The tumoral and stromal immune microenvironment in malignant pleural mesothelioma: A comprehensive analysis reveals prognostic immune markers

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Abbreviations: CI, confidence interval; FoxP3, forkhead box P3; HR, hazard ratio; IL-7R, interleukin-7 receptor; MDSCs, myeloid-derived suppressor cells; MPM, malignant pleural mesothelioma; OS, overall survival; TAMs, tumor-associated macrophages; TILs, tumor-infiltrating lymphocytes; Tregs, regulatory T cells

Antitumor immune responses against solid malignancies correlate with improved patient survival. We conducted a comprehensive investigation of immune responses in tumor and tumor-associated stroma in epithelioid malignant pleural mesothelioma with the goal of characterizing the tumor immune microenvironment and identifying prognostic immune markers. We investigated 8 types of tumor-infiltrating immune cells within the tumor nest and tumor-associated stroma, as well as tumor expression of 5 cytokine/chemokine receptors in 230 patients. According to univariate analyses, high densities of tumoral CD4- and CD20-expressing lymphocytes were associated with better outcomes. High expression of tumor interleukin-7 (IL-7) receptor was associated with worse outcomes. According to multivariate analyses, stage and tumoral CD20 detection were independently associated with survival. Analysis of single immune cell infiltration for CD163+ tumor-associated macrophages did not correlate with survival. However, analysis of immunologically relevant cell combinations identified that: (1) high CD163+ tumor-associated macrophages and low CD8+ lymphocyte infiltration had worse prognosis than other groups and (2) low CD163+ tumor associated macrophages and high CD20+ lymphocyte infiltration had better prognosis than other groups. Multivariate analyses demonstrated that CD163/CD8 and CD163/CD20 were independent prognostic factors of survival. With a recent increase in immunotherapy investigations and clinical trials for malignant pleural mesothelioma patients, our observations that CD20+ B lymphocytes and tumor-associated macrophages are prognostic markers provide important information about the tumor microenvironment of malignant pleural mesothelioma.

Introduction

Malignant pleural mesothelioma (MPM) is a highly aggressive and rare primary pleural malignancy with median survival of 9–12 months.1 In epithelioid MPM, which is the most common histological type of MPM, median survival is only 17 months, even with trimodality therapy—chemotherapy, surgical resection, and thoracic radiation.2,3 Despite poor prognosis, few studies have reported that MPM patients with antitumor immune responses survived longer and that their improved survival was associated with increased CD8+ tumor-infiltrating lymphocytes (TILs).4,5 Immunosuppressive cytokines and regulatory T cells (Tregs) are also hypothesized to infiltrate the tumor microenvironment, dampening antitumor immune function and promoting MPM tumor growth.6 With recent successes, both preclinical7 and clinical,8 in immunotherapy for MPM, understanding the interplay between protumorigenic and antitumorigenic immune factors in the MPM tumor microenvironment is vital to developing novel therapies for MPM patients. Although the prognostic utility of tumor-infiltrating immune cells for MPM has been previously investigated,4-6,9 these study cohorts were heterogeneous in histologic subtypes.

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and included only a small number of patients. In our study here, we sought to elucidate the prognostic significance of the tumor immune microenvironment, so we qualitatively and quantitatively investigated immune cells infiltrating the tumor nest and tumor-associated stroma, as well as tumoral cytokine (receptor) expression, in a large cohort of patients with epithelioid MPM.

### Results

#### Clinicopathologic variables

Demographical, clinical, and histopathological variables for 230 patients are shown in Table 1. Median follow-up time for survivors was 37.3 months (range, 0.5–92.5 months). Median overall survival (OS) was 16.3 months (95% confidence interval [CI]: 14.6–19.3 months), with 2-year OS of 33.2%, and 5-year OS of 11.6%. Univariate analysis revealed that male sex ($P = 0.032$), advanced stage (III, IV vs. I, II; $P = 0.001$), lymphatic invasion ($P = 0.038$), vascular invasion ($P = 0.009$), and pleomorphic histology ($P = 0.015$) were significantly associated with worse OS (Table 1).

#### Epithelioid MPM tumor immune microenvironment

The tumor microenvironment of MPM was characterized by examining tumor-associated expression of CD4, CD68, and CD163, as well as stromal-associated expression of CD163, CXCR4, and IL-12Rβ2. Immune and stromal markers were evaluated as described below to detect prognostic markers for MPM.

#### Survival analysis for tumor-infiltrating lymphocytes and cytokine (receptor) expression

Each immune parameter in the tumor nest and tumor-associated stroma was independently assessed for its associations with survival (Table 2). A high density of CD4-expressing cells in tumors was significantly associated with favorable survival (median OS, 15.2 months for low vs. 17.0 months for high level; $P = 0.04$; Fig. 1A), whereas a high density of CD8$^+$ lymphocytes in tumors reflected tendency for longer survival (median OS, 14.0 months for high level; $P = 0.04$; Table 2). Elevated levels of the B cell marker CD20 in tumors were also significantly associated with favorable survival (median OS, 14.5 months for low vs. 20.7 months for high level; $P = 0.003$; Fig. 1C).

Of the 5 cytokines and cytokine receptors, interleukin-7 receptor (IL-7R) was determined to be a prognostic factor (Table 2). Higher-level expression of IL-7R was associated with increased risk of death (median OS, 19.3 months for low level vs. 14.0 months for high level; $P = 0.007$; Fig. 1D).

Multivariate analyses were performed for the 3 prognostic immune markers—tumor CD4, tumor CD20, and IL-7R—and these models adjusted for the prognostic clinicopathologic factors from univariate analysis, including sex, disease stage (III, IV vs. I, II), vascular invasion, and pleomorphic morphology. The final multivariate model confirmed that stage (hazard ratio [HR] 1.72, 95% CI 1.26–2.35; $P < 0.001$) and tumor CD20 (HR 0.69, 95% CI 0.51–0.93; $P = 0.015$) remained independently associated with survival. Higher-level expression of IL-7R reflected a tendency for increased risk of death (HR 1.34, 95% CI 1.00–1.81; $P = 0.052$) (Table 3A).

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**Table 1.** Univariate analysis of overall survival and clinicopathologic factors of epithelioid MPM patient cohort

| Variables               | N (%) | Median OS | 95% CI     | P-value |
|-------------------------|-------|-----------|------------|---------|
| All Patients            | 230   | 16.3      | (14.6, 19.3)|         |
| Age                     |       |           |            |         |
| <65                     | 136 (59) | 16.3      | (14.1, 22.0)| 0.27    |
| >65                     | 94 (41)  | 16.3      | (14.5, 19.4)|         |
| Gender                  |       |           |            |         |
| Female                  | 63 (27)  | 22.4      | (17.4, 28.8)| 0.032   |
| Male                    | 167 (73) | 15.0      | (12.4, 17.5)|         |
| Asbestos                |       |           |            |         |
| Yes                     | 100 (43) | 16.3      | (14.3, 21.0)| 0.71    |
| No                      | 66 (29)  | 19.4      | (15.0, 23.8)|         |
| Unknown                 | 64 (28)  | 14.7      | (10.3, 19.9)|         |
| Smoking                 |       |           |            |         |
| Yes                     | 137 (60) | 16.8      | (15.0, 20.7)| 0.29    |
| No                      | 45 (20)  | 19.1      | (14.1, 33.3)|         |
| Unknown                 | 48 (21)  | 12.3      | (8.9, 19.3)|         |
| Laterality              |       |           |            |         |
| Right                   | 129 (56) | 16.8      | (13.8, 19.9)| 0.79    |
| Left                    | 100 (43) | 16.1      | (14.1, 22.0)|         |
| T stage                 |       |           |            |         |
| T1,2                    | 116 (50) | 19.2      | (16.3, 23.8)| 0.025   |
| T3,4                    | 114 (49) | 14.3      | (9.5, 17.4)|         |
| N stage                 |       |           |            |         |
| N0                      | 162 (70) | 18.9      | (16.3, 22.4)| 0.077   |
| N1, 2                   | 68 (28)  | 9.8       | (7.4, 17.5)|         |
| Stage                   |       |           |            |         |
| I, II                   | 75 (33)  | 23.4      | (19.1, 32.6)| 0.001   |
| II, IV                  | 155 (67) | 14.1      | (9.8, 17.0)|         |
| Procedure               |       |           |            |         |
| EPP                     | 124 (54) | 15.0      | (12.4, 20.7)| 0.59    |
| P/D                     | 90 (39)  | 18.1      | (15.1, 21.9)|         |
| Others (Surgical biopsy)| 16 (7)  | 12.9      | (4.8, 38.0)|         |
| Lymphatic invasion      |       |           |            |         |
| Absent                  | 121 (53) | 19.3      | (16.2, 23.8)| 0.038   |
| Present                 | 109 (47) | 14.5      | (11.4, 16.9)|         |
| Vascular invasion       |       |           |            |         |
| Absent                  | 177 (77) | 17.6      | (15.4, 21.0)| 0.009   |
| Present                 | 53 (23)  | 11.4      | (8.2, 16.2)|         |
| Induction Chemotherapy  |       |           |            |         |
| Yes                     | 63 (28)  | 20.1      | (15.1, 27.5)| 0.15*   |
| No                      | 164 (71) | 15.2      | (12.4, 18.1)|         |
| Unknown                 | 3 (1)   | 25.0      | (4.1, NA) |         |
| Morphology              |       |           |            |         |
| Pleomorphic             | 38 (17)  | 14.6      | (8.1, 17.5)| 0.015   |
| Non-pleomorphic         | 192 (83) | 17.5      | (14.9, 20.9)|         |

Abbreviations: CI = confidence interval; EPP = extrapleural pneumonectomy; OS = overall survival (months); P/D = pleurectomy/decortication.

Statistical analysis was performed by log-rank test; significant P-values (<0.05) are shown in bold type.

*A excludes unknown.

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Survival analysis for tumor-associated macrophages levels and ratio to tumor-infiltrating lymphocytes

Analysis of single immune cell infiltration revealed that CD163⁺ M2-polarized tumor-associated macrophages (TAMs) were not correlated with OS (tumor: \( P = 0.49 \), stroma: \( P = 0.12 \)). Next, we investigated immunologically relevant combinations—the relative proportion of protumorigenic CD163⁺ TAMs to antitumorigenic tumor-infiltrating lymphocytes (TILs) (Fig. 2). On the basis of this observation, patients with high CD163⁺ TAMs and low CD8⁺ lymphocyte infiltration had worse prognosis (median OS, 8.8 months for high risk index) than other groups (median OS, 17.0 months for low risk index; \( P = 0.009 \); Fig. 2A) and, conversely, patients with low CD163⁺ TAMs and high CD20⁺ lymphocyte infiltration had better prognosis (median OS, 25.0 months for low risk index) than other groups (median OS, 15.0 months for high risk index; \( P < 0.001 \); Fig. 2B).

In the final multivariate model, stage (HR 1.57; 95% CI 1.15–2.14; \( P = 0.005 \)), tumoral CD163/CD8 (HR 1.64; 95% CI 1.01–2.66; \( P = 0.044 \)), and CD163/CD20 (HR 1.64; 95% CI 1.10–2.14; \( P = 0.015 \)) expression remained independent predictors of worse OS (Table 3B).

### Table 2. Univariate analysis of overall survival and immune parameters

| Marker   | Tumor N (%) | Median OS  | 95%CI     | P-value* | Stroma N (%) | Median OS  | 95%CI     | P-value* |
|----------|-------------|------------|-----------|----------|--------------|------------|-----------|----------|
| CD3      | Low 54 (25) | 14.3       | (9.4, 24.9) | 0.57     | 143 (67)     | 16.2       | (13.8, 19.4) | 0.52     |
|          | High 162 (75) | 16.8       | (15.0, 19.8) |         | 72 (33)     | 17.4       | (15.0, 23.3) |         |
| CD4      | Low 113 (52) | 15.2       | (10.6, 19.2) | 0.04     | 28 (13)     | 15.0       | (7.4, 21.9)  | 0.26     |
|          | High 105 (48) | 17.0       | (14.5, 24.8) |         | 188 (87)    | 17.0       | (14.9, 20.1) |         |
| CD8      | Low 86 (40)  | 14.7       | (12.0, 19.8) | 0.061    | 94 (44)     | 18.9       | (14.7, 23.0) | 0.68     |
|          | High 131 (60) | 17.0       | (14.5, 21.0) |         | 120 (56)    | 15.3       | (12.5, 18.1) |         |
| CD45RO   | Low 93 (61)  | 16.3       | (14.5, 23.8) | 0.98     | 133 (86)    | 16.2       | (14.6, 19.4) | 0.72     |
|          | High 60 (39)  | 15.0       | (9.8, 20.9)  |         | 21 (14)     | 17.4       | (10.1, 28.9) |         |
| FoxP3    | Low 128 (59) | 14.5       | (10.5, 16.8) | 0.003    | 94 (44)     | 15.0       | (11.4, 18.9) | 0.21     |
|          | High 89 (41)  | 20.7       | (16.3, 26.9) |         | 119 (56)    | 18.1       | (15.2, 23.3) |         |
| CD68     | Low 142 (65) | 16.1       | (14.0, 19.9) | 0.58     | 140 (66)    | 16.2       | (14.3, 20.1) | 0.87     |
|          | High 75 (35)  | 16.8       | (11.4, 21.1) |         | 72 (34)     | 17.0       | (15.0, 21.1) |         |
| CD163    | Low 172 (79) | 16.3       | (14.3, 19.8) | 0.87     | 22 (10)     | 22.0       | (16.2, 61.1) | 0.067    |
|          | High 47 (21)  | 16.9       | (10.9, 23.4) |         | 193 (90)    | 16.3       | (14.5, 19.2) |         |
| IL-7R    | Low 112 (53) | 19.1       | (15.1, 23.4) | 0.49     | 133 (67)    | 18.1       | (15.1, 22.4) | 0.12     |
|          | High 98 (47)  | 15.0       | (10.6, 18.1) |         | 66 (33)     | 14.5       | (10.3, 17.0) |         |
| IL-12Rβ2 | Low 116 (53) | 19.3       | (16.3, 26.4) | 0.007    |              |            |           |          |
|          | High 103 (47) | 14.0       | (10.6, 18.1) |         |              |            |           |          |
| CCR7     | Low 112 (75) | 15.4       | (13.8, 19.1) | 0.067    |              |            |           |          |
|          | High 38 (25)  | 19.9       | (14.5, 41.6) |         |              |            |           |          |
| CXCL12   | Low 123 (79) | 15.2       | (14.0, 18.1) | 0.16     |              |            |           |          |
|          | High 33 (21)  | 28.1       | (15.0, 45.5) |         |              |            |           |          |
| CXCR4    | Low 55 (36)  | 15.4       | (11.4, 18.1) | 0.49     |              |            |           |          |
|          | High 99 (64)  | 16.8       | (15.0, 23.4) |         |              |            |           |          |

Abbreviations: CI = confidence interval; FoxP3 = forkhead box P3; IL-7R = interleukin-7 receptor; IL-12Rβ2 = interleukin-12 receptor β2; OS = overall survival (months).

Statistical analysis was performed by log-rank test; significant P-values (<0.05) are shown in bold type.

*Stroma not applicable for this marker.

Adjusted for search of optimal cut-point.
Subgroup analysis of non-induction chemotherapy cases

In our cohort, there were 63 (27%) cisplatin-based induction chemotherapy cases and 164 (71%) non-induction chemotherapy cases. We performed subgroup analyses of immune markers for the non-induction chemotherapy cases. In this subgroup, higher-level expression of IL-7R was associated with increased risk of death (median OS, 12.3 months for high vs. 19.1 months for low level; \( P = 0.006 \)). Low density of CD68 in the stroma was significantly associated with better survival (median OS, 23.4 months for low vs. 15.0 months for high level; \( P = 0.003 \)). Low density of CD163 in the stroma was also significantly associated with better survival (median OS, 17.5 months for low vs. 11.0 months for high level; \( P = 0.041 \)) (Table 4). The sample size of induction therapy patients was insufficient to analyze separately. However, immunohistochemical scoring information for the expression of each immune marker in all epithelioid MPM patients-(both with and without induction chemotherapy)-is provided in Supplemental Table 2.

Discussion

In our investigation of TILs and expression of cytokine receptors on tumor cells in MPM, we found that tumor CD20, IL-7R, CD163/CD8, and CD163/CD20 were significant prognostic factors of epithelioid MPM.

It has been demonstrated that the presence of tumor-infiltrating immune cells correlate with the clinical outcome of multiple solid tumors, and with outcomes predicated on type, density, and location of immune cell infiltrates. \(^{10-14}\) While our initial investigations supported the prognostic role of inflammatory responses as independent predictors of survival in 175 epithelioid MPM patients, \(^{15}\) detailed investigations in this study using specific cellular markers highlights the influence of protumor and antitumor immune responses within the tumor and associated stroma.

Other investigators previously reported correlations between the presence of CD8\(^+\) TILs and MPM patient survival in a smaller cohort of 32 patients, as demonstrated by immunohistochemical analysis of extrapleural pneumonectomy specimens. \(^{4,5}\) In our study, patients with a high density of CD8\(^+\) TILs in tumors tended to exhibit improved survival; although, these results were not statistically significant (\( P = 0.061 \); Fig. 1B).

Furthermore, Anraku et al. \(^{4}\) demonstrated protective association of CD8\(^+\) TILs in patients who received induction chemotherapy and a high density of CD8\(^+\) TILs was found to be an independent predictor of prolonged survival and correlated with reduced frequency of mediastinal lymph node metastases. These results suggested that strategies promoting tumor-infiltrating CD8\(^+\) T cells may clinically benefit MPM patients.

Various prior studies have investigated the potential effects of the presence of distinct T-cell subpopulations in MPM. \(^{4,5}\) In a preclinical orthotopic mouse model of MPM, we have shown that adoptive T-cell therapy-induced immune responses are
predominantly mediated by CD4-expressing T cells that provide beneficial and durable immunity. By contrast, there was a distinct lack of data regarding B cell infiltrate in MPM. B lymphocytes are effector cells that have humoral immunity and can terminally differentiate into antibody secreting plasma cells upon stimulation. Moreover, B cells contributed to cellular immunity by serving as antigen-presenting cells and/or by providing stimulatory signals to T cells. The role of B lymphocytes during tumor immunity remains controversial. On one hand, antigen-presenting B cells were found to induce tumor-specific cytotoxic T-cell activation, and B cell deficient mice exhibited significantly reduced tumor-specific, T-cell immunity. On the other hand, B cell antibody response may potentiate chronic inflammation that could enhance tumor development. Tumor-infiltrating CD20+ B lymphocytes were found to associate with improved patient survival in primary breast cancer, non-small cell lung cancer, epithelial ovarian cancer, and pancreatic cancer. Our study provides the first evidence that B cells, perhaps as part of the humoral immune response, may have a role in constraining epithelioid MPM.

In our study, M2-polarized TAMs (i.e., CD163+) and their ratio with biologically relevant TILs (i.e., CD8+ and CD20+ lymphocytes) were independent predictors of survival in epithelioid MPM. In the non-induction chemotherapy group, high stromal CD163+ TAMs were associated with poor survival. Emerging evidence has pointed to the clinical significance of TAMs in several malignant tumors. To our knowledge, the association between CD163+ TAMs and clinical outcome has not been fully investigated in patients with MPM. In various solid tumors, while in the presence of appropriate cytokines or ligands, macrophages polarized into 2 types—M1 and M2 TAMs. A hallmark of macrophages is their plasticity—the ability to either aid or fight tumors depending on the tumor microenvironment—which has given them the reputation in tumor biology of being a "double-edged sword." M1 TAMs demonstrated immunostimulatory properties and conferred enhanced tumor resistance and cytotoxicity, while M2 TAMs (CD163) secreted immunosuppressive cytokines, promoted angiogenesis, and supported tumor progression, invasion, and metastasis. Interaction with MPM cells appeared to shift mature macrophages toward the M2 phenotype, which was characterized by poor antigen presentation and increased immunosuppressive activity. Upon co-cultivation with MPM cells, macrophages released significant amounts of prostaglandin E2, an arachidonic acid metabolite with considerable anti-inflammatory properties. Production of this prostaglandin has been shown to stimulate the development of regulatory T cells, promoting an immunosuppressive tumor microenvironment. Tumor-associated macrophages have also been reported to upregulate interleukin-10 and B7-H3 on tumor cells which, in turn, inhibit antitumor T-cell responses. Worse prognosis correlating with elevated CD163+ TAMs in the current non-induction chemotherapy cohort supports the premise that the adaptive humoral immune response may play a crucial role in disease progression.

In our study, high expression levels of tumoral IL-7R were associated with unfavorable prognosis, even after excluding the induction chemotherapy group. We previously reported that IL-7R was a poor prognostic marker in early-stage lung adenocarcinoma patients. The role of IL-7R expression in MPM remains unknown, although in lung and breast cancers, IL-7R was shown to induce tumor growth and lymphangiogenesis via upregulation of vascular endothelial growth factor D. The ligand of IL-7R, IL-7, is produced by bone marrow and thymic stromal and epithelial cells, as well as a variety of tumor cells. Most human FoxP3 Tregs express low levels of IL-7R as IL-7 signaling plays a role in the development and function of these cells. Therefore, IL-7 may promote tumor progression via the activation of IL-7R on both tumor cells and Tregs. Therapeutic
strategies that target the IL-7/IL-7R axis may decrease tumor growth and lymphangiogenesis while limiting the immuno-suppressive effects of Tregs.33

Based on our observation that higher levels of tumor-infiltrating lymphocytes were associated with improved survival among MPM patients, we investigated the therapeutic utility of chimeric antigen receptor (CAR)-directed adoptive T-cell therapy in clinically relevant MPM mouse models, work now being translated into a Phase I clinical trial.7

A side note, as the number of patients receiving neoadjuvant therapy in our cohort was low (n = 63) and the chemotherapy regimen and cycles varied among patients, we did not conduct a separate analysis of this cohort. Similarly, since the number of mesothelioma patients with biphasic (n = 26) and sarcomatoid (n = 23) histologies were low, both subsets of patients displayed shorter survival (median survival was 7.6 months in biphasic patients and 4 months in sarcomatoid patients) and inadequate tissue was available from patients due to lack of surgical resections, such that we also did not perform a separate analysis on this cohort.

**Conclusion**

Our findings shed light on the complex tumor immune microenvironment in epithelioid MPM. First, we demonstrated the association of CD4-, CD8-, and CD20-expressing lymphocytes with favorable prognosis. Second, we demonstrated that M2-polarized TAMs (CD163+) and their ratio to biologically relevant TILs (CD8+ T cells and CD20+ B cells) were independent markers of prognosis. Third, IL-7R expression on tumor cells was associated with worse patient survival.

To our knowledge, we are among the first to identify prognostic immune factors dictating the tumor immune microenvironment in a large-scale study of epithelioid MPM patients. Our findings provide the foundation for future investigations into immunomodulatory therapies for epithelioid MPM.

**Materials and Methods**

**Patients**

Clinical and pathological data for 620 patients diagnosed with MPM between 1989 and 2010 at Memorial Sloan Kettering Cancer Center (MSK) were obtained from the prospectively maintained Thoracic Surgery Mesothelioma Database upon MSK’s Internal Review Board approval.15,37-40

From this cohort, we reviewed 395 MPM cases with available hematoxylin and eosin-stained slides. All slides were re-reviewed by 2 pathologists (K.K. and W.D.T.) yielding 301 epithelioid, 59 biphasic, and 35 sarcomatoid MPMs. All slides were evaluated
for lymphatic and vascular invasion.37,38 This study focused on epithelioid MPM cases with tissue available for the construction of tissue microarray (TMA). A total of 230 epithelioid MPM cases with TMAs were included in the analysis. Staging was based on the seventh edition of the American Joint Committee on Cancer TNM Cancer Staging Manual.41 All patients were observed until death or end of study (January 2014).

Immunohistochemical analysis
Formalin-fixed, paraffin-embedded tumor specimens were used for TMA construction. For each tumor, the area with the most severe inflammatory reaction was chosen. From each tumor, 9 representative cores with the most abundant inflammatory reaction, 0.6 mm in size, were marked—6 from the tumor nest and 3 from the tumor-associated stroma.42 We constructed TMAs from 230 epithelioid MPM cases. Standard avidin-biotin-peroxidase complex technique was used for immunohistochemical staining of human-specific antibodies (Table 5).32

### Scoring of immunohistochemistry
Representative images and immunohistochemical scoring are shown in Supplemental Figure 1. Under a high-power field (magnification, ×200), each core was scored semi-quantitatively

| Marker           | Tumor N (%) | Median OS | 95% CI       | P-value* |
|------------------|-------------|-----------|--------------|----------|
| CD3              | Low 38 (25) | 14.4      | (9.4, 28.8)  | 0.24     |
|                  | High 115 (75)| 15.4     | (12.3, 19.2) |          |
| CD4              | Low 87 (56) | 14.7      | (9.8, 18.9)  | 0.13     |
|                  | High 68 (44)| 16.1     | (12.4, 23.8) |          |
| CD8              | Low 63 (41) | 14.7      | (12.0, 19.8) | 0.17     |
|                  | High 91 (59)| 16.1     | (11.4, 19.3) |          |
| CDH5RO           | Low 70 (60) | 15.4      | (12.4, 23.4) | 0.9      |
|                  | High 46 (40)| 15.0     | (9.5, 20.9)  |          |
| CD20             | Low 97 (63) | 15.0      | (10.6, 18.1) | 0.18     |
|                  | High 57 (37)| 16.9     | (11.4, 23.8) |          |
| FoxP3            | Low 97 (63) | 16.1      | (12.5, 19.9) | 0.75     |
|                  | High 58 (37)| 14.5     | (9.4, 19.4)  |          |
| CD68             | Low 125 (81)| 15.2     | (12.3, 19.2) | 0.79     |
|                  | High 30 (19)| 15.0     | (8.1, 23.4)  |          |
| CD163            | Low 81 (54) | 18.9      | (14.7, 22.0) | 0.26     |
|                  | High 70 (46)| 12.0     | (8.8, 16.9)  |          |
| IL-7R            | Low 80 (52) | 19.1      | (15.2, 28.1) | 0.006    |
|                  | High 75 (48)| 12.3     | (9.0, 16.2)  |          |
| IL-12Rβ2         | Low 90 (78) | 15.2      | (12.3, 19.1) | 0.27     |
|                  | High 26 (22)| 14.7     | (8.2, 38.0)  |          |
| CCR7             | Low 94 (80) | 14.9      | (12.0, 17.6) | 0.19     |
|                  | High 24 (20)| 28.1     | (9.5, 45.5)  |          |
| CXCL12           | Low 40 (34) | 14.0      | (9.8, 18.1)  | 0.68     |
|                  | High 77 (66)| 16.3     | (14.5, 22.0) |          |
| CXCR4            | Low 82 (72) | 15.0      | (12.3, 19.1) | 0.67     |
|                  | High 32 (28)| 16.2     | (8.8, 28.8)  |          |

Abbreviations: CI = confidence interval; FoxP3 = forkhead box P3; IL-7R = interleukin-7 receptor; IL-12Rβ2 = interleukin-12 receptor β2; OS = overall survival (months).
Statistical analysis was performed by log-rank test; significant P-values (<0.05) are shown in bold type.
*Stroma not applicable for this marker.
*Adjusted for search of optimal cut-point.
for the degree of immune cell infiltration into the tumor nest and tumor-associated stroma. Scores for each core were averaged to give a single score for each patient. For expression of cytokine and chemokine receptors, we scored tumor stains on the basis of intensity and distribution, as previously described.32,43

Statistical analysis

Associations between variables were analyzed using the Fisher’s exact test for categorical variables and the Wilcoxon test for continuous variables.44 CD marker expressions were dichotomized into high versus low using optimal cut-points, which were found using a maximally selected log-rank statistic (Table S1). Overall survival was estimated using the Kaplan-Meier method starting at time of surgery. Patients who did not die during study follow-up were censored at last time they were known to be alive. Differences in OS between patient subgroups were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model to estimate the effect of immune markers of interest on OS, with adjustments for clinico-pathologic factors that were found to be significantly associated with OS on univariate analyses. A separate multivariate model was built for each immune marker that was significant on univariate analyses. The final multivariate model included immune markers that were significantly associated with OS in their separate multivariate models. All significance tests were 2-sided and used a 5% level of significance. Statistical analyses were conducted using the “survival” and “maxstat” packages in R version 3.0.1 (R Development Core Team).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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Table 5. Antibodies used for immunohistochemistry

| Marker     | Source          | Type          | Manufacturer                               | Dilution |
|------------|-----------------|---------------|-------------------------------------------|----------|
| CD3        | Mouse           | Monoclonal    | Dako (Glostrup, Denmark)                  | 1:1,600  |
| CD4        | Goat            | Polyclonal    | R&D Systems (Minneapolis, MN)             | 1:100    |
| CD8        | Mouse           | Monoclonal    | Dako                                      | 1:200    |
| CD20       | Mouse           | Monoclonal    | Dako                                      | 1:4,000  |
| CD45RO     | Mouse           | Monoclonal    | Dako                                      | 1:4,000  |
| FoxP3      | Mouse           | Monoclonal    | Abcam (Cambridge, United Kingdom)         | 1:2,000  |
| CD68       | Mouse           | Monoclonal    | Dako                                      | 1:2,000  |
| CD163      | Mouse           | Monoclonal    | Vector (Burlingame, CA)                   | 1:100    |
| CCR7       | Rabbit          | Monoclonal    | Epitomics (Burlingame, CA)                | 1:100    |
| CXCL12     | Mouse           | Monoclonal    | Epitomics (Burlingame, CA)                | 1:100    |
| CXCR4      | Mouse           | Monoclonal    | R&D Systems                               | 1:1,000  |
| IL-7R      | Mouse           | Monoclonal    | Santa Cruz Biotechnology (Santa Cruz, CA) | 1:2,000  |
| IL12Rβ2    | Goat            | Polyclonal    | Santa Cruz Biotechnology                  | 1:100    |

Abbreviations: FoxP3 = forkhead box P3; IL-7R = interleukin-7 receptor; IL-12Rβ2 = interleukin-12 receptor β2.
