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Expansion of Effector Memory Regulatory T Cells Represents a Novel Prognostic Factor in Lower Risk Myelodysplastic Syndrome

Adam W. Mailloux,*1 Chiharu Sugimori,†1 Rami S. Komrokji,‡ Lili Yang,* Jaroslav P. Maciejewski,§ Mikkael A. Sekeres,* Ronald Paquette,* Thomas P. Loughran, Jr.,1 Alan F. List,‡ and Pearlie K. Epling-Burnette§

Myelodysplastic syndromes (MDS) refer to a group of pathophysiologically diverse premalignant hematopoietic diseases characterized by inflammation-associated cytopenias, myeloid dysplasia, autoimmunity, and variable risk 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 reclassified a large number of IPSS-defined lower risk patients, was found to be inferior OS. The prognostication of the MDAS, which reclassified a large number of IPSS-defined lower risk patients, was also improved by the inclusion of TregEM in the analysis. These findings suggest that expansion of a specific Treg subset, rather than expansion of Tregs as a whole, identifies a subset of higher risk patients. Inclusion of Treg memory phenotype analysis into prognostic models may indicate the initiation of an immunosuppressive microenvironment with importance to disease pathobiology and treatment.

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 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 (Amersham Pharma Biotech, Piscataway, NJ) gradient centrifugation as per the manufacturer’s recommendations. Following collection of PBMCs, cell pellets were resuspended, washed thoroughly with PBS, and then frozen at −80°C in FBS containing 10% DMSO (Sigma-Aldrich, St. Louis, MO) in a polycarbonate container insulated with isopropanol before storage in liquid nitrogen. Cells were then thawed in culture medium (RPMI 1640 medium; Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.02 M HEPES buffer) and then rinsed in PBS prior to immunofluorescent staining.

Immunofluorescent staining of Tregs and Treg subsets

Nonspecific staining was first blocked for 30 min at 4°C with 300 μl 2% FBS in PBS per 1.0 × 10^6 cells. The cells were then labeled with fixable Live/Dead Yellow after thorough PBS washes (Invitrogen) so that nonviable cells could be excluded during flow cytometric acquisition. Cell surface staining was accomplished by 30-min incubation at 4°C with 1 μl/1.0 × 10^6 cells of the following Abs: Pacific Blue-CD3, PE-Cy7-CD25, PE-CD127, alloglycoprotein-CD27 (BD Pharmingen, San Diego, CA); and PerCP-Cy5.5-CD45RA (eBioscience, San Diego, CA). For experiments comparing CD127 and FOXP3 expression, the intracellular detection of FOXP3 required fixation and permeabilization with Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA) followed by a 30-min incubation with alloglycoprotein-FOXP3 (BD Pharmingen) Ab at 4°C. Analysis of Treg populations was performed on an LSRII cytometer (BD Biosciences) harboring a custom configuration for the H. Lee Moffitt Cancer Center & Research Institute. Using the gating schema outlined in Fig. 1A, viable cells were selected using cells gated on fixable Live/Dead Yellow, and then CD4+FOXP3+CD25dimCD127+ T cells were discriminated into naive (TregN), central memory (TregCM), TregEM, or terminal memory Tregs based on CD27 and CD45RA expression (Fig. 1A). TregN expressed both CD27 and CD45RA, TregCM cells were CD27CD45RA−, TregEM cells were double negative for CD27 and CD45RA, and Tregs were considered CD27+CD45RA+. Intracellular FoxP3 staining and surface analysis confirmed that CD4+CD25dimCD127dim cells accurately defined Tregs based on FOXP3 expression. To visualize these cells, CD4+ FOXP3+ and CD4+FOXP3− Tregs were gated and shown in blue, and TregEM/FOXP3− effector cells are shown in orange. FOXP3+ cells are largely contained within the CD25green and CD127dim region (Fig. 1A). For functional studies, fixable Live/Dead Yellow could not be used due to the amine-reactive nature of this dye. Instead viability staining was performed using 7-aminoactinomycin D (BD Biosciences) prior to functional assays, and populations were sorted based on phenotypic markers on specific subpopulations as shown in Supplemental Fig. 1. Analysis of cytometry data was achieved using FlowJo software version 7.6.1 (Tree Star, Ashland, OR).

T cell suppression assay

Tregs with distinctive naive and memory phenotypes were isolated following immunofluorescent staining by FACS using a FACSARia cell sort (BD Biosciences). Following isolation, increasing ratios of each Treg population were mixed with 1.0 × 10^6 conventional CD4+CD25− (responder) T cells labeled with 0.5 μM CFSE according to the manufacturer’s recommendations (Invitrogen). Each mixture was then placed in a round-bottom 96-well plate coated with 5 μM anti-CD3 Ab (eBioscience), and 2 μM anti-CD28 Ab (eBioscience) was then added to each well for costimulation. Proliferation of responding T cells was assessed by CFSE dilution after 5 d using flow cytometry on an LSRII cytometer (BD Biosciences) harboring a custom configuration for the H. Lee Moffitt Cancer Center & Research Institute. The amount of proliferation in each assay was quantified using the proliferation algorithm available with FlowJo analysis software (Tree Star).

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 Pearson correlation 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 Treg/μl) and each of the Treg subsets: TregN cells (1–9 Treg/μl), TregCM cells (19–53 TregCM/μl), effector memory Tregs (0–6 TregEM/μl), and terminal memory Tregs (0 to 2 TregEM/μl). MDS patients with absolute total Treg or Treg subset numbers above the range of healthy age-matched controls were
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 (TregTM) 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).
considered to have high levels, whereas patients within or below this range were considered to have normal levels. Because complete blood count data were not available for each individual healthy control subject, an estimated WBC count of 7 k/μl was used to determine the normal range for the absolute number of Tregs in the peripheral blood of controls.

### 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 excess 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 TregN, TregCM, TregEM, 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. 1C). 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 TregEM or TregCM subsets, as shown in Fig. 1C–F. Treg in 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>
<thead>
<tr>
<th>Characteristic</th>
<th>MDS (n = 52)</th>
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<tr>
<td>Age (y)$^a$</td>
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</tr>
<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
<td>26</td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
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<tr>
<td>WHO$^b$</td>
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<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>
<td></td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Abnormal: unfavorable</td>
<td>17</td>
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</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 (≥3 abnormalities) based on IPSS criteria (1).

### The TregEM phenotype is more suppressive than the TregCM 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. TregN and TregEM cells represent, on average, 13 and 2% of the entire Treg population (23), but suppress at a 1:2 ratio, showing that these individual sorted populations display different suppressive activity in vitro.

#### High TregEM 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 (n = 0) were not included in this analysis because of low sample size. Characteristics of patients with high Tregs (n = 9) and high TregCM (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
TregEM subsets was then compared among patients in these two groups. A total of 16 patients (31%) in this cohort had died at the time of the study, demonstrating that the expansion of TregEM correlates with negative prognostic features of MDS.

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 by univariate Cox regression analysis and log-rank test based on IPSS criteria (1).
Treg<sup>EM</sup> cells had significantly worse OS compared with MDS cases with normal numbers of Treg<sup>EM</sup> 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 Treg<sup>EM</sup> expansion represents an

**FIGURE 3.** Association with increased blast percentage is unique to MDS patients with elevated Treg<sup>EM</sup>. The absolute numbers (A) or percentages (B) of total Tregs, Treg<sup>N</sup>, Treg<sup>CM</sup>, and Treg<sup>EM</sup> 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 Treg<sup>EM</sup> 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), Treg<sup>EM</sup> numbers (C), and total Treg numbers (D). OS was also analyzed between patients stratified by normal or high Treg<sup>EM</sup> cell numbers in regard to higher-risk (E) and lower-risk (F) MDAS. mOS, Median OS (mo); nr, not reached.
independent prognostic factor in MDS in multivariate analyses including total Tregs, Treg<sup>N</sup>, and Treg<sup>Cm</sup> numbers (Treg<sup>Em</sup>, HR 3.8, 95% CI 1.3–11.1; p = 0.017), higher-risk IPSS (Treg<sup>Em</sup>, HR 4.9, 95% CI 1.8–13.6; p = 0.002), higher-risk MDAS (Treg<sup>Em</sup>, HR 2.9, 95% CI 1.0–8.1; p = 0.047), platelet counts <50 × 10<sup>9</sup>/L (Treg<sup>Em</sup>, HR 4.6, 95% CI 1.6–13.1; p = 0.004), Hg <10 g/dl (Treg<sup>Em</sup>, HR 3.2, 95% CI 1.2–9.0; p = 0.025), WBC >20 × 10<sup>9</sup>/L (Treg<sup>Em</sup>, HR 3.4, 95% CI 1.2–9.7; p = 0.022), and increased blasts ≥5% (Treg<sup>Em</sup>, HR 3.2, 95% CI 1.1–9.2; p = 0.029) when adjusting for each factor individually or when adjusting for all four factors (Treg<sup>Em</sup>, HR 3.7, 95% CI 1.1–12.2; p = 0.036). The presence of high Treg<sup>Em</sup> 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<sup>Em</sup> numbers...
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 subpopulation (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 natural 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). Arousing 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 immunosurveillance 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, 35–42) 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.
Several Food 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 immunosuppressive 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 prognostic 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.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Cell Sorting of Treg Subsets. (A) Flow cytometry gating schema for sorting Treg subsets. Cells (pre-enriched for CD4⁺ T-cells using negative selection) were first gated on CD3 and CD4 positivity, and then gated on CD127⁺CD25⁻ cells (conventional CD4 T-cells) or on CD127⁻CD25⁺(Tregs). Treg subsets were then identified using CD45RA and CD27 staining. Treg\(^N\) cells were considered CD45RA⁺CD27⁺, Treg\(^{CM}\) cells were considered CD45RA⁻CD27⁺, and Treg\(^{EM}\) cells were considered CD45RA⁻CD27⁻. No significant population of Treg\(^{TM}\) cells was observed (CD45RA⁺CD27⁻). (B) FoxP3 expression was then confirmed \textit{post hoc} in each Treg subpopulation following sorting.