CD68- and CD163-positive tumor-associated macrophages in triple negative cancer of the breast

Tsengelmaa Jamiyan1,2 · Hajime Kuroda1,3 · Rin Yamaguchi4 · Akihito Abe5,6 · Mitsuhiro Hayashi5

Abstract
Tumor-associated macrophages (TAMs) have recently been reported as an important factor in tumor growth and the progression of cancer. The prognostic significance of localizations and densities of TAMs in triple negative cancer (TNC) of the breast is not well understood. The aim of this study was to assess the localizations and densities of the TAMs subtype in TNC and examine their clinicopathological features. The study was based on 107 TNC cases operated on at Dokkyo Medical University Hospital using the pan-macrophage marker CD68 and the M2 macrophage marker CD163 in the tumor stroma (TS) and tumor nest (TN), respectively, and examined the clinicopathological significance. Multivariate Cox regression analyses revealed that age and CD163+ TAMs in both the TS and TN were independent prognostic factors for relapse-free survival and overall survival. No correlation was found between the number of CD68+ cells or the CD163/CD68 ratio either in TS or TN, or clinicopathological features. Our study found that infiltration of CD163+ TAMs, rather than CD68+, in both TS and TN was associated with poor prognosis in TNC patients by multivariate analysis. This suggests that CD163+ TAMs may affect the prognosis of TNC by not only regulating the immune reaction by TAMs in TS, but also because of their direct influence on TN.

Keywords Breast · Triple-negative cancer · Tumor-associated macrophages · CD68 · CD163

Introduction
Tumor-associated macrophages (TAMs) have recently been reported as an important factor in tumor growth and the progression of cancer. Recently, two processes were proposed for TAMs activation: Classically-activated type 1 (M1-like) macrophages and alternatively-activated type 2 (M2-like) macrophages. M1-like macrophages, characterized by CD68 expression, produce free radicals that can lead to DNA damage with the potential to contribute to tumoricidal activity [1]. In contrast, M2-like macrophages, characterized by both CD68 and CD163 expression, are considered to promote tumor growth and metastasis by releasing chemokines, which are inflammatory growth factors [2, 3]. Previous studies confirmed that TAMs are associated with cancer survival in several organs such as hepatoma [4], gastric cancer [5], and lung cancer [6]. In breast cancer, several studies have demonstrated that TAMs are related to hormonal status, stage, lymph node (LN) status, and prognosis [7–10]. Therefore, TAMs in different regions and at different densities may have different prognostic value in breast cancer. In general, triple-negative cancer (TNC) is
characterized by a lack of expression of the estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) protein; this type is well known to have a poor prognosis [11, 12]. However, we should note that TNCs do not always correlate with poor prognosis. Therefore, to confirm the association of TAMs and TNC, a larger cohort using different statistical methods should be evaluated. Moreover, the prognostic significance of localizations and densities of CD68+ and CD163+ TAMs in TNC is not well understood. The aim of this study was to assess the localizations and densities of the macrophage markers CD68+ and CD163+ TAMs in TNC and examine their clinicopathological features.

Materials and methods

Patients

The study was based on 107 TNC cases operated on at Dokkyo Medical University Hospital between 2006 and 2018. Patient and tumor characteristics, including patient age at the time of diagnosis, tumor size, histologic grade, LN status, and follow-up data, were determined from patients' medical records and pathology reports. Relapse-free survival (RFS) was defined as the number of months from surgical resection to the development of documented relapse, including recurrence or distant metastasis. Overall survival (OS) was recorded from the date of curative surgery to the date of breast cancer-specific death.

The present study was approved by the Ethics Committee of Dokkyo Medical University (Tochigi, Japan; registration number: 28009) and was conducted according to the Declaration of Helsinki.

Immunohistochemistry (IHC)

Surgical sections were immunostained for ER (clone SP1, Novocastra (Leica), prediluted, nuclear), PgR (clone 1E2, Novocastra (Leica), prediluted, nuclear), HER2 (clone 4B5, Roche (VENTANA), prediluted, membranous), CD68 (CD68, clone PG-M1, Dako (Agilent), 1:50), and CD163 (CD163, clone 10D6, Novocastra (Leica), 1:50). Counterstaining was performed with hematoxylin. The percentages of nuclei stained for ER and PgR were calculated, as stated by the guideline, and a patient was considered to be “positive” if the breast tumor contained at least 1% positive cells [13]. HER2 status was assessed according to the guidelines defined by the American Society of Clinical Oncology/College of American Pathologists [14]. We estimated the TILs on hematoxylin and eosin (H&E) stained sections according to the criteria proposed by the International Immuno-Oncology Biomarkers Working Group [15]. TILs levels were categorized as high (≥ 30%) and low (< 30%) adopting previously validated cut-offs [16].

TAMs were evaluated by adapting the previously reported hotspot quantitative method [7, 10, 17–19]. The CD68+ and CD163+ staining was assessed by counting the number of positive macrophages. TAMs were scored as the infiltration density of CD68+ or CD163+ cells with a macrophage morphology that showed strong membranous or cytoplasmic staining. Each specimen was screened at low magnification (× 100), and the five areas with the greatest number of positively stained cells (hot spot area) were selected for further analysis. The mean macrophage count in these areas for each case was estimated at high power (× 400) magnification. The CD68+ and CD163+ macrophages were counted in the tumor stroma (TS) and tumor nest (TN) separately. The definition of TS in this study was the stromal tissue surrounding the tumor nest. TAMs in TN were defined as intraepithelial tumor-infiltrating macrophages. For statistical analyses, the number of positive cells was divided into lower and higher groups based on cut-off points according to the median. As a result, the cut-off for CD68 in TS was 26.2, CD68 in TN was 11.2, CD163 in TS was 26.6, CD163 in TN was 8.6 CD163/CD68 in TS was 1.0, CD163/CD68 in TN was 0.99 (Table 1). Two pathologists (TJ and HK) did the evaluations without access to any clinical information.

Statistical analysis

Spearman’s Rho and χ2 tests were used to compare CD68 and CD163 expression and patient and tumor characteristics. Kaplan-Meier analysis and log-rank tests were used to illustrate differences in RFS and OS according to CD163 and CD68 expression. Cox regression proportional hazards models were used to estimate hazard ratios (HR) for death from breast cancer according to CD68 and CD163 expression in both uni- and multivariate analysis. Covariates with a P value ≤ 0.05 in the

| Table 1 Distribution pattern of TAMs in TNC |
|--------------------------------------------|
| Variables                  | Mean | SE  | Median | Range   |
|-----------------------------|------|-----|--------|---------|
| CD68+ TAMs                  |      |     |        |         |
| Tumor stroma                | 28.85| 1.2 | 26.2   | 5–96.2  |
| Tumor nest                  | 13.36| 0.74| 11.2   | 1.6–43  |
| CD163+ TAMs                 |      |     |        |         |
| Tumor stroma                | 29.58| 1.45| 26.6   | 4.2–78.2|
| Tumor nest                  | 10.58| 0.88| 8.6    | 0–44.8  |
| Ratio of CD163 and CD68     |      |     |        |         |
| Tumor stroma                | 0.99 | 0.007| 1      | 0.79–1.2|
| Tumor nest                  | 0.98 | 0.005| 0.99   | 0.84–1.12|

TAMs tumor-associated macrophages, TNC triple-negative cancer, SE standard error.
univariate analysis were included in the multivariate analysis. All statistical tests were two-sided and $P \leq 0.05$ was considered significant. Statistical analysis was performed using IBM SPSS Statistics 25 (IBM, Armonk, NY, USA).

Results

The CD68 and CD163 expressions in TS and TN were determined for all 107 samples. CD68+ (Fig. 1a, b) and CD163+ (Fig. 1c, d) macrophages were detected in both the TS and TN of TNC. The relationship between the density of TAMs (CD68+, or CD163+) and clinicopathological features is shown in Table 2. The study demonstrated that a high density of CD68+ TAMs in both TS and TN was significantly associated with larger tumor size ($p=0.036$; $p=0.004$). Whereas a high density of CD163+ TAMs in TN was also significantly related to larger tumor size ($p=0.002$), however, not in TS ($p=0.634$). Moreover, a high density of CD163+ TAMs in TS and TN were correlated with higher histological grade ($p<0.001$; $p=0.010$), higher recurrence rate ($p<0.001$; $p=0.004$), and higher breast cancer mortality ($p=0.004$, $p=0.012$). In contrast, no significant correlations were found between the infiltration densities of TAMs (CD68+, CD163+, CD163/CD68 ratio) and TILs in both TS ($p=0.635$, $p=0.382$, and $p=0.670$, respectively) or TN ($p=0.635$, $p=0.861$, and $p=0.670$, respectively). No correlation was found between the CD163/CD68 ratios for either TS or TN or in terms of clinicopathological features.

Univariate and multivariate Cox regression analysis of RFS and OS were performed using clinicopathological prognostic factors and expressions of CD68 and CD163 (Table 3). Multivariate Cox regression analyses revealed that age and CD163+ TAMs in both TS and TN were independent prognostic factors for RFS (HR = 0.164, 95% CI 0.048–0.560, $p=0.004$; HR = 9.059, 95% CI 1.160–70.76, $p=0.036$; HR = 4.476, 95% CI 1.028–22.08, $p=0.046$) and OS (HR = 0.095, 95% CI 0.024–0.374, $p=0.001$; HR = 10.69, 95% CI 1.313–87.18, $p=0.027$; HR = 5.017, 95% CI 1.065–23.64, $p=0.041$).

We investigated survival rate with regard to the different expressions of TAMs status using the Kaplan-Meier method and log-rank test. No correlation was found in the higher CD68+ TAMs density in both TS and TN with RFS ($p=0.119$, $p=0.957$) or OS ($p=0.104$, $p=0.911$) (Fig. 2a, b, c, and d). A higher CD163+ TAMs density in both TS and TN was correlated with unfavorable RFS ($p=0.003$, $p=0.022$) and OS ($p=0.005$, $p=0.026$) (Fig. 2e–h). However, no correlation was identified between high CD163/CD68+ ratios in both TS and TN with RFS ($p=0.085$, $p=0.782$) or OS ($p=0.102$, $p=0.891$) (Fig. 2i–l).

Discussion

TAMs can contribute to tumor destruction and influence tumor growth and progression. M1-like macrophages, characterized by CD68 expression, produce free radicals that can lead to DNA damage with the potential to contribute to


| Clinicopathological features | CD68 Tumor stroma | CD68 Tumor nest | CD163 Tumor stroma | CD163 Tumor nest | CD163:CD68 Tumor stroma | CD163:CD68 Tumor nest | p value | p value | p value | p value | p value | p value |
|-----------------------------|------------------|----------------|-------------------|-----------------|------------------------|------------------------|---------|---------|---------|---------|---------|---------|
| Age (years)                 |                  |                |                   |                 |                        |                        |         |         |         |         |         |         |
| < 50                        | 0.364            | 0.719          | 0.183             | 0.541           | 0.627                  | 0.072                  |         |         |         |         |         |         |
| ≥ 50                        | 0.036*           | 0.564          | 0.004*            | 0.002*          | 0.634                  | 0.208                  |         |         |         |         |         |         |
| Tumor size (cm)             |                  |                |                   |                 |                        |                        |         |         |         |         |         |         |
| ≤ 2                         | 0.063            | 0.564          | < 0.001*          | 0.010*          | 0.26                   | 0.924                  |         |         |         |         |         |         |
| > 2                         | 0.501            | 0.629          | 0.298             | 0.148           | 0.298                  | 0.408                  |         |         |         |         |         |         |
| Histological grade          |                  |                |                   |                 |                        |                        |         |         |         |         |         |         |
| I and II                    | 0.794            | 0.789          | 0.115             | 0.054           | 0.212                  | 0.499                  |         |         |         |         |         |         |
| III                         | 0.635            | 0.635          | 0.382             | 0.861           | 0.382                  | 0.670                  |         |         |         |         |         |         |
| Lymph node status           |                  |                |                   |                 |                        |                        |         |         |         |         |         |         |
| Absent                      | 0.053            | 0.185          | < 0.001*          | 0.004*          | 0.185                  | 0.485                  |         |         |         |         |         |         |
| Present                     | 0.179            | 0.970          | 0.004*            | 0.012*          | 0.059                  | 0.736                  |         |         |         |         |         |         |

TNC triple-negative cancer, TAMs tumor-associated macrophages, IBC-NST invasive breast carcinoma of no special type, ca carcinoma, ILC invasive lobular carcinoma, IMP invasive micropapillary carcinoma, N/A not applicable, TILs tumor-infiltrating lymphocytes

*p value is significant

*χ² test
Table 3  Univariate and multivariate Cox regression analyses for relapse-free survival (RFS) and overall survival (OS) of triple-negative cancer (TNC)

| Clinicopathological features | Univariate analysis | Multivariate analysis | Univariate analysis | Multivariate analysis |
|-----------------------------|---------------------|----------------------|---------------------|----------------------|
|                             | RFS                 | p value              | RFS                 | p value              |
|                             | HR (95% CI)         |                      | OS                  | HR (95% CI)         | p value |
| Age (< 50 vs. ≥ 50)         | 0.169 (0.051–0.568) | 0.004*               | 0.164 (0.048–0.560) | 0.004*               |
| Tumor size (2 cm vs. > 2 cm) | 2.535 (0.809–7.936) | 0.110                |                      | 3.012 (0.962–9.431) | 0.058 |
| Histological grade (I, II vs. III) | 2.233 (0.488–10.22) | 0.360                | 2.274 (0.497–10.41) | 0.29                |
| Histological type (IBC-NST vs. other types) | 0.770 (0.413–1.438) | 0.413                | 0.799 (0.431–1.481) | 0.476                |
| Lymph node status (absent vs. present) | 1.683 (0.818–3.465) | 0.158                | 1.820 (0.868–3.816) | 0.113                |
| TILs (low vs. high)         | 0.449 (0.121–1.660) | 0.230                | 0.433 (0.117–1.606) | 0.211                |
| TS CD68 (low vs. high)      | 1.021 (0.984–1.059) | 0.268                | 2.619 (0.783–8.756) | 0.118                |
| TN CD68 (low vs. high)      | 0.969 (0.312–3.008) | 0.957                | 0.936 (0.302–2.911) | 0.912                |
| TS CD163 (low vs. high)     | 11.50 (1.481–89.29) | 0.020*               | 10.597 (1.366–82.192) | 0.024*               |
| TN CD163 (low vs. high)     | 4.952 (1.084–22.61) | 0.039*               | 4.735 (1.037–21.623) | 0.045*               |
| TS CD163/CD68 (low vs. high) | 2.972 (0.804–10.98) | 0.102                | 2.829 (0.765–10.45) | 0.119                |
| TN CD163/CD68 (low vs. high) | 0.853 (0.275–2.646) | 0.783                | 0.924 (0.298–2.868) | 0.892                |

Multivariate Cox regression analyses were performed for all potential variables that were significantly associated with survival in univariate analysis. RFS relapse-free survival and OS overall survival. TNC triple-negative cancer, HR hazard ratio, CI confidence interval, IBC-NST invasive breast carcinoma of no special type, TILs tumor-infiltrating lymphocytes, TS tumor stroma, TN tumor nest.

*p value is significant
p = 0.119, log-rank

p = 0.003, log-rank

p = 0.022, log-rank

p = 0.085, log-rank

p = 0.782, log-rank

p = 0.102, log-rank

p = 0.026, log-rank

p = 0.005, log-rank
CD163+ TAMs affect the prognosis of TNC by not only regulating the immune reaction by TAMs in TS, but also through their direct influence on TN.

We also examined the correlation between TAMs and TILs which have recently been highlighted as prognostic markers and potential targets for adjuvant therapy [31–34]. TILs have antitumor activity and a favorable prognostic effect in breast cancer, especially in TNC [16, 35–37]. In our study, no significant correlations were found between the infiltration densities of TAMs and TILs. However, we could not draw any conclusion on the basis of our small number of cases.

There is a limitation in this study. First, the methods and subtypes of breast cancer patients were different in other studies, including our own. Yang et al. examined cases in which Basal-like carcinoma was defined by not only TNC, but also by EGFR and/or CK5/6 expression [7]. Second, although CD163 is regarded as a highly specific M2 macrophage marker, it can also be expressed by myeloid dendritic cells (MDCs). Both macrophages and MDCs are members of the mononuclear phagocyte system, these cells are considered distinct cell types based on their morphology and functions. Macrophages are defined as large vacuolar cells that have oval or rounded nuclei, while MDCs are characterized as stellate migratory cells. Therefore, we could have excluded the majority of the CD68+ and CD163+ MDCs with morphological features. Nevertheless, it could not be confirmed whether or not CD68+ and CD163+ MDCs are located in TS and TN. Of the different cell characteristics, surface markers are often used to distinguish MDCs from macrophages, but phenotypic analysis has been considered insufficient to define MDCs subsets. Some specific markers have been suggested to detect M1/M2 macrophages, but they remain controversial. In the future, more studies on larger sample sizes and TAMs labeling new, reliable macrophage markers are needed to evaluate the clinical value. Further, Medrek et al. observed some CD163+ areas that lacked CD68 expression. They suggested this result was due to a CD163-expressing subset of immature myeloid cells with prognostic impact [28]. Here, we confirmed TAMs not only by immunohistochemical staining, but also H&E staining, then estimated the number of typical macrophages and excluded the possibility that MDCs cells or myeloid-derived cells expressed CD163. However, further investigation is needed to identify TAMs’ roles in TNC with new, specific markers in future studies.

Conclusions

We examined the prognostic value of TAMs in TNC. Our study found that infiltration of CD163+ TAMs, rather than CD68, in both TS and TN was associated with poor prognosis in TNC patients by multivariate analysis. This suggested that CD163+ TAMs may affect the prognosis of TNC by not only regulating the immune reaction by TAMs in TS, but also through their direct influence on TN.

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Author contributions

T Jamiiyan and H Kuroda contributed equally to this work; A Abe and M Hayashi collected clinical information; T Jamiiyan and Kuroda H reviewed the pathological diagnosis; T Jamiiyan and H Kuroda analyzed the data and wrote the manuscript; R Yamaguchi made critical revisions to the manuscript; T Jamiiyan and H Kuroda designed the study; H Kuroda gave the final approval of the manuscript for publication. All authors read and approved the final manuscript.

Compliance with ethical standards

The authors adhered to institutional ethical standards.

Conflict of interest

The authors declare no potential competing interests.

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