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A study of multinucleated giant cells in esophageal cancer

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ABSTRACT

Objectives: To evaluate the occurrence, abundance, distribution, nature and clinical significance of multinucleated giant cell (MGC) in esophageal cancer.
Materials and methods: MGCs were examined with conventional pathology, immunohistochemistry and immunofluorescence in 107 esophageal cancer tissues. The findings were correlated to pathological diagnosis and clinical behavior of the cancers.
Results: MGCs were identified in 31.7% (34/107) of the cases. MGCs were positive for CD11c, CD11b, CD32, CD16, HLA-DR and MMP9, and negative for CD163, CD206 and CD64 giving a molecular profile of proinflammatory M1 but not immunosuppressive M2. MGCs were significantly related to decreased lymph node metastasis ($p = 0.011$), low pTNM stage ($p = 0.044$), favorable survival ($p = 0.04$), squamous cell cancer type rather than other histopathological subtypes ($p = 0.020$) and associated to better differentiation ($p = 0.063$).
Conclusions: MGCs belong to M1 macrophage and perform phagocytosis and scavenging of cancer cells that would benefit patients' survival and could serve as a prognostic marker.

1. Introduction

Multinucleated giant cell (MGC) is usually observed in granulomatous diseases that are common in many conditions including infections, vasculitis, immunological disorders, leucocyte oxidase defect, hypersensitivity, chemicals and neoplasia [1–3]. MGCs are the main cell type in granulomatous inflammation that are formed by fusion of circulating monocytes infiltrating into local tissues [4–6]. It was reported that granulomatous reaction might exist in 4.4% of carcinomas, including breast cancer, gastric cancer, lung cancer, laryngeal cancer and lower lip cancer [7–11]. However, detailed study of the nature and significance of MGC in cancer has not been reported.

Esophageal cancer has a high incidence in China, where about 90% of esophageal cancer are squamous cell carcinomas in contrast to adenocarcinoma in Western countries. Shantou, China where this study was conducted has one of the highest incidences at about 40/100,000 with a 5-year survival of 30.3% (2012–2015) [12,13]. At present, there is no effective therapy for esophageal cancer. It is imperative to

Abbreviations: MGC, multinucleated giant cell; IHC, immunohistochemistry; IF, immunofluorescence; ESCC, esophageal squamous cell cancer; EC, esophageal cancer; TAM, tumor associated macrophage; M1, type1 macrophage; M2, type2 macrophage; MMP9, matrix metalloproteinase 9; GM-CSF, granulocyte-macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; HLA-DR, human leukocyte antigen-DR isotype; ADCC, antibody dependent cellular cytotoxicity; ADCP, antibody dependent cellular phagocytosis; PBS, phosphate-buffered saline; CK, cytokeratin; pTNM, pathological tumor-node-metastasis; AJCC, American Joint Committee on Cancer; CR, complement receptor

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investigate the pathologic mechanisms including the immune mechanisms of MGCs in esophageal cancer [14,15].

Macrophage is a multi-functional cell of the immune system, participating in antigen presentation to T cells, digestion and phagocytosis of cellular debris, foreign substances and microbes. It was reported that macrophages have poor ability to phagocytize tumor cells once they migrate to cancer tissues and develop into TAMs [16,17], and TAMs in tumor microenvironment are mainly M2 type [18,19]. A large number of studies have shown that the higher the number of macrophages infiltrated into tumor tissue, the worse the prognosis of the patients [20–23]. Polarizations of macrophages are induced by different cytokines in different microenvironment [24,25]. M1 macrophages are polarized by Th1 cytokines including GM-CSF and IFN-γ. M1 macrophages after differentiation and maturation produce high levels of reactive oxygen species, IL-2, IL-23 etc., participating in the killing of pathogenic microorganisms and promote inflammatory response. M2 macrophages are mainly polarized by cytokines including M-CSF, IL-4 and IL-10 after differentiation and maturation, and mainly produce IL-10, VEGF, CD163 and CD206. These cytokines and receptors enable M2 to play a role in tissue remodeling, antigen clearance, wound healing and tumor progression [26–33]. HLA-DR, CD11c, iNOS, TNFα, IL-1β, and IL-12 are recognized as M1 macrophage markers, and CD163, CD206, IL-10, and Arg1 are M2 macrophage markers [18,26].

Overall there is little understanding of whether MGC in esophageal cancer belongs to M1 or M2 family. There has been no detailed report about the prevalence of MGC, its phagocytic ability and expression and distribution of MGC surface receptors including Fc gamma receptor (FcγR) and complement receptor (CR) in cancer [34,35]. In addition, the abilities of MGC to produce matrix metalloproteinases, and their secretion to tumor microenvironment have been completely unknown.

Therefore, we undertook this study to investigate the prevalence, pathological features and prognosis in patients with MGC infiltration in esophageal cancers and evaluated the immune phenotypes of MGC and its possible biological behavior in cancer microenvironment.

2. Materials and methods

2.1. Tissue samples

Pathological tissue samples of 107 esophageal cancer patients were obtained from the Department of Pathology, Shantou University Medical College Affiliated Tumor Hospital. The pTNM grades were determined under the guidance of esophageal cancer AJCC 8th edition guidelines. Clinical pathological diagnoses were based on radiological, clinical and histopathological criteria. Of the 107 patients, one was also diagnosed as tuberculosis, and none underwent preoperative radiotherapy or chemotherapy.

2.2. Ethical statement

The fixed and embedded tissue samples were obtained from tissue archive of Department of Pathology, Shantou University Medical College. All patients gave consent for research use of their surgically removed tumor tissue after pathological diagnosis. Additional ethical approval is not required.

2.3. Histopathology and immunohistochemistry

Fresh surgical specimens were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Four (4) μm consecutive sections were cut and stained with both hematoxylin & eosin (H&E) and immunohistochemistry (IHC). Sections were mounted on poly-L-lysine coated slides and fixed in acetone. Briefly, after dewaxing and rehydration, slides were stained with H&E following a standard protocol. For IHC, after dewaxing and rehydration, the endogenous peroxidase activity was blocked by incubation for 20 min in 3% hydrogen peroxide. Antigen retrieval was performed by heating the tissue sections at 96 °C in 0.01 M citrate buffer (pH = 6.0) or Tris-EDTA buffer (pH = 9) for15 min and then cooled to room temperature. Following incubation with 4% BSA in 0.01 M PBS for 1 h, the sections were incubated with primary antibodies overnight at 4 °C. Rabbit anti-human CD68 (Abcam, Cat#ab213363), and mouse anti-human CK (ZSBIO, Cat#ZM-0069) were used as primary antibodies. PBS was used as a parallel negative control. Following primary antibodies incubation and rinsing, the sections were incubated with a secondary antibody (ZSBIO, Cat#PV9000 reacting with both rabbit and mouse immunoglobulins) conjugated with peroxidase at 37 °C for 40 min. Following every step, the sections were rinsed with 0.01 M PBS 3 times for 5 min each. Positive signal was visualized with AEC (ZSBIO, Cat#ZLI-9036), which gave a red staining signal. Between steps, the slides were rinsed for three times 5 min each in phosphate-buffered saline (PBS). All sections were counterstained lightly with hematoxylin, dehydrated and mounted.

2.4. Immunofluorescence staining

After dewaxing, rehydration and antigen retrieval, slides were blocked with 10% horse serum, sections were incubated with indicated primary antibodies at 4 °C overnight, followed by sections-paired secondary antibodies at room temperature for 1 h (see in Supplementary data, Table S1). PBS was used as a negative control. Slides were mounted with DAPI mounting medium and then imaged with a ZEISS Axio Imager A2 fluorescence microscope.

2.5. Statistical analysis

Clinical data were analyzed with IBM SPSS Statistics software. Descriptive statistics was used for characterization of the patients. The chi-square test and trend chi-square test were employed to analyze the difference between the MGC (+) and MGC (−) groups. The possible relationship between MGC occurrence and patient survival durations of 107 cases was analyzed statistically with Kaplan-Meier method. Cox proportional hazards model was used to test hazard factors in survival.

3. Results

3.1. Clinical and pathological information of patients

The clinical features of the 107 patients are summarized in Table 1. The average age was 61.61 years, ranging from 41 to 80 years. Male patients comprised the majority of the cases (87 of 107), and female the minority (20 of 107). The cases were classified as grade II (26 of 107), III (51 of 107), and IV (29 of 107). Tumor recurrence and distant metastasis to the adjacent organs were identified in only 2 cases. 24 cases were graded as well differentiated, 46 as moderately differentiated and 19 as poorly differentiated. Most of the pathological phenotype is squamous cell (87 of 107), while the rest are small cell carcinoma (5 of 107), adenocarcinoma (7 of 107), adenosquamous carcinoma (2 of 107), adenoid cystic carcinoma (1 of 107), mucoepidermoid carcinoma (2 of 107), and basaloid squamous carcinoma (3 of 107). Most of the tumors were developed from the middle (51.4%) or lower (37.4%) segments of the esophageal. All patients underwent radical resection of esophageal cancer in thoracic surgery. None of the patients received preoperative radiotherapy or chemotherapy.

3.2. MGC infiltration in esophageal cancer

Light microscopy examination of the 107 cases of esophageal cancer tissues, adjacent cancer tissues and distant normal tissues unveiled that 34 (31.7%) of cases contained MGC infiltration in tumor tissue while no MGC was seen in adjacent tissues or normal esophageal tissues. An MGC usually has three or more nuclei surrounded by abundant pink

Table 1
Clinical information of 107 esophageal cancer patients.

Features	Patients		MGC occurrence		
	No.	%	(−)(n = 73)	(+)(n = 34)	p
Mean age (year)	61.61		61.07	62.76	
Gender					0.470
Male	87	81.3	58	29	
Female	20	18.7	15	5	
Tumor localization					0.793
L	40	37.4	26	14	
M	55	51.4	38	17	
U	12	11.2	9	3	
Tumor size					0.866
≤4 cm	39	36.4	27	12	
> 4 cm	68	63.6	46	22	
Differentiation	(Squamous)				0.063
Good	24	26.9	13	11	
Moderate	46	51.6	29	17	
Poor	19	21.3	15	4	
Pathological type					0.020
Squamous	87	81.3	55	32	
Other type	20	18.7	18	2	
T stage					0.761
T1	2	1.9	1	1	
T2	7	6.5	5	2	
T3	44	41.1	28	16	
T4	54	50.5	39	15	
N stage					0.011
N0	35	32.7	18	17	
N1	34	31.8	22	12	
N2	22	20.6	20	2	
N3	16	15.0	13	3	
Metastasis					0.330
Yes	2	1.9	2	0	
No	105	98.1	71	34	
pTNM stage					0.044
I	1	0.9	0	1	
II	26	24.3	15	11	
III	51	47.7	33	18	
IV	29	27.1	25	4	

L: lower segment of esophagus, M: middle segment of esophagus, U: upper segment of esophagus. P: *P* value of Chi square tests.

eosinophilic cytoplasm in H&E staining. These cells were positive for macrophage maker CD68 with medium intensity as shown in Fig. 1. Thirty two of the 34 cases containing MGCs were ESCC, and other 2 cases were ESCC combined with regional adenocarcinoma or differentiation of basal-like cells.

3.3. Correlation between MGC infiltration and patients' clinical data

We analyzed the clinical data of esophageal cancer patients with chi-square test and found that there were significant correlations between MGC occurrence and lymph node metastasis (p = 0.011), tumor pathological type (p = 0.020) and pTNM stage (p = 0.044). However, the correlation between MGC and tumor differentiation (p = 0.063), tumor stage (p = 0.843), or distant metastasis (p = 0.32) was not statistically significant. The frequency of MGC presence was 7 times higher in well/moderately differentiated tumors (n = 28) than in poorly differentiated tumors (n = 4) (Table 1, Fig. 2A). In addition, with Kaplan-meier survival analysis we found that EC patients with MGC tended to have better prognoses than patients without (p = 0.04), and patients with higher lymph node metastasis grade (p = 0.006) and distant metastasis (p = 0.0002) tended to have worse prognosis than those without (Fig. 2B). However, MGC had no statistical significance in multivariate Cox proportional hazards model, although lymph node grade and distant metastasis were still risk factors in predicting survival (Supplementary Table S2). Patients with well or moderately differentiated squamous cell carcinoma had better prognosis than poorly differentiated cases (p = 0.016). When the parameters of MGC infiltration and tumor differentiation were combined, patients with MGC and better differentiation had better prognosis in comparison to other cases (p = 0.025) (Supplementary Fig. S1).

3.4. MGC belongs to M1 type macrophage but not M2

We investigated whether MGC is more inclined to M1 or M2 macrophage polarization. We examined the polarization markers of M1 (HLA-DR, CD11c) and M2 (CD163, CD206), and found that both CD163 and CD206 were negative, while HLA-DR and CD11c were positive on MGC (Fig. 3A). All MGC were positively stained for HLA-DR and CD11c,

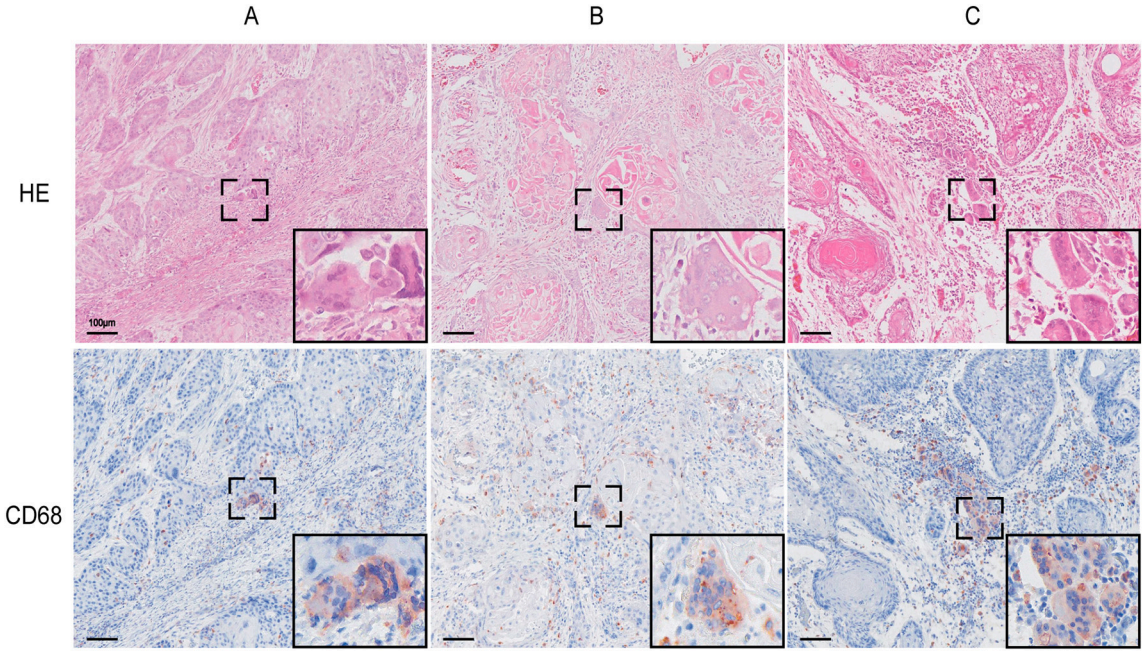

Fig. 1. MGC infiltration in esophageal cancer tissues. A, B, C are H&E staining showing typical MGC cells from three patients. The results of H&E (upper row) and CD68 immunostaining (lower row) were obtained from consecutive sections of the same tumor tissue. The enlarged image at the lower right corner show CD68 positive MGC cells. Scale bar = 100 μm.

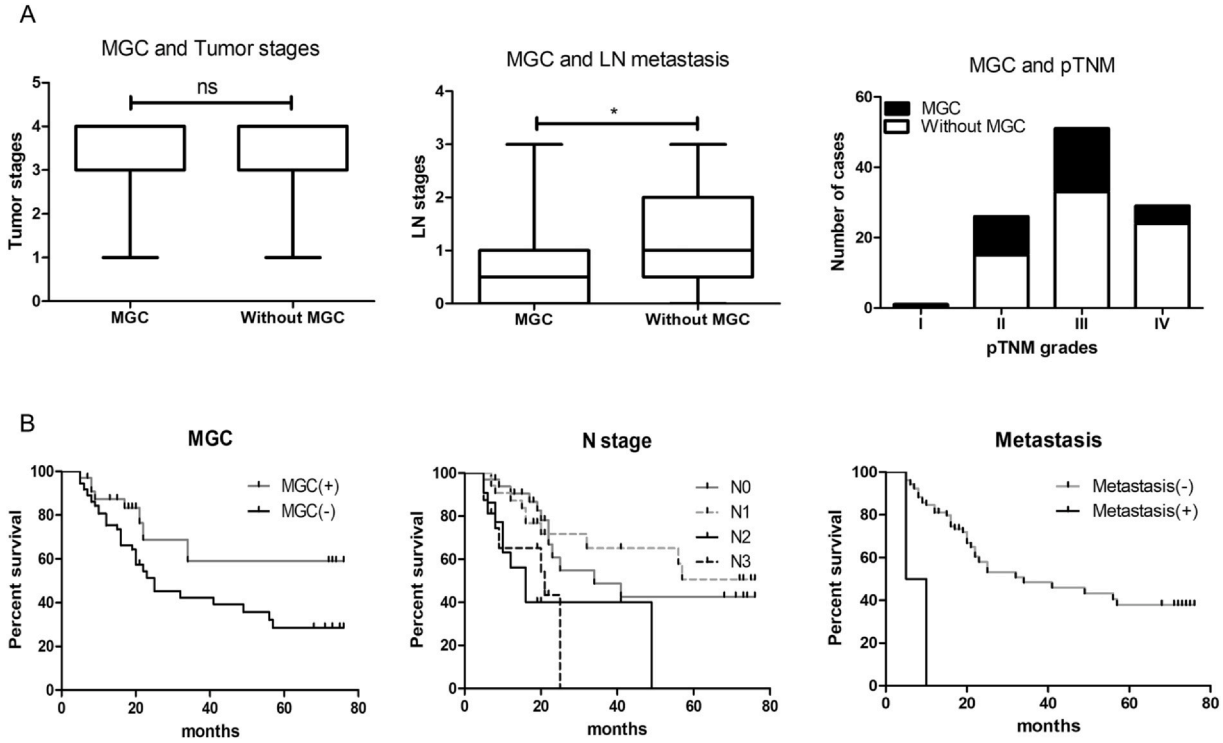

Fig. 2. A. Differences between MGC positive group and MGC negative group was statistically analyzed for Tumor stages ($p = 0.761$), lymph node metastasis ($p = 0.011$), and pTNM stages ($p = 0.044$) with Chi square test. B, Survival time of esophageal cancer patients with MGC was significantly different from those without MGC patients ($P = 0.04$). Patients with lower lymph node grade showed better prognosis than those with higher grade ($P = 0.006$). Patients without distant metastasis showed longer survival than those with distant metastasis ($P = 0.0002$).

but only a portion of single nucleated CD68 (+) macrophages showed positive staining of these two antigens (Fig. 3B). Although MGCs were negative for M2 markers CD163 or CD206, numerous single nucleated macrophages were positive for either M1 or M2 markers infiltrating in and around tumor stroma (Fig. 4C). In addition, co-expression of MMP9 (green fluorescence) and CD68 (red fluorescence) in MGCs was evident with a change of color (yellow or orange fluorescence) (Fig. 4B), suggesting a high concentration of MMP9 in MGC cells.

We further examined the expressions of MGC surface receptors including Fc gamma receptors and complement receptors. The expressions of CD32 and CD16 were positive on the surface of MGC, but CD64 was weak or negative (Fig. 4D, E, F). The two complement receptors CD11b and CD11c were both positive on the surface of MGC (Figs. 3, 4A), indicating that MGC may have the proper ligands to bind to complements. Positive CD11b staining may also suggest that MGCs are of myeloid origin.

3.5. Evidence of MGC phagocytosis in tumor

MGCs were mainly distributed along the edge of tumor nests (Fig. 4C) and gave an appearance of close interaction with squamous cancer cells and keratinized pearls. Among these positive MGC cases, 3 showed MGC next to keratinized pearls (Fig. 5C, D), 12 showed phagocytosis of cancer cells (Fig. 5A, B), 1 showed both features and other 19 cases showed neither.

4. Discussion

Previously it has been reported that M2 type macrophage constituted the majority of TAM in several cancer types and higher number of M1 type of macrophage was positively associated to favorable patients' survival [36–38]. In this study we found that the polarization profile of MGC surface markers (CD11c+, HLA-DR+, CD163-, CD206-) were similar to M1 macrophage, indicating that MGC belongs to anti-tumor M1 macrophage. The morphologic phagocytosis feature of CK positive neoplastic epithelial cells by MGC was evident in tumor tissues, suggesting that MGC plays an active role in destroying and scavenging cancer cells, while evidences of phagocytosis by individual macrophages in the same areas were rare. In addition, there is statistical significance between the MGC positive and MGC negative group in the survival of these cancer patients. For other clinical and pathological parameters such as tumor cell differentiation and tumor type, the association of MGC were not statistically significant but the relevance can be seen. This is not surprising as apart from MGC, there are many other factors playing roles in determining cancer behavior. It is also possible that with increased number of cases, the relationship between MGC and other clinical parameters would be more clearly demonstrated.

FcγRs are important for immune cells to perform phagocytosis and cytotoxic functions in antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP) [35,39]. There are activating and inhibitory Fc receptors that mediate targeted immune responses of immune effector cells. The process helps to neutralize toxins and remove antigens [40]. The complement system enhances the ability of macrophage to clear bacteria and tumor cells and evoke inflammatory responses by binding to IgG or complement receptors and attacking pathogens [34,41,42]. Complement mediated killing effect is one of the important mechanisms of tumor destruction [43–45]. In this study, MGC were found to be CD32 (+), CD16 (+), CD11b (+), and CD11c (+), suggesting that MGCs express FcγRII, FcγRIII, CR3, and CR4 receptors on their surface and may be able to perform antibody- or complement-mediated cytotoxicity and phagocytosis.

MGC produces MMP9 which induces matrix degradation. Matrix metalloproteinases play an important role in embryo development and growth, tissue remodeling, angiogenesis and tumor progression [46–49]. In 2000 Coussens et al. found that MMP9 could promote keratinization of epithelial cancer. In a MMP9 gene knockout mouse model, the incidence of malignant tumors decreased, and with the

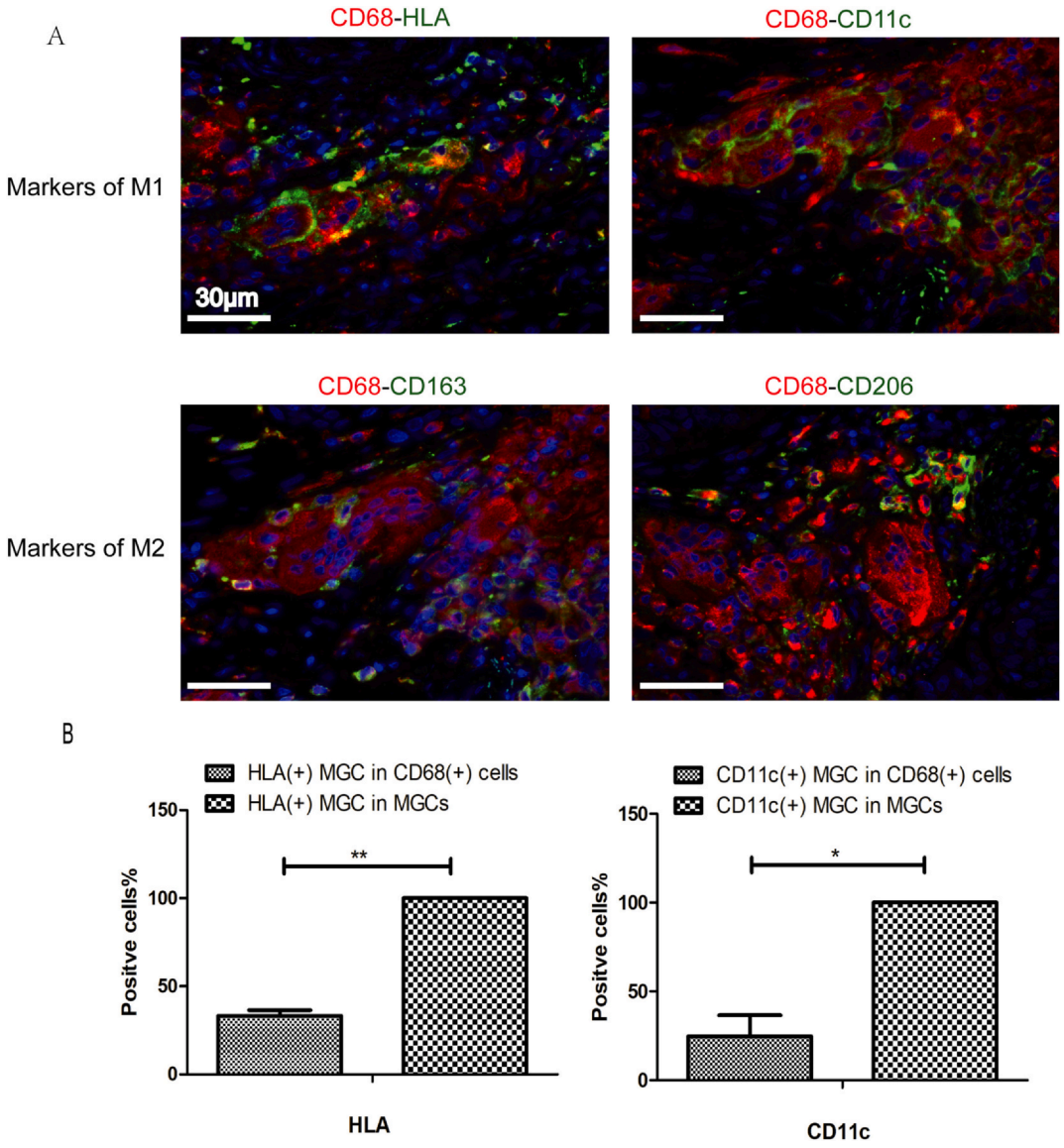

Fig. 3. A. Double immunostaining of CD68 positive macrophages (red fluorescence) with other markers on tissue sections of ESCC. Immunofluorescence staining markers of M1 (HLA-DR, CD11c), M2 (CD163, CD206) (green fluorescence) and nuclei stained with DAPI (blue color), showing MGCs are positive for HLA and CD11c, negative for CD163 and CD206. Many single nucleated macrophages showing positive for CD163 and CD206 are distributed alongside MGCs. Scale bar = 30 μm. B. HLA-DR and CD11c positive cell percent in CD68(+) macrophages and positive MGCs percent in MGCs (n = 3). Student's t-tests were used in the comparison. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

decrease of keratinization, the invasiveness of tumor increased [30]. These findings in animals are similar to our observation in ESCC patients. Since MGC produces MMP9 in esophageal cancer, it may create a high concentration of MMP9 in cancer microenvironment. We found that MGC mainly appeared in highly or moderately differentiated squamous cell carcinomas rather than in poorly differentiated squamous cell or small cell carcinoma. CD11b (+) MGC derived from bone marrow in tumor microenvironment may actively participate in tumor transformation [50] and involve in the differentiation and progression of squamous cell carcinoma. In addition, we established the relationship between biological behavior and MGC in tumor. In this study, only one patient was confirmed to have tuberculosis infection. In this case, MGC were found but showed no particular difference with other positive MGC cases.

MGC is a common feature in granulomatous inflammation or sarcoid reaction but has been rarely noticed in cancer and has not been reported in esophageal cancer. This is the first study of MGC in a relatively large cohort of esophageal cancer. Previously MGC has been reported in breast cancer, lung cancer, gastric cancer and lymphoma. Dagaonkar et al. observed 19 cases of inflammatory granuloma in 127 cases of lung cancer, and analyzed clinical parameters and prognosis of lung cancer patients with granuloma [9]. No statistical significance between the MGC presence and age, pathological type or survival was found in these patients. Our study focused on two aspects, i.e., first, the characteristic of these MGC cells, and second the relationship of these cells to clinical behavior of the cancers. In our study, MGC was found to have a positive correlation to lesser lymph node metastasis and better survival. We found that MGC was a M1-type macrophage but not M2. Previously, in one case of thyroid cancer, MGC was said to be CD163 positive [51] which was inconsistent to our observation. In our study, all MGC cells in all cases showed M1 characteristics. This is consistent with a case report of a tongue cancer [52]. We also obtained evidence of cancer cell phagocytosis by MGC, suggesting the nature of proinflammatory characteristic of these cells.

In conclusion, multinucleated giant cells are a group of specialized macrophages exhibiting M1 characteristics in immunophenotype in

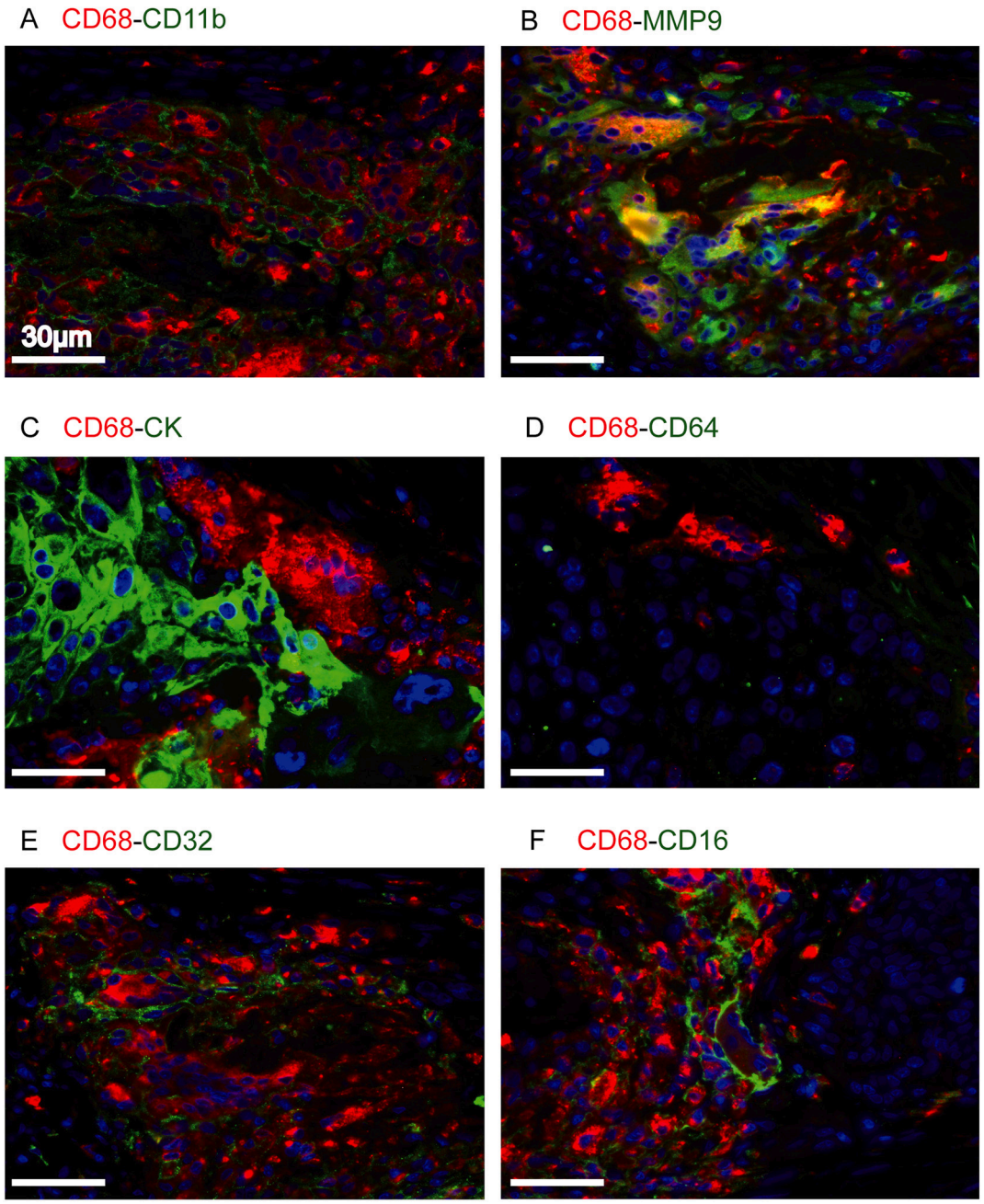

Fig. 4. All macrophages are shown in red fluorescence and double-stained with CD11b, MMP9, CK, CD64, CD32, and CD16 in green fluorescence. MGCs are CD11b + positive on cell membrane suggesting MGCs are myeloid in origin. Matrix metalloproteinase 9 in MGCs were colocalized with CD68 showing yellow to orange color. MGCs are mainly distributed around nests of cancer cells which are CK (+). Fc gamma receptors staining showed CD64 negative and CD32, CD16 positive on the membrane of MGCs and other individual macrophages. Scale bar = 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

esophageal cancer. MGCs have phagocytic ability of cancer cell and debris. This is the first time that tumor cells were observed of being phagocytized by MGCs in cancer. Moreover, MGC has a beneficial effect on preventing lymph node metastasis and prolonging patients' survival. These properties of MGC unveil a new perspective of the plasticity of monocyte macrophage lineage and may provide new possibilities for cancer therapy.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2020.108600.

Statement of author contributions

Hui Wang and Junjie Zhou performed most of the experiments and analysis. Jun Li helped with statistical analysis and experiments. Yiqun Geng, Pei Meng, Changchun Ma, Ziqi Zhu, Weifeng Zhang, Liangli Hong, Yan Quan, Jiacong Wei, Qiongyi Huang, You Zhou, Zuoqing Su, and Xiaoqing Zhu helped with clinical sample and data collection. Chuangzhen Chen and Shaobin Chen helped with clinical sample collection. Jiang Gu contributed to conceptualization, result analysis, and manuscript writing.

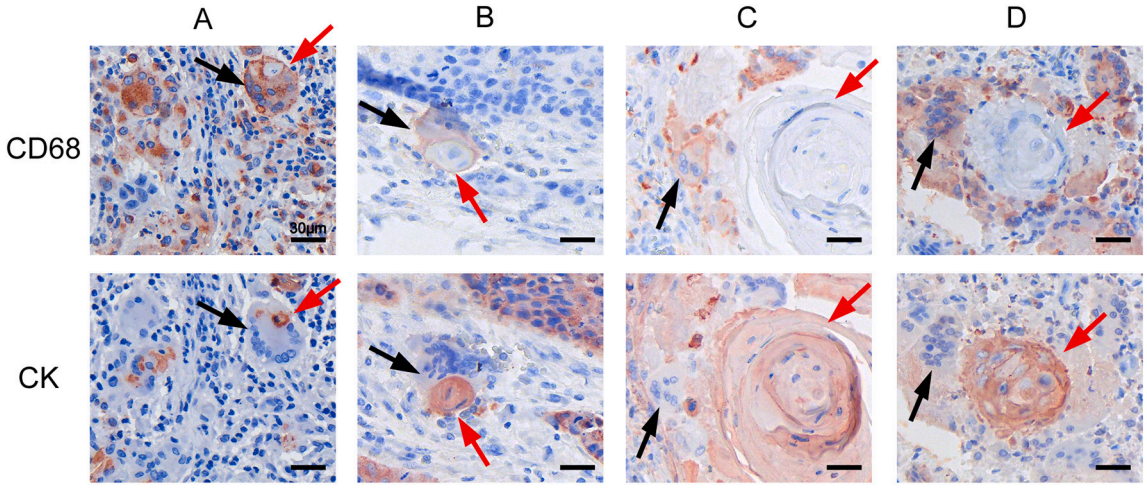

Fig. 5. Two consecutive sections (upper and lower rows) from four different patients of ESCC showed phagocytosis of CK positive tumor cells by CD68 positive multinuclear giant cells. A&B show that CK positive cancer cells (red arrows) are engulfed by CD68 positive MGCs (black arrows). C&D show that CD68 positive MGCs (black arrows) surround CK positive keratin pearls (red arrows). Scale bar = 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Statement of author contributions

Hui Wang and Junjie Zhou participated in the conceptualization, project administration and data curation. Jun Li helped with methodology and software. Yiqun Geng, Pei Meng, Changchun Ma, Ziqi Zhu, Weifeng Zhang, Liangli Hong, Yan Quan, Jiacong Wei, Qiongyi Huang, You Zhou helped with investigation and resources during their study or working time in Shantou Univerdity medical college. Zuoqing Su, and Xiaoqing Zhu helped with investigation and validation. Chuangzhen Chen and Shaobin Chen helped with resources. Jiang Gu contributed to conceptualization, formal analysis, funding acquisition, and manuscript writing.

Declaration of Competing Interest

There is no conflict of interest by any of the authors.

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