Research Article

Cancer Immunology Research

Pretreatment Immune Status Correlates with Progression-Free Survival in Chemotherapy-Treated Metastatic Colorectal Cancer Patients

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Abstract

It remains unclear whether the immunologic status of cells in peripheral blood can be used as a prognostic indicator of response to treatment for patients with unresectable metastatic colorectal cancer (MCRC). We therefore investigated the relationship between the pretreatment immunologic status of 40 patients with MCRC who planned to receive the first-line chemotherapy and their progression-free survival. Twenty-five immune cell subsets, including monocytic myeloid-derived suppressor cells (M-MDSC) and effector memory T cells (T_{EM}), were measured by multicolor-flow cytometry. We divided patients into high and low (above and below the median, respectively) groups based on the median value for each immune cell subset and compared progression-free survival of the two groups. Patients with high M-MDSC, low $CD4^+$ T_{EM}, or low $CD8^+$ T_{EM} quantities had significantly shorter progression-free survival ($P = 0.004$, 0.005, and 0.002, respectively). Patients were classified into two prognostic groups based on numbers of adverse factors; having two or three adverse factors ($n = 21$, 52.5%) was correlated with significantly shorter progression-free survival compared with none or one ($n = 19$, 47.5%; $P < 0.001$). The presence of two or three adverse factors was an independent poor prognostic factor for progression-free survival (HR, 9.2; 95% confidence interval, 2.5–34.2; $P < 0.001$). These results provide evidence that pretreatment peripheral immune status can inform the outcome of patients with MCRC treated with first-line chemotherapy. *Cancer Immunol Res; 4(7); 592–9. ©2016 AACR.*

Introduction

Effector T cells in cancer patients respond to tumor cells. Cytotoxic $CD4^+$ or $CD8^+$ T cells recognize tumor-specific antigens or tumor-associated antigens and exert direct cytotoxic actions against tumor cells. Colorectal cancer was the first neoplasia found to be under immune surveillance (1, 2). An increased quantity of tumor-infiltrating lymphocytes (TIL), $CD3^+$ cells, $CD8^+$ cells, and Th1 cells in surgically resected colorectal tumor specimens is associated with an improved prognosis (1, 3–11). In a more detailed analysis of the infiltrating T cells, the quantity of $CD45RO^+$ cells (memory T cells) or effector memory (T_{EM}) cells in a colorectal tumor was shown to have strong prognostic significance after surgery (12, 13).

However, it has not been determined whether the quantity of T cells or memory T cells correlates with the outcome of unresectable metastatic colorectal cancer (MCRC) patients treated with systemic chemotherapy. Because of the limited opportunities for obtaining resected specimens in MCRC patients, TIL analyses are difficult to conduct, and thus alternative immunologic parameters for determination of prognosis are needed. Peripheral blood is easily obtained with little burden on patients. Our preliminary study, which quantified the immune cells in peripheral blood of patients with gastrointestinal cancer treated with a peptide vaccine, suggested that patients with a high quantity of memory T cells in peripheral blood at pretreatment had durable stable disease, whereas patients with a low quantity had early progressed disease (unpublished data). Memory T cells are subdivided into effector memory (T_{EM}) and central memory (T_{CM}) cells. The former play an important role in antitumor immunity (12, 14, 15). Therefore, we hypothesized that the quantity of T_{EM} cells in peripheral blood might correlate with prognosis in patients with MCRC.

Immune-suppressive cells, including myeloid-derived suppressor cells (MDSC), inhibit the activation and proliferation of effector T cells, such that the tumor can evade the host immune response. Large numbers of MDSCs are a poor prognostic factor

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for various cancer patients (16–20). Therefore, we also hypothesized that the quantity of MDSCs might affect the prognosis of patients with MCRC.

In the current prospective study, the primary objective was to investigate the relationship between the quantity of T_{EM} cells or MDSCs in peripheral blood and the progression-free survival (PFS) of patients with unresectable MCRC who had received first-line chemotherapy. We also carried out an exploratory investigation of the relationship between the quantity of other immune cells and PFS of those patients.

Materials and Methods

Patients

Patients ($N = 40$) were prospectively enrolled to this study if they met the following criteria: age at least 20 years; histologically proven adenocarcinoma of the colorectum; curatively unresectable, metastatic, or recurrent disease; treatment plan to receive oxaliplatin-based first-line chemotherapy; and no active viral infection such as with the human immunodeficiency virus or hepatitis B and/or hepatitis C. Attending physicians chose either the 5-fluorouracil, folinic acid, and oxaliplatin (FOLFOX) regimen or the capecitabine plus oxaliplatin (XELOX) regimen combined with bevacizumab (BV). The FOLFOX regimen consisted of a simultaneous intravenous infusion of 85 mg/m^2 of oxaliplatin (2 hours), 200 mg/m^2 l-leucovorin (2 hours), and 400 mg/m^2 bolus 5-fluorouracil on day 1, followed by a continuous infusion of 2,400 mg/m^2 of 5-fluorouracil (46 hours), repeated every 2 weeks (21). The XELOX regimen consisted of 130 mg/m^2 of oxaliplatin (2 hours) on day 1 plus oral capecitabine, 1,000 mg/m^2 twice daily for 2 weeks, repeated every 3 weeks (22). BV was administered before oxaliplatin at a dose of 5 mg/kg (FOLFOX) or 7.5 mg/kg (XELOX) on day 1 of each cycle. These treatments were repeated until disease progression, unacceptable toxicities, or patient's refusal. The FOLFOX-without-BV regimen was used only when a patient had a history of vascular complications. To evaluate treatment efficacy, systemic CT was repeated every 2 months. All of the patients provided fully informed consent for study registration and blood collection. The study protocol was reviewed and approved by the institutional ethics committee of the National Cancer Center.

Staining of peripheral blood mononuclear cells

Peripheral blood was collected within 5 days before initial chemotherapy administration. The second and third blood collection was performed just before the fifth cycle (ninth week) in the FOLFOX ± BV cohort or the third cycle (seventh week) in the XELOX + BV cohort, and at 6 months after initial chemotherapy, respectively. If chemotherapy was terminated because of disease progression or an adverse event before the second or third blood collection, blood was collected at that time.

Blood samples were centrifuged, and the separated plasma was cryopreserved. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by density gradient centrifugation. MDSCs were measured in fresh PBMC samples, because a previous study demonstrated that the MDSC fraction is decreased by cryopreservation (23). Dendritic cells (DC) were measured at the same time as measurement of the MDSC because a common flow cytometry panel was used for both cell subsets. The remaining PBMCs were cryopreserved and used for measurement of T cells, regulatory T cells (Treg), and natural killer (NK) cells.

Aliquots containing 2.5×10^5 to 5×10^5 PBMCs were suspended in 100 μL staining buffer (PBS containing 2% FBS). The antibodies for surface markers were then added followed by a 30-minute incubation at 4°C. For staining of intracellular proteins (Ki-67, FOXP3, perforin, and granzyme B), FOXP3/Transcription Factor for Staining Buffer Set (eBioscience) was used according to the manufacturer's protocol. The antibodies used were as follows: Lineage (Lin, CD3/CD16/CD19/CD20/CD56) cocktail FITC, CD14–PerCP (peridinin chlorophyll protein)-Cy5.5, CD11b–APC (allophycocyanin)-Cy7, CD33–PE (phycoerythrin)-Cy7, CD11c–Alexa Fluor700, CD123–Brilliant Violet 421, CD15–V500, CD3-APC, Ki-67–Alexa Fluor700, CD8–APC-Cy7, granzyme B–FITC, CD56–PE-CF594 (BD Pharmingen), CD4–Brilliant Violet 650, CD16–PerCP-Cy5.5, CCR7–PerCP-Cy5.5 (Biolegend), FOXP3–PE, CD66b–APC (eBioscience), CD45RA–FITC, HLA-DR–ECD (Beckman Coulter), and perforin-PE (Cell Sciences). Isotype controls included the appropriate fluorochrome-conjugated mouse IgG1, IgG1/κ, or IgG2a/κ. The stained cells were detected using an LSR II Fortessa with FACS Diva software (BD Biosciences). All analyses were carried out using FlowJo software (TreeStar).

Definition and analysis of immune cell subsets

We analyzed 25 immune cell subsets in this study. The immune cell subsets were defined as follows: monocytic MDSC (M-MDSC), Lin$^-$CD14$^+$CD33$^+$CD11b$^+$HLA-DR$^{low/-}$, granulocytic MDSC (Gr-MDSC), CD33^{dim}CD15$^+$CD66$^+$CD11b$^+$, naïve Treg, CD3$^+$CD4$^+$CD45RA$^+$FOXP3^{low}, effector Treg, CD3$^+$CD4$^+$CD45RA$^-$FOXP3^{high}, plasmacytoid DC, Lin$^-$CD14$^-$CD123$^+$HLA-DR^{high}, myeloid DC, and Lin$^-$CD14$^-$CD11c$^+$HLA-DR^{high}. T cells were classified as naïve (CD45RA$^+$CCR7$^+$), central memory (CD45RA$^-$CCR7$^+$), effector memory (CD45RA$^-$CCR7$^-$), and terminally differentiated effector cells (CD45RA$^+$CCR7$^-$) in CD4$^+$ or CD8$^+$ cells. Expression of granzyme B, perforin, and Ki-67 was also assessed in CD4$^+$ or CD8$^+$ T cells. Appropriate isotype controls served as the cutoff levels between positivity and negativity. A positive gate was set to include less than 0.1% cells in each specimen with a matched isotype control.

The proportion of lymphoid subsets was obtained by dividing the cell number of each subset by the cell number of the lymphocyte fraction based on the results of flow cytometric analysis. Absolute counts of lymphoid subsets (/μL) were estimated using the following formula: (Proportion of each lymphoid subset) × [absolute count of lymphocytes (/μL)]. The M-MDSC proportion in the monocytes was obtained by dividing the cell number of HLA-DR$^{low/-}$ by the cell number of Lin$^-$CD14$^+$CD33$^+$CD11b$^+$. The absolute count of M-MDSCs (/μL) in peripheral blood was estimated using this formula: (Proportion of M-MDSCs) × (absolute count of monocytes/μL; ref. 19).

Measurement of cytokines in plasma

Cryopreserved plasma was used for measuring VEGF-A, VEGF-C, VEGF-D, IL2, IL6, IL8, IL10, IFNγ, TNFα, and GM-CSF. These cytokines were simultaneously measured on an MSD SECTOR Imager 2400 instrument (Meso Scale Discovery, Inc.) according to the manufacturer's protocol.

Statistical analysis

The primary objective of the analysis was to describe the relationship between pretreatment peripheral immune status and

PFS. Based on the median values for proportion of or absolute counts for each immune cell subset or cytokine value, the patients were divided into high (above median) and low (below median) groups. PFS was defined as the time period from the day of initial chemotherapy until disease progression or death from any cause. Disease progression was judged according to the RECIST guidelines (Version 1.1).

Cox proportional hazards models were used to estimate the effect of immune status. The proportional hazards assumption was confirmed by a scored test for proportional hazards (24); no relevant violations of the assumption were found. The following model building process was done. Step 1: Variables included 25 immune cell subsets. Variables with a P value < 0.05 on univariate analysis were considered candidate variables. Finally, the candidate variables were combined into a single variable of immune status, because the absolute values of these coefficients were similar. Step 2: As the final analysis, a multivariate Cox proportional hazards model was used after adjustment for important covariates. An important covariate is defined as a characteristic that meets any of the following criteria: It is a known prognostic factor (e.g., use of BV) if removal of the covariate has produced an important change (more than 10%) in the coefficient of the immune status. This final model was chosen based on a clinical and statistical perspective by reference to a previously published approach (25).

For the patient characteristics, summary statistics comprise frequencies and proportions for categorical variables, and median and range for a continuous variable. Between-group differences in patient characteristics were analyzed with the Fisher exact test. The Kaplan–Meier method was used to estimate the PFS, and the differences were compared using the log-rank test. The Student t test was used for comparisons of measured values of cytokines. Pearson correlation was used to evaluate for relationships between two immune cell subsets.

All P values are two-sided. P values of < 0.05 were considered to indicate statistical significance, and 95% confidence intervals (CI) were calculated. All statistical analyses were performed using SAS software version 9.4 (SAS Institute).

Table 1. Patient characteristics

	***N* = 40**	**%**
Age, median (range)	62.5	(28–85)
Sex		
Female	23	57
Male	17	43
History of adjuvant chemotherapy	11	28
Chemotherapy regimens		
FOLFOX	4	10
FOLFOX + BV	21	52.5
XELOX + BV	15	37.5
ECOG PS		
0	16	40
1	23	57.5
2	1	2.5
Primary lesion		
Right hemicolon	17	42.5
Left hemicolon	11	27.5
Rectum	12	30
Number of metastatic lesions		
1	23	57.5
2	12	30
3	5	12.5

Results

Patient characteristics

Patient characteristics are presented in Table 1: 21 (52.5%) and 15 (37.5%) patients were treated with FOLFOX+BV and XELOX+BV, respectively; 4 (10%) patients received FOLFOX without BV because they had past history of vascular complication; and 39 (97.5%) patients had good performance status (0–1).

Associations between the quantity of each immune cell subset and PFS

The gating strategy and representative dot plots for M-MDSCs and effector memory cells in $CD4^+$ or $CD8^+$ T-cell fractions ($CD4^+$ T_{EM} or $CD8^+$ T_{EM}) are shown in Fig. 1A and B. The median values of the proportion and absolute count for each immune cell subset were 13.3% (range, 0.7–59.0) and 40.5/μL (range, 1.5–536.3) for M-MDSC; 7.1% (range, 2.8–21.5) and 86.5/μL (range, 36.9–326.8) for $CD4^+T_{EM}$, and 9.9% (range, 1.5–14.3); and 75.1/μL (range, 13.9–274.0) for $CD8^+T_{EM}$, respectively. The gating strategy and representative dot plots for other immune cell subsets (Gr-MDSCs, Tregs, DCs, NK cells, granzyme B^+, perforin$^+$, or Ki-67^+ cells) are shown in Supplementary Fig. S1.

Patients were divided into high (>median) and low (<median) groups based on the median value of the proportion for each immune cell subset, and PFS was then compared between each pair of groups (Table 2). A high proportion of M-MDSCs, low proportion of $CD4^+$ T_{EM}, and low proportion of $CD8^+$ T_{EM} were associated with significantly shorter PFS (median PFS, not reached vs. 8.5, not reached vs. 8.5, not reached vs. 9.0 months; $P = 0.004$, 0.005, and 0.002, respectively; Fig. 1C). A high absolute count of M-MDSCs, low absolute count of $CD4^+$ T_{EM}, and low absolute count of $CD8^+$ T_{EM} were also associated with significantly shorter PFS ($P = 0.005$, 0.03, and 0.046, respectively).

Correlations between quantities of M-MDSCs, $CD4^+$ T_{EM}, and $CD8^+$ T_{EM}

Because the quantities of M-MDSCs, $CD4^+$ T_{EM}, or $CD8^+$ T_{EM} each correlated with PFS, correlations between each pair among the proportions of M-MDSC, $CD4^+$ T_{EM}, and $CD8^+$ T_{EM} were investigated (Fig. 2A). A weak inverse correlation between M-MDSC and $CD4^+$ T_{EM} was observed ($r = -0.33$, $P = 0.03$): 14 (35%) patients presented with high M-MDSC and low $CD4^+$ T_{EM}, whereas 14 (35%) patients had low M-MDSC and high $CD4^+$ T_{EM}. This inverse correlation is reasonable because MDSCs are immune-suppressive cells that inhibit the proliferation and activation of T cells. However, the quantities of M-MDSCs and $CD4^+$ T_{EM} were somewhat discrepant: 6 (15%) patients presented with low M-MDSCs with low $CD4^+$ T_{EM}, whereas 6 (15%) had high M-MDSC with high $CD4^+$ T_{EM}. Similarly, there was a trend toward an inverse correlation between M-MDSC and $CD8^+$ T_{EM}, although the difference did not reach statistical significance ($r = -0.28$, $P = 0.08$): 13 (32.5%) patients each presented with high M-MDSC and low $CD8^+$ T_{EM}, or low M-MDSC and $CD8^+$ T_{EM}, whereas 7 (17.5%) each had low M-MDSC with low $CD8^+$ T_{EM}, and high M-MDSC with high $CD8^+$ T_{EM}. In addition, a weak positive correlation between $CD4^+$ T_{EM} and $CD8^+$ T_{EM} was observed ($r = 0.32$, $P = 0.045$): 14 (35%) patients each presented with high $CD4^+$ T_{EM} and high $CD8^+$ T_{EM}, or low $CD4^+$ T_{EM} and low $CD8^+$ T_{EM}, whereas 6 (15%) each had low $CD4^+$ T_{EM} with high $CD8^+$ T_{EM}, and high $CD4^+$ T_{EM} with low $CD8^+$ T_{EM}.

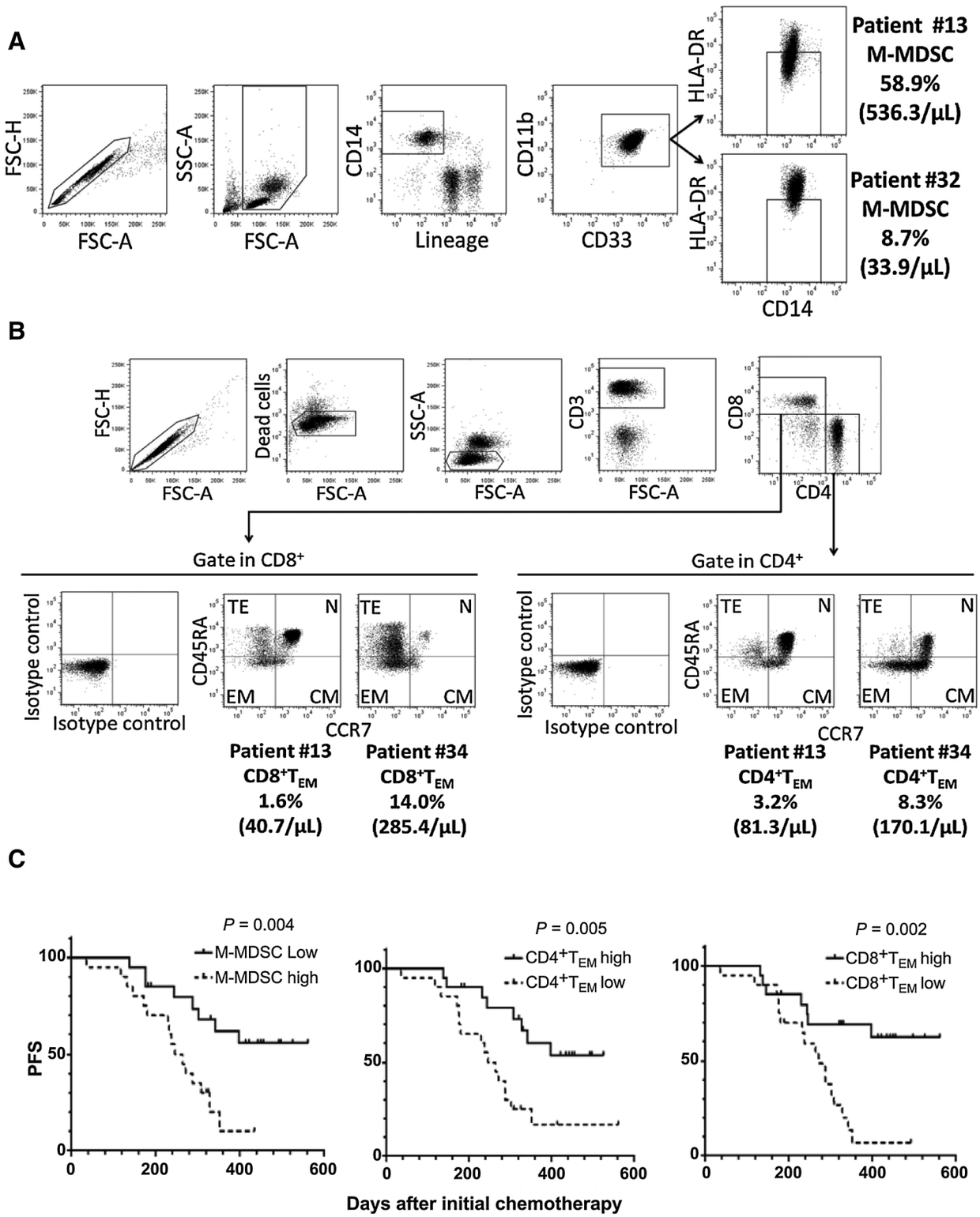

Figure 1.
A and B, gating strategy and representative dot plots for M-MDSCs (A) and naïve/memory cells in T-cell fractions (B). Two independent dot plots are shown as high and low M-MDSCs (A), and high and low effector memory T cells (B). C, the Kaplan–Meier curves for PFS are shown according to the pretreatment quantities of M-MDSCs, effector memory $CD4^+$ T cells, and $CD8^+$ T cells. CM, central memory; EM, effector memory; N, naïve; TE, terminally differentiated effector.

Table 2. Associations between the proportion of each immune cell subset and PFS

Cell subsets	HR (95% CI)	*P* value
Suppressor cells		
M-MDSCs	3.4 (1.4–8.3)	0.006
Granulocytic MDSCs	1.3 (0.6–2.9)	0.5
$CD4^+$ $FOXP3^+$	1.4 (0.6–3.2)	0.4
Naïve Tregs	0.9 (0.4–2.1)	0.8
Effector Tregs	1.6 (0.7–3.5)	0.3
Effector cells		
$CD3^+$	0.7 (0.3–1.6)	0.4
$CD4^+$	1.0 (0.5–2.3)	1
Naïve	0.9 (0.4–2.1)	0.9
Central memory	1.3 (0.6–3.0)	0.5
Effector memory	0.3 (0.1–0.7)	0.007
Terminally differentiated effector	0.6 (2.9–1.5)	0.3
Granzyme B^+	1.0 (0.4–2.1)	1
$Perforin^+$	1.3 (0.6–2.9)	0.5
$Ki\text{-}67^+$	1.6 (0.7–3.6)	0.2
$CD8^+$	0.4 (0.2–1.03)	0.06
Naïve	0.7 (0.3–1.6)	0.4
Central memory	1.5 (0.7–3.5)	0.3
Effector memory	0.3 (0.1–0.6)	0.004
Terminally differentiated effector	0.6 (0.3–1.5)	0.3
Granzyme B^+	0.5 (0.2–1.1)	0.09
$Perforin^+$	0.7 (0.3–1.5)	0.3
$Ki\text{-}67^+$	1.1 (0.5–2.5)	0.8
NK cell	1.4 (0.6–3.1)	0.4
Antigen-presenting cells		
Myeloid dendritic cells	0.9 (0.4–1.9)	0.7
Plasmacytoid dendritic cells	0.7 (0.3–1.6)	0.4

NOTE: Patients were divided into high (>median) and low (<median) groups based on the median value of the proportion of each immune cell subset, and univariate analyses for PFS were performed by using Cox proportional hazards models. The reference for the HR is a low group of each immune cell subset.

Based on this grouping, univariate analyses were performed to estimate the HR of each immunologically distinct subgroup (Supplementary Table S1). Patients with high M-MDSC and low $CD4^+$ T_{EM} values had significantly higher HR than those with low M-MDSC and high $CD4^+$ T_{EM} (HR, 6.9; 95% CI, 2.1–22.9; $P = 0.001$). Similarly, patients with high M-MDSC and low $CD8^+$ T_{EM} values had significantly higher HR than those with low M-MDSC and high $CD8^+$ T_{EM} values (HR, 9.1; 95% CI, 2.4–34.7; $P = 0.001$). In addition, patients with low $CD4^+$ T_{EM} and low $CD8^+$ T_{EM} values had significantly higher HR than those with high $CD4^+$ T_{EM} and high $CD8^+$ T_{EM} values (HR, 7.2; 95% CI, 2.2–23.8; $P = 0.001$).

Combined M-MDSC, $CD4^+$ T_{EM}, and $CD8^+$ T_{EM} assessments improved PFS prediction

High M-MDSC, low $CD4^+$ T_{EM}, or low $CD8^+$ T_{EM} values were adverse immunologic factors. We also noted that approximately 70% of patients presented with concordance between the quantity of M-MDSCs and that of $CD4^+$ T_{EM} or $CD8^+$ T_{EM}, whereas approximately 30% showed discrepant results for these quantities. Therefore, we investigated whether a combined assessment of the immune-suppressive cells (M-MDSCs) and effector memory cells ($CD4^+$ T_{EM} and $CD8^+$ T_{EM}) would increase the accuracy of predicting clinical outcomes for the different patient groups. Patients were divided into four groups based on the numbers of adverse immunologic factors: Group 1, no adverse factors ($n = 11$, 27.5%); Group 2, one adverse factor ($n = 8$, 20%); Group 3, two adverse factors ($n = 11$, 27.5%); and Group 4, three adverse factors ($n = 10$, 25%; Fig. 2B). The Kaplan–Meier curves for the PFS of each group are shown in Fig. 2C. The curve of Group 1 overlapped that of Group 2, and the curve of Group 3 overlapped that of Group 4. These findings suggest that Groups 1 and 2 have an equivalent prognosis, as do Groups 3 and 4. Therefore, Groups 1 and 2 were combined, as were Groups 3 and 4. The Kaplan–Meier curves of Groups 1 and 2 and Groups 3 and 4 are shown in Fig. 2D. Groups 3/4 had significantly shorter PFS than Groups 1 and 2 (median PFS, not reached vs. 8.0 months, $P < 0.001$).

Next, patient characteristics were compared between Groups 1 and 2 and Groups 3 and 4 (Supplementary Table S2). Groups 3 and 4 included a significantly higher number of patients with elevated alkaline phosphatase values (> normal range; $P = 0.02$), but the differences between Groups 1 and 2 and Groups 3 and 4 with regard to other characteristics such as age, sex, history of adjuvant chemotherapy, use of BV, Eastern Cooperative Oncology Group (ECOG) PS, primary lesion, and number of metastatic lesions, were not statistically significant.

Finally, the univariate and multivariate analyses for PFS were performed on different groups, patient characteristics, and cytokine values (Table 3 and Supplementary Table S3). The multivariate analysis showed Groups 3 and 4 to have a significantly higher HR than Groups 1 and 2 (HR, 9.2; 95% CI, 2.5–34.2; $P < 0.001$) after adjusting for important covariates, which were chosen based on the criteria as described in Materials and Methods.

Comparison of plasma cytokine concentrations between Groups 1/2 and Groups 3/4

We measured the concentration of 10 plasma cytokines to assess any differences between Groups 1 and 2 and Groups 3 and 4 (Supplementary Table S4). Concentrations of VEGF-A in Groups 3 and 4 were significantly higher than those in Groups 1 and 2 (mean ± SD, 87.0 ± 62.5 vs. 35.6 ± 21.4; $P = 0.002$; Fig. 2E), and IL6 was also significantly higher in Groups 3 and 4 than in Groups 1 and 2 (mean ± SD, 5.2 ± 4.7 vs. 1.9 ± 1.9; $P = 0.007$; Fig. 2E). The two groups had no statistically significant differences in other cytokines, such as VEGF-C, VEGF-D, IL2, IL8, IL10, IFNγ, TNFα, and GM-CSF.

Quantitative changes in M-MDSC and T_{EM} after chemotherapy and their impact on PFS

We also analyzed the quantity of M-MDSC and T_{EM} cells after chemotherapy. Although a second blood collection was performed in all patients ($N = 40$), a third blood collection was done only in 31 patients because of discontinuance of chemotherapy or patients' withdrawal. The means of %M-MDSC or $\%T_{EM}$ were compared between before (first blood collection) and after chemotherapy (second or third blood collection) using a paired *t* test; however, no tendency for an increase or decrease of any immune cell subset was noted (Supplementary Fig. S2A). Regarding changes in immune cell subsets from before chemotherapy to the second blood sample, M-MDSC increased in 18 (45%) patients and decreased in 22 (55%), $CD4^+T_{EM}$ increased in 18 (45%) and decreased in 22 (55%), and $CD8^+T_{EM}$ increased in 25 (62.5%) and decreased in 15 (37.5%). PFS was then compared between patients who showed an increase or decrease in those cells; however, no statistically significant differences were found (patients with

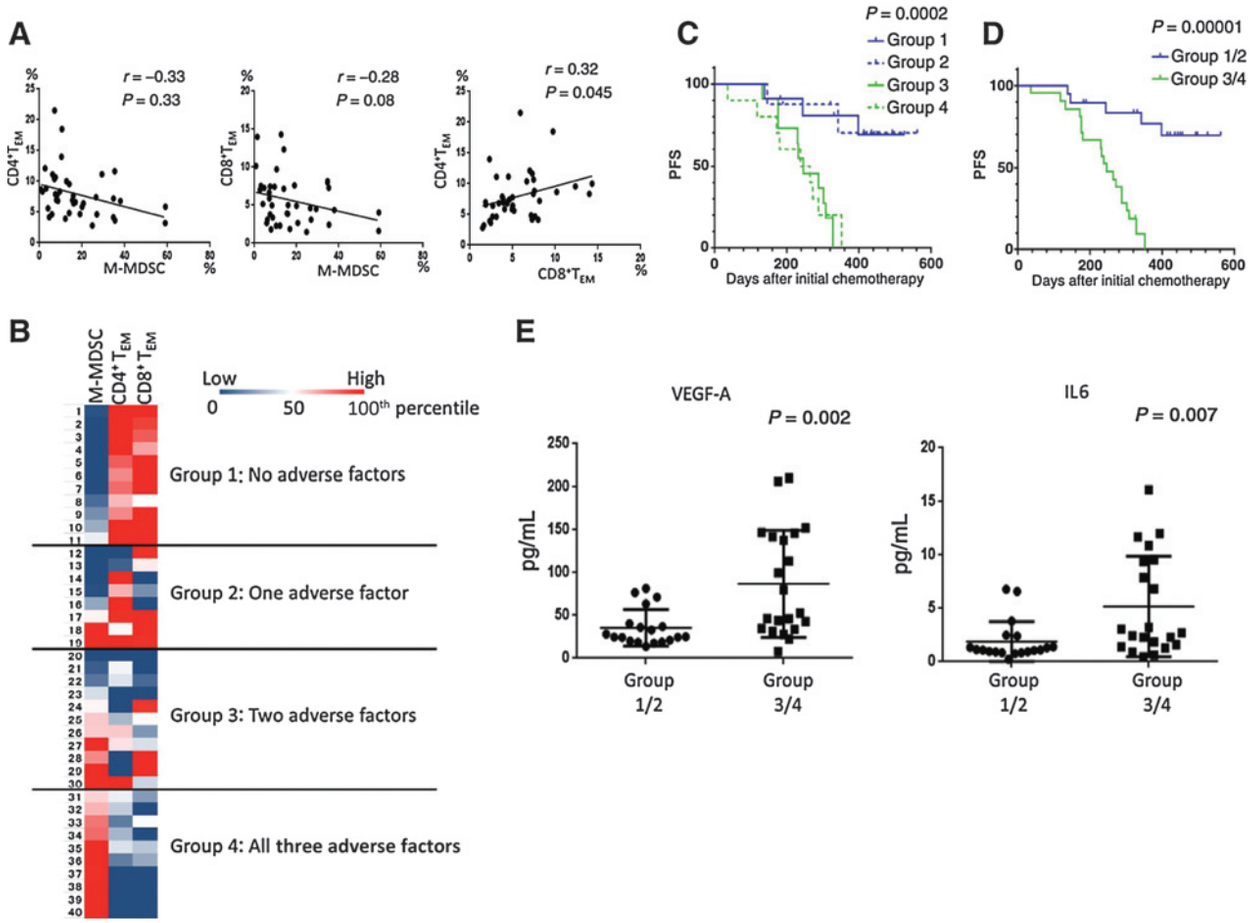

Figure 2.

Combined M-MDSC, $CD4^+$ T_{EM}, and $CD8^+$ T_{EM} assessments. A, correlations between each pair of the proportions of M-MDSCs, $CD4^+$ T_{EM}, and $CD8^+$ T_{EM} cells are shown in dot plots. Pearson correlation coefficient is indicated as "r." B, analysis of patient groups with different numbers of the adverse factors of high M-MDSC, low $CD4^+$ T_{EM}, and low $CD8^+$ T_{EM}, which we identified as adverse immunologic factors as shown in Fig. 1C and Table 2. Patients were divided into four groups based on the number of adverse factors of each patient: Group 1, no adverse factor ($n = 11$); Group 2, one ($n = 8$); Group 3, two ($n = 11$); Group 4, three ($n = 10$). Blue and red colors in the heat map indicate that a patient has low (<median value) or high (>median value) quantities, respectively, of the corresponding immune cell. C and D, Kaplan–Meier curves for PFS of the four different immunologic groups are shown in C. Because the curve of Group 1 overlapped with that of Group 2, and the curve of Group 3 overlapped with that of Group 4, Groups 1 and 2 were combined, as were Groups 3 and 4. The Kaplan–Meier curves of Groups 1 and 2 and Groups 3 and 4 are shown in D. *P* values were calculated by the log-rank test. E, values of VEGF-A and IL6 were compared between Groups 1 and 2 and 3 and 4 with a *t* test. The long and short horizontal lines in the figure indicate the means and SDs, respectively. Comparison of other cytokine values are shown in Supplementary Table S4.

increased vs. decreased M-MDSC, $CD4^+T_{EM}$, and $CD8^+T_{EM}$; $P =$ 0.3, 0.9, and 0.3, respectively; Supplementary Fig. S2B). Comparison of PFS between before chemotherapy and the third blood sample was not performed because patients who underwent a third blood collection were selected patients who could continue initial chemotherapy for up to 6 months.

Discussion

The present study demonstrated that pretreatment immune status correlates with the PFS of patients with unresectable MCRC given first-line chemotherapy. We analyzed 25 immune cell subsets and identified high M-MDSC, low $CD4^+$ T_{EM}, and low $CD8^+$ T_{EM} values as adverse prognostic factors for PFS. In addition, combined assessment of all three adverse factors showed the outcomes of patients who had two or three of these factors (Groups 3 and 4) to be significantly poorer than those of patients who had zero or one adverse factor (Groups 1 and 2). This negative impact remained statistically significant in multivariate analysis. Although many retrospective studies have already shown that the quantity of TILs in surgically resected specimens correlates with the outcomes of patients with resectable colorectal cancer (1, 3–13, 26), this prospective study has demonstrated that the quantity of immune cells in peripheral blood correlates with the outcomes of those with unresectable tumors.

Approximately 27.5% of the patients in this study had low M-MDSC, high $CD4^+T_{EM}$, and high $CD8^+T_{EM}$ values (Group 1), whereas 25% of patients had high M-MDSC, low $CD4^+T_{EM}$, and low $CD8^+T_{EM}$ values (Group 4). This inverse correlation

Table 3. Multivariate analysis for PFS

	Multivariate analysis ($N = 40$)	
Covariates	**HR (95% CI)**	***P* value**
Group		
1/2	Reference	
3/4	9.2 (2.5–34.2)	<0.001
Use of bevacizumab		
Used	Reference	
Not used	2.5 (0.7–9.3)	0.2
Primary lesion		
Right hemicolon	Reference	
Left hemicolon	0.5 (0.1–1.7)	0.3
Rectum	0.5 (0.2–1.7)	0.3
IFNγ		
Low	Reference	
High	1.6 (0.6–4.6)	0.4
IL8		
Low	Reference	
High	2.7 (0.9–8.7)	0.09

NOTE: Multivariate analysis for PFS was performed on different immune groups, patient characteristics, and cytokine values by using a Cox proportional hazards model. Covariates were chosen based on the criteria described in Materials and Methods.

between M-MDSCs and effecter memory T cells is reasonable because MDSCs are immune-suppressive cells that inhibit the proliferation and activation of T cells. However, the remaining 47.5% of patients (Groups 2 and 3) showed discrepant results for the quantities of M-MDSCs, $CD4^+T_{EM}$, and $CD8^+T_{EM}$. These results suggest that the quantities of M-MDSCs, $CD4^+T_{EM}$, and $CD8^+T_{EM}$ are specific for each patient (Fig. 2B and Supplementary Fig. S2). Therefore, combined assessment of the immune-suppressive cells (M-MDSC) and the cytotoxic effector cells ($CD4^+T_{EM}$ and $CD8^+T_{EM}$) may provide a more appropriate reflection of the immune status of each patient and would also, presumably, illustrate the correlation between immune status and prognosis more accurately than would individual assessments of these cell subsets. For example, a patient with a high quantity of M-MDSCs would generally have a short PFS, but the negative impact might be canceled out in the presence of high quantities of $CD4^+T_{EM}$ and/or $CD8^+T_{EM}$. Similarly, although a patient with a low quantity of $CD8^+T_{EM}$ might be expected to have a short PFS, the negative impact could be canceled out in the presence of a low M-MDSC and a high $CD4^+T_{EM}$ value. In fact, we demonstrated that PFS in Group 2, which consisted of such patients, is equivalent to that in Group 1, comprised of patients with low M-MDSC, high $CD4^+T_{EM}$, and high $CD8^+T_{EM}$ values (Fig. 2C). Our results demonstrate that individual assessments of M-MDSC and effector memory T cells have potential prognostic value for PFS and that the combined assessment of these cell subsets predicts PFS with greater accuracy than that of any one cell subset alone.

We demonstrated that the immune status at pretreatment correlated with PFS; however, changes of those cells after chemotherapy did not correlate with PFS. It is very likely that change of immune status after chemotherapy is influenced by several factors, such as direct cytotoxicity from therapeutic agents, disease progression or regression, incidence of adverse event, and so on. These various factors may make it difficult to interpret the correlation between change of immune status and PFS.

We also analyzed plasma cytokines that affect the formation of immune cell subsets. We found that VEGF-A and IL6 were significantly higher in Groups 3 and 4 than in Groups 1 and 2. VEGF-A contributes not only to tumor angiogenesis but also to formation of the immunosuppressive microenvironment in tumors (27). VEGF-A augments MDSCs (28, 29) and inhibits DC maturation (30, 31), directly inhibits the activation and proliferation of T cells (32), and upregulates expression of the programmed death-1 molecule on T cells (33). IL6 is a multifunctional cytokine with pro- and anti-inflammatory activity. Under certain pathologic circumstances, IL6 augments MDSCs. Based on these findings, increased VEGF-A and IL6 concentrations in our cohort may have contributed to the adverse immune status, which resulted in shorter PFS.

Our present prospective study included a rather small number of patients. Nevertheless, we identified statistically significant prognostic factors for PFS. Assessment of the impact on overall survival requires an additional follow-up period because only 8 of our patients did not survive. Despite this limitation, our results have meaningful clinical implications: Antitumor immunity may be helpful for the effects of chemotherapy and thus provide a rationale for developing a regimen combining chemotherapy with immunotherapy. An immunotherapeutic approach that reduces M-MDSCs or increases effector memory T cells might overcome immunologically mediated adverse impacts on prognosis.

In conclusion, we analyzed 25 immune cell subsets in peripheral blood from patients with unresectable MCRC before first-line chemotherapy and identified high M-MDSC, low $CD4^+T_{EM}$, and $CD8^+T_{EM}$ quantities as significant adverse factors for PFS. Combining the assessment of these three adverse factors gave greater accuracy of PFS prediction for the immunologically different patient subgroups. These results suggest that pretreatment peripheral immune status correlates with the outcomes of patients with unresectable MCRCs receiving first-line chemotherapy. Further studies involving patients with other types of cancer are warranted to assess our results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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