



Positive correlation between the density of macrophages and T-cells in undifferentiated sarcoma

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Abstract

Undifferentiated sarcoma (US) is a frequent soft tissue sarcoma. Although the 10-year survival rate is around 60%, advanced US is highly resistant to chemo/radiotherapy. The tumor microenvironment (TME) is closely associated with tumor progression. However, few studies of infiltrated immune cells in US have been published. In this study, we evaluated tumor-associated macrophages (TAMs) and CD8-positive cytotoxic T lymphocytes (CTLs) in 28 cases of US. Iba1, CD163, and CD204 were used as markers for TAMs. The density of CTLs was positively correlated with the density of TAMs. However, a negative correlation was seen between the density of CTLs and the percentage of CD204-positive TAMs. We found no significant association between the density of Iba1-/CD204-/CD8-positive cells and clinicopathological factors. No significant correlation between immune cell infiltration and clinical outcome was observed. Although we found no significant association between immune cells and clinicopathological factors, these findings may provide new insight into the characterization of immune cells in the TME of US.

Keywords TAM · CD163 · CD204 · Iba1 · CD8

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Introduction

Undifferentiated pleomorphic sarcoma (UPS, changed to undifferentiated sarcoma, US, according to recent classification) is a frequent soft tissue sarcoma, and the 10-year survival rate is around 60% [1–3]. A larger tumor size and older age are well-known factors of worse clinical prognosis. Conversely, expression of PTEN and STAT3 is associated with a favorable clinical course [4, 5]. Advanced sarcomas including UPS show resistance to chemo/radiotherapy [6]. Notably, immunotherapy that blocks immune checkpoint molecules is a promising new therapy for advanced UPS, and significant tumor reduction was seen in 40% of UPS patients following treatment with anti-programmed death-1 (PD-1) antibodies [7].

The tumor microenvironment (TME) is closely associated with not only tumor progression but also resistance to chemo/radiotherapy and immunotherapy [8–10]. Macrophages that have infiltrated into the TME are associated with tumor progression via the secretion of soluble factors related to tumor cell growth, tumor invasion, metastatic niche formation, neovascularization, maintenance of stem-like cells, and immunosuppression [8–10]. Tumor-associated

macrophages (TAMs) with heterogeneous phenotypes infiltrate into the TME. Over the last decade, the M1/M2 concept of macrophage phenotypes has been described, and heterogeneity of macrophage phenotypes is now considered to be involved in various diseases including malignant tumors and non-tumor diseases [11, 12]. Although a more complex activation status and phenotypical heterogeneity has been suggested by recent studies [13], CD163 and CD204, which are specific markers for macrophages, are highly expressed on TAMs in human malignant tumors with the protumor phenotype [14]. Animal studies have shown that CD163 and CD204 are involved in protumor activation of TAMs [15–17]. In addition, CD8-positive cytotoxic T lymphocytes (CTLs) infiltrating into tumor tissues are associated with anti-tumor immune responses and influence the clinical course in several types of tumors including colorectal cancer [18]. TAMs have immunosuppressive functions on CTLs in several malignant tumors [19, 20]. However, few studies have investigated the relationship between TAMs and CTLs in UPS. Therefore, we investigated TAMs and CTLs, as well as their correlations with clinicopathological factors using tumor samples diagnosed as UPS.

Materials and methods

Patients

We evaluated 28 tumors diagnosed as UPS that were registered in the Department of Anatomic Pathology, Kyushu University (Fukuoka, Japan). All samples were primary cases, and radiation-induced sarcomas and secondary sarcomas after chemotherapy were excluded. The reassessed diagnosis of UPS was made according to the World Health Organization 2013 classification [2]. Studies using these UPS cases were previously published. We evaluated the extent of necrosis and mitosis to define each tumor's French Federation of Cancer Centers grade. Each tumor was staged according to the seventh edition of the American Joint Committee on Cancer staging system. The Institutional Review Board at Kyushu University approved this retrospective study (#27-78).

Immunohistochemistry (IHC)

ICH for Iba1, CD204, CD163, and CD8 was performed as described in previous studies. Reactions were visualized using a diaminobenzidine substrate system (Nichirei). Positively stained cells were counted in five randomly selected areas in a high-power field in a microscope by two pathologists who were blinded to information about the patients' backgrounds or their prognosis. The Iba1 and CD163 data were the same as those published in a previous study [17].

Statistics

Statistical analyses were carried out using JMP10 (SAS Institute, Chicago, IL, USA) and StatMate III (ATOMS, Tokyo, Japan). The Kaplan–Meier method and the Cox hazard test were used to analyze associations with the clinical course. Differences between two groups were examined for statistical significance using the Mann–Whitney *U* test. A value of $P < 0.05$ was considered statistically significant.

Results

The densities of TAMs and CTLs in UPS

First, we evaluated the densities of CD204-positive TAMs and CD8-positive CTLs in tumor tissues using IHC (Fig. 1); these densities were 479 ± 390 and $381 \pm 433/\text{mm}^2$, respectively (Fig. 2a). The densities of Iba1-positive TAMs and CD163-positive TAMs in the same tissue samples, which were reported in our previous study, were 814 ± 515 and $670 \pm 368/\text{mm}^2$, respectively. The densities of Iba1-, CD163-, and CD204-positive TAMs were significantly and positively correlated with each other (Fig. 2b).

The correlations between TAMs and CTLs

Next, we tested the association between the densities of TAMs and CTLs. The densities of CTLs were positively correlated with the density of Iba1-, CD163-, and CD204-positive TAMs (Fig. 3a). Although a slightly negative correlation was seen between the density of CTLs and the percentage of CD204-positive TAMs, it was not statistically significant ($P = 0.10$) (Fig. 3b). We also found no significant association between the density of CTLs and the percentage of CD163-positive TAMs (Fig. 3b).

The density of macrophages and CTLs and their correlations with clinicopathological factors

As shown in Table 1, the density of CD163-positive macrophages was high in older patients, and the percentage of CD163-positive cells among Iba1-positive total macrophages was higher in patients with a smaller tumor size. However, we found no significant association between the density of Iba1-/CD204-/CD8-positive cells and clinicopathological factors (Table 1). We also found no association between expression of these immune cell markers and clinical outcome, although a high density of CD163- or CD204-positive cells tended to be associated with a lower

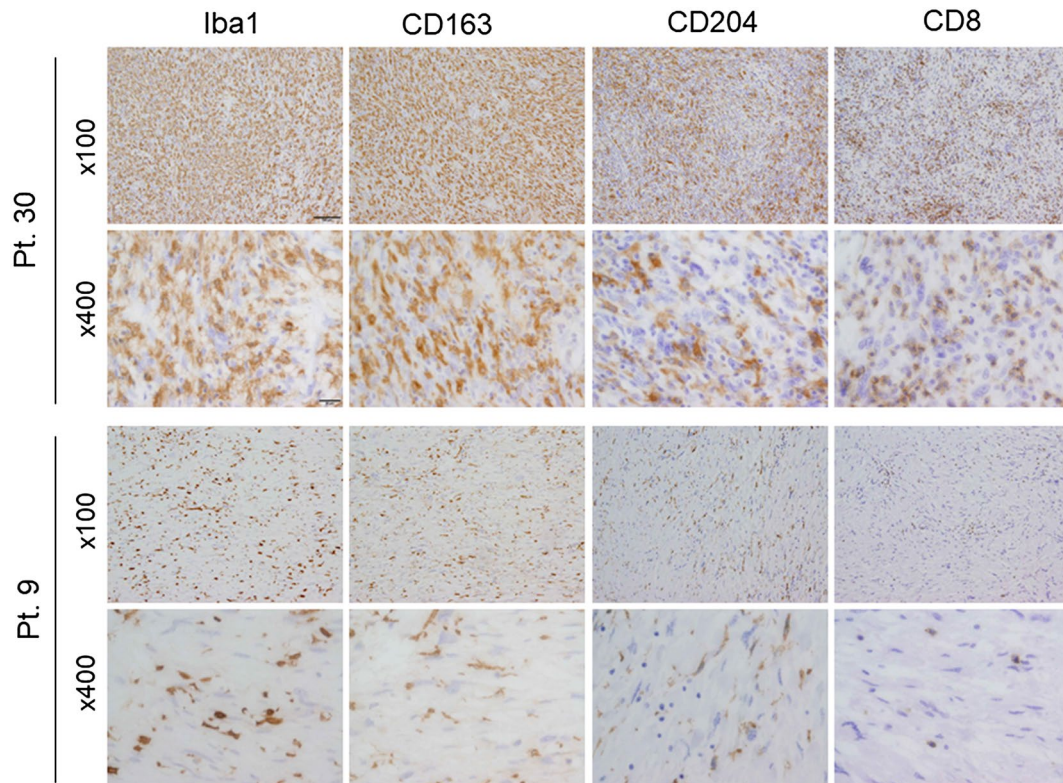


Fig. 1 Immunohistochemistry (IHC) of Iba1, CD163, CD204, and CD8. Representative results of IHC in two cases. Scale bar: 100 μ m (\times 100 magnification) and 20 μ m (\times 400 magnification)

disease-free survival rate and lower overall survival rate (Fig. 4; Table 2).

Discussion

In this study, we found no correlation between clinical outcome and infiltrating immune cells, especially CTLs. We believe this study is the first report to investigate the correlation between infiltrating CTLs and clinical outcome in UPS. Although we found no significant involvement of CTL density in the clinical course, we found that the density of CTLs was well correlated with the density of TAMs.

The density of CTLs was positively correlated with the density of Iba1-, CD163-, and CD204-positive TAMs, and interestingly, we found negative correlations between the density of CTLs and the ratio of CD163- or CD204-positive cells among Iba1-positive TAMs. Iba1 is a well-known marker of macrophages, whereas CD163 and CD204 are promising markers for protumor TAMs as described below. High infiltration of CTLs into the TME is well correlated with a better clinical outcome in several malignant tumors such as colorectal cancer and liver cancer [18, 19], whereas no association with outcome is present with other types of tumors such as breast cancer and ovarian cancer [20, 21].

CTL infiltration into the tumor cell nest has been focused on as a preferential marker for better prognosis and is considered to reflect anti-tumor immune responses in some types of tumors including lung squamous cell carcinoma [22]. Recent studies indicate that TAMs suppress the cytotoxic functions and chemotaxis of CTLs in the TME [23, 24]. Whether TAMs affect CTL functions in UPS will be an interesting topic for a future study.

Macrophages have multiple functions and have been broadly classified into classically activated macrophages (M1/kill/inflammatory macrophages) and alternatively activated macrophages (M2/repair/anti-inflammatory macrophages) according to their biological functions and expression markers [23]. In this study, we investigated CD163 and CD204 as markers for M2-like or protumor TAM subpopulations [25]. An in vitro study revealed that CD163 is specifically up-regulated by stimulation with interleukin-10 and can be considered a marker for M2c-like macrophages [25]. CD204 is highly expressed in both M1-like and M2-like macrophages in vitro, and the CD204⁺ subpopulation is thought to differ somewhat from the CD163⁺ subpopulation [25]. In this study, the number of CD204-positive cells was slightly lower than that of CD163-positive cells. Similar results were seen in kidney cancer [26], and these indicated the existence of CD163-positive and CD204-negative TAMs

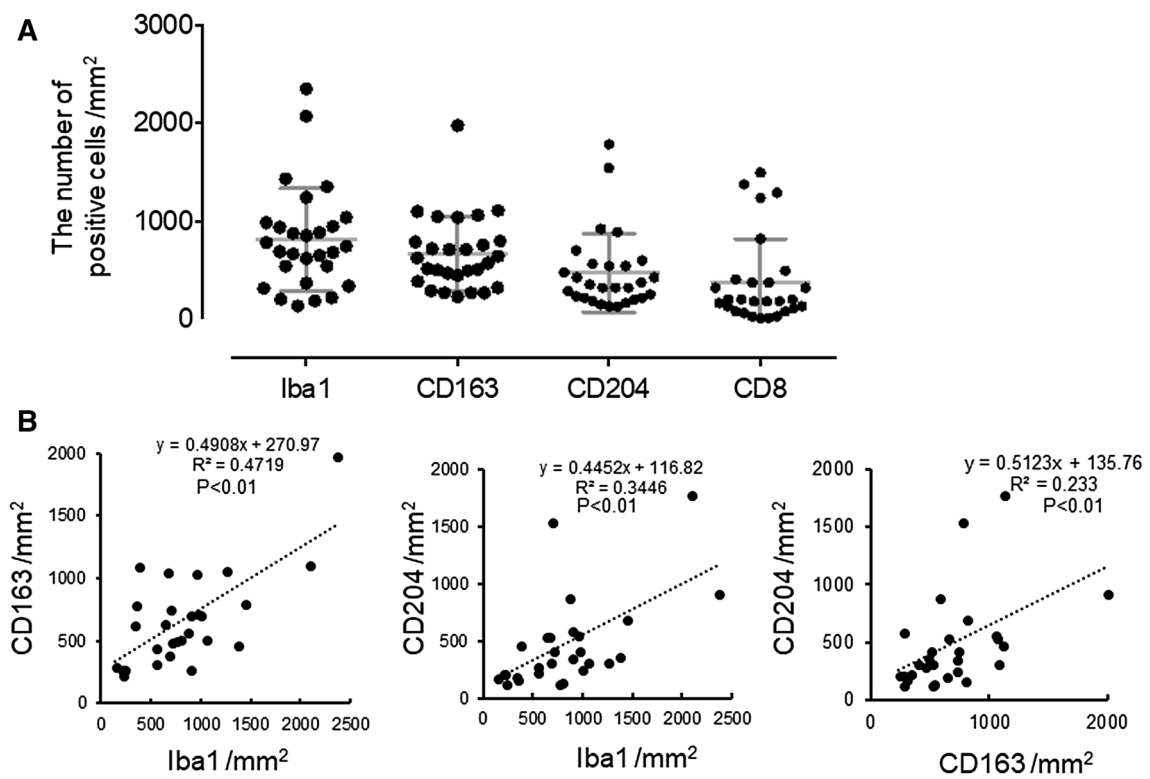


Fig. 2 The density of Iba1-, CD163-, and CD204-positive TAMs, and CD8-positive CTLs. The numbers of Iba1-, CD163-, CD204-, and CD8-positive cells were counted, and the density (the number per 1 mm²) is shown

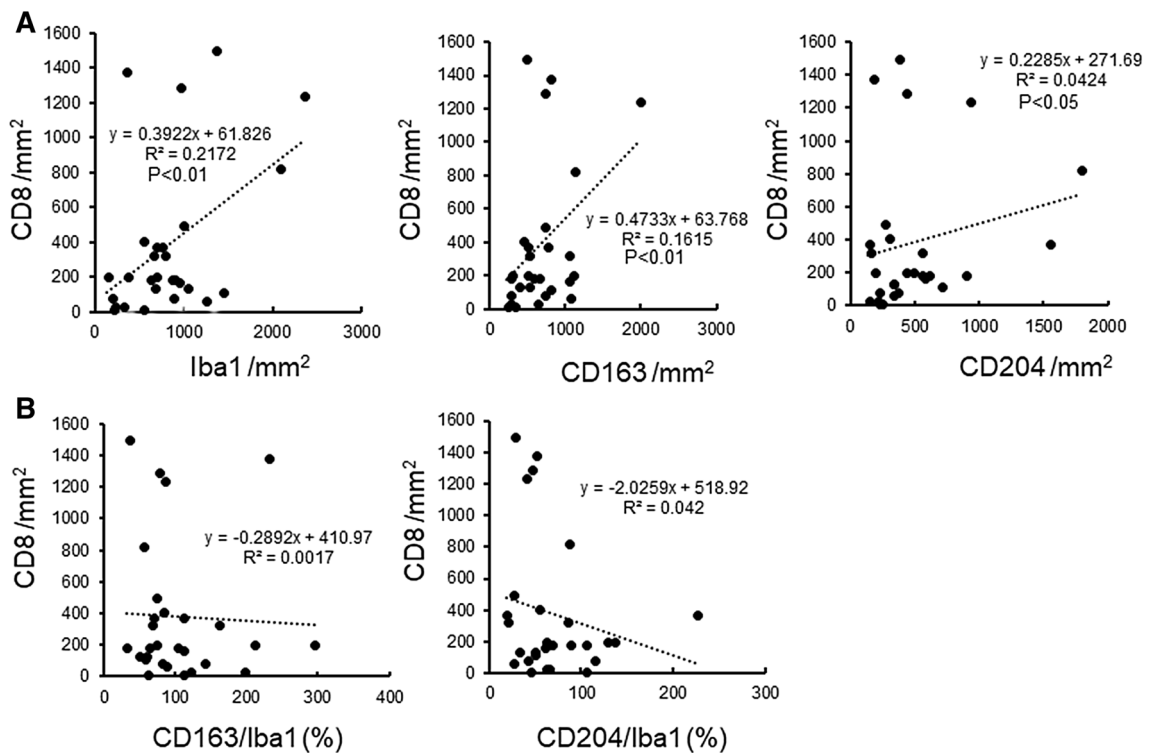


Fig. 3 The correlations between TAMs and CTLs. The correlations between the densities of Iba1-, CD163-, and CD204-positive TAMs and the density of CD8-positive CTLs were analyzed by Spearman’s correlation test

Table 1 The densities of macrophages and lymphocytes and clinicopathological factors

	CD163 Mean/mm ²	P value	CD204 Mean/mm ²	P value	Iba1 Mean/mm ²	P value	CD163 (%)	P value	CD204 (%)	P value	CD8 Mean/mm ²	P value
Gender												
Male	560	N.S.	377	N.S.	671	N.S.	111	N.S.	64.9	N.S.	450	N.S.
Female	781		582		958		94.9		71		313	
Age												
≥ 65	742	0.0273	448	N.S.	826	N.S.	111	N.S.	62	N.S.	451	N.S.
< 65	576		521		798		91.7		76		289	
Size												
≥ 5 cm	693	N.S.	534	N.S.	894	N.S.	93	0.0357	67.7	N.S.	381	N.S.
< 5 cm	589		278		366		139		68.9		382	
Location												
Deep	643	N.S.	544	N.S.	830	N.S.	96.3	N.S.	72.7	N.S.	348	N.S.
Superficial	728		344		780		117		58.1		450	
Metastasis												
Positive	778	N.S.	614	N.S.	932	N.S.	102	N.S.	77.4	N.S.	356	N.S.
Negative	563		345		697		104		58.5		407	
Necrosis												
Positive	680	N.S.	557	N.S.	893	N.S.	97.3	N.S.	69.8	N.S.	423	N.S.
Negative	661		401		735		108		66.2		340	
FNCLCC												
3	755	N.S.	592	N.S.	964	N.S.	87.8	N.S.	66.7	N.S.	393	N.S.
2	558		329		614		123		69.6		365	
AJCC(ed7)												
IV, III	723	N.S.	567	N.S.	889	N.S.	98.5	N.S.	72.5	N.S.	357	N.S.
II	561		294		657		112		58.4		433	

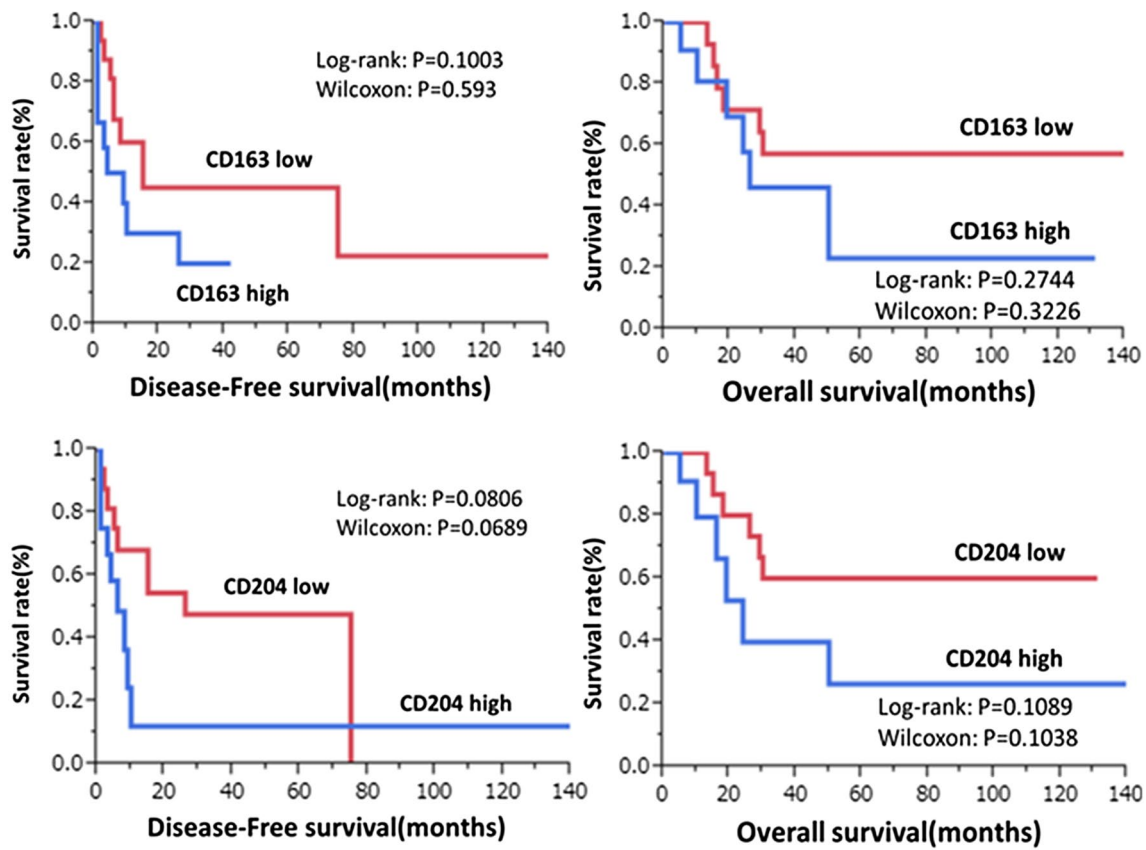


Fig. 4 The correlation between TAMs and survival rate. The correlations between disease-free or overall survival and the densities of CD163- or CD204-positive cells were analyzed by the Kaplan–Meier method

Table 2 Univariate analysis of disease free survival (DFS) and overall survival (OS)

	DFS			OS		
	HR	95% CI	P value	HR	95% CI	P value
CD163/mm ² ≥ 700/< 700	2.15	0.817–5.77	0.465	1.87	0.581–6.01	0.535
CD204/mm ² ≥ 400/< 400	2.29	0.856–6.2	0.0978	2.47	0.765–7.99	0.126
Iba1/mm ² ≥ 800/< 800	0.894	0.324–2.33	0.82	0.73	0.215–2.3	0.592
CD163% ≥ 100/< 100	1.77	0.662–4.66	0.247	1.59	0.469–5.02	0.437
CD204% ≥ 60/< 60	2.26	0.873–6.25	0.0933	1.62	0.514–5.52	0.408
CD8/mm ² ≥ 400/< 400	0.61	0.140–1.87	0.414	0.444	0.0681–1.70	0.256

in TME. The significance of this population in TME has never been clarified; however, macrophages positive for both CD163 and CD204 preferentially express high levels of PD-L1 and have strong immunosuppressive function [27]. Studies using knock-out mice have shown that CD163 and CD204 are involved in protumor activation of TAMs [15–17]. TAMs engulf tumor cell-derived soluble factors via CD204, and CD204 also inhibits Toll-like receptor-mediated macrophage activation by scavenging Toll-like receptor ligands [28]. CD163 binds the hemoglobin/haptoglobin complex and is associated with the production of protumor cytokines such as interleukin-6 and CXCL2 by TAMs [17].

High expression of CD163 on TAMs was significantly associated with a poor clinical outcome in our previous study of 62 patients [17], but we found no association between TAMs and clinical outcome in this study. This discrepancy may be due to the limitation of the small number of enrolled cases in this study.

Taken together, we found positive correlations between the densities of TAMs and CTLs in UPS. The density of CTLs was negatively correlated with the percent of CD204-positive TAMs. Although we found no significant correlation between immune cells and clinical outcome in the present cases, these findings may provide new insight

into the characterization of immune cells in the TME of UPS.

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Compliance with ethical standards

Conflict of interest All authors have no financial competing interests to declare.

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