ Tregs and Tregs express immunosuppressive factor COX-2

ST2 is the functional receptor for IL-33, and the effect of IL-33 on the recruitment of Tregs may be through ST2 receptor expressed in target cells [23]. One of the potential mechanisms for Tregs in inhibiting host immunity is to release immunosuppressive factors such as COX-2 [29, 32]. We have therefore examined the expression of ST2 in Tregs and COX-2 expression in Tregs.

Double immunofluorescence images demonstrated that the immunoreactivity of ST2 was co-expressed with FoxP3$^+$ Tregs (see Fig. 3a–c), which confirmed that ST2 is expressed in Tregs in the ESCC microenvironment. Moreover, many FoxP3$^+$ Tregs located in the ESCC stroma could express immunosuppressive factor COX-2 (Fig. 3c, d), which suggests a possibility of Tregs in producing COX-2.

Both IL-33 and its functional receptor, ST2, are expressed in FoxP3$^+$ Tregs in the ESCC microenvironment

Multicolor immunofluorescence staining showed that both IL-33 (red cell in Fig. 4a) and its functional receptor, ST2 (green cell in Fig. 4b), were co-expressed with the FoxP3$^+$ Tregs (blue cell in Fig. 4c) located in the ESCC stroma (see merged image in Fig. 4d); this finding indicates that Tregs are not only the target, but also the cellular sources for IL-33, and IL-33 might exert its biological effect on Tregs via a potential autocrine/paracrine action pathway in the ESCC microenvironment.

Discussion

Several previous studies have shown that IL-33 participates in the modulation of Treg expansion and function [10, 24, 27, 33, 40]. In this study, we have for the first time demonstrated that the tumor- and stroma-derived IL-33 is associated with the presence of FoxP3$^+$ Tregs in the ESCC microenvironment and these FoxP3$^+$ Tregs were also positive for ST2 and COX-2. In addition, multicolor immunofluorescence images revealed that both IL-33 and its receptor ST2 are highly expressed in FoxP3$^+$ Tregs. Our findings suggest that microenvironmental IL-33 plays an essential role in the recruitment of FoxP3$^+$ Tregs in the human ESCC.

In this study, the examination of IL-33 in the ESCC microenvironment revealed an abundant expression of IL-33 in both the ESCC epithelium and the tumor stroma. In addition,

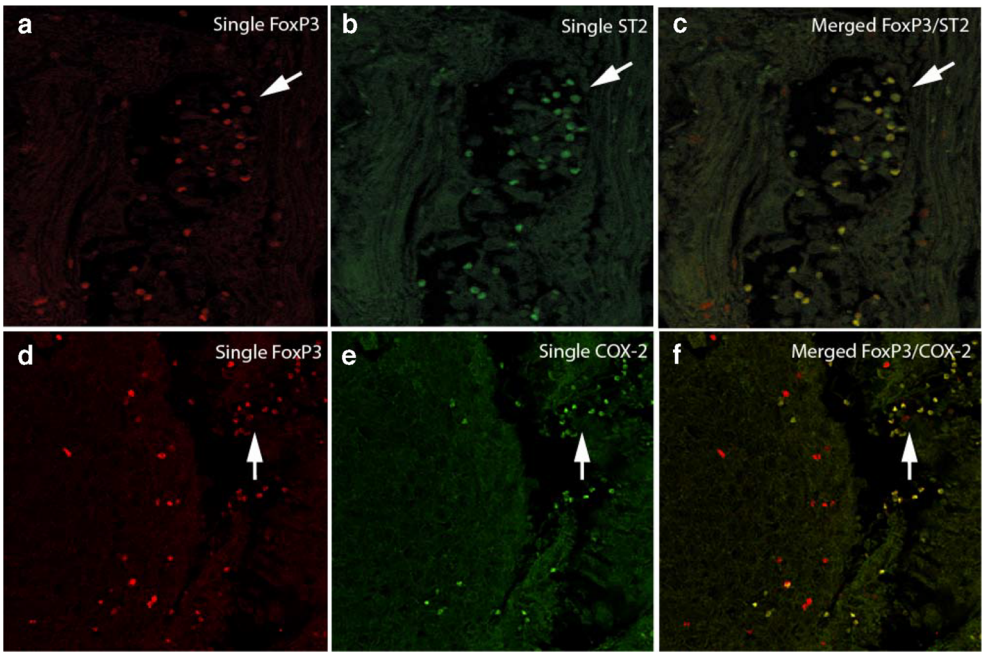

Fig. 3 Double immunofluorescence staining with confocal microscopy to characterize the expressions of IL-33's functional receptor ST2 and immunosuppressive factor cyclooxygenase-2 (COX-2) in FoxP3$^+$ Tregs in the ESCC microenvironment. Double immunofluorescence images revealed the co-localization (merged images in (**c**)) of FoxP3 immunoreactivity (IR) (*red* cells in (**a**)) with IL-33's functional receptor ST2 IR (*green* cells in (**b**)) in the ESCC stroma. Similarly, the co-localization (merged images (**f**)) of COX-2 IR (*red* cells in (**d**)) with FoxP3 IR (*green* cells in (**e**)) in the ESCC stroma was also observed (**a**–**f** images, original magnification × 200; counterstaining was not applied)

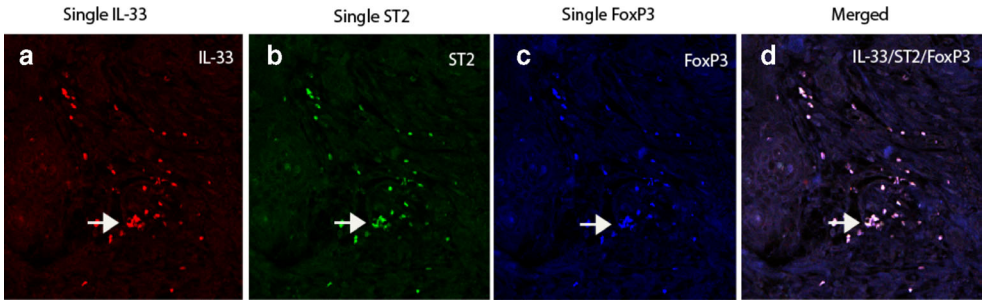

Fig. 4 Multicolor immunofluorescence staining with confocal microscopy to evaluate the autocrine/paracrine loop of IL-33/ST2 axis in FoxP3$^+$ Treg cells in the ESCC stroma. Images showed that the co-localization of IL-33 IR (*red* cells in (**a**)) and ST2 IR (*green* cells in (**b**)) with FoxP3 IR (*blue* cells in (**c**)) was observed (merged image in (**d**)) (**a**–**d** images, original magnification × 200; counterstaining was not applied)

FoxP3$^+$ Tregs were highly enriched in both the ESCC epithelium and stroma. More importantly, Spearman's *R* coefficient analysis revealed that IL-33 expressed in both ESCC cells and stromal cells was associated with the density of FoxP3$^+$ Tregs in the ESCC stroma. Since the important impact of Tregs on the suppression of host immunity has been previously demonstrated [36], such a significant correlation between stromal-expressing IL-33 and FoxP3$^+$ Tregs might logically support the notion that microenvironmental-derived IL-33 is an essential contributor for the suppression of host immunity during the process of esophageal tumorigenesis. Given the fact of infiltration of Tregs in the ESCC epithelium, we have performed an analysis for the impact of IL-33$^+$ ESCC cells and stromal cells on the infiltration of FoxP3$^+$ Tregs in the ESCC epithelium. However, the results showed that the density scores of neither tumor-derived nor stroma-derived IL-33 are associated with the density of intraepithelial FoxP3$^+$ Tregs in the ESCC; this result might imply that IL-33 does not play a major role in activating or tracking FoxP3$^+$ Tregs into the ESCC epithelium.

Furthermore, accumulative scientific evidence is now to suggest that the modulating effect of IL-33 on the generation and function of Tregs is mediated by IL-33 receptor ST2 expressed in Tregs [22, 24, 27]. We therefore assess the potential action pathway of IL-33 on Tregs in the ESCC microenvironment. Double immunofluorescence with ST2/FoxP3 antibodies showed that IL-33 functional receptor ST2 is frequently expressed in FoxP3$^+$ Tregs in the ESCC stroma, suggesting that ST2 expressed in Tregs is the main pathway for IL-33 to exert its biological effect on the recruitment of Tregs in the tumor microenvironment. It has been widely recognized that the progression of tumors is attributed to an immunosuppressive privileged microenvironment, which protects tumor cells from host immune attack. Many immunosuppressive factors released from tumor cells and inflammatory cells contribute to the formation of such immunosuppressive microenvironment in patients with cancers [2, 15]. COX-2 is a key enzyme that catalyzes the conversion of arachidonic acid into prostaglandins (PGs). Whereas PGs are the critical immunosuppressive factors that suppress host immunity and stimulate tumor cell growth [15]. Several studies assessed the association of increased tumor-infiltrating Tregs with immunosuppressive formation and revealed that one of the possible mechanisms for Tregs inhibiting host anti-tumor immunity is via the production of COX-2 that enhances the synthesis of PGs in the tumor microenvironment. There have been several reports demonstrating that the density of tumor-infiltrating Tregs was significantly correlated with COX-2 expression in human cancers [32, 39]. Therefore, in the present study, the potential of FoxP3$^+$ Tregs in expressing immunosuppressive factor COX-2, taking as an example, was examined. We found that COX-2 is highly expressed in FoxP3$^+$ Tregs in the ESCC stroma. Taken together, all these results imply an important role of COX-2 in Treg-mediated immunosuppression. Thus, a proposed mechanism for IL-33-elicited Tregs to help tumor cells to evade immune system control is by producing immunosuppressive factors as the critical contributors that inhibit the activation or/and function of dendritic cells and T effector cells, and finally induce immune tolerance [19, 28].

It is now well accepted that autocrine/paracrine manners are the important regulatory strategies for tumor- and stroma-derived factors in participating the modulation of immune cell function [27]. To identify the autocrine/paracrine loop for IL-33/ST2 in Tregs, we have conducted a multicolor immunofluorescence staining with IL-33/ST2/FoxP3 in the ESCC sections. In line with a previous study performed in the intestine [27], we were able to show that both IL-33 and ST2 were expressed in the same FoxP3$^+$ Tregs in the ESCC microenvironment. This observation supports the hypothesis that an IL-33/ST2 autocrine/paracrine pathway in the modulation of Tregs' function might exist in the ESCC microenvironment.

In conclusion, we report here, for the first time, that tumor- and stroma-derived IL-33 is crucial for the accumulation of FoxP3$^+$ Tregs in the ESCC microenvironment. These results extend our understanding of IL-33's role in modulating Treg accumulation in patients with ESCC. In the future, additional studies are needed to further refine the exact mechanisms of IL-33 signals contributing to the modulation of Treg expansion and function in the ESCC microenvironment.

Contributions Cui G designed, analyzed data, and wrote most parts of the manuscript. Yuan A did experiments, analyzed data, and wrote the manuscript. Ren J did pathological review and analysis, and Li Z joined the data analysis, discussion, and writing.

Funding This research was financially supported by the programs of Innovation Scientists and Technicians Troop Construction Projects of Henan Province (Program No. CXTD20150009), Nature and Science Foundation of Henan Province (Program No. 182300410326), and Scientific and Technological Research Project of Henan Province, China (Program No. 182102310383).

Compliance with ethical standards

Usage of human materials was approved by the Local Ethic Committee of the Second Affiliated Hospital, Zhengzhou University, and written concerns were obtained from all the participants.

Conflict of interest The authors declare that they have no conflict of interest.

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