ARTICLE

Thrombomodulin-expressing monocytes are associated with low-risk features in myelodysplastic syndromes and dampen excessive immune activation

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ABSTRACT

The bone marrow of patients with low-risk myelodysplastic syndromes (MDS) is often an inflammatory environment and associated with an active cellular immune response. An active immune response generally contributes to antitumor responses and may prevent disease progression. However, chronic immune stimulation can also induce cell stress, DNA damage and contribute to the pathogenesis of MDS. The protective mechanisms against excessive immune activation are therefore an important aspect of the pathophysiology of MDS and characterizing them may help us to better understand the fine balance between protective and destabilizing inflammation in lower-risk disease. In this study we investigated the role of thrombomodulin (CD141/BDCA-3) expression, a molecule with anti-inflammatory properties, on monocytes in the bone marrow and peripheral blood of MDS patients in different risk groups. Patient-derived classical monocytes showed high expression levels of thrombomodulin, whereas monocytes from healthy donors hardly expressed any thrombomodulin. The presence of thrombomodulin on monocytes from MDS patients correlated with lower-risk disease groups and better overall and leukemia-free survival. Using multidimensional mass cytometry, in an *in-vitro* setting, we showed that thrombomodulin-positive monocytes could polarize naïve T cells toward cell clusters which are closer to T helper type 2 and T regulatory cell phenotypes and less likely to contribute to effective immune surveillance. In conclusion, the expression of thrombomodulin on classical monocytes is a favorable and early prognostic marker in patients with low-risk MDS and may represent a new mechanism in the protection against disproportionate immune activation.

Introduction

The immune system plays an important role in the pathogenesis and disease course of myelodysplastic syndromes (MDS). The immune status can be markedly different between MDS prognostic risk groups. Low-risk disease is often characterized by an increased number and activation state of pro-inflammatory immune cells [i.e. T helper (Th)17, natural killer (NK) and $CD8^+$ cytotoxic T cells[1–6]] whereas in high-risk disease an immunosuppressive response is the dominant feature [i.e. expansion of T regulatory cells (Treg)[7–10] and myeloid-derived suppressor cells[11]] which could facilitate immune escape and eventually progression to acute myeloid leukemia. Although an "activated" immune system and associated tumor-specific immune responses are crucial for effective immune surveillance and elimination of the malignant clone, in the longer term chronic immune stimulation may enhance the risk of genomic instability and development of MDS/acute myeloid leukemia.[12] Smoldering inflammation as a result of aberrant activation of inflammatory path-

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ways, e.g. Toll-like receptor (TLR) signaling, can induce malignant transformation and disease progression by causing genotoxic cell stress. Indeed, in low-risk MDS elevated levels of several stress-inducing molecules, such as the damage-associated molecular pattern molecules S100A8/A9, are actively secreted from mesenchymal niche cells in the bone marrow (BM) microenvironment, thereby causing niche-induced DNA damage in hematopoietic stem and progenitor cells.[13] High S100A9 levels in MDS BM also result in inflammasome assembly and subsequent initiation of pyroptosis, an immunogenic form of cell death, which could potentially explain the high rate of cell death in low-risk MDS BM.[14–16] These soluble inflammatory molecules are able to bind to TLR on the surface of hematopoietic stem and progenitor cells and immune cells. Constitutively activated TLR-signaling and downstream mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) activation are evident and have been implicated in the pathogenesis of MDS.[17–24] Besides active secretion of stress-inducing molecules, passive release from cells undergoing immunogenic cell death has also been described in MDS. Levels of high mobility group box 1 (HMGB1), a mediator strongly involved in inflammatory processes and a ligand for TLR4, were found to be increased in the BM of MDS patients due to impaired clearance of apoptotic cells causing secondary necrosis and leakage of this molecule into the BM environment.[25]

As a result of this vicious circle of inflammation and cell death, immune-inhibitory mechanisms that interfere with this excessive inflammatory process kick in. While these immune-inhibitory pathways may control the inflammatory response to some extent, they also facilitate the expansion of immunosuppressive cells, such as Treg and myeloid-derived suppressor cells, which further suppress the already weakened immune surveillance against the malignant clone. A delicate balance between immune activation and inhibition is, therefore, required to maintain effective immunosurveillance. Thrombomodulin (TM) is known for its anticoagulant function by serving as a cofactor for thrombin. Notably, the lectin-like domain of the TM molecule has marked anti-inflammatory activities and interferes with the complement pathway.[26–28] Several studies have shown strong correlations between disease severity and TM levels in, for instance, autoimmune and infectious diseases as well as in cancer.[29–31] In the immune system, TM, also known as CD141 or BDCA-3, is mainly expressed on dendritic cells.[32–34] We have previously described elevated expression of TM/BDCA-3 on tumor-conditioned and immunosuppressive monocyte-derived dendritic cells that acquire a M2-like macrophage phenotype.[35,36] The anti-inflammatory potential of TM has also been assigned to the fact that TM is able to bind HMGB1, thereby inhibiting the strong pro-inflammatory effect of this molecule. Since high levels of this molecule were found in low-risk MDS BM, this interactive mechanism may be relevant in keeping excessive immune activation to a minimum. The aim of this study was to evaluate the possible role and prognostic value of TM in regulating the inflammatory immune response in MDS. The expression of TM was evaluated on different monocyte subsets (classical, intermediate and non-classical) in the peripheral blood (PB) and BM within different MDS risk groups. Multidimensional mass cytometry was used to investigate the putative impact of TM+ monocytes on the T-cell phenotype. The cell surface expression of TM was higher on classical monocytes in both the PB and BM of MDS patients than on healthy donor-derived monocytes. The expression of TM was related to a more favorable prognosis and functional skewing of the T-cell response toward a more tolerized state.

Methods

Patient and control samples

Twenty-nine PB and 154 BM samples from newly diagnosed MDS patients were collected in this study. Patients were assigned to different risk categories using the Revised International Prognostic Scoring System (IPSS-R) and the 2016 World Health Organization (WHO) classification (details are given in the *Online Supplementary Methods* file and Table 1). A set of 25 age-matched control BM samples was obtained after written informed consent from hematologically healthy patients who were undergoing cardiac surgery at Amsterdam University Medical Center (the Netherlands). For the PB analysis, 31 control samples were collected. The study was approved by the local ethical committee and was conducted in accordance with the declaration of Helsinki.

Flow cytometry and fluorescence *in situ* hybridization

PB and BM cells were analyzed on a flow cytometer (FACSCanto™, BD Biosciences, San Jose, CA, USA) after incubation with a panel of monoclonal antibodies (see *Online Supplementary Methods* for details). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Monocyte subsets were identified based on the differential expression of CD14, CD16 and M-DC8 [anti-6-Sulfo LacNAc (Slan)], using recent recommendations (*Online Supplementary Figure S1*).[37,38] Classical monocytes were characterized by high CD14 expression, and CD16 and M-DC8 negativity. Intermediate and non-classical monocytes were both defined as $CD16^+$. However, only intermediate monocytes expressed CD14. We used M-DC8 as a marker to discriminate between intermediate and non-classical monocytes as suggested by Hofer *et al.* (Figure 1A).[38]

Three samples containing monocytes with high TM expression and a known cytogenetic aberrancy were used for the isolation of classical monocytes and subsequent interphase fluorescence *in situ* hybridization (FISH) analysis (details are given in the *Online Supplementary Methods*).

T-cell cultures and multidimensional mass cytometry

A multiparameter deep-phenotyping strategy, known as cytometry by time-of-flight (CyTOF), was used for T cells cultured in the presence of MDS-derived TM^- or TM^+ monocytes (culture details are provided in the *Online Supplementary Methods*). Data were analyzed using a combination of automated dimension reduction and clustering methods including t-distributed stochastic neighbor embedding (t-SNE)[39] to visually (viSNE) identify cell populations.[40] This was followed by spanning-tree progression analysis of density-normalized events (SPADE)[41] for the clustering of T cells as published before.[42,43] The deep immunophenotyping of T-cell clusters was performed using our in-house pipeline (publicly available here: *https://github.com/kordastilab/cytoClustR*) followed by marker enrichment modeling (MEM) to calculate MEM scores of the identified subpopulations.[44]

Statistical analysis

Significant differences for two-group comparisons were analyzed by applying a non-parametric Mann-Whitney U test, whereas for multi-group comparisons a Kruskal-Wallis test with

the Dunn multiple comparisons test was used. A Spearman correlation was computed for PB and BM comparisons. *P* values <0.05 were considered statistically significant. Graphpad Prism 6 software (San Diego, CA USA) was used for graphic display and statistical calculations. A multivariate Cox regression analysis for overall and leukemia-free survival was performed using IBM SPSS Statistics software version 22 (New York, NY, USA).

Results

Classical monocytes in patients with myelodysplastic syndromes express thrombomodulin

Monocyte subsets were identified by flow cytometric analysis based on the expression of CD14, CD16 and M-DC8 according to recently published recommendations.[38] Classical, intermediate and non-classical monocytes were characterized by using the above mentioned markers (Figure 1A). Subsequently, the expression of TM and HLA-DR, a major histocompatibility complex (MHC) molecule class II, was assessed on all monocyte subsets derived from normal bone marrow (NBM) and MDS BM samples. Monocyte subsets from MDS patients showed high levels of TM expression on their cell surface, whereas NBM-derived monocytes showed very low levels of TM. HLA-DR expression, too, was higher on all monocyte subsets in MDS BM than it was in NBM (Figure 1A). The levels of expression on different monocyte subsets were quantified in a larger cohort of patients (NMB samples, n=10 and MDS BM samples, n=24). Total percentages of monocyte subsets in MDS BM compared to NBM were not significantly different. However, the percentage of monocytes that expressed TM was significantly higher for MDS-derived classical monocytes compared to the same monocyte subset in NBM (37.3% *vs.* 9.9%; *P*<0.0001) (Figure 1B). The percentages of TM expression on intermediate and non-classical monocytes were equally distributed between MDS BM and NBM (Figure 1B). The median fluorescent intensity of TM and HLA-DR was evaluated on the three distinct monocyte subsets using the same set of samples. Classical monocytes from MDS BM showed higher levels of TM expression compared to classical monocytes fron NMB (3.7-fold higher; *P*<0.0001). HLA-DR expression levels were higher for all MDS-derived monocyte subsets (classical monocytes: 2.0-fold, *P*=0.0015; intermediate monocytes: 1.8-fold, *P*=0.0154; non-classical monocytes: 1.5-fold, *P*<0.0001) (Figure 1B). TM expression remained unchanged upon overnight stimulation with TLR ligands in a preliminary set of samples (*Online Supplementary Figure S2*).

Classical monocytes were then analyzed in a larger set of samples, since this subset forms the most prevalent subset in NBM as well as in MDS BM, and revealed the most prominent difference in TM expression. The PB compartment was also included in the analysis. The cohort was extended with 130 MDS BM-derived samples and 15 NBM samples. The PB and BM samples in the extended control and patient cohorts (normal PB, n=31; MDS PB, n=29; NBM, n=25 and MDS BM, n=154) were screened for the presence of TM on classical monocytes (Figure 1C). MDS-derived classical monocytes in both the PB and BM compartment showed higher expression of TM compared to the expression on classical monocytes from normal PB and NBM samples (PB: 33.6% *vs.* 17.8%, *P*=0.015; BM: 37.0% *vs.* 8.6%, *P*<0.0001). Furthermore, a strong positive correlation was found for the percentage of classical monocytes expressing TM in the two compartments in 25 paired MDS samples (r=0.83, *P*<0.0001) (Figure 1C). The same flow cytometric panel was used to study TM expression on other cell types present in the PB and BM,. We were able to identify granulocytes, eosinophils and B cells. TM was exclusively expressed on MDS-derived monocytes (*Online Supplementary Figure S3*) and none of the other cell types in PB and BM showed positivity for TM, including the non-B cell lymphocytic compartment consisting of T and NK cells (*data not shown*).

Table 1. Characteristics of the patients and controls.

Characteristics	Value	[min-max]
Total number		
HD	56	
MDS	183	
Peripheral blood samples		
Number	60	
HD	31	
MDS	29	
Age – mean, years		
HD	-	
MDS	69	[45-85]
Sex		
HD – male/female	-	
MDS – male/female	20/9	
Bone marrow samples		
Number	179	
HD	25	
MDS	154	
Age – mean,y		
HD	62	[20-86]
MDS	69	[36-94]
Sex		
HD – male/female	18/7	
MDS – male/female	110/44	
IPSS-R		
Very low risk		
Low	20	
Low risk	34	
Intermediate risk	22	
High risk	8	
Very high risk	8	
WHO classification		
MDS-SLD	5	
MDS-MLD	48	
MDS-RS-SLD	8	
MDS-RS-MLD	27	
MDS-EB1	22	
MDS-EB2	20	
Del5q	12	
% Blasts		
<5%	97	
≥5%	44	
Ring sideroblasts		
No	106	
Yes	40	

HD: healthy donor; MDS: myelodysplastic syndrome; IPSS-R: Revised International Prognostic Scoring System; WHO, World Health Organization; SLD, single lineage dysplasia; MLD, multilineage dysplasia; RS, ring sideroblasts; EB, excess blasts; Del5q: deletion 5q.

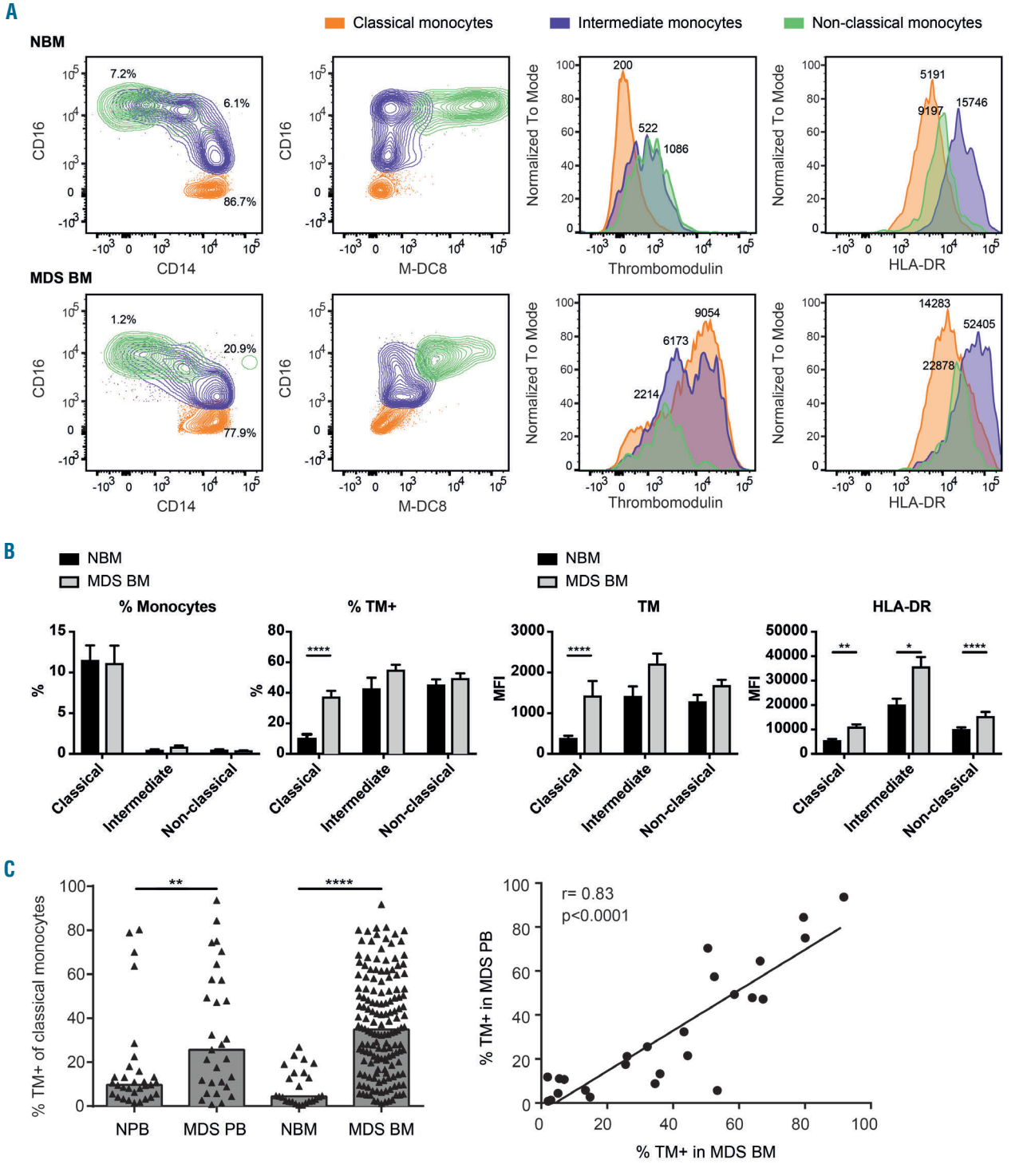


Figure 1. Thrombomodulin-expressing monocyte subsets in normal and myelodysplastic syndrome bone marrow and peripheral blood samples. (A) Identification of different monocyte subsets in normal bone marrow (NBM) and myelodysplastic syndrome (MDS) bone marrow (BM). Three markers, CD14, CD16 and M-DC8, were used to identify classical ($CD14^{++}CD16^{-}M\text{-}DC8^{-}$, in orange), intermediate ($CD14^{+}CD16^{+}M\text{-}DC8^{-}$, in purple) and non-classical monocytes ($CD14^{+/-}CD16^{+}M\text{-}DC8^{+}$, in green). The levels of expression of thrombomodulin (TM) and HLA-DR were assessed on all separate monocyte subsets. The median fluorescence intensity (MFI) value for each subset is shown for a representative sample. (B) Frequencies of monocyte subsets in the BM of ten healthy individuals and 24 MDS patients. Percentages were calculated from the total $CD45^{+}$ mononuclear cell fraction. Mean frequencies ± standard error of mean (SEM) are given (NBM *vs.* MDS BM: classical monocytes 11.47% ± 1.86 *vs.* 11.11% ± 2.20, intermediate monocytes 0.45% ± 0.12 *vs.* 0.85% ± 0.17, non-classical monocytes 0.46% ± 0.11 *vs.* 0.38% ± 0.05). Furthermore, the percentage of monocytes that express TM is displayed (NBM *vs.* MDS BM: classical monocytes 9.94% ± 2.82 *vs.* 37.27% ± 4.00, intermediate monocytes 42.60% ± 7.27 *vs.* 54.90% ± 3.48, non-classical monocytes 45.11% ± 3.72 *vs.* 49.46% ± 3.34). Expression levels of TM and HLA-DR on NBM and MDS BM monocyte subsets are also shown. Mean MFI values ± SEM are shown for ten NBM and 24 MDS BM samples (TM in NBM *vs.* MDS BM: classical monocytes 382 ± 65 *vs.* 1425 ± 367, intermediate monocytes 1414 ± 245 *vs.* 2208 ± 259, non-classical monocytes 1279 ± 169 *vs.* 1676 ± 144. HLA-DR in NBM *vs.* MDS BM: classical monocytes 5426 ± 715 *vs.* 11010 ± 1056, intermediate monocytes 20062 ± 2529 *vs.* 35639 ± 3989, non-classical monocytes 10117 ± 856 *vs.* 15255 ± 1915). (C) Percentages of TM-expressing classical monocytes in peripheral blood (PB) and BM. Bars indicate mean frequencies [normal PB (NPB, n=31) *vs.* MDS PB (n=29): 17.8% *vs.* 33.6%. normal BM (NBM, n=25) *vs.* MDS BM (n=154): 8.6% *vs.* 37.0%]. TM expression was correlated in PB- and BM-derived classical monocytes. In total, 25 paired MDS samples were included. **P*<0.05, ***P*<0.01, *****P*<0.0001.

Thrombomodulin-expressing monocytes are clonally involved and are associated with the features of low-risk myelodysplastic syndromes

In order to investigate the clonal involvement of TM+ monocytes in MDS, cells from three different patients were sorted and screened for the presence of a known cytogenetic aberration according to their karyotype. Almost all monocytes of these patients showed high expression of TM.

One patient had a deletion of chromosome 5q (del5q) in all cells (karyotype: 46,XY,del(5)(q22q33)[10]), one showed trisomy for chromosome 8 in 65% of the cells (karyotype: 47,XY,+8[13]/46,XY[7]) and one case had a monosomy 7 (karyotype: 45,XY,-7[10]). In all cases, TM+ monocytes were highly involved in the dysplastic clone and showed a high percentage of cells with the respective cytogenetic abnormality, suggesting that they did not come from healthy CD34+ cells. The isolated CD34+ progenitor cells showed a similar pattern, since the known cytogenetic aberration was present in most analyzed cells. As expected, B cells were not involved. Partial involvement was found in whole BM samples (Figure 2A).

To further correlate TM expression with different MDS subgroups, the patients' clinical data were collected and their IPSS-R scores, reflecting survival and the risk of disease progression, were calculated. Furthermore, patients were categorized using the 2016 WHO classification system which incorporates clinical characteristics, PB and BM findings and cytogenetic analysis. The percentage of monocytes displaying TM expression was higher for patients who had a very low- or low-risk score in the IPSS-R than for patients in higher-risk groups and healthy controls (very low/low 40.1% *vs*. intermediate 22.7% *vs*. high/very high 28.3% *vs*. NBM 11.3%) (Figure 2B). Additionally, TM expression was elevated in WHO categories related to lower-risk disease, such as MDS single and multiple lineage dysplasia with or without ring sideroblasts, compared to categories related to higher-risk MDS, i.e. MDS with excess blasts 1 and 2. The percentage of monocytes expressing TM was higher in all WHO subgroups than in NBM (single/multiple lineage dysplasia with/without ring sideroblasts, 40.9% *vs*. MDS with excess blasts 1 and 2, 24.2% *vs*. NBM, 11.3%) (Figure 2B). Using the percentage of BM blast cells as a reflection of disease stage, patients with blast percentages below 5% harbored higher numbers of TM+ monocytes than patients with 5% or more blast cells (41.3% *vs*. 25.7%, respectively; $P<0.0001$) (Figure 2C). Finally, a relation between the percentage of monocytes expressing TM and the presence of ring sideroblasts (erythroblasts with mitochondrial iron accumulation) was found (present 45.4% *vs*. absent 33.2%; $P=0.003$) (Figure 2C). As an "indirect" indication of the presence of a *SF3B1* mutation, the percentage of TM+ monocytes was compared between subtypes with ring sideroblasts and other MDS subtypes (*Online Supplementary Figure S4*). Significantly higher percentages of TM+ monocytes were found in cases with ring sideroblasts than in those with MDS with excess blasts 1 or 2. A trend to higher frequencies was observed for the comparisons with other subtypes.

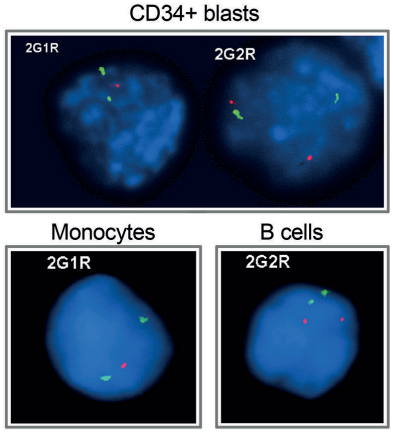
Myelodysplastic syndrome-derived thrombomodulin-positive monocytes polarize CD4+ T cells to an immunosuppressive phenotype

The next research focus was to study the effect of TM+ monocytes on the phenotype of CD4+ T cells and to determine whether they could induce an anti-inflammatory T-cell phenotype. Healthy donor-derived CD4+ T cells were co-cultured with sorted TM- or TM+ monocytes from two MDS patients (*Online Supplementary Figure S5*). After 5 days, T cells were harvested and labeled with a comprehensive panel of metal-tagged antibodies (*Online Supplementary Table S1*) for mass cytometry (CyTOF). Using our data analysis pipeline, T-cell subsets were identified (*Online Supplementary Figure S6*) and compared between the conditions (i.e. day 0, control stimulated and cultures with TM- or TM+ monocytes) (Figure 3A). Compared to day 0, all conditions showed expansion of specific T-cell subsets. To further characterize these cell islands, T cells were clustered by SPADE on tSNE (see Methods). Using selected T-cell markers various subsets could be characterized (Figure 3B). Then frequencies of clusters between the conditions were compared (Figure 3C). Nodes highlighted with red circles refer to the most frequent T cell clusters in the TM- condition whereas black circles indicate a higher percentage in the TM+ condition. For both conditions the top five of highest frequencies were selected. The expression profiles of T cells in the identified clusters were evaluated next, using MEM (see Methods). T-cell clusters were divided into two groups for comparison: clusters with highest frequency in the TM+ condition (called "up") and clusters with highest frequency in the TM- condition (called "down"). MEM scores were calculated for each marker in each group for further comparison.[44] Interestingly, T cells that were predominantly present upon culturing with TM+ monocytes (group called "up") showed an anti-inflammatory profile (Figure 3C). They were polarized toward Th2, Treg and PD-1-expressing clusters of T cells, since they expressed high levels of FoxP3, GATA3 and CD279 (PD-1) and had elevated concentrations of intracellularly measured interleukin-4 and interleukin-10. In contrast, T cells cultured in the presence of TM- monocytes (group called "down") were mainly positive for interferon-γ and hardly for immunosuppressive cytokines.

The presence of thrombomodulin-positive monocytes is related to a better overall and leukemia-free survival

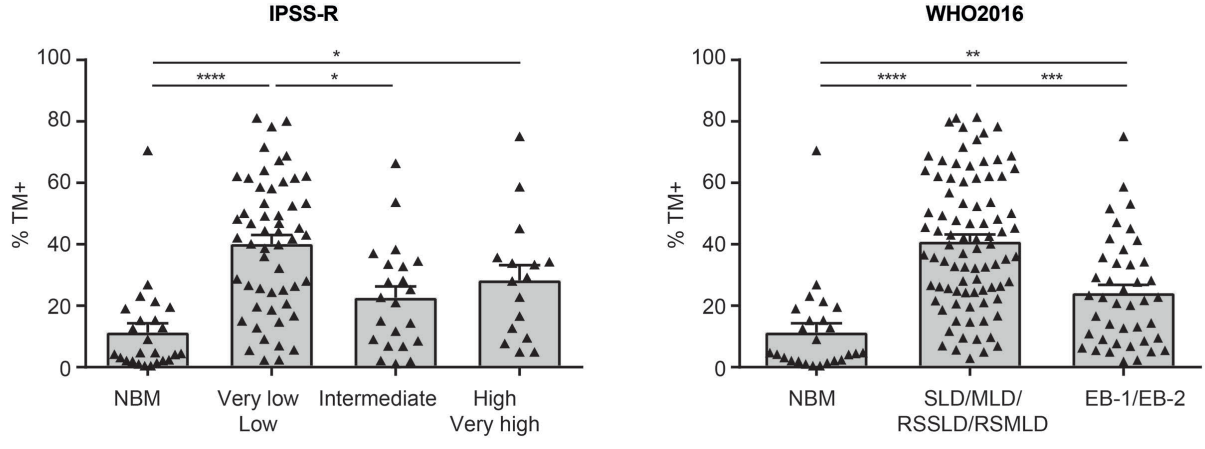
In order to determine the clinical significance of the presence of TM+ monocytes, overall survival and leukemia-free survival were calculated. As a cut-off for TM expression monocytes from the healthy donor cohort were used. The mean TM percentage plus two standard deviations was calculated resulting in a cut-off of 25.53%. In total, overall survival data for 122 MDS patients and leukemia-free survival data for 102 patients were available. Interestingly, the presence of TM on MDS monocytes was significantly associated with a better overall survival ($P=0.006$) as well as a better leukemia-free survival ($P=0.029$) (Figure 4A). The median overall survival for patients with TM+ monocytes was 58 months, whereas that of patients without TM+ monocytes was 30 months. For a subgroup of patients, data were available for further risk stratification into IPSS and IPSS-R risk groups. Following the hypothesis that TM is mainly present in an inflammatory environment, survival and leukemia-free survival data were also analyzed in low-risk MDS groups (i.e. IPSS low and intermediate groups or IPSS-R very low, low and intermediate groups). The presence of TM+ monocytes resulted in better overall survival in this sub-

A



Cell type	FISH results: number of cells	
	Monosomy 7	**Two chromosome 7**
Whole bone marrow	57	43
Monocytes	99	1
B cells	0	100
CD34+ blasts	96	4
	Del 5q	**Normal chromosome 5**
Whole bone marrow	163	37
Monocytes	93	7
B cells	2	98
CD34+ blasts	96	4
	Trisomy 8	**Two chromosome 8**
Whole bone marrow	56	44
Monocytes	81	19
B cells	0	91
CD34+ blasts	77	23

B



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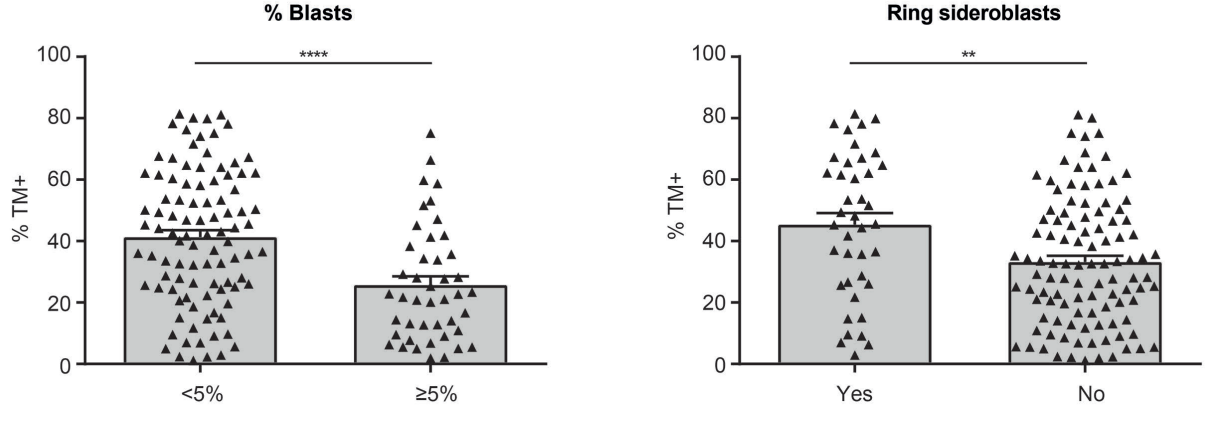

Figure 2. Classical monocytes are clonally involved and the presence of thrombomodulin correlates with disease states. (A) Sorted cells, including B cells and CD34$^+$ blast cells, from patients with a known cytogenetic aberrancy were subjected to fluorescence *in situ* hybridization (FISH) analysis. Furthermore, whole bone marrow (BM) samples were used for degree of cytogenetic load. Representative interphase cells hybridized with the chromosome 5q probe, showing loss of 5q in CD34$^+$ blasts and monocytes, and no loss of 5q in B cells. In three tested cases (monosomy 7, deletion 5q and trisomy 8), classical monocytes and CD34$^+$ blast cells were highly involved in the dysplastic clone, whereas B cells were not involved. Interphase FISH on whole BM samples showed both an aberrant and a normal cell line. (B) Thrombomodulin-positive (TM$^+$) classical monocytes in different myelodysplastic syndrome (MDS) risk groups defined according to the Revised International Prognostic Scoring System and the World Health Organization (WHO) 2016 classification. The percentage of classical monocytes that express TM was highest in the very low/low-risk groups: the percentage of TM expression was significantly reduced in higher-risk groups compared to the lower-risk group [mean ± standard error of mean (SEM): normal BM (n=25) 11.3% ± 3.0% *vs.* very low/low-risk MDS BM (n=54) 40.1% ± 2.9% *vs.* intermediate-risk MDS BM (n=22) 22.7% ± 3.7% *vs.* high/very high-risk MDS BM (n=16) 28.3% ± 4.9%). Patients with low-risk MDS according to the WHO 2016 classification (single/multiple lineage dysplasia with or without ring sideroblasts, n=87) had higher percentages of TM expression on monocytes (40.9% ± 2.3%) compared to patients in higher-risk groups (excess blasts-1 and -2, n=42) (24.2% ± 2.7%) and normal BM (n=25) (11.3% ± 3.0%). (C) Percentages of classical monocytes that are positive for TM in patients with low and higher blast counts. The percentage of monocytes expressing TM was significantly higher in the group of patients with blast counts below 5% (n=97) than in the group of patients with blast counts ≥5% (n=44) (blast count <5%, 41.3% ± 2.2% *vs.* blast count ≥5%, 25.7% ± 2.8%). Patients with ring sideroblasts (RS) (n=40) had higher percentages of TM$^+$ monocytes than patients who did not have RS (n=106) (RS yes, 45.4% ± 3.7% *vs.* RS no, 33.2% ± 2.0%). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. IPSS-R: Revised International Prognostic Scoring System; NBM: normal bone marrow; SLD: single lineage dysplasia; MLD: multilineage dysplasia;.RS: ring sideroblasts; EB, excess blasts.

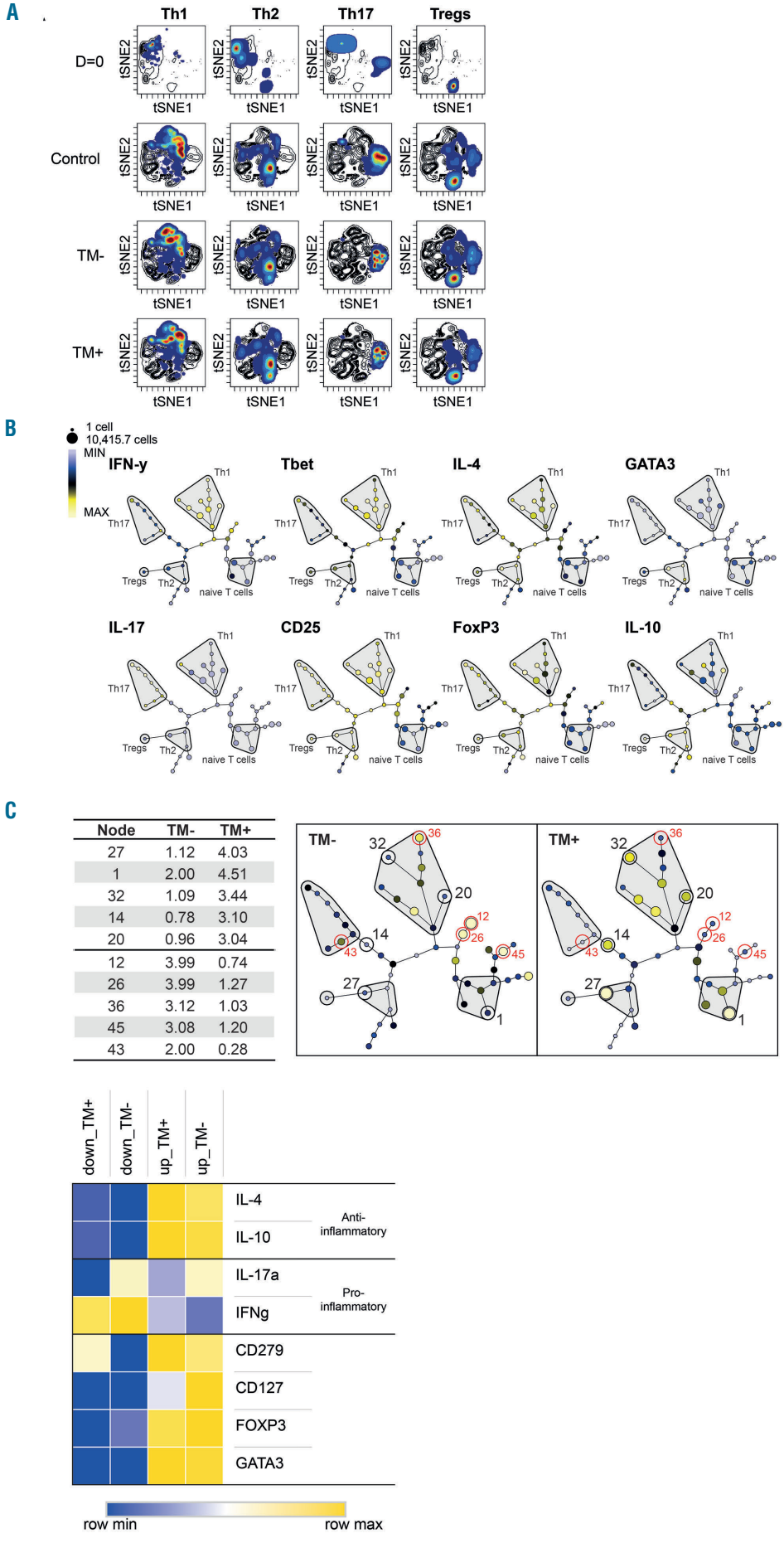

Figure 3. Deep phenotyping of T cells after co-culture with thrombomodulin-negative or –positive monocytes from patients with myelodysplastic syndromes. Healthy donor-derived CD4$^+$ T cells were co-cultured with thrombomodulin (TM)-negative or -positive monocytes from two patients with myelodysplastic syndromes (MDS), or in the stimulated control condition with plate-bound anti-CD3 antibody only. After culture for 5 days, T cells were stained with an extensive panel of surface and intracellular markers as well as stains for transcription factors and cytokines and analyzed using time of flight mass cytometry. (A) Viable T cells were identified and visualized using stochastic neighbor embedding (viSNE). Different T-cell subsets were identified based on interferon (IFN)-γ, GATA3, interleukin (IL)-17, IL-4, FoxP3 and CD127 (Th1 were considered to be IFN-γ$^+$, IL-17 and GATA3$^-$; Th2 were GATA3$^+$IL-4$^+$; Th17 were IL-17$^+$; Treg were CD127$^-$ and FoxP3$^+$CD25$^+$ and IFN-γ) (*Online Supplementary Figure S6*). Overlays were created from condition-specific biaxial viSNE contour plots and each individual T-cell subset. T-cell populations at the start of the experiment (D=0) and after 5 days of culture with anti-CD3 only, TM$^-$ monocytes or TM$^+$ monocytes are shown for a representative sample. (B) Cells were then further clustered with spanning-tree progression analysis of density-normalized events (SPADE) into 50 nodes using the clustering channels tSNE1 and tSNE2. Different clusters of nodes representing various T-cell subsets could be identified in the stimulated control condition by using selected markers. The color intensity in each node reflects the expression level of the indicated marker and the size of the node reveals the number of cells involved. (C) Frequencies of all clusters were compared between the TM$^-$ and TM$^+$ conditions. T-cell clusters that were most prevalent in the TM$^-$ condition are highlighted in red. Black circles represent clusters that show higher percentages in the TM$^+$ condition. The top five of highest frequencies for both conditions are shown. Using marker enrichment modeling (MEM), profiles of T-cell clusters with highest frequencies in TM$^-$ or TM$^+$ cells were characterized. Two subgroups of clusters were generated: (i) five nodes with highest frequency in the TM$^+$ condition (called "up") and (ii) five nodes with highest frequency in TM$^-$ condition (called "down"). Expression levels of given markers are shown for the TM$^+$ as well as the TM$^-$ conditions, showing that global expression in the identified set of clusters for the different markers is nearly similar in both conditions. MEM scores were calculated for the markers and results are presented in a heat map. The group of nodes that are present in a higher percentage in cultures with TM$^+$ monocytes compared to cultures with TM$^-$ monocytes reflect an anti-inflammatory profile. T cells in this group (all within "non-Treg nodes") express higher levels of FoxP3, GATA3, CD279 (PD-1), IL-4 and IL-10, a phenotype which suggests they are polarizing toward a Treg phenotype. tSNE, t-distributed stochastic neighbor embedding.

group of patients as well (Figure 4B). A difference in leukemia-free survival did not reach statistical significance, probably because of the small numbers of patients (Figure 4C). To test whether TM as a single marker has an independent prognostic value in overall and leukemia-free survival a multivariate Cox regression analysis with backward stepwise elimination was performed. Covariates that were included in this analysis were hemoglobin level, absolute neutrophil count, platelet count, bone marrow blast percentage, cytogenetic risk group and percentage of TM expression on monocytes. Information on all variables was available for 60 patients (not shown). Covariates with a *P* value >0.10 were removed. Both the cytogenetic risk group as well as the percentage of TM expression were predictive markers for overall survival (*P*=0.001 and *P*=0.064, respectively). For leukemia-free survival, the percentage of blasts and TM expression had predictive value (*P*=0.010 and *P*=0.077, respectively).

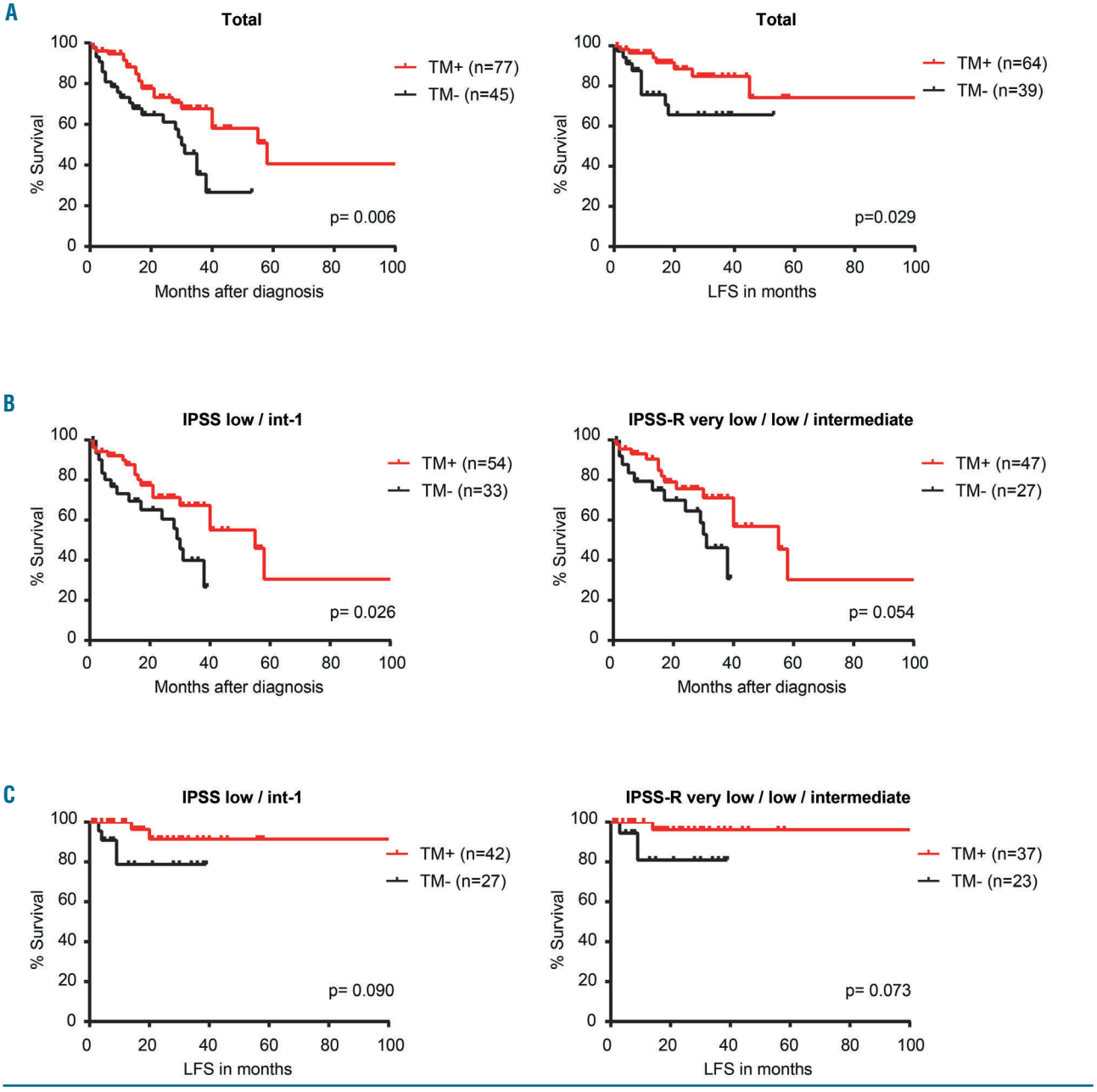

Figure 4. Overall and leukemia-free survival is related to the presence of thrombomodulin-positive monocytes. As a cut-off percentage for the presence of thrombomodulin (TM) on myelodysplastic syndrome (MDS) monocytes, the expression rate in the healthy donor cohort was used. The mean percentage + two standard deviations was calculated, resulting in a cut-off of 25.53%. Statistical differences were calculated using the log-rank test. (A) Overall survival data for 122 MDS patients and leukemia-free survival (LFS) data for 102 patients. A significant difference in overall survival was found between MDS patients with or without TM on BM monocytes (*P*=0.006). The median overall survival for patients with TM$^+$ monocytes was 58 months while that for patients without TM was 30 months. The time to development of leukemia was significantly longer for patients with TM expression than for patients without TM expression (*P*=0.029). (B) Patients were further selected based on their low-risk status. Survival curves are shown for low-risk patients (according to the International Prognostic Scoring System and its revision) with or without TM$^+$ monocytes. (C) LFS of low-risk patients in the TM$^+$ and TM$^-$ groups. IPSS: International Prognostic Scoring System; IPSS-R: Revised International Prognostic Scoring System.

Discussion

The BM microenvironment in MDS, and particularly in low-risk groups, is often characterized by the presence of pro-inflammatory cells and molecules. While increased inflammation and the subsequent cellular immune response are crucial to eliminate malignant cells, continuous immune stimulation could lead to genomic instability and inevitable malignant transformation.[12] Identifying the factors that could maintain a healthy overall immune response in MDS is important and clinically relevant. In mice, it has been shown that lack of the lectin-like domain of TM leads to reduced survival after exposure to endotoxins, whereas a recombinant form diminished NF-κB and MAPK activation.[45] In the current study, we therefore investigated the presence of TM on immune cells in MDS-derived BM and PB samples and its immune-modulatory role in MDS. TM was mainly expressed on monocyte subsets and not on granulocytes, lymphocytes or eosinophils. The difference in TM expression between monocytes from healthy donors and those from patients with MDS was most evident for classical monocytes. FISH analysis showed clonal involvement for these TM+ monocytes. This subset showed hardly any TM expression on cells derived from either healthy BM or healthy PB. In MDS patients there was marked variation in monocytes expressing TM. Some patients showed expression levels similar to those on healthy donor-derived monocytes, whereas for others nearly all monocytes expressed TM. We found that the percentage of TM expression was clearly higher in low-risk MDS groups than in higher-risk groups, suggesting that the presence of TM may be primarily observed in a pro-inflammatory environment (i.e. low-risk MDS). Interestingly, Talati *et al.* previously described a correlation between the presence of classical monocytosis in MDS and favorable prognostic factors such as increased white blood cell counts and absolute neutrophil counts.[46] Furthermore, an increased percentage of monocytes was associated with lower MDS risk groups and good-risk cytogenetics. SF3B1 was present at a higher frequency in this MDS group and overall survival of these patients tended to be better. In this perspective, it would be interesting to investigate the presence of TM in a same set of samples in future research.

Besides active secretion and passive release of inflammatory molecules in the MDS BM environment, certain T-cell subsets, particularly Th1 and Th17 type T cells, contribute to an immune-active state by the secretion of high amounts of interferon-γ and interleukin-17. In order to investigate the effect of monocytes on T-cell skewing, healthy T cells were cultured in the presence of TM+ or TM- MDS monocytes. Using mass cytometry, we were able to utilize a comprehensive panel of surface and intracellular markers for this purpose. Interestingly, T cells cultured in the presence of TM+ monocytes showed an anti-inflammatory skewed profile. Compared to T cells cultured with TM- monocytes, they showed less interferon-γ positivity and higher concentrations of interleukin-4 and interleukin-10 measured intracellularly. While the number of patients' samples was limited and data need to be interpreted with caution, our findings suggest that TM+ monocytes polarize T cells toward Th2 and/or Treg phenotypes.

Collectively, our data point to an interesting function for TM-expressing monocytes in the highly inflammatory environment of low-risk MDS patients. They could play an essential role in dampening disproportionate immune activation by inducing anti-inflammatory T-cell subsets. In keeping with this notion, overall and leukemia-free survival was better for patients in whom the BM contained TM+ monocytes than for patients lacking TM expression, supporting a clinically relevant mechanism. Nevertheless, in the longer term, this mechanism could lead to a profound immunosuppressive state which would prevent effective immune surveillance; a common condition in higher-risk MDS.

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