# M2 tumor-associated macrophages promote tumor progression in non-small-cell lung cancer

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**Abstract.** Tumor-associated macrophages (TAMs) are key components of the tumor microenvironment that can be polarized into different phenotypes, including tumor-inhibiting M1 macrophages and tumor-promoting M2 macrophages. To elucidate the biological and clinical significance of M2 TAMs in non-small-cell lung cancer (NSCLC), a comprehensive clinical assessment of the tissue distribution of M2 TAMs was performed. The tissue distribution of M2 TAMs was retrospectively analyzed using CD163 immunohistochemistry in 160 consecutive patients who underwent NSCLC resection. Tumor proliferation was evaluated via the Ki-67 proliferation index. The results revealed that the stromal density of M2 TAMs was significantly associated with the C-reactive protein (CRP) level (P=0.0250), the Ki-67 proliferation index (P=0.0090) and invasive size (P=0.0285). Furthermore, the stromal M2 TAM density was significantly associated with tumor differentiation (P=0.0018), lymph node metastasis (P=0.0347) and pathological stage (P=0.0412). The alveolar M2 TAM density was also significantly associated with the CRP level (P=0.0309), invasive size (P<0.0001), tumor differentiation (P=0.0192), tumor status (P=0.0108) and pathological stage (P=0.0110). By contrast, no association was observed between islet M2 TAM density and the aforementioned biological and clinical factors. In regards to prognosis, disease-free survival rate was significantly lower in patients with stromal M2 TAM-high tumors (P=0.0270) and in those with alveolar M2 TAM-high tumors (P=0.0283). Furthermore, the overall survival rate was also significantly lower in patients with stromal M2 TAM-high tumors (P=0.0162) and in those with alveolar M2 TAM-high tumors (P=0.0225). Therefore, during NSCLC progression, M2 TAMs may induce tumor cell aggressiveness and proliferation and increase metastatic potential, resulting in a poor prognosis in patients with NSCLC.

## Introduction

Lung cancer is the leading cause of cancer-related mortality in developed countries (1,2). Based on the treatment strategy, lung cancer is clinically classified into non-small-cell lung cancer (NSCLC) and small-cell lung cancer. NSCLC accounts for 85% of all lung cancer cases, and it includes several types of histological subtypes, including adenocarcinoma and squamous cell carcinoma. Due to the advances in molecular biology, several molecular-targeted therapies have been developed for lung adenocarcinomas. Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), such as gefitinib, have been proven to be effective against lung adenocarcinomas with activating EGFR mutations (3). Anaplastic lymphoma kinase (ALK) inhibitors, such as alectinib, have also been reported to be effective against lung adenocarcinomas with the ALK fusion gene (4). However, molecular-targeted therapies are not applicable to cancers without mutations of these target genes. Recently, immune checkpoint inhibitors (ICIs), such as nivolumab, have been demonstrated to exhibit prominent clinical efficacy against various types of cancer, including NSCLC (5). However, these ICIs have been reported to be less effective against patients with programmed death-ligand 1 (PD-L1)-negative tumors (6). Therefore, it is crucial to fully elucidate tumor biology in order to develop novel treatment strategies against NSCLC without these mutations of target genes or PD-L1-positive expression.

The evaluation of infiltrating macrophages in tumors, referred to as tumor-associated macrophages (TAMs), has been reported to be important. TAMs are key components of the tumor microenvironment (TME) that influence tumor growth and progression (7,8). Tumor cells release various chemokines to attract macrophages, as well as other inflammatory cells, into the tumor stroma, and a number of substances secreted by TAMs may stimulate the proliferation and metastasis of tumor cells (9,10). Several clinical studies on TAMs have been reported in various human cancers, including NSCLC, colon and breast cancer (11-14). However, previous studies using only immunostaining for CD68, the most common pan-macrophage

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marker, yielded confusing results regarding its prognostic potential in NSCLC. For example, these studies reported that increased levels of TAMs in tumor islets were associated with good prognosis (15-19), whereas the increased levels of TAMs in the tumor stroma were found to be associated with poor prognosis (15,18). There could be several factors responsible for such confusing results.

First, macrophages are particularly heterogeneous in their phenotype and function. Under physiological or pathological conditions, macrophages can be polarized into different phenotypes, namely tumor-inhibiting M1 and tumor-promoting M2 macrophages (11,20,21). M1 TAM-derived cytokines have the ability to kill pathogens. On the other hand, M2 macrophages are pro-angiogenic and participate in wound healing by downregulating inflammatory response to promote connective tissue remodeling (14). Th2-derived cytokines, such as interleukin (IL)4, IL10 and IL13, transforming growth factor-β, prostaglandin E2 or colony-stimulating factor 1, may promote M2 differentiation of macrophages (8,21). During tumor progression, these signals originating from tumor and stromal cells may induce the production of M2 TAMs in the TME. M2 TAMs may induce angiogenesis by secretion of cytokines, such as vascular endothelial growth factor (VEGF) (22), and promote tumor growth and metastasis. Experimental studies also demonstrated that M2 macrophages may promote tumor cell proliferation (23).

Second, macrophages are distributed in various tissue compartments in lung cancer, such as tumor stroma, tumor islets and alveolar space, and the TAMs present in different tissue components may display different biological properties (11,12). In fact, previous clinical studies in NSCLC demonstrated that high infiltration of tumor islets by M1 TAMs was associated with increased survival (20,24), whereas infiltration of tumor islets and tumor stroma by high numbers of M2 TAMs was associated with reduced survival (20,25). In addition, the levels of M2 macrophages were reported to be higher compared with those of M1 macrophages in NSCLC (24).

Taking these factors into consideration, in order to elucidate the biological and clinical significance of M2 TAMs in NSCLC, a comprehensive clinical study on M2 TAMs in terms of tissue distribution was performed, using immunostaining for CD163, an M2 macrophage marker (20,24,25).

## Materials and methods

*Patients*. A total of 160 consecutive NSCLC patients who underwent surgery at the Department of Thoracic Surgery of Kitano Hospital between November 2011 and October 2014 were retrospectively investigated. The study protocol was approved by the Institutional Ethics Committee of Kitano Hospital (P181200300), and written informed consent was obtained from all the patients. Pathological staging was determined using the 8th tumor-node-metastasis (TNM) classification system (26). Invasive size was defined as the maximum dimension of the invasive component, excluding the lepidic growth component (26). The histological type and grade of differentiation of the tumors were determined according to the World Health Organization classification system (27). The patients' medical records and histopathological diagnoses were fully documented. The patient records included follow-up data as of August 2018. The overall median follow-up period was 42.8 months.

*Immunohistochemistry*. Immunohistochemical studies were performed to evaluate the M2 TAM distribution by CD163 staining and the tumor proliferation rate by Ki-67 proliferation index, using the Ventana BenchMark GX system (Roche/Ventana Medical Systems), according to the recommended protocol. The following antibodies were used: Mouse monoclonal anti-human CD163 antibody (clone 760-4437, Roche/VentanaMedical Systems), and rabbit monoclonal anti-human Ki-67 antibody (clone 30-9, Roche/VentanaMedical Systems). Formalin-fixed paraffin-embedded tissues were cut into 4-$\mu$m sections and mounted on poly-L-lysine-coated slides. The sections were deparaffinized and rehydrated, and antigen retrieval was performed with Cell Conditioner 1 (32 min for CD163 and 64 min for Ki-67). The sections were then incubated with the specific primary antibody against CD163 (16 min) and Ki-67 (8 min). Subsequently, the sections were treated with the OptiView HQ Linker for 8 min and the OptiView HRP Multimer for 8 min. Finally, counterstaining was performed with Mayer's hematoxylin and Scott's tap water bluing reagent.

*Evaluation of immunohistochemistry*. Evaluation of stained tissue sections was performed by two investigators (RS and TH) who were blinded to the patients' clinical status. Cases with discrepancies were jointly re-evaluated and a consensus was reached. For CD163 staining, the five most representative high-power fields (magnification, x400; 0.0625 mm$^2$) of the tumor stroma, tumor islets and alveolar space per tissue section were selected (Fig. 1). Tumor stroma was defined as the area where tumor stromal cells accounted for >70% of the total cells (28). In adenocarcinoma *in situ* cases with a scant stromal component, the perivascular or peribronchiolar stromal tissue inside the tumor was analyzed as tumor stroma. Tumor islets were defined as areas where tumor cells accounted for >70% of the total cells. The alveolar space was defined as the air space inside the main tumor or outside within three alveoli. The number of CD163-positive cells in each area was counted manually, and the mean number of fields in each area was calculated. Finally, the CD163-positive macrophage (M2 TAM) density was defined as cell number per mm$^2$ in the tumor stroma (stromal M2 TAM), tumor islets (islet M2 TAM) and alveolar space (alveolar M2 TAM). The percentage of carcinoma cells with a positive staining for Ki-67 in a given specimen was defined as the Ki-67 proliferation index (29).

*Statistical analysis*. As stromal M2 TAM density (P=0.1648), islet M2 TAM density (P=0.2845), alveolar M2 TAM density (P=0.1936), C-reactive protein (CRP) level (P=0.3056), total tumor size (P=0.1387), invasive size (P=0.1211) and Ki-67 proliferation index (P=0.1734) exhibited normal distribution (Kolmogorov-Smirnov analysis), statistical significance was assessed by the t-test, ANOVA with Bonferroni/Dunn test or Pearson's correlation coefficient. Categorical variables were compared using $\chi^2$ test. As previous clinical studies reported that the level of CRP, a marker of inflammatory response, was related to cancer risk and prognosis (30,31), receiver operating characteristic (ROC) curve analysis was performed to determine the optimal cut-off value of each M2 TAM density with maximal sensitivity and specificity for distinguishing between <1 mg/l and ≥1 mg/l of CRP (30,31). The sample was classified as stromal M2 TAM-high when the stromal M2 TAM density
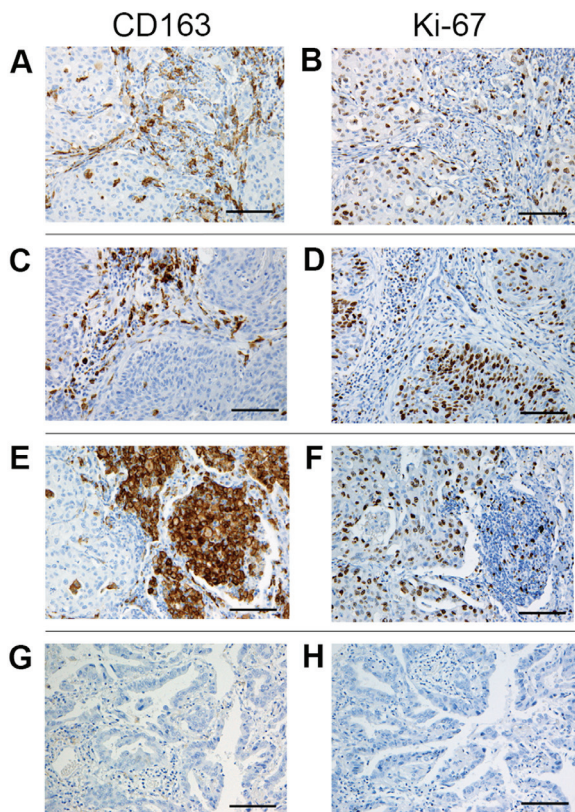
Figure 1. Lung cancer immunostaining. (A) Carcinoma with a high density of CD163-positive TAMs in the tumor stroma and the tumor islets with (B) a high Ki-67 index. (C) Carcinoma with a high density of CD163-positive stromal TAMs and a low density of CD163-positive islet TAMs with (D) a high Ki-67 index. (E) Carcinoma with a high density of CD163-positive TAMs in the tumor stroma and alveolar space, with (F) a high Ki-67 index. (G) Carcinoma with a low density of CD163-positive TAMs in the tumor stroma and tumor islets, with (H) a low Ki-67 index. Scale bars, 100 $\mu$m. TAM, tumor-associated macrophage.
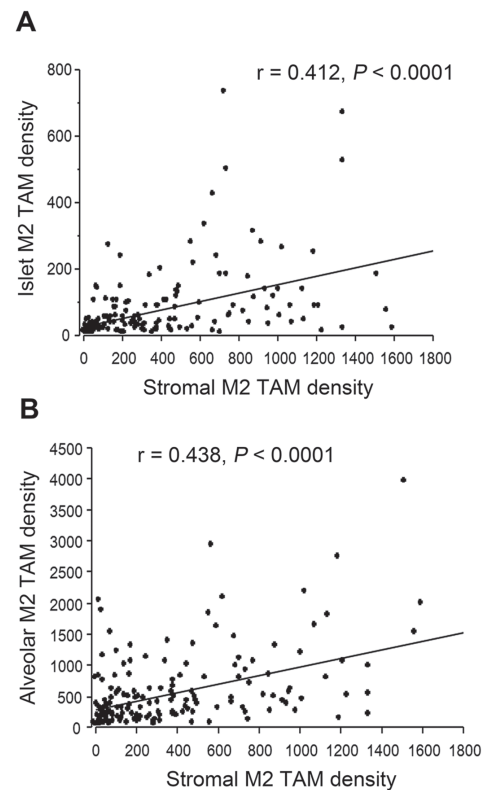


Figure 2. Correlation analysis. (A) Association between stromal M2 TAM density and islet M2 TAM density. (B) Association between stromal M2 TAM density and alveolar M2 TAM density. TAM, tumor-associated macrophage.

was >380 [area under the curve (AUC)=0.521]. The sample was classified as alveolar M2 TAM-high when the alveolar M2 TAM density was >400 (AUC=0.628). On the other hand, the sample was classified as islet M2 TAM-high when the islet M2 TAM density was >35, its median value, because the islet M2 TAM density was not associated with the CRP level. Disease-free survival (DFS) was defined as the time from treatment initiation (surgical resection, chemotherapy or radiation) to the date of disease recurrence or death from any cause. Overall survival (OS) was defined as the time from treatment initiation to the date of death from any cause. The Kaplan-Meier method was used to estimate the probability of DFS and OS as a function of time, and differences in the survival of subgroups of patients were compared using Mantel's log-rank test. The univariate analysis using the Cox regression model was used to evaluate the effects on survival. P-values obtained used a t-test, Mantel's log-rank test or Bonferroni/Dunn post-hoc test were based on the two-sided statistical analysis, and a P<0.05 was considered to indicate a statistically significant difference.

## Results

*Distribution of M2 TAMs in the tumor stroma, tumor islets and alveolar space.* The stromal M2 TAM density varied

greatly among the 160 tumor tissues investigated (mean, 407.0±389.2). A total of 93 tumors (58.1%) were classified as stromal M2 TAM-low tumors, and 67 (41.9%) as stromal M2 TAM-high tumors. In addition, the islet M2 TAM density also varied greatly among the 160 tumor tissues (mean, 82.3±143.4). A total of 80 tumors (50.0%) were islet M2 TAM-low tumors, and 80 (50.0%) were islet M2 TAM-high tumors. The stromal M2 TAM density was moderately correlated with the islet M2 TAM density (r=0.412; Fig. 2A). However, the islet M2 TAM density was significantly lower compared with the stromal M2 TAM density in each tumor tissue (P<0.001).

The alveolar M2 TAM density also varied greatly among the 160 tumor tissues (mean, 560.6±612.9). A total of 88 tumors (55.0%) were alveolar M2 TAM-low tumors, and 72 (45.0%) were alveolar M2 TAM-high tumors. The alveolar M2 TAM density was also moderately correlated with the stromal M2 TAM density (r=0.438; Fig. 2B). However, the correlation between the islet M2 TAM density and the alveolar M2 TAM density was low (r=0.212).

*Biological and clinical significance of M2 TAMs in the tumor stroma among resected NSCLC.* The biological significance of the M2 TAMs in the tumor stroma is shown in Fig. 3. The CRP level was significantly higher in the stromal M2 TAM-high group compared with that in the stromal M2 TAM-low group (4.41±7.88 vs. 2.29±3.52 mg/l, P=0.0250; Fig. 3A). In addition, the Ki-67 proliferation index was significantly higher in the stromal M2 TAM-high group compared with that in the stromal M2 TAM-low group (34.8±30.0 vs. 23.2±25.1%,
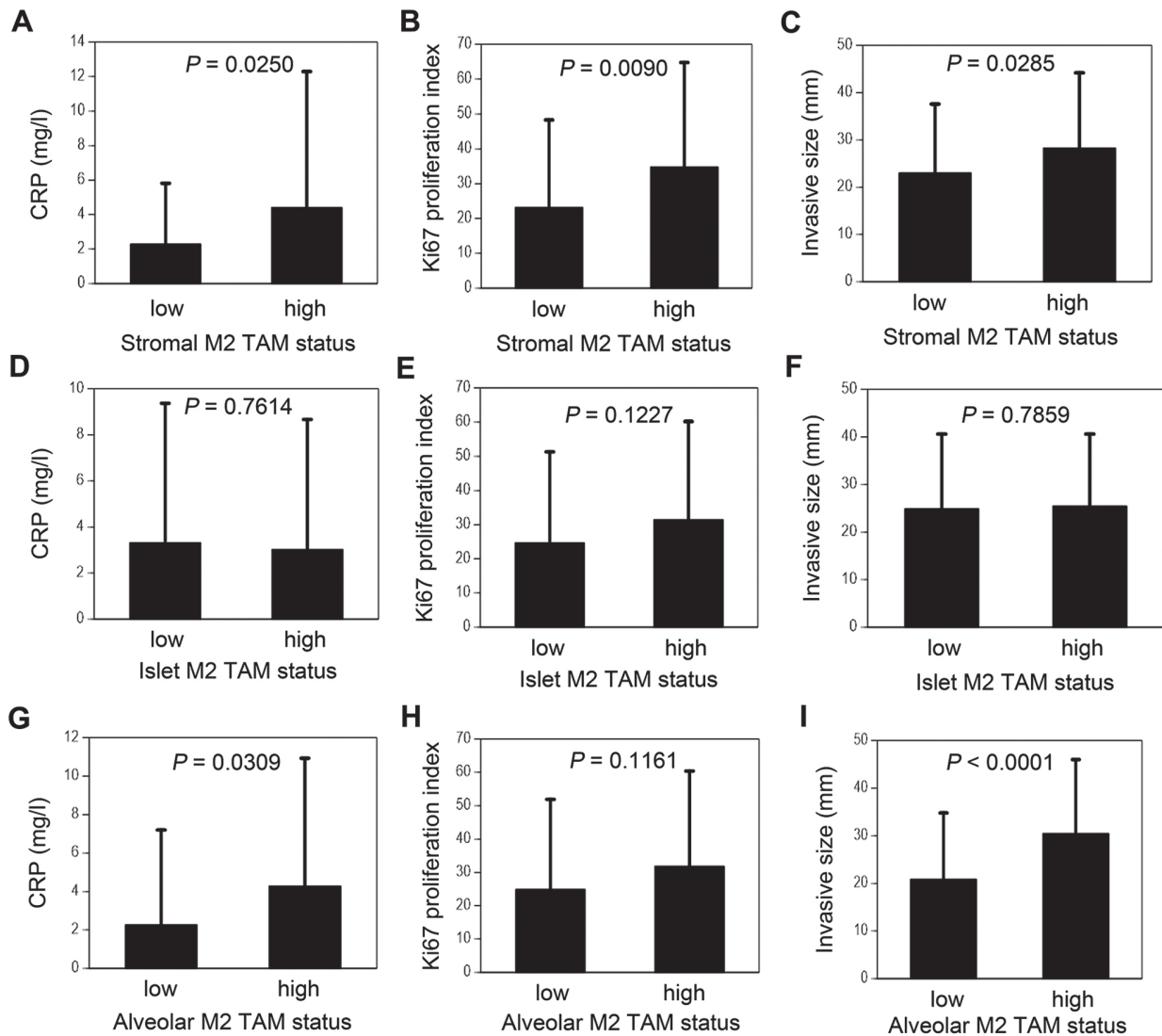
Figure 3. Biological significance of M2 TAMs. Biological significance of stromal M2 TAM density and (A) CRP (B) Ki-67 proliferation indices, and (C) invasive size. Biological significance of islet M2 TAM density and (D) CRP, (E) Ki-67 proliferation index and (F) invasive size. Biological significance of alveolar M2 TAM density and (G) CRP, (H) Ki-67 proliferation index and (I) invasive size. Data are presented as the mean ± standard deviation. *P*, t-test. TAM, tumor-associated macrophage; CRP, C-reactive protein.

P=0.0090; Fig. 3B). The invasive size was also significantly higher in the stromal M2 TAM-high group compared with that in the stromal M2 TAM-low group (28.3±15.9 vs. 23.0±14.6 mm, P=0.0285; Fig. 3C).

The distribution of the stromal M2 TAM density according to clinicopathological characteristics is shown in Table I. With respect to tumor histology, the stromal M2 TAM density was significantly higher in squamous cell carcinomas compared with that in adenocarcinomas (P=0.0034). In addition, the stromal M2 TAM density was significantly associated with tumor differentiation (P=0.0018), and was significantly higher in poorly differentiated tumors compared with that in well-differentiated and moderately differentiated tumors (P=0.0004 and P=0.0149, respectively). With respect to nodal status, the stromal M2 TAM density was significantly higher in node-positive tumors compared with that in node-negative tumors (P=0.0347). Furthermore, the stromal M2 TAM density was significantly associated with pathological stage (P=0.0412).

*Biological and clinical significance of M2 TAMs in the tumor islets among resected NSCLC.* The islet M2 TAM was not associated with the CRP level, the Ki-67 proliferation index or the invasive size (Fig. 3D-F). In addition, the islet M2 TAM density was not associated with tumor histology, tumor differentiation, tumor status, nodal status or pathological stage (Table I).

*Biological and clinical significance of M2 TAMs in the alveolar space among resected NSCLC.* With respect to biological significance, the CRP level was significantly higher in the alveolar M2 TAM-high group compared with that in the alveolar M2 TAM-low group (4.29±6.64 vs. 2.27±4.93 mg/l, P=0.0309; Fig. 3G). On the other hand, the alveolar M2 TAM was not significantly associated with the Ki-67 proliferation index (Fig. 3H). However, the total tumor size was significantly higher in the alveolar M2 TAM-high group compared with that in the alveolar M2 TAM-low group (31.7±14.8 vs. 23.4±12.8 mm, P=0.0005). In addition, the invasive size

Table I. Distribution of M2 tumor-associated macrophage density in patients with NSCLC according to clinicopathological characteristics.

| Characteristics | n | Tumor stroma (cells/mm$^2$) | P-value | Tumor islet (cells/mm$^2$) | P-value | Alveolar space (cells/mm$^2$) | P-value |
|---|---|---|---|---|---|---|---|
| Smoking | | | | | | | |
| Non-smoker | 85 | 370.6±367.4 | 0.2092[a] | 76.6±102.2 | 0.5960[a] | 525.1±684.3 | 0.4374[a] |
| Smoker | 75 | 448.2±411.0 | | 88.7±179.7 | | 600.8±522.1 | |
| Tumor status | | | | | | | |
| T0 | 8 | 207.0±330.1 | 0.1726[b] | 52.2±52.1 | 0.0714[b] | 239.0±168.3 | 0.0108[b] |
| T1 | 72 | 379.7±386.6 | | 56.8±67.7 | | 442.9±625.8 | |
| T2-4 | 80 | 451.6±392.6 | | 108.3±188.9 | | 698.7±599.4 | |
| Nodal status | | | | | | | |
| N0 | 123 | 371.4±344.9 | 0.0347[a] | 74.1±106.7 | 0.1864[a] | 517.1±575.0 | 0.1017[a] |
| N1-3 | 37 | 525.2±497.1 | | 109.7±226.5 | | 705.2±714.8 | |
| Pathological Stage | | | | | | | |
| 0 | 7 | 95.4±104.6 | 0.0412[b] | 43.8±50.1 | 0.8020[b] | 241.1±181.7 | 0.0110[b] |
| I | 100 | 385.3±359.4 | | 89.1±166.9 | | 472.2±594.7 | |
| II | 24 | 548.0±428.9 | | 66.9±83.5 | | 851.3±657.4 | |
| III | 29 | 440.3±453.5 | | 80.9±106.4 | | 701.9±611.6 | |
| Differentiation | | | | | | | |
| Well | 33 | 251.8±278.7 | 0.0018[b] | 80.4±123.2 | 0.0522[b] | 405.9±393.6 | 0.0192[b] |
| Moderately | 93 | 397.7±369.4 | | 59.2±68.5 | | 526.1±561.4 | |
| Poorly | 34 | 499.9±154.0 | | 147.3±255.9 | | 805.1±832.0 | |
| Histology | | | | | | | |
| Adenocarcinoma | 128 | 357.3±365.5 | 0.0034[b] | 71.8±95.1 | 0.0692[b] | 535.6±637.2 | 0.3053[b] |
| Squamous cell carcinoma | 25 | 635.3±469.4 | | 106.0±261.9 | | 726.4±534.0 | |
| Large cell carcinoma | 7 | 499.9±154.0 | | 190.0±247.6 | | 424.9±266.8 | |
| Total number of patients | 160 | 407.0±389.2 | | 82.3±143.4 | | 560.6±612.9 | |

[a]P-value determined using a t-test. [b]P-value determined using ANOVA followed by a Bonferroni/Dunn test. NSCLC, non-small-cell lung cancer.

was also significantly higher in the alveolar M2 TAM-high group compared with that in the alveolar M2 TAM-low group (30.5±15.5 vs. 20.9±13.9 mm, P<0.0001; Fig. 3I).

With respect to clinical significance, the alveolar M2 TAM density was significantly associated with tumor differentiation (P=0.0192) (Table I), and the alveolar M2 TAM density was significantly higher in poorly differentiated tumors compared with that in well-differentiated and moderately differentiated tumors (P=0.0073 and P=0.0219, respectively). In addition, the alveolar M2 TAM density was significantly associated with tumor status and pathological stage (P=0.0108 and P=0.0110, respectively).

*DFS of patients with resected NSCLC in relation to M2 TAM status.* With respect to the stromal M2 TAM status, the 5-year DFS rate was significantly lower in patients with stromal M2 TAM-high tumors compared with stromal M2 TAM-low tumors (54.7 vs. 72.9%, P=0.0270; Fig. 4A). In particular, among patients with early-stage disease (stage 0 and I), the 5-year DFS rate was significantly lower in patients with stromal M2 TAM-high tumors compared with those with stromal M2 TAM-low tumors (64.0 vs. 84.5%, P=0.0233; Fig. 4B).

However, among patients with advanced disease (stage II and III), no significant difference was observed in the DFS of patients with resected NSCLC patients in relation to the stromal M2 TAM status (Fig. 4C). In addition, no difference was observed in the DFS of patients with resected NSCLC according to islet M2 TAM status (Fig. 4D). With respect to the alveolar M2 TAM status, the 5-year DFS rate was significantly lower in patients with alveolar M2 TAM-high tumors compared with those with alveolar M2 TAM-low tumors (54.0 vs. 76.2%, P=0.0283; Fig. 4E). However, among patients with early-stage disease (stage 0 and I), no significant difference was observed in the DFS of patients with resected NSCLC patients in relation to the alveolar M2 TAM status (Fig. 4F). Univariate analyses using the Cox regression model also demonstrated that the stromal M2 TAM status [HR=1.869 (95% CI: 1.064-3.283); P=0.0296] and the alveolar M2 TAM status [HR=1.873 (95% CI: 1.059-3.311); P=0.0310] were significant factors for predicting the DFS of patients with resected NSCLC.

*OS of patients with resected NSCLC in relation to M2 TAM status.* With respect to the stromal M2 TAM status, the 5-year
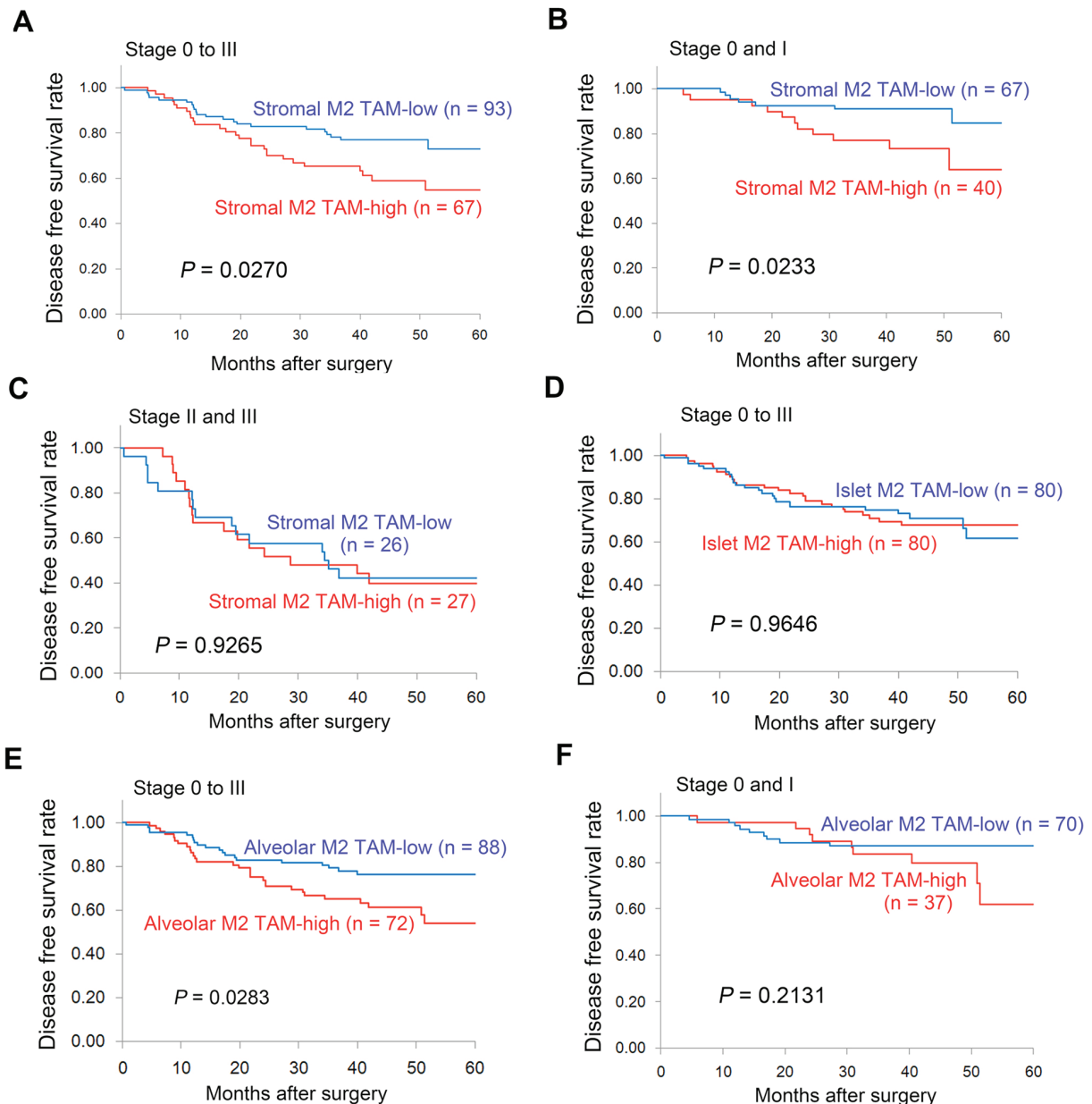
Figure 4. Disease-free survival. (A) Stromal M2 TAM density of 160 patients with NSCLC. (B) Stromal M2 TAM density of 107 patients with stage 0 and I NSCLC. (C) Stromal M2 TAM density of 53 patients with stage II and III NSCLC. (D) Islet M2 TAM density of the 160 patients with NSCLC. (E) Alveolar M2 TAM density of the 160 patients with NSCLC. (F) Alveolar M2 TAM density of 107 patients with stage 0 and I NSCLC. *P*, Mantel's log-rank test. TAM, tumor-associated macrophage; NSCLC, non-small-cell lung cancer.

OS rate was significantly lower in patients with stromal M2 TAM-high tumors compared with those with stromal M2 TAM-low tumors (74.5 vs. 85.8%, P=0.0162; Fig. 5A). In particular, for early-stage disease (stage 0 and I), the 3-year OS was significantly lower in patients with stromal M2 TAM-high tumors compared with those with stromal M2 TAM-low tumors (87.3 vs. 98.5%, P=0.0204; Fig. 5B). However, for advanced disease (stage II and III), no difference was observed in the OS of patients with resected NSCLC in relation to the stromal M2 TAM status (Fig. 5C). In addition, no difference was observed in the OS of patients with resected NSCLC according to islet M2 TAM status (Fig. 5D). With respect to the alveolar M2 TAM status, the 5-year OS was significantly lower in patients with alveolar M2 TAM-high tumors compared with those with alveolar M2 TAM-low tumors (72.7 vs. 89.5%,

P=0.0225; Fig. 5E). However, among patients with early-stage disease (stage 0 and I), no significant difference was observed in the OS of patients with resected NSCLC patients in relation to the alveolar M2 TAM status (Fig. 5F). Univariate analyses using the Cox regression model also demonstrated that the stromal M2 TAM status [HR=2.630 (95% CI: 1.160-5.964); P=0.0207] and the alveolar M2 TAM status [HR=2.573 (95% CI: 1.109-5.972); P=0.0278] were significant factors for predicting OS in patients with resected NSCLC.

## Discussion

In order to elucidate the biological and clinical significance of M2 TAMs in NSCLC, a comprehensive clinical study on M2 TAMs with respect to tissue distribution was
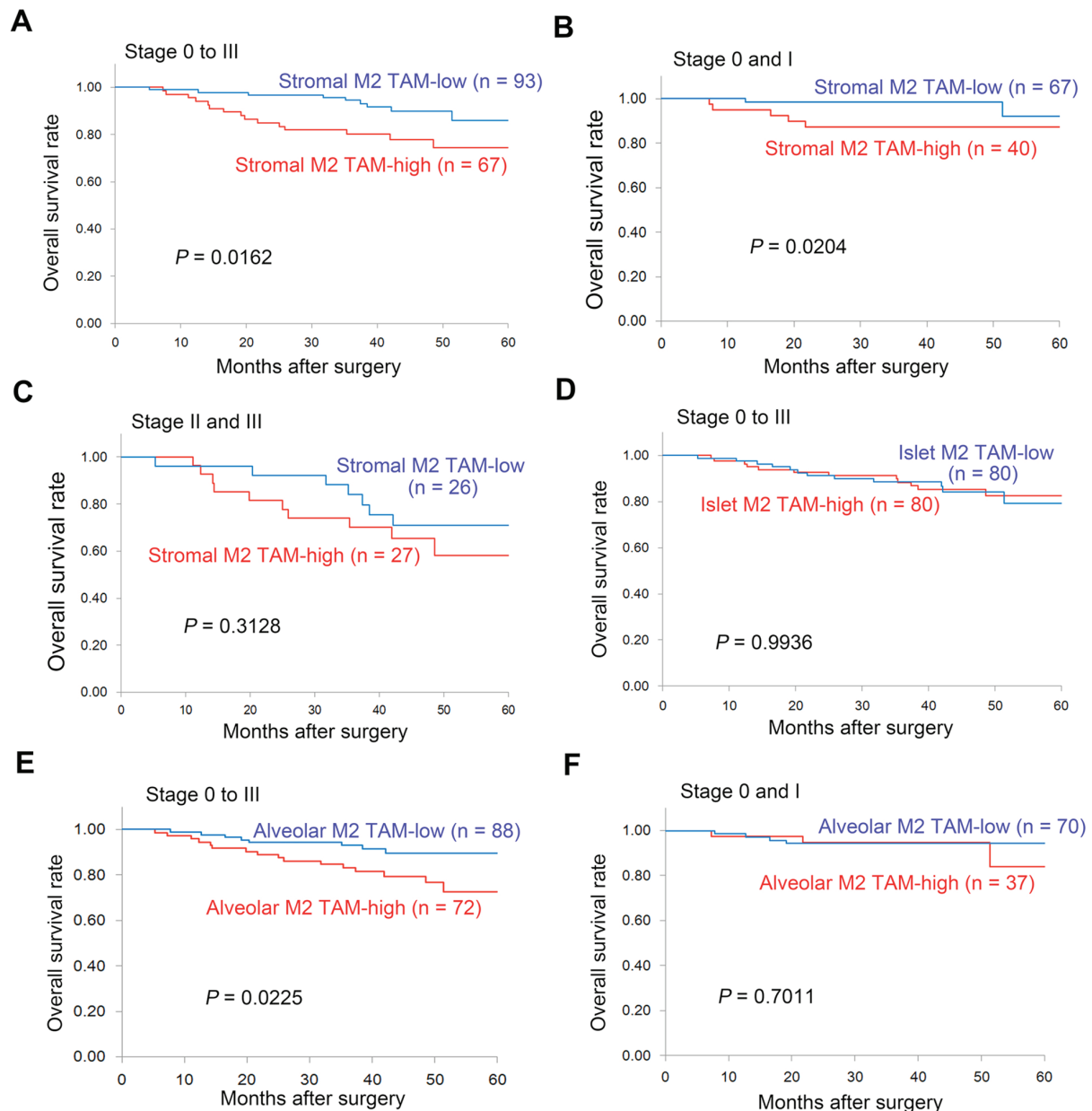
Figure 5. Overall survival. (A) Stromal M2 TAM density of 160 patients with NSCLC. (B) Stromal M2 TAM density of 107 patients with stage 0 and I NSCLC. (C) Stromal M2 TAM density of 53 patients with stage II and III NSCLC. (D) Islet M2 TAM density of the 160 patients with NSCLC. (E) Alveolar M2 TAM density of the 160 patients with NSCLC. (F) Alveolar M2 TAM density of 107 patients with stage 0 and I NSCLC. *P*, Mantel's log-rank test. TAM, tumor-associated macrophage; NSCLC, non-small-cell lung cancer.

performed. Regarding M2 macrophage markers, such as CD163 and CD204 (28), CD163 immunostaining was used, as previous clinical studies using this immunostaining demonstrated the clinical significance of M2 TAMs in NSCLC patients (20,24,25). The findings of the present study demonstrated that the stromal M2 TAM density in NSCLC was associated with tumor differentiation, CRP level, tumor growth, invasive size, lymph node metastasis, pathological stage and poor prognosis. In addition, the alveolar M2 TAM density was also associated with tumor differentiation, CRP level, tumor status, invasive size, pathological stage and poor prognosis. By contrast, the islet TAM density was significantly lower compared with the stromal M2 TAM density, and no association was observed between the islet M2 TAM and the abovementioned biological and clinical factors.

First, TAMs originate from circulating blood cells, such as monocytes. Chemotactic signals originating from tumor or stromal cells in the TME could recruit monocytic precursors to the tumor site. The present study demonstrated that the CRP level was associated with both the stromal and alveolar M2 TAM density. A previous clinical study also reported that a higher density of CD163-positive macrophages was associated with elevated CRP levels (32). These results suggest the existence of crosstalk between cancer-related inflammation and M2 TAMs in TME (8). During tumor progression, this crosstalk may generate more aggressive tumors. In fact, previous clinical studies reported that an elevated CRP level was a poor prognostic factor in NSCLC patients (33,34).

Next, the present study demonstrated that the stromal M2 TAM density was associated with tumor proliferation and

invasive size, and that the alveolar M2 TAM density was also associated with invasive size. M2 TAMs produce various tumor-promoting cytokines, including VEGF, which affect tumor growth and metastasis (8,21). In addition, invasive size was found to be correlated with various prognostic pathological factors (35), and it is an important factor for TNM classification (26). To the best of our knowledge, the present study was the first to demonstrate that stromal M2 TAM density is correlated with tumor proliferation and invasive size, which indicate a more aggressive malignant potential.

Therefore, the present study revealed that the stromal M2 TAM density is associated with lymph node metastasis, pathological stage, reduced DFS and reduced OS. The alveolar M2 TAM density was also associated with reduced DFS and reduced OS. Previous clinical studies also reported that stromal M2 macrophages are associated with poor prognosis in lung cancer patients (20,25,36). Former clinical studies using immunostaining for only CD68 also demonstrated that the stromal macrophages, which were primarily M2 TAMs, were associated with a poor prognosis in NSCLC (15,18). Although the prognostic significance of M2 TAMs was found only on univariate analysis using the Cox regression model in the present study, the statistical analyses regarding prognosis did not reach statistical significance on multivariate analysis using the Cox regression model. These results may be partly due to the relatively small number of patients, which was a limitation of the present study. Further clinical studies using a higher number of patients are required.

Considering the results of the present study, during NSCLC progression, TME may produce M2 TAMs, thereby promoting tumor aggressiveness. M2 TAMs are predominantly located in the tumor stroma, and may move into the alveolar space. The stromal M2 TAM density is a potential marker for predicting malignant potential and clinical outcome. Therefore, postoperative adjuvant chemotherapy may be required for patients with stromal M2 TAM-high tumors, even in the early stages. In addition, further investigation on TAMs may enable the development of novel treatments, such as TAM repolarization strategies using 'M2-to-M1' macrophage reprogramming molecules (8,21).

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## Funding

## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

RS, MF and CH designed the present study. RS, TH and CH designed and performed the experiments. RS, HM and YO collected the data. RS, HM, CH and YO analyzed and interpreted the data and wrote the manuscript. All authors have read and approved the final version of the manuscript for publication.

## Ethics approval and consent to participate

The current study was approved by the Institutional Ethics Committee of the Kitano Hospital (approval no. P181200300), and written informed consent was obtained from each patient. The research was conducted in compliance with the principles outlined in the Declaration of Helsinki.

## Patient consent for publication

Written informed consent for publication of patient data/accompanying images was obtained.

## Competing interests

The authors declare that they have no competing interests.

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