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ORIGINAL ARTICLE

Mast cells expressing interleukin 17 in the muscularis propria predict a favorable prognosis in esophageal squamous cell carcinoma

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Abstract The proinflammatory cytokine interleukin 17 (IL-17) is considered to play a crucial role in diverse human tumors; however, its role in disease progression remains controversial. This study investigated the cellular source and distribution of IL-17 in esophageal squamous cell carcinoma (ESCC) in situ and determined its prognostic value. Immunohistochemistry, immunofluorescence and immunoelectron microscopy were used to identify IL-17-expressing cells in ESCC tissues, paying particular attention to their anatomic localization. Kaplan–Meier analysis and Cox proportional hazards regression models were applied to estimate overall survival in 215 ESCC patients with long-term follow-up (>10 years). The results showed that mast cells, but not T cells or macrophages, were the predominant cell type expressing IL-17 in ESCC tissues. Unexpectedly, these IL-17$^+$ cells were highly enriched in the muscularis propria rather than the corresponding tumor nest ($p < 0.0001$). The density of IL-17$^+$ cells in muscularis propria was inversely associated with tumor invasion ($p = 0.016$) and served as an independent predictor of favorable survival ($p = 0.007$). Moreover, the levels of IL-17$^+$ cells in muscularis propria were positively associated with the density of effector CD8$^+$ T cells and activated macrophages in the same area (both $p < 0.0001$). This finding suggested that mast cells may play a significant role in tumor immunity by releasing IL-17 at a previously unappreciated location, the muscularis propria, in ESCC tissues, which could serve as a potential prognostic marker and a novel therapeutic target for ESCC.

Keywords Mast cells · Muscularis propria · Interleukin 17 (IL-17) · Esophageal squamous cell carcinoma (ESCC) · Overall survival

Abbreviations

IL-17	Interleukin 17
ESCC	Esophageal squamous cell carcinoma
TRY	Tryptase
CHY	Chymase
MU_N	Normal mucosa
MP_N	Muscularis propria in non-tumor tissues
TN_T	Tumor nest
MP_T	Muscularis propria in tumor tissues
$IL\text{-}17^+_{MP}$ cells	IL-17$^+$ cells in MP_T
TRY^+_{MP} cells	TRY$^+$ cells in MP_T
TRY^+_{MP} cells	TRY$^+$ cells in TN_T

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Introduction

Esophageal squamous cell carcinoma (ESCC) accounts for more than 90 % of esophageal cancer cases worldwide, ranking eighth in incidence and sixth in mortality [1]. Despite the improved diagnostic and treatment strategies, the overall survival of patients with ESCC remains poor [2, 3]. Tumor invasion into the muscularis propria is a key step in disease progression, is associated with an increase in regional lymph node metastasis and serves as a critical parameter in ESCC staging [4].

Tumor progression is now recognized as the product of evolving crosstalk between different cell types within tumors [5, 6]. Emerging evidence has demonstrated that the infiltrating immune cells and the cytokine milieu at the tumor site can largely determine the biological behavior of human tumors [7]. In ESCC, it has been shown that high densities of intratumoral $CD4^+$ and $CD8^+$ T cells are associated with prolonged patient survival [8], whereas infiltrating dendritic cells can promote ESCC progression by inducing T-cell tolerance [9]. These findings are in accordance with the general view that the inflammatory reaction at a tumor site can either inhibit or promote disease progression, depending on the context [10, 11].

The proinflammatory cytokine interleukin (IL)-17 has recently been identified as a crucial mediator in the pathogenesis of diverse human tumors and is capable of being pro- or antitumorigenic [12–14]. Although the precise underlying mechanisms are not yet clear, this paradox may be accounted for by the IL-17 concentration or the nature and density of IL-17-expressing cells. IL-17 secreted by different cell types may have distinct functional effects on tumor progression due to their varying levels of IL-17 production and other contexts, including localization, the associated cytokine profile and cellular environment [13]. In addition to the well-studied IL-17-producing T cells, IL-17 can be produced in tissues by diverse cell types, such as γδ T cells, NK/NKT cells, macrophages, neutrophils, eosinophils and mast cells [15, 16]. The net IL-17 expression in situ may arise from a broad array of adaptive and innate immune cells. Thus, to understand the complex tissue context, it is important to define the cellular sources of IL-17 in tumors in situ and evaluate their clinical and pathological associations.

Human solid tumors can be anatomically classified into different areas with distinct compositions and functional properties [17–19]. We have recently shown that, in hepatocellular carcinoma, IL-17-expressing cells were predominantly enriched in the area of peritumoral stroma, where they promote disease progression by recruiting neutrophils and stimulating angiogenesis at the edge of adjacent invading tumors [20–22]. In this study, we examine the cellular source and clinical significance of IL-17 in tumors in situ from 215 ESCC patients, paying particular attention to their microlocalization. Unexpectedly, we found that mast cells in the muscularis propria are the predominant sources of IL-17-expressing cells in ESCC tumors in situ, and the density of $IL\text{-}17^+$ cells predicts a favorable prognosis and is positively associated with effector $CD8^+$ T cells and activated macrophages in the muscularis propria. These data suggest that the muscularis propria may represent a previously unrecognized region in tumor immunity in esophagus.

Materials and methods

Patients and tissue specimens

Tissue specimens were obtained between January 1996 and December 2002 from 215 patients with pathologically confirmed ESCC at the Cancer Center of Sun Yat-sen University. All patients underwent curative resection for ESCC, which was defined as complete removal of the tumor with no residual cancer [2, 23]. None of the patients had distant metastasis or any neoadjuvant therapies, such as radiotherapy or chemotherapy, before surgery. Individuals with concurrent autoimmune diseases were also excluded. Tumor stages were classified according to the TNM classification (6th edition, 2002). The clinicopathological characteristics of the patients are summarized in Supplementary material Table 1. In addition, fresh muscularis propria associated with tumor tissue (1 mm^3) was excised from three patients and used for immunoelectron microscopy. All specimens were anonymously coded in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki), and written informed consent was obtained. The protocol was approved by the Review Board of the Cancer Center.

Data on patient follow-up were obtained by the Cancer Center Tumor Registry as described previously [17, 20, 23, 24]. Of the 215 patients examined, 149 (69.3 %) died as a direct result of their disease before the end of the observation period. Overall survival was defined as the interval between surgery and death or last observation. The range of overall survival was 2.2–156.7 months. The overall survival rate was 80 % at 1 year, 43.3 % at 3 years, 34.9 % at 5 years and 30.6 % at 10 years.

Immunohistochemistry and immunofluorescence

Specimens were obtained immediately after surgical resection and fixed in 10 % neutral formalin, paraffin-embedded and used for histological assays as previously described [17, 20, 25]. Immunohistochemistry of paraffin sections was carried out using a two-step protocol (Dako A/S, Glostrup, Copenhagen, Denmark). Briefly, 5-μm paraffin

Table 1 Univariate and multivariate analysis of factors associated with overall survival in ESCC

Variables	Univariate			Multivariate		
	HR[a]	95 % CI	*p*	HR[a]	95 % CI	*p*
Age, years (>56 vs. ≤56)	1.274	0.923–1.758	0.14			NA
Gender (female vs. male)	0.622	0.418–0.924	**0.019**	0.663	0.446–0.987	**0.043**
Histology (poor vs. well + moderate)	1.236	0.855–1.787	0.259			NA
pT (pT3 + pT4 vs. pT1 + pT2)	2.009	1.404–2.875	**<0.001**	1.518	1.046–2.203	**0.028**
pN (pN1 vs. pN0)	2.646	1.903–3.679	**<0.001**	2.347	1.667–3.305	**<0.001**
Stage (IIb + III + IV vs. I + IIa)	2.639	1.895–3.676	**<0.001**			
Muscularis propria (high vs. low)						
IL-17$^{+}_{MP}$ cells	0.633	0.458–0.875	**0.006**	0.636	0.459–0.881	**0.007**
TRY$^{+}_{MP}$ cells	0.827	0.597–1.146	0.254			NA
Tumor nest (high vs. low)						
TRY$^{+}_{TN}$ cells	1.226	0.876–1.714	0.235			NA

Cox proportional hazards regression model. Variables used in multivariate analysis were adopted by univariate analysis. Significant *p* values (<0.05) are shown in bold

ESCC, esophageal squamous cell carcinoma; pT, depth of tumor invasion; pN, lymph node metastasis; IL-17$^{+}_{MP}$ cells, IL-17^{+} cells in muscularis propria of tumor tissues; TRY$^{+}_{MP}$ cells, TRY^{+} mast cells in muscularis propria of tumor tissues; TRY$^{+}_{TN}$ cells, TRY^{+} mast cells in tumor nest; HR, hazard ratio; CI, confidence interval; NA, not applicable

[a] HR > 1, risk for death increased; HR < 1, risk for death decreased

sections were first deparaffinized and hydrated, and endogenous peroxidase activity was blocked by incubating the slides in 0.3 % H_2O_2. Antigen retrieval was performed by microwave treatment in citrate buffer pH 6.0. Sections were blocked with normal sera from the same species from which the secondary antibodies were derived. After overnight incubation at 4 °C with antibodies against human IL-17 (1:300 dilution, R&D Systems, Minneapolis, MN, USA), tryptase (1:500 dilution, Thermo Fisher Scientific, Waltham, MA, USA), CD8 (1:300 dilution, Thermo Fisher Scientific, Waltham, MA, USA), CD68 (1:500 dilution, Dako A/S, Glostrup, Copenhagen, Denmark), CD169 (1:200 dilution, R&D Systems, Minneapolis, MN, USA), or control antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the sections were incubated with the secondary antibodies conjugated with horseradish peroxidase (Envision + Dual Link Kit, DAKO, for mouse/rabbit antibodies; donkey anti-goat secondary antibody from Santa Cruz Biotechnology; or R&D Systems for donkey anti-sheep secondary antibody) for 30 min. The enzymatic reaction was developed with peroxidase-labeled secondary antibody and stained with 3,3′-diaminobenzidine tetrahydrochloride using the Envision System (Dako A/S, Glostrup, Copenhagen, Denmark). Sections were then counterstained with hematoxylin (Zymed Laboratories Inc., San Francisco, CA, USA) and mounted in non-aqueous mounting medium.

For double-color immunofluorescence, paraffin-embedded tissue sections were first incubated with goat anti-human IL-17 and rabbit anti-human CD3, mouse anti-human CD68, tryptase or chymase, or sheep anti-human CD169 and mouse anti-human CD68, followed by specimen-paired immunofluorescence secondary antibodies (Life Technologies Inc., CA, USA). For triple-color immunofluorescence, sections were first incubated with goat anti-human IL-17, mouse anti-human chymase and rabbit anti-human tryptase; or with goat anti-human IL-17, mouse anti-human tryptase and rabbit anti-human IL-17, followed by specimen-paired immunofluorescence secondary antibodies (Life Technologies Inc.). Slides were mounted with Vectashield containing DAPI (Vector Laboratories) and analyzed on a fluorescent imaging microscope (BX50; Olympus, Essex, UK). Isotype-matched primary antibodies were used as negative controls (Santa Cruz Biotechnology, Inc.). Images were captured and analyzed using FV10-ASW 1.7 Viewer (Olympus, Essex, UK). Full details of the primary antibodies are listed in Supplementary material Table 2.

Immunoelectron microscopy

Tissues were processed for immunoelectron microscopy as previously described [26]. Ultrathin sections of gold interference color were cut using an ultramicrotome (EMUC6; Leica, Wetzlar, Germany) and collected on formvar-coated nickel slot grids. All immunolabeling steps were performed in a humid chamber at room temperature. Grids were floated on blocking solution, followed by incubation with goat anti-human IL-17 (R&D systems) or control antibody (Santa Cruz Biotechnology, Inc.), and then subjected to secondary labeling with donkey anti-goat IgG coupled with

10-nm gold nanoparticles (Sigma-Aldrich, Missouri, USA). Sections were post-stained in uranyl acetate and with lead stain. Grids were viewed using a JEM 1400 (JEOL, Mitaka, Tokyo) operating at 80 kV.

Quantification of immunofluorescence

Quantification methods were as previously described [27]. Numbers of single-positive or double-positive cells of interest in each of two representative fields at × 100 magnification (0.5 mm^2 per field) were counted manually by two independent, blinded observers. From these numbers, the proportions of cells in different populations were calculated [e.g., the proportion of all tryptase$^+$ (TRY$^+$) mast cells which were IL-17$^+$ was calculated as: (number of IL-17$^+$ TRY$^+$ cells)/(number of IL-17$^+$ TRY$^+$ mast cells + IL-17$^-$ TRY$^+$ mast cells)].

Evaluation of immunohistology

Analysis was performed by two independent observers who were blinded to the clinical outcome [17, 21]. To evaluate the density of IL-17$^+$ cells, TRY$^+$ mast cells, CD68$^+$ macrophages, CD169$^+$ macrophages and CD8$^+$ T cells, areas of normal mucosa, tumor nest and muscularis propria were screened at low power (100× magnification) and the five most representative high-power fields were then selected at 400× magnification (0.0768 mm^2 per field) using a Hitachi HV-C20A CCD camera (Hitachi, Tokyo, Japan) installed on a Leica AS LMD light microscope (Leica Microsystems) for each area of every specimen. The number of IL-17$^+$ cells or TRY$^+$ mast cells in each area were counted manually and expressed as the mean ± SEM cells per field. There was a significant linear correlation between the counting data of two independent observers, and the average counting by two investigators was applied in the following analysis to minimize interobserver variability.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The statistical significance of differences between groups was determined by the Wilcoxon signed-rank test. Cumulative survival time was calculated by the Kaplan–Meier method and analyzed by the log-rank test. A multivariate Cox proportional hazards model was used to estimate adjusted hazard ratios, 95 % confidence intervals (CI) and to identify independent prognostic factors. For categorical analysis, the median value was used as a cutoff to dichotomize the continuous variables (for clinical applications). Analysis of the association between variables was conducted using Spearman's rank correlation coefficient for continuous variables and χ^2 tests for categorical variables. For the above comparisons, two-tailed $p < 0.05$ was considered statistically significant.

Results

IL-17$^+$ cells are enriched in the muscularis propria of ESCC tissues

Previous studies of IL-17 expression in tumor tissues focused primarily on T cells stimulated ex vivo with PMA and ionomycin for subsequent flow cytometric analysis [13, 28]. However, such an approach does not define the identity and anatomic localization of the cells expressing intracellular IL-17 in vivo. We therefore examined the tissue location of IL-17-expressing cells in paired tumor and non-tumor tissues from 60 untreated ESCC patients. We divided the tissues into four anatomical regions: normal mucosa (MU_N) and muscularis propria (MP_N) in non-tumor tissues, and tumor nest (TN_T) and muscularis propria (MP_T) in tumor tissues (Fig. 1a, c). As shown in Fig. 1, IL-17$^+$ cells were present throughout the tissue, but predominantly located in MP_N and MP_T (8.5 ± 0.7 and 11.7 ± 0.9 cells/field, respectively) rather than MU_N or TN_T (0.3 ± 0.1 and 0.4 ± 0.1/field, respectively). Moreover, the number of IL-17$^+$ cells was significantly higher in MP_T than in the corresponding MP_N ($p = 0.003$; Fig. 1d).

Mast cells, but not T cells or macrophages, constitute the majority of IL-17-expressing cells in ESCC tissues

To identify the cellular source of IL-17 in ESCC tumor tissues, we performed colocalization studies and calculated the proportion of IL-17$^+$ cells within each cellular subset. We first investigated the colocalization of intracellular IL-17 with the T-cell marker CD3 (Fig. 2a). Unexpectedly, T cells represented only a minority of all IL-17$^+$ cells in both the TN_T and MP_T of ESCC tissues (2.1 ± 0.6 and 1.3 ± 0.5 %, respectively; $n = 9$; Fig. 2d).

In addition to T cells, recent studies have shown that IL-17 can be produced by innate immune cells, such as macrophages and mast cells in inflammatory tissues [27, 29–31]. We therefore performed double-color immunofluorescence for IL-17 with the macrophage marker CD68 or with the mast cell-specific enzyme tryptase (TRY). Although macrophages were abundant in ESCC tissues, most did not express IL-17 (Fig. 2b, e). Macrophages represented a minority of all IL-17$^+$ cells in both TN_T and MP_T of ESCC tissues (3.6 ± 1.2 and 5.0 ± 0.8 %, respectively; $n = 10$; Fig. 2e). In contrast, most IL-17$^+$ cells costained brightly with TRY (Fig. 2c and Supplementary material Fig. 1). Although the levels of TRY$^+$ mast cells were significantly higher in MP_T

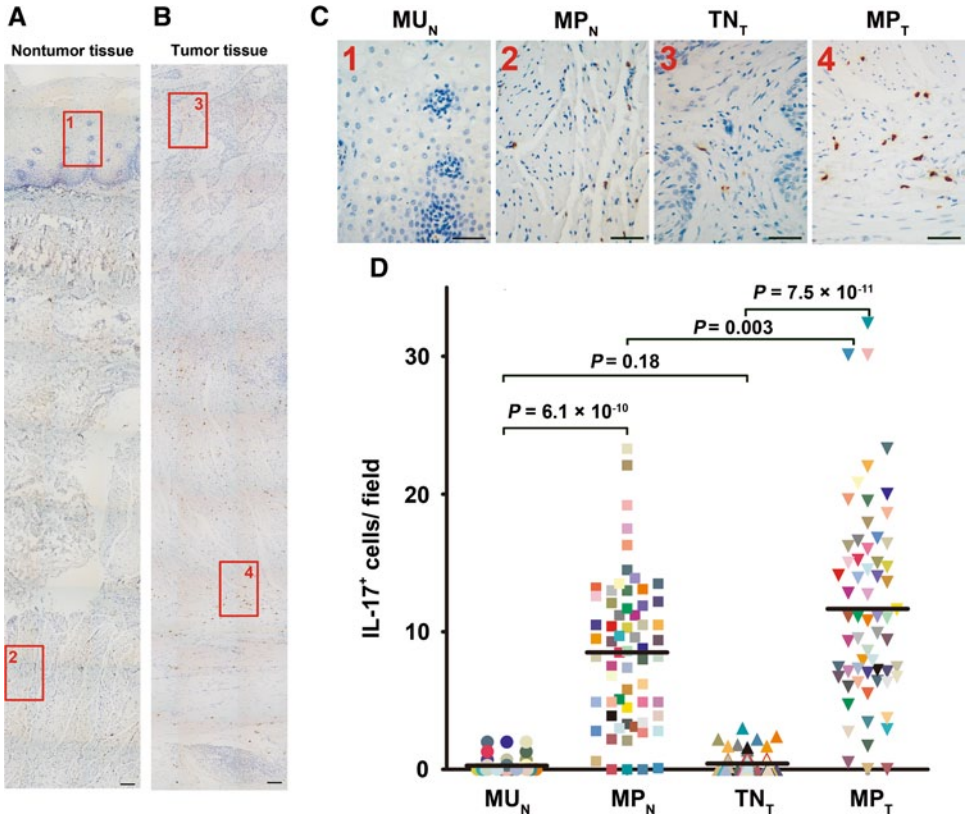


Fig. 1 IL-17$^+$ cells were enriched in the muscularis propria of ESCC tissues. Paraffin-embedded ESCC sections were stained with a goat anti-human IL-17 antibody in paired **a** non-tumor tissues and **b** tumor tissues. *Scale bar* 100 μm. **c** Higher magnification micrographs of: *1*, normal mucosa (MU_N); *2*, muscularis propria in non-tumor tissues (MP_N); *3*, tumor nest (TN_T); *4*, muscularis propria in tumor tissues (MP_T). *Scale bar* 100 μm. **d** The density of IL-17$^+$ cells was higher in MP_N and MP_T than in MU_N or TN_T from paired non-tumor and tumor tissues ($n = 60$). Results are expressed as mean ± SEM (*bars*). $p < 0.05$ was considered significant difference (Wilcoxon signed-rank test)

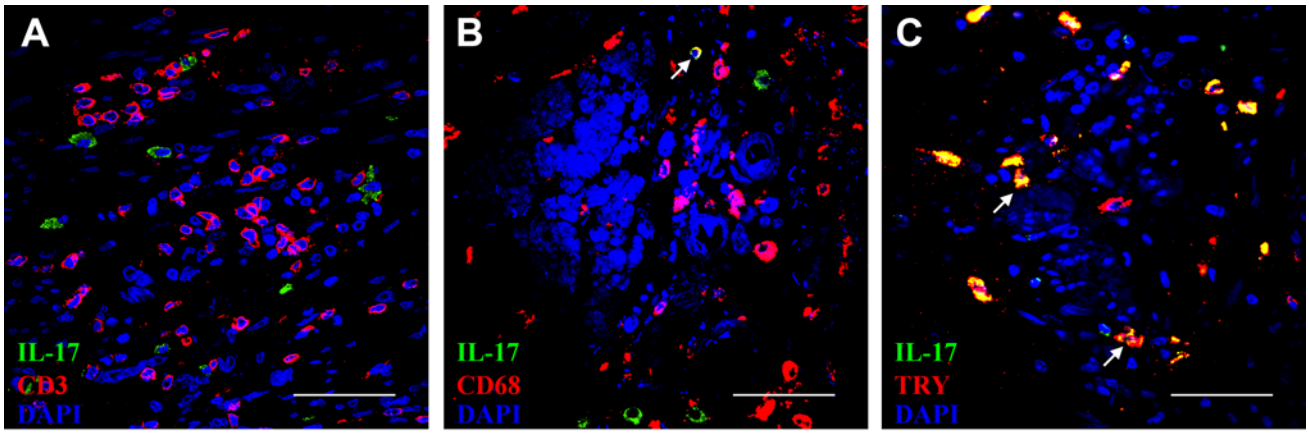
(292 ± 33/mm^2) than in TN_T (155 ± 26/mm^2; $n = 19$; $p = 0.001$), mast cells represented the majority of IL-17$^+$ cells in both the MP_T and TN_T of ESCC tissues (97 ± 6.2 and 71.9 ± 7.0 %, respectively; Fig. 2f). Moreover, both the number and proportion of IL-17$^+$ TRY$^+$ mast cells were significantly higher in MP_T (224 ± 26/mm^2 and 77 ± 3.2 %) than the corresponding TN_T tissues (28 ± 6/mm^2 and 19.1 ± 3.7 %; Fig. 2f), which is consistent with the finding that IL-17$^+$ cells were enriched in the MP_T of ESCC tissues (Fig. 1d). The specificity of IL-17 staining was further confirmed by triple-color immunofluorescence with mouse anti-TRY and two antibodies from goat and rabbit that recognize different epitopes of the human IL-17 protein, showing good colocalization of IL-17 in TRY$^+$ mast cells in ESCC tissues (Supplementary material Fig. 2).

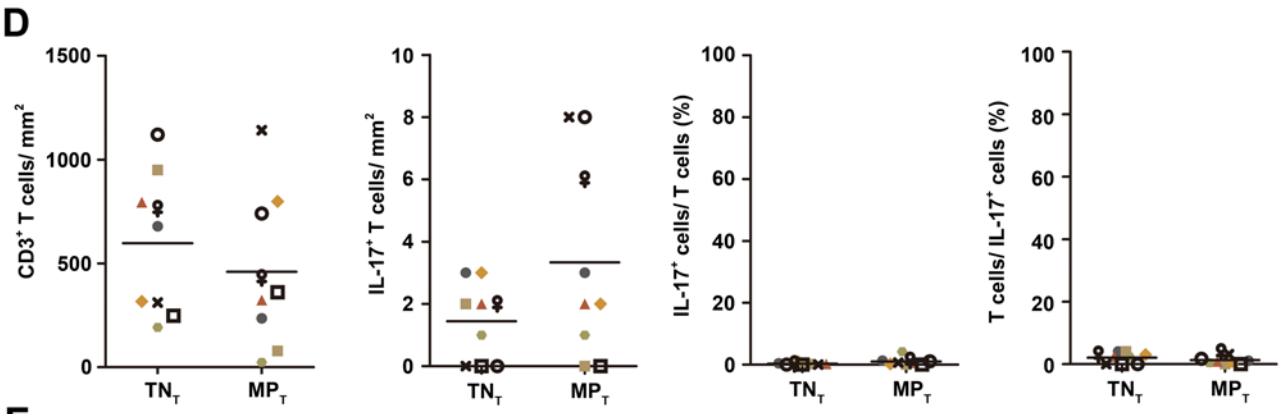
The results described above indicate that the majority of IL-17-expressing cells in ESCC tissues in situ are mast cells. We next determined the subcellular localization of IL-17 within mast cells by immunogold staining and transmission electron microscopy. The mast cells were identified on the basis of their polygonal morphology and abundant, densely packed specific granules in the cytoplasm [32]. As shown in Fig. 3a, most of the gold particles were closely associated with secretory granules, demonstrating constitutive expression of IL-17 in mast cells.

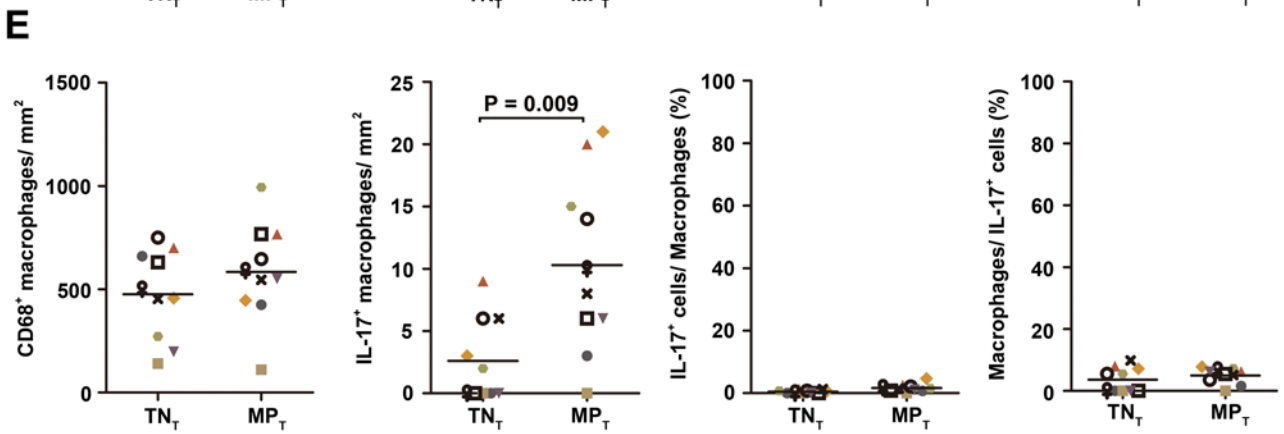
Human mast cells can be divided into two subsets depending on the expression of proteases in their granules. Mast cells expressing only tryptase are typically located in the respiratory and intestinal mucosa, whereas mast cells that contain tryptase, chymase and other proteases are found in connective tissues and are defined as connective tissue mast cells (MC_{TC}) [33]. We therefore used triple-color immunofluorescence to identify the mast cell subsets. The results showed that most IL-17-expressing cells costained brightly with tryptase and chymase, suggesting that MC_{TC} represented the majority of IL-17-expressing cells in ESCC tissues (Supplementary material Fig. 3).

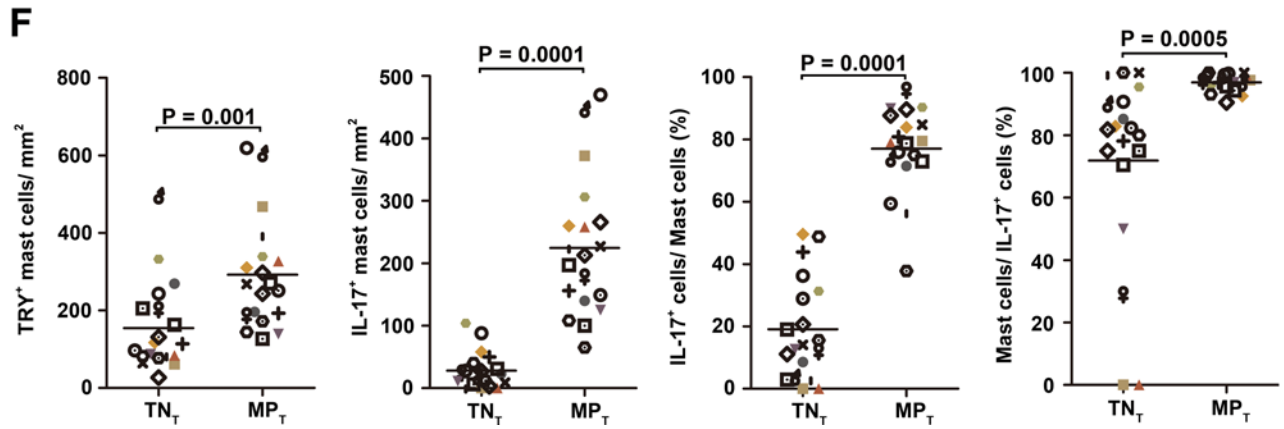
A high density of IL-17$^+$ cells in MP_T is inversely associated with tumor invasion and predicts a favorable prognosis in ESCC patients

Based on our observations that most IL-17-expressing cells were mast cells and that they were highly enriched in MP_T, but not in the corresponding TN_T in ESCC tumor tissues, we predicted that these cells might be associated with disease progression. To test this assumption, 215 ESCC patients who had received curative resection with long-term follow-up data (>10 years) were divided into two groups according to the median value of IL-17$^+$ cells in MP_T (IL-17$^+_{MP}$ cells), TRY$^+$ cells in MP_T (TRY$^+_{MP}$ cells)


A
IL-17
CD3
DAPI
B
IL-17
CD68
DAPI
C
IL-17
TRY
DAPI


D
CD3+ T cells/ mm2
IL-17+ T cells/ mm2
IL-17+ cells/ T cells (%)
T cells/ IL-17+ cells (%)
TNT
MPT


E
CD68+ macrophages/ mm2
IL-17+ macrophages/ mm2
P = 0.009
IL-17+ cells/ Macrophages (%)
Macrophages/ IL-17+ cells (%)
TNT
MPT


F
TRY+ mast cells/ mm2
P = 0.001
IL-17+ mast cells/ mm2
P = 0.0001
IL-17+ cells/ Mast cells (%)
P = 0.0001
Mast cells/ IL-17+ cells (%)
P = 0.0005
TNT
MPT

◀ **Fig. 2** Mast cells, but not T cells or macrophages, constitute the majority of IL-17-expressing cells in ESCC tissues. Paraffin-embedded ESCC sections ($n \geq 9$) were subjected to double-color immunofluorescence for IL-17 (*green*) and **a** the T-cell marker CD3, **b** macrophage marker CD68 or **c** mast cell marker TRY (*red*), with DAPI counterstain (*blue*). These high-power fields at × 600 magnification in the muscularis propria illustrate the colocalization of IL-17 with the indicated markers (*yellow color*; *white arrows*). *Scale bar* 50 μm. **d–f** Numbers and proportions of IL-17$^+$ cells in tumor nest (TN_T) and muscularis propria (MP_T) of ESCC tumor tissues. Cell numbers were calculated as cells per mm^2. **d** The numbers of CD3$^+$ T cells, IL-17$^+$ T cells and the percentages of all CD3$^+$ T cells or IL-17$^+$ cells which were double-positive for CD3 and IL-17 (from *left* to *right*, $n = 9$). **e** The numbers of CD68$^+$ macrophages, IL-17$^+$ macrophages and the percentages of all CD68$^+$ macrophages or IL-17$^+$ cells which were double-positive (from *left* to *right*, $n = 10$). **(f)** The number of TRY$^+$ mast cells, IL-17$^+$ mast cells and the percentages of all TRY$^+$ mast cells or IL-17$^+$ cells which were double-positive (from *left* to *right*, $n = 19$). Results are expressed as mean ± SEM (*bars*). $p < 0.05$ was considered significant difference (Wilcoxon signed-rank test)

or TRY$^+$ cells in TN_T (TRY^+_{TN} cells). Kaplan–Meier analysis revealed a positive association between the density of IL-17$^+_{MP}$ cells and overall survival ($n = 215$; $p = 0.005$; Fig. 4a). Patients with higher IL-17$^+_{MP}$ cell density had significantly longer overall survival (median 41.95 months) than those with lower IL-17$^+_{MP}$ cell density (median 19.73 months). In addition, the density of IL-17$^+_{MP}$ cells was inversely associated with tumor invasion ($p = 0.016$; Supplementary material Table 3). However, mast cell numbers in MP_T or TN_T did not correlate with the survival of ESCC patients (Fig. 4b, c). When the clinicopathological variables that were significant in univariate analysis were adopted as covariates, multivariate analysis revealed that the density of IL-17$^+_{MP}$ cells was an independent prognostic factor for overall survival ($p = 0.007$; Table 1). These results suggest that IL-17$^+$ cell density is inversely associated with progression of ESCC and might serve as an independent predictor of good survival.

IL-17$^+_{MP}$ cells are positively associated with effector immune cells in the same microenvironment

It is generally agreed that IL-17 may not directly mediate antitumor activity, but can indirectly promote antitumor immunity by facilitating the recruitment of other effector immune cells, such as CD8$^+$ T cells [12, 13]. A recent study in mouse showed that the CD169$^+$ macrophage subset dominates antitumor immunity by cross-presenting tumor antigens to CD8$^+$ T cells [34]. To investigate whether such a mechanism is also involved in tumor immunity in the muscularis propria, we examined the distribution of these effector cells and their association with IL-17$^+$ cells in ESCC tissues. The number of CD8$^+$ T cells was indeed significantly higher in the MP_T than the TN_T of the same ESCC patients (Fig. 5a). Although there was no obvious difference in macrophage density (using CD68 as a pan-macrophage marker) between MP_T and TN_T, CD169$^+$ macrophages were more prominent in MP_T (Fig. 5b, c, Supplementary material Fig. 4).

These data prompted us to further examine the correlations between the densities of IL-17$^+_{MP}$ cells and CD8$^+$ T cells or macrophages in serial sections of ESCC tissues. Spearman's rank correlation coefficient tests revealed that the level of IL-17$^+$ cells in muscularis propria was positively correlated with the density of CD8$^+$ T cells, CD169$^+$ macrophages and CD68$^+$ macrophages in the same anatomic location (all $p < 0.001$; Fig. 5d, f). However, the density of IL-17$^+_{MP}$ cells showed no correlation with the

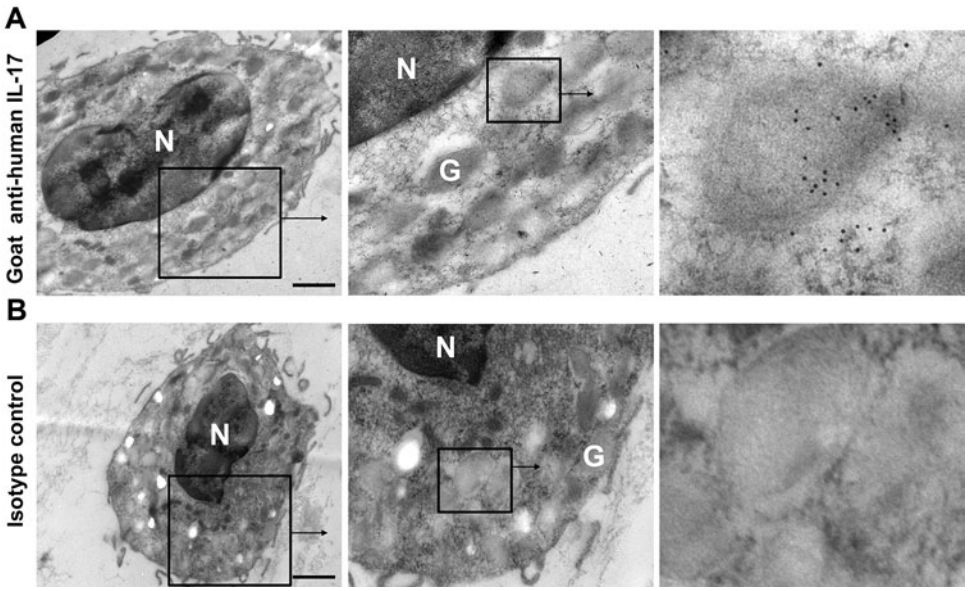


Fig. 3 IL-17 is expressed in mast cells. Specimens were dissected from ESCC patients immediately after surgery and examined by immunoelectron microscopy. One of three representative micrographs is shown in the *left panels*. *Boxes* show area enlarged in the *right panels*. **a** Immunogold detection of IL-17 was closely associated with secretory granules. **b** Isotype control showed no signal. N, nuclear; G, secretory granules. *Scale bar* 500 nm

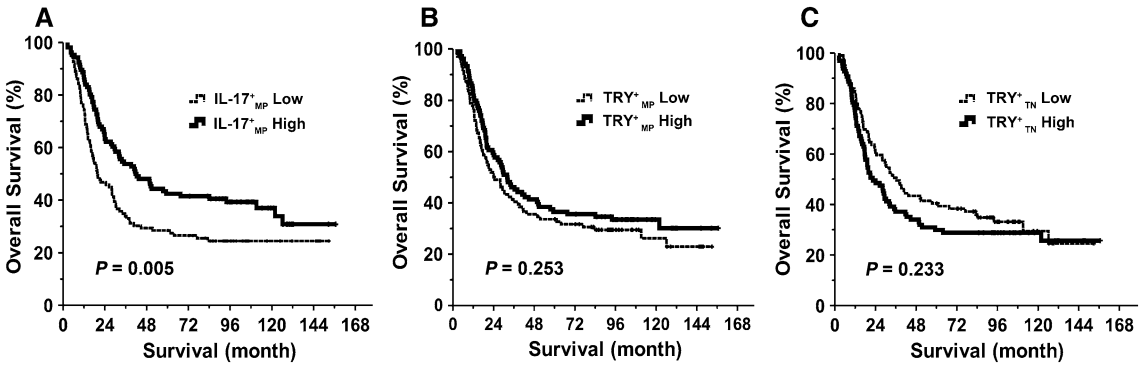


Fig. 4 Accumulation of IL-17$^+$ cells in the muscularis propria predicts favorable prognosis in ESCC patients. The patients ($n = 215$) were divided into two groups (high or low) according to the median values for: **a** IL-17$^+$ cell density in muscularis propria (IL-17$^+_{MP}$ cells, median = 8.6); **b** TRY$^+$ cell density in the muscularis propria (TRY$^+_{MP}$ cells, median = 13.6); or **c** TRY$^+$ cell density in the tumor nest (TRY$^+_{TN}$ cells, median = 3). Cell densities were obtained in ×400 fields (0.0768 mm^2 per field) from different anatomic locations in tumor tissues. *Dashed lines* low-density groups; *solid lines* high-density groups. Overall survival was calculated by the Kaplan–Meier method and analyzed by the log-rank test

levels of CD8$^+$ T cells, CD169$^+$ macrophages or CD68$^+$ macrophages in the tumor nest (Fig. 5g, i). These results suggest that the antitumor activity of IL-17$^+$ cells in the muscularis propria of ESCC tissues may be mediated by recruiting CD8$^+$ T cells and the CD169$^+$ macrophage subset into the same microenvironment.

Discussion

Although production of the proinflammatory cytokine IL-17 has been investigated in patients with diverse types of cancer, its role in tumor progression is still controversial [13]. The present study showed that mast cells, but not T cells, were the predominant IL-17-expressing cells in ESCC tissues in situ. These cells accumulated in the muscularis propria rather than the corresponding tumor nest, and increased IL-17$^+_{MP}$ cell density was associated with prolonged survival in ESCC patients. Moreover, we have provided evidence that the density of IL-17$^+$ cells in the muscularis propria was positively associated with the numbers of effector immune cells in the same area of ESCC tissues, and inversely associated with tumor invasion. These data suggest that mast cells play a significant role in tumor immunity by releasing IL-17 at a previously unappreciated location, the muscularis propria, in ESCC tissues.

The opposing effects reported for IL-17 on tumor progression may be partly due to its diverse cellular sources in different tumor microenvironments [13]. IL-17-expressing T cells may contribute to protective human tumor immunity in ovarian cancer, whereas IL-17-expressing macrophages could promote the invasiveness of tumor cells in breast cancer [30, 35]. In the present study, we found that although the densities of CD3$^+$ T cells are significantly higher than mast cells in ESCC tissues, T cells represented only a minority of all IL-17-expressing cells in both tumor nest and muscularis propria of ESCC tissues in situ. However, we observed that IL-17 colocalized with tryptase, a highly specific marker for mast cells, in ESCC tissues. Moreover, electron microscopy provided further evidence for subcellular localization of IL-17 within mast cells. The specificity of IL-17 staining was further confirmed by triple-color immunofluorescence with mouse anti-TRY and two Abs from goat and rabbit that recognize different epitopes of the human IL-17 protein.

Mast cells can promote or inhibit the development of tumors, due to their production of a variety of cytokines [36–38]. However, production of IL-17 by mast cells has not previously been investigated in human tumors [36]. Our present study therefore suggests a more important role for mast cells in tumor progression than previously appreciated, mediated by the production of IL-17. In line with our results, IL-17-producing mast cells have recently emerged as crucial mediators in the pathogenesis of diverse inflammatory diseases [16, 39]. In vitro studies have also revealed that mast cells can produce IL-17 in response to various inflammatory stimuli [29].

Anatomic localization may be another crucial context, which determines the effects of IL-17-producing cells on tumor progression [17, 18]. It is generally agreed that the mucosa and the submucosa, but not the muscularis propria, are the predominant sites of chronic inflammation in gastrointestinal tissues [40, 41]. We were therefore surprised to find that a significant number of leukocytes, including IL-17$^+$ cells, CD8$^+$ T cells and CD169$^+$ macrophages, were enriched in the muscularis propria of ESCC tissues. Moreover, we found that the number of IL-17$^+$ cells in the muscularis propria served as an independent predictor for

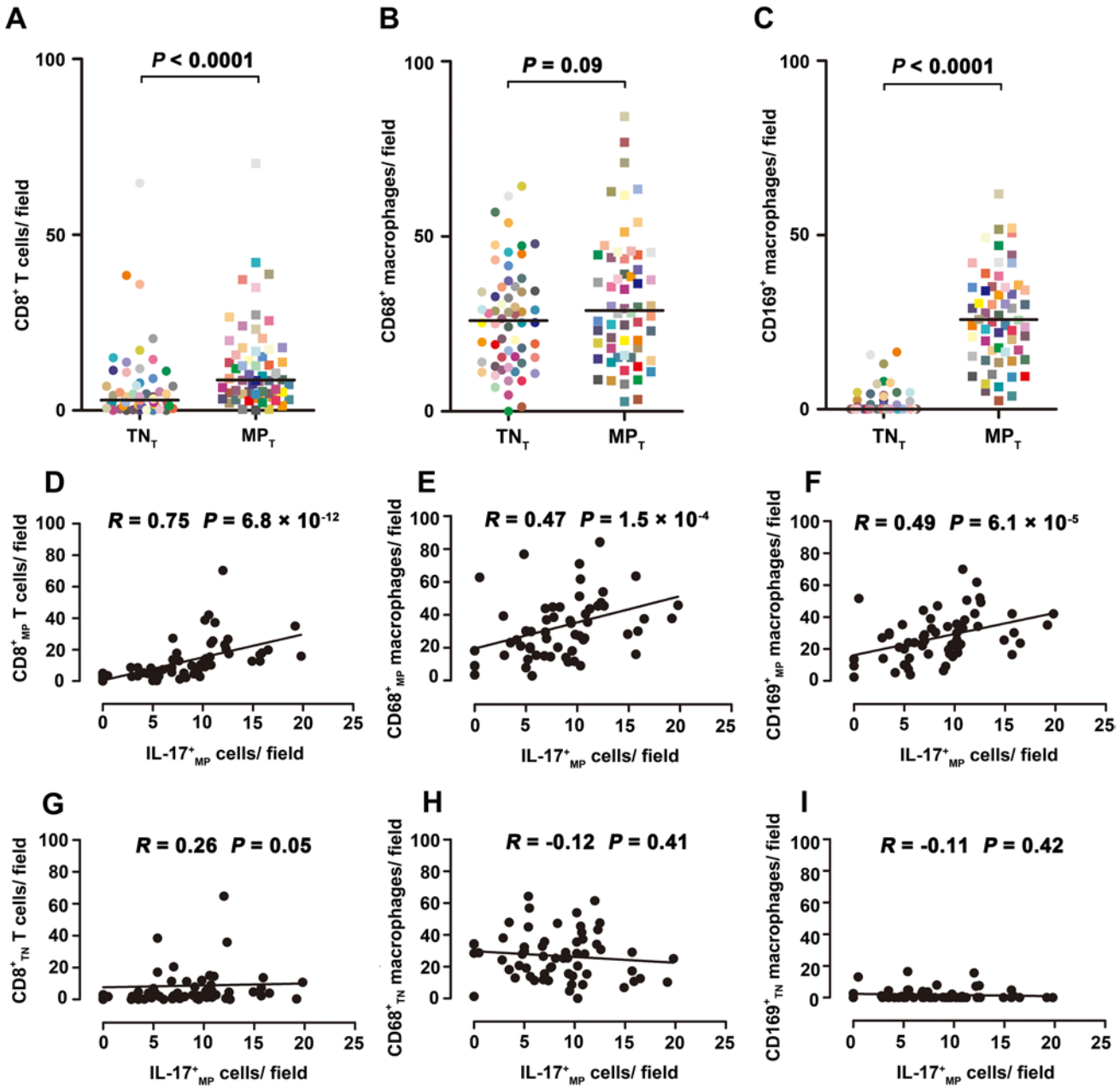


Fig. 5 IL-17$^+$ cells are positively associated with effector immune cells in the muscularis propria of ESCC tumor tissues. **a–c** Analysis of the density of CD8$^+$ T cells, CD169$^+$ macrophages or CD68$^+$ macrophages in paired tumor nest (TN_T) and muscularis propria (MP_T) of ESCC tumor tissues ($n = 60$). *Bars* show the mean values from five most representative ×400 fields (0.0768 mm^2 per field). **d–i** The association between IL-17$^+$ cells and other effector immune cells in ESCC tumor tissues ($n = 60$). **d–f** Positive associations between IL-17$^+_{MP}$ cells and CD8$^+_{MP}$ T cells, CD169$^+_{MP}$ macrophages or CD68$^+_{MP}$ macrophages in the same anatomic location (MP_T). **g–i** No associations between IL-17$^+_{MP}$ cells and CD8$^+_{TN}$ T cells, CD169$^+_{TN}$ macrophages or CD68$^+_{TN}$ macrophages in different anatomic locations (MP_T vs. TN_T). $p < 0.05$ was considered significant difference (Spearman's rank correlation coefficient test)

favorable survival in ESCC. These data suggested that the muscularis propria microenvironment might also affect tumor immunity and regulate the progression of ESCC. In accordance with our results, previous studies have shown that mature dendritic cells are predominantly distributed in the muscularis propria, rather than the mucosa or submucosa areas of the mouse intestine [42]. In addition, Lv et al. [43] reported that a high density of IL-17$^+$ cells was associated with improved survival in ESCC, but was not an independent predictor in multivariate analysis. This discrepancy is probably due to their study not evaluating IL-17$^+$ cells in distinct anatomic locations, whereas our

studies have shown that the mean density of IL-17$^+$ cells in the muscularis propria is about 20-fold higher than in the tumor nest (data not shown).

Although antitumor effects of IL-17 have been observed in human tumors, the mechanisms are presently unclear [12]. It has been suggested that IL-17$^+$ cells might mediate their antitumor activity indirectly, by facilitating the recruitment of other effector immune cells [13]. In line with this possibility, we observed that IL-17RA, the predominant IL-17 receptor, which can be expressed by both tumor and stromal cells throughout the tissue (data not shown), and the levels of IL-17$^+_{MP}$ cells were inversely associated with tumor invasion, and positively associated with effector CD8$^+_{MP}$ T cells and activated CD169$^+_{MP}$ macrophages in the same microenvironment. These data are consistent with several lines of evidence. In patients with prostate cancer, a high level of involvement of IL-17$^+$ cells in inflammation is associated with a lower pathologic stage [44]. In primary ovarian cancer, IL-17 plays an indirect role in antitumor immunity by promoting effector T cell and NK cell trafficking to the tumor microenvironment [35]. Moreover, IL-17 can induce the production of antitumor cytokines from macrophages [45].

Emerging evidence indicates that it is not inflammation per se but inflammatory "context" that determines the ability of proinflammatory factors to facilitate or prevent tumor growth [10, 11, 13]. Our results suggest that mast cells may play a more prominent role than previously appreciated, by releasing IL-17 at the site of the muscularis propria in ESCC tissues. Moreover, increased IL-17$^+_{MP}$ cells are a potential prognostic marker in ESCC and were positively associated with other effector immune cells in the same location. This study confirms that the relationship between IL-17-producing cells and tumor immunopathology is highly dependent on context, including cellular source, anatomic location and the associated cellular microenvironment. A better understanding of these contexts could be used to develop new cancer immunotherapies.

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Conflict of interest The authors declare no potential conflicts of interest.

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