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*J Immunol* 2012; 189:3198-3208; Prepublished online 8 August 2012; doi: 10.4049/jimmunol.1200602

http://www.jimmunol.org/content/189/6/3198

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/08/08/jimmunol.1200602.DC1

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Expansion of Effector Memory Regulatory T Cells Represents a Novel Prognostic Factor in Lower Risk Myelodysplastic Syndrome

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Myelodysplastic syndromes (MDS) refer to a group of pathophysiologically diverse premalignant hematopoeitic diseases characterized by inflammation-associated cytopenias, myeloid dysplasia, autoimmunity, and variable risk of acute myeloid leukemia (AML) progression (1). Several prognostic models have been developed to gauge the risk of AML transformation and overall survival (OS) and thus play a large role in disease management. The International Prognostic Scoring System (IPSS) (2, 3) represents the most widely used model segregating patients into low, intermediate-1 (int-1), intermediate-2 (int-2), and high-risk based on the number of cytopenias, bone marrow blast percentage, and karyotype. The IPSS was validated in newly diagnosed and untreated patients (1), but newer prognostic risk models, such as the MD Anderson Scoring System (MDAS), incorporate a broader range of factors that refine prognostic precision and may better reflect changes that occur during disease progression (4–6). Although both systems accurately assess risk and disease outcome, neither system accurately predicts response to Food and Drug Administration-approved drugs, making treatment decisions difficult and nonstandardized.

Overall, ~30–40% of lower IPSS risk patients experience hematopoietic improvement with T cell-depleting therapy with either antithymocyte globulin or cyclosporine (7–10). Autoreactivity against abnormally expressed self-Ags in the bone marrow is now widely suspected to play a role in MDS pathogenesis (7–11). However, not all patients respond to such treatment, and clinical benefit seems limited to those with less advanced disease (7–10). This suggests that the role of the T cell compartment may change...
over time as MDS progresses from the early autoimmune stages (9) into more advanced stages, in which it is likely that classic immune-suppressive mechanisms prevail. Unfortunately, none of the clinical parameters encompassed by prognostic scoring systems like the IPSS or MDAS reflect T cell reactivity or suppressive state.

Regulatory T cells (Tregs) have become the quintessential suppressive population within the T cell compartment and have been extensively studied for their role in tumor-induced immune suppression (12). Like most cancers, increased numbers of Tregs were found in MDS patients, but were restricted to those with higher risk as defined by IPSS, likely reflecting the immune suppressive state of more advanced disease (13). It is now known that self-Ag–induced TCR signaling is required for Treg-suppressive activity (14) and that this activation results in memory populations similar to conventional T cells (15). We hypothesize that shifts in naive or memory phenotype within the Treg compartment may provide an earlier indicator of active immune suppression, which may result in better prognostic value compared with total Treg numbers alone.

Using phenotypic markers commonly employed to define conventional T cell memory pools, we demonstrate different suppressive capacities among distinct Treg subpopulations. In a cohort of 52 MDS patients, an increase in a Treg subset with more suppressive capacity (i.e., effector memory Tregs [TregEM]) was independently associated with increased bone marrow myeloblasts and inferior OS. The prognostication of the MDAS, which re-}

**Materials and Methods**

**Patients and healthy controls**

Fifty-two previously untreated patients diagnosed with MDS at the University of California Los Angeles, Penn State University Cancer Institute, Cleveland Clinic Cancer Institute, or the Malignant Hematology Clinic at the H. Lee Moffitt Cancer & Research Institute were studied retrospectively based on data collected by the Bone Marrow Failure Rare Disease Clinical Research Network (BMF-RDCRN). All diagnoses were confirmed at enrollment by experienced hematopathologist through centralized standards implemented at participating institutions, and patients were classified according to World Health Organization (WHO) criteria. Metaphase cytogenetic testing was obtained using standard banding techniques. Patients were categorized into lower-risk (low/int-1) or higher-risk (int-2/high) groups based on IPSS (2, 3) and MDAS (4–6). Following consent, 40 mL peripheral blood was obtained from each patient in heparin tubes. Blood samples from 41 healthy subjects who donated to the Southwest Florida Blood Services, and consented therein, were used as controls. This protocol was approved by the Institutional Review Boards at participating institutions in accordance with the Declaration of Helsinki, and all human participants gave written informed consent.

**Isolation of PBMCs**

PBMCs were isolated from blood samples using Ficoll-Hypaque (Amer-}

**Immunofluorescent staining of Tregs and Treg subsets**

Nonspecific staining was first blocked for 30 min at 4°C with 300 μg/mL PBS in PBS per 1.0 × 106 cells. The cells were then labeled with fixable Live/Dead Yellow after thorough PBS washes (Invitrogen) so that nonvi-}

**T cell suppression assay**

Tregs with distinctive naive and memory phenotypes were isolated fol-

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism software version 5.03 (GraphPad Software, La Jolla, CA) or IBM SPSS Statistics software (version 19). Descriptive statistics such as the mean, median, and SD were determined for continuous variables including the total number and percentage of Tregs, Treg phenotypes, and age. Comparisons between cases and controls were made using Mann–Whitney analysis. Correlation tests were performed using the Spearman analysis. The D’Agostino and Pearson omnibus normality statistic was used to assess normality of the data. The statistical methods used included simple linear regression and analysis of covariance. Kaplan-Meier estimates were used for OS rates, and log-rank method was used to compare between groups. Univariate and multivariate survival analyses were performed using the Cox proportional hazards model. Associations of MDS characteristics and Treg subgroups were determined using Fisher’s exact test. All analyses were performed at the 95% confidence interval (CI), and a p value <0.05 was considered statistically significant.

To compare subsets of patients with high total Tregs or high Treg subsets, dichotomous cut points were used based on the normal ranges established in age-matched controls. In peripheral blood, the normal range (mean ± 1 SD) was defined for the absolute number of total Tregs (28–77 Tregs/μL) and each of the Treg subsets: TregN cells (1–9 TregN/μL), TregM cells (19–53 TregM/μL), effector memory Tregs (0–6 TregEM/μL), and terminal memory Tregs (0 to 2 TregTM/μL). MDS patients with absolute total Treg or Treg subset numbers above the range of healthy age-matched controls were

**FlowJo software version 7.6.1** (Tree Star, Ashland, OR).

Analysis of cytometry data was achieved using FlowJo software (version 19).

**Statistical analysis**

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FIGURE 1. Treg expansion in distinct subsets of lower-risk MDS patients. (A) Flow cytometry gating schema for defining Tregs and Treg subsets. Cells were first gated on CD3 expression, and then on CD4 and FOXP3 expression. The resulting Treg (CD4+FOXP3+) or conventional T cell (CD4+FOXP3-) populations were then analyzed for CD45RA and CD27 expression. TregN were considered CD45RA+CD27+, TregCM were considered CD45RA-CD27+, and TregEM were considered CD45RA-CD27-. No significant population of terminal memory Tregs (TregT) were observed (CD45RA+CD27-). CD4+FOXP3+ Tregs or conventional CD4+ T cells were then analyzed for CD127 and CD25 expression. (B) Tregs were reported as a percentage of the CD4+ compartment in MDS patients and age-matched controls. (C-G) The normal range of Tregs in peripheral blood was established in age-matched healthy donors. To establish this cut point for normal versus high expression, the mean ± 1 SD (gray areas) was determined based on the ALC. Graphs indicate the data from control and MDS patients with normal and high levels of total Tregs or of each Treg subtype calculated. (H) A Venn diagram demonstrating the amount of overlap of the 18 MDS patients with high total Treg or high Treg subtype numbers. The frequency of TregCM cells negatively correlates with the frequency of TregEM cells within the Treg compartment in age-matched controls (I) and MDS patients (J).
Results

Clinical characteristics of MDS patients

Fifty-two consecutive MDS patients enrolled into the BMF-RDCRN were examined for novel aspects of Treg biology with prognostic significance. Median age was 68 y (range 42–82 y) at the time of sample acquisition. Forty-one control subjects were included with a median age of 65 y (range 45–83 y). There was no statistical difference in age or gender between MDS patients and controls ($p = 0.097$ for age and $p = 0.530$ for gender). Two MDS patients (4%) were classified as isolated deletion 5q- [del (5q)], 12 (24%) as refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS), 8 (16%) as RA with excessive blasts (RAEB), 18 (35%) as refractory cytopenia with multilineage dysplasia (RCMD) or as RCMD with ringed sideroblasts (RCMD-RS), and 7 (13%) patients were classified as MDS unclassified (MDS-U). Five patients (10%) had the myelodysplastic/myeloproliferative neoplasm chronic myelomonocytic leukemia (CMMML) (1). In this study, 45 out of 52 (85%) patients were classified as lower risk (low/int-1) based on IPSS, as shown in Table I, and the majority of patients studied (69%) retained a lower-risk classification using the MDAS. Subsets of patients displayed thrombocytopenia (35%), neutropenia (56%), and/or anemia (56%), with cytopenias defined by IPSS standards. Thirty-five (67.3%) had a normal karyotype, whereas 17 (32.7%) had an abnormal karyotype.

Treg subset expansion in a group of MDS patients

Studies suggest that TCR activation is required for suppressive activity from Tregs (14), raising the possibility that Tregs, like conventional T cells, may change their phenotype when induced to expand. The percentage of Tregs in phenotypically distinct subsets (Fig. 1A) was determined by flow cytometry, and the absolute lymphocyte count (ALC) was used to estimate the total number in peripheral blood. Tregs were phenotypically discriminated into Treg$^N$, Treg$^{CM}$, Treg$^{EM}$, and terminal memory Tregs based on CD27 and CD45RA expression, as described in the Materials and Methods. Using simultaneous overlays of conventional T cells (shown in orange) and FOXP3$^+$ Tregs (shown in blue), all four phenotypic subtypes were evident within the conventional CD4$^+$ T cell population (16–19) (Fig. 1A). The vast majority of Tregs in patients and controls had a central memory phenotype, and a significantly higher percentage of Tregs within the CD4$^+$ T cell compartment was observed in some MDS cases compared with controls ($p = 0.026$) (Fig. 1B). Examining the absolute number of total Tregs and/or Treg subsets estimated from the ALC, 18 of the 52 (34.6%) patients had changes within the Treg compartment compared with normal ranges established in healthy controls. This included abnormally high absolute numbers of Tregs and/or an increase in the absolute number of Treg$^{EM}$ or Treg$^{CM}$ subsets, as shown in Fig. 1C–F. Tregs were low or undetectable in both cases and controls (Fig. 1G). To display the profile of the 18 patients with an altered Treg compartment, a Venn diagram of individual MDS patients is shown in Fig. 1H. Tregs primarily express high-affinity TCRs against self-Ags as a result of positive selection in the thymus (20–22). We correlated the frequency of Treg$^{EM}$ and Treg$^{CM}$ by linear regression, and a strong negative correlation is shown in Fig. 11 and 1J in both healthy donors and MDS patients ($p < 0.001$), suggesting that recent Ag activation may favor central-to-effector memory transition, similar to conventional T cells (23).

Table I. Characteristics of MDS patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MDS (n = 52)</th>
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<tr>
<td>Age (y)$^a$</td>
<td>68 ± 10</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>26</td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
</tr>
<tr>
<td>WHO$^b$</td>
<td></td>
</tr>
<tr>
<td>5q-</td>
<td>2</td>
</tr>
<tr>
<td>RA/RARS</td>
<td>12</td>
</tr>
<tr>
<td>RAEB</td>
<td>8</td>
</tr>
<tr>
<td>MDS-U</td>
<td>7</td>
</tr>
<tr>
<td>RCMD/RCMD-RS</td>
<td>18</td>
</tr>
<tr>
<td>CMMML</td>
<td>5</td>
</tr>
<tr>
<td>IPSS$^c$</td>
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</tr>
<tr>
<td>High (int-2/high)</td>
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<tr>
<td>Low (low/int-1)</td>
<td>45</td>
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<td>MDAS$^d$</td>
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<td>High (int-2/high)</td>
<td>16</td>
</tr>
<tr>
<td>Low (low/int-1)</td>
<td>36</td>
</tr>
<tr>
<td>Neutropenia (&lt;1 × 10$^3$/l)</td>
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<td>29</td>
</tr>
<tr>
<td>No</td>
<td>23</td>
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<tr>
<td>Thrombocytopenia (&lt;100 × 10$^3$/l)</td>
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<td>Yes</td>
<td>18</td>
</tr>
<tr>
<td>No</td>
<td>34</td>
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<td>Anemia (Hg &lt; 9 g/dl)</td>
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<td>29</td>
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<tr>
<td>Karyotype$^e$</td>
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<td>Normal</td>
<td>33</td>
</tr>
<tr>
<td>Abnormal: favorable</td>
<td>22</td>
</tr>
<tr>
<td>Abnormal: unfavorable</td>
<td>17</td>
</tr>
</tbody>
</table>

$^a$Age is not statistically different from the control group.

$^b$WHO includes RA, RARS, RCMD, RCMD-RS, RAEB, CMMML, and MDS-U (1).

$^c$IPSS: low risk (IPSS score low or int-1) or high risk (IPSS score int-2 or high).

$^d$MDAS classification: lower-risk (low or int-1) or higher-risk (int-2 or high) (2, 3).

$^e$Karyotype was performed by standard cytogenetics and available for all 52 patients. Favorable karyotype includes del(5q), -Y, and del(20q), and an unfavorable karyotype includes chromosome 7 abnormalities or complex ($\geq$3 abnormalities) based on IPSS criteria (1).

The Treg$^{EM}$ phenotype is more suppressive than the Treg$^{CM}$ phenotype

Because cohorts of MDS patients displayed Treg compartmental skewing, we determined the suppressive capacity of each Treg subset. To study the functional differences, sorted cells were cultured at increasing ratios with conventional CFSE-labeled responder T cells stimulated with plate-bound anti-CD3/soluble anti-CD28 Abs. And, CFSE dilution was assessed after 5 d using flow cytometry (Fig. 2) in Tregs isolated from source leukocyte-enriched (buffy coat) blood from healthy donors. Treg$^N$ and Treg$^{EM}$ cells represent, on average, 13 and 2% of the entire Treg population, respectively, and both cell populations suppress at a 1:8 ratio. Treg$^{CM}$ cells, however, represent >80% of all Tregs, but suppress at a 1:2 ratio, showing that these individual sorted populations display different suppressive activity in vitro.

High Treg$^{EM}$ numbers are associated with higher-risk MDS characteristics and increased blast percentage

To test the clinical relevance of Tregs in MDS, we investigated the association of Tregs to clinical outcomes and classification. Clinical characteristics were compared among MDS cases stratified into high and normal Treg groups based on the mean + 1 SD of healthy donors, as shown in Fig. 1 and Table II. Treg$^N$ (n = 2) and Treg$^0$ (n = 0) were not included in this analysis because of low sample size. Characteristics of patients with high Tregs (n = 9) and high Treg$^{CM}$ (n = 8) were similar (Table II). Higher numbers of
total Tregs and high TregEM (n = 12) were correlated with a higher-risk MDAS score (p = 0.023) (Table II). Additionally, the TregEM high group was distinguished by several poor prognostic features, including a history of anemia (p = 0.046), lower hemoglobin (Hg) (p = 0.038 <10 g/dl), and increased percentage of bone marrow myeloblasts (≥5%; p = 0.006), suggesting that the
presence of these cells correlates with worse prognosis (Table II). Patients were stratified into two groups on the basis of bone marrow myeloblast percentage, <5% (n = 10) and ≥5% (n = 42), and the absolute number of total Tregs or of TregCM, TregN, and TregEM subsets was then compared among patients in these two groups. Higher TregEM number (Fig. 3A) and percentage (Fig. 3B) were uniquely found to be associated with myeloblast accumulation, demonstrating that the expansion of TregEM correlates with negative prognostic features of MDS.

**MDS patients with elevated TregEM have reduced OS**

The impact of total Tregs and Treg subsets on OS was then examined. A total of 16 patients (31%) in this cohort had died at the time of retrospective analysis, and the median survival of the 52 patients in total was not reached. The median duration of follow-up was 3.1 y (range 2.7–4.9 y) from sample acquisition. In this cohort, there was a trend, but no statistical difference detected in OS by univariate Cox regression analysis or log-rank test based on IPSS risk (hazard ratio [HR] 2.0, 95% CI 0.6–7.0; p = 0.287) (Fig. 4A, Table III) possibly related to sample size and due to the primary focus on IPSS lower-risk patients in this study. The MDAS model revealed subgroups with different OS (HR 6.3, 95% CI 2.2–18.1; p < 0.001) (Fig. 4B) and confirmed the ability of this system to refine survival estimates in IPSS lower-risk MDS patients. Cox regression survival analyses were then performed to determine variables that impacted OS in this cohort of patients (Table III), and platelet count <50 k/μl (p = 0.008), WBC count (WBC) >20 × 10^9/l (p = 0.007), Hg <10 g/dl (p = 0.018), and blast count ≥5% (p = 0.005) (Table III) were negatively correlated with high TregEM counts. Hg levels were unavailable for two patients. One of these had high total Tregs, and one had high TregCM numbers.

*WHO includes RA, RARS, RCMD, RCMD-RS, RAEB, CMMML, and MDS-U (1).

*IPSS: low risk (IPSS score low or int-1) or high risk (IPSS score int-2 or high).

*MDAS classification: lower risk (low or int-1) or higher risk (int-2 or high) (2, 3).

*IPSS: low risk (IPSS score low or int-1) or high risk (IPSS score int-2 or high).

*WHO includes RA, RARS, RCMD, RCMD-RS, RAEB, CMML, and MDS-U (1).

*p-values were calculated using Fisher’s exact test (2-sample test for counts).

*p-values were calculated using Chi-square test.

*p-values were calculated using the Wilcoxon rank-sum test.

*p-values were calculated using the Kruskal-Wallis test.

*p-values were calculated using the Mann-Whitney U test.

*p-values were calculated using the Student’s t-test.

*p-values were calculated using the Pearson’s chi-square test.

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*p-values were calculated using the Student’s t-test.

*p-values were calculated using the Pearson’s chi-square test.

*p-values were calculated using the Fisher’s exact test.

*p-values were calculated using the chi-square test.

*p-values were calculated using the Mann-Whitney U test.

*p-values were calculated using the Student’s t-test.

*p-values were calculated using the Pearson’s chi-square test.

*p-values were calculated using the Fisher’s exact test.

*p-values were calculated using the chi-square test.

*p-values were calculated using the Mann-Whitney U test.

*p-values were calculated using the Student’s t-test.

*p-values were calculated using the Pearson’s chi-square test.

*p-values were calculated using the Fisher’s exact test.

*p-values were calculated using the chi-square test.
TregEM cells had significantly worse OS compared with MDS cases with normal numbers of TregEM cells (HR 4.3, 95% CI 1.6–11.6; \( p = 0.004 \)) (Fig. 4C, Table III). Although the difference based on total Tregs was not significant (Fig. 4D, Table III), a negative trend was observed (HR 2.6, 95% CI 0.9–7.6; NS). As shown in Table IV, we show that TregEM expansion represents an

---

**FIGURE 3.** Association with increased blast percentage is unique to MDS patients with elevated TregEM. The absolute numbers (A) or percentages (B) of total Tregs, TregN, TregCM, and TregEM were compared between patients with normal blast percentage (<5%) and patients with increased blast percentage (≥5%).

**FIGURE 4.** Reduced OS in MDS patients with high TregEM numbers. OS data of 52 MDS patients was analyzed using the log-rank method and visualized using Kaplan-Meier plots as stratified by IPSS (A), MDAS (B), TregEM numbers (C), and total Treg numbers (D). OS was also analyzed between patients stratified by normal or high TregEM cell numbers in regard to higher-risk (E) and lower-risk (F) MDAS. mOS, Median OS (mo); nr, not reached.
Table III. Kaplan-Meier and univariate Cox regression analyses for OS

<table>
<thead>
<tr>
<th>Variables</th>
<th>n (%)</th>
<th>HR</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treg and Treg phenotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal total Tregs</td>
<td>43 (83)</td>
<td>2.6</td>
<td>0.9–7.6</td>
<td>NS</td>
</tr>
<tr>
<td>High total Tregs</td>
<td>9 (17 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Treg^N</td>
<td>48 (91)</td>
<td>1.7</td>
<td>0.4–7.5</td>
<td>NS</td>
</tr>
<tr>
<td>High Treg^N</td>
<td>4 (8 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Treg^CM</td>
<td>44 (85)</td>
<td>2.2</td>
<td>0.7–6.9</td>
<td>NS</td>
</tr>
<tr>
<td>High Treg^CM</td>
<td>8 (15 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Treg^EM</td>
<td>40 (77)</td>
<td>4.3</td>
<td>1.6–11.6</td>
<td>0.004</td>
</tr>
<tr>
<td>High Treg^EM</td>
<td>12 (23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk scoring systems</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPSS (low/int-1)^a</td>
<td>45 (87)</td>
<td>2.0</td>
<td>0.6–7.0</td>
<td>NS</td>
</tr>
<tr>
<td>IPSS (int-2/high)</td>
<td>7 (13 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDAS (low/int-1)^b</td>
<td>35 (67)</td>
<td>6.3</td>
<td>2.2–18.1</td>
<td>0.001</td>
</tr>
<tr>
<td>MDAS (int-2/high)</td>
<td>17 (33)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Established risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;65 y</td>
<td>17 (33)</td>
<td>4.0</td>
<td>0.9–17.7</td>
<td>NS</td>
</tr>
<tr>
<td>Age ≥65 y</td>
<td>35 (67)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No transfusion</td>
<td>28 (54)</td>
<td>2.3</td>
<td>0.8–6.3</td>
<td>NS</td>
</tr>
<tr>
<td>Prior transfusion</td>
<td>24 (46)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets ≥50 × 10^9/L</td>
<td>36 (75)</td>
<td>4.0</td>
<td>1.4–11.1</td>
<td>0.008</td>
</tr>
<tr>
<td>Platelets &lt;50 × 10^9/L</td>
<td>12 (25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hg ≤10 g/dl</td>
<td>31 (62)</td>
<td>3.4</td>
<td>1.2–9.3</td>
<td>0.018</td>
</tr>
<tr>
<td>Hg &gt;10 g/dl</td>
<td>19 (38)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ECOG &lt;2</td>
<td>51 (98)</td>
<td>1.2</td>
<td>0.4–3.2</td>
<td>NS</td>
</tr>
<tr>
<td>ECOG ≥2</td>
<td>1 (2 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC &lt;20 × 10^9/L</td>
<td>48 (96)</td>
<td>8.4</td>
<td>1.8–39.2</td>
<td>0.007</td>
</tr>
<tr>
<td>WBC ≥20 × 10^9/L</td>
<td>2 (4 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cytopenias &lt;2</td>
<td>33 (63)</td>
<td>1.2</td>
<td>0.4–3.2</td>
<td>NS</td>
</tr>
<tr>
<td>No. of cytopenias ≥2</td>
<td>19 (37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favorable/normal karyotype^c</td>
<td>43 (84)</td>
<td>2.6</td>
<td>0.9–7.7</td>
<td>NS</td>
</tr>
<tr>
<td>Unfavorable karyotype^c</td>
<td>9 (16 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myeloblasts &lt;5%</td>
<td>42 (81)</td>
<td>4.2</td>
<td>1.5–11.2</td>
<td>0.005</td>
</tr>
<tr>
<td>Myeloblasts ≥5%</td>
<td>10 (19)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^aIPSS: low risk (IPSS score low or int-1) or high risk (IPSS score int-2 or high).

^bMDAS classification: lower risk (low or int-1) or higher risk (int-2 or high) (2, 3).

^cData were not available for all 52 patients for this risk factor.

^dUnivariate analysis was not performed due to an insufficient number of patients meeting the criteria for this risk factor.

^eKaryotype was performed by standard cytogenetics and was available for all 52 patients. Favorable karyotype includes del (5q), -Y, and del(20q), and an unfavorable karyotype includes chromosome 7 abnormalities or complex (≥3 abnormalities) based on IPSS criteria (1).

^fBone marrow myeloblast count.

The presence of high Treg^EM cells improved upon the MDAS system and was able to independently refine risk estimates of patients with higher MDAS risk (int-2/high) classification (Fig. 4E, Table IV), but did not impact OS estimates in lower MDAS risk patients (Fig. 4F, Table IV). These data indicate that high Treg^EM numbers are an independent prognostic factor, but that it may improve upon already established risk models.

**Discussion**

A role for Tregs in tumor immune evasion is well defined in animal models and in established solid tumors, but there is limited information about the factors or conditions that contribute to their accumulation in premalignant human diseases. Events that precede diagnosis such as inflammation, constant assault by autoreactive tumor-associated Ags, and establishment of the suppressive microenvironment may contribute to Treg expansion. We hypothesized that studies in a well-defined premalignant neoplasm, such as MDS, would identify the prognostic importance of Treg phenotypic changes that may indicate unique factors that contribute to their expansion in humans. MDS is associated with a heterogeneous clinical presentation and variable rates of leukemia transformation.
allowing concrete investigations into mechanisms governing disease progression, and a role for Tregs in MDS evolution is well established (24). Our investigations demonstrate that expansion of a phenotypically unique suppressive Treg subset (TregEM cells) is associated with malignant progression. The phenotypic markers expressed by Tregs in MDS suggest that they may be recently activated in a similar manner to conventional effector memory T cells (16–19) because they lose CD27 expression. Markers expressed by Tregs in MDS suggest that they may be recently activated in a similar manner to conventional effector memory T cells (16–19) because they lose CD27 expression. The emergence of TregEM cells may originate from either naturally occurring or inducible populations, but in either case, this phenotypic change may reflect cellular activation, as their presence in MDS patients is associated with myeloblast accumulation. A mechanistic explanation for the observed differences in the suppressive capacity of the individual Treg subsets would be beneficial, and experiments are currently ongoing to understand these principles.

During carcinogenesis, developing neoplasms elicit cytotoxic responses from conventional T cells through the presentation of immunogenic autoantigens (25–27). A rise in the thymus, naturally occurring Tregs (nTregs) activate in response to self-Ag presentation in the context of MHC class II (20–22), and activated Tregs control autoreactive effector T cells that escape central tolerance and become responsive to autoantigens (28, 29). Overexpressed autoantigen presentation, well defined in the bone marrow of IPSS lower-risk MDS patients, may also activate Tregs, leading to their expansion and the escape of the developing neoplasm from immune surveillance and ultimately to leukemia progression. In addition to nTregs, conventional T cells can be induced to express FOXP3 in the periphery after activation in polarizing conditions (inducible Tregs) (30). Following development, all Tregs persist in secondary lymph tissue and in the periphery (31–34), where their numbers are tightly maintained. Significant alteration in the balance of the nTregs or inducible Treg compartments has pathologic consequences. Accumulation of nTregs is demonstrable in patients with solid tumors (31, 35–37) and in some hematologic malignancies (43) and is associated with antitumor immune suppression in animal models (12, 44). The emergence of TregEM cells may originate from either naturally occurring or inducible populations, but in either case, this phenotypic change may reflect cellular activation, as their presence in MDS patients is associated with myeloblast accumulation. A mechanistic explanation for the observed differences in the suppressive capacity of the individual Treg subsets would be beneficial, and experiments are currently ongoing to understand these principles.

Current factors used in prognostic MDS models reflect progressive changes inherent to the dysplastic myeloid clone including bone marrow morphology, cytogenetics, mutations, transfusion dependency, the number of cytopenias, as well as age and other comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prognostic precision by adding age, Eastern Cooperative Oncology Group (ECOG) performance status, and weighted comorbidities. The newer models, such as MDAS, have successfully refined prog

Table IV. Multivariate Cox regression analyses for OS

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR</th>
<th>95% CI</th>
<th>p Value</th>
<th>0.95 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High total Tregs</td>
<td>1.9</td>
<td>0.1–36.7</td>
<td>NS</td>
<td>9 (17)</td>
</tr>
<tr>
<td>High Treg&lt;EM&gt;</td>
<td>0.8</td>
<td>0.1–6.9</td>
<td>NS</td>
<td>4 (8)</td>
</tr>
<tr>
<td>High TregN&lt;EM&gt;</td>
<td>0.8</td>
<td>&lt;0.1–13.0</td>
<td>NS</td>
<td>8 (15)</td>
</tr>
<tr>
<td>High TregEM</td>
<td>3.8</td>
<td>1.3–11.1</td>
<td>0.017</td>
<td>12 (23)</td>
</tr>
<tr>
<td>n = 52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Treg&lt;EM&gt;</td>
<td>4.9</td>
<td>1.8–13.6</td>
<td>0.002</td>
<td>12 (23)</td>
</tr>
<tr>
<td>IPSS (int-2/High)*</td>
<td>2.8</td>
<td>0.7–10.1</td>
<td>NS</td>
<td>7 (13)</td>
</tr>
<tr>
<td>n = 48^B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Treg&lt;EM&gt;</td>
<td>2.9</td>
<td>1.0–8.1</td>
<td>0.047</td>
<td>12 (23)</td>
</tr>
<tr>
<td>MDAS (int-2/High)*</td>
<td>4.9</td>
<td>1.6–14.8</td>
<td>0.005</td>
<td>17 (33)</td>
</tr>
<tr>
<td>n = 5^B</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>High Treg&lt;EM&gt;</td>
<td>4.6</td>
<td>1.6–9.0</td>
<td>0.004</td>
<td>11 (23)</td>
</tr>
<tr>
<td>Platelets &lt;100 × 10^9/l</td>
<td>4.9</td>
<td>1.7–13.8</td>
<td>0.003</td>
<td>12 (25)</td>
</tr>
<tr>
<td>n = 50^B</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Treg&lt;EM&gt;</td>
<td>3.2</td>
<td>1.2–9.0</td>
<td>0.025</td>
<td>12 (24)</td>
</tr>
<tr>
<td>Hg &lt;10 g/dl</td>
<td>2.6</td>
<td>0.9–7.5</td>
<td>NS</td>
<td>19 (38)</td>
</tr>
<tr>
<td>n = 52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Treg&lt;EM&gt;</td>
<td>3.4</td>
<td>1.2–9.7</td>
<td>0.022</td>
<td>12 (24)</td>
</tr>
<tr>
<td>WBC ≥20 × 10^9/l</td>
<td>4.0</td>
<td>0.8–20.3</td>
<td>NS</td>
<td>2 (4)</td>
</tr>
<tr>
<td>n = 48^B</td>
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<td></td>
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<tr>
<td>High Treg&lt;EM&gt;</td>
<td>3.2</td>
<td>1.1–9.2</td>
<td>0.029</td>
<td>12 (23)</td>
</tr>
<tr>
<td>Myeloblasts ≥5%</td>
<td>2.9</td>
<td>1.1–8.4</td>
<td>0.045</td>
<td>10 (19)</td>
</tr>
<tr>
<td>High Treg&lt;EM&gt;</td>
<td>3.7</td>
<td>1.1–12.2</td>
<td>0.036</td>
<td>11 (23)</td>
</tr>
<tr>
<td>Platelets &lt;50 k/μl</td>
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<td>2.0–21.1</td>
<td>0.002</td>
<td>12 (25)</td>
</tr>
<tr>
<td>Hg &lt;10 g/dl</td>
<td>1.2</td>
<td>0.3–4.1</td>
<td>NS</td>
<td>18 (38)</td>
</tr>
<tr>
<td>WBC ≥20 × 10^9/l</td>
<td>8.9</td>
<td>1.4–54.4</td>
<td>0.019</td>
<td>2 (42)</td>
</tr>
<tr>
<td>Myeloblasts ≥5%</td>
<td>4.2</td>
<td>1.2–14.4</td>
<td>0.023</td>
<td>9 (19)</td>
</tr>
</tbody>
</table>

^aIPSS: low risk (IPSS score low or int-1) or high risk (IPSS score int-2 or high).
^bData for at least one variable were not available for all 52 patients.
^cMDAS classification: lower risk (low or int-1) or higher risk (int-2 or high) (2, 3).
Several Journal and Drug Administration-approved drugs for MDS display variable response rates with preferential activity in select disease subsets including erythroid-stimulating agents, hypomethylating agents azacitidine and decitabine (45), immunomodulatory drug lenalidomide (46, 47), and immuno-suppressive therapy such as antithymocyte globulin and cyclosporine (8, 10, 48). Patients included in this study received no prior disease-modifying therapy other than growth factors such as G-CSF for cytopenias. Current prognostic models are incapable of discriminating response to therapies, so Treg phenotyping may be a useful tool to segregate MDS patients who are responsive to various drug classes. Therefore, inclusion of Treg status into the current prognostic and treatment models may improve prognostication and better inform therapeutic decisions in MDS. Our study sheds light into unique aspects of T cell-mediated pathophysiology as it relates to human immunity in a premalignant model of disease and implicates specific TregEM expansion in disease progression in MDS.

Acknowledgments
We thank the H. Lee Moffitt Cancer Center Flow Cytometry Core Facility for advice and assistance with acquisition and analyses of flow cytometry data. Statistical analysis was performed in conjunction with the Biostatistics Program at H. Lee Moffitt Cancer Center. Samples were obtained from the BMF-RDCRN. We also thank the reviewer from Gynme Corporation for comments.

Disclosures
The authors have no financial interests of interest.

References


