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High frequency of CD95$^+$/CD45RA$^-$ regulatory T cells defines an immunosuppressive profile associated with MDS progression

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Summary

Dynamic interactions between mutated haematopoietic cells and immune cells are key drivers of myelodysplastic neoplasms (MDS) initiation and progression. Regulatory T cells (Tregs) are central mediators of immunosuppression in MDS. We thus aimed to characterize Treg subpopulations in the bone marrow (BM) of MDS patients and to explore their clinical significance. Using mass cytometry and an unbiased multidimensional analytical approach, we first confirmed the presence in MDS BM of two Treg subsets, including the highly activated CD95$^+$/CD45RA$^-$ subpopulation previously reported in aplastic anaemia. We then prospectively analysed Tregs distribution in BM of 113 MDS patients at diagnosis and during follow-up. While Treg proportions among CD4$^+$ T cells were unchanged, CD95$^+$/CD45RA$^-$ Tregs were significantly expanded in the BM of both low- and high-risk MDS patients. CD95$^+$/CD45RA$^-$ Tregs accumulated during disease evolution but remained stable in patients responding to hypomethylating agents. At diagnosis, CD95$^+$/CD45RA$^-$ Tregs levels correlated with specific clinical features: low CD95$^+$/CD45RA$^-$ Tregs with *TET2/IDH* mutations and autoimmune manifestations; high CD95$^+$/CD45RA$^-$ Tregs with an increased risk of disease progression independently of the IPSS-R and the IPSS-M. Our findings suggest that profiling Treg subpopulations at diagnosis could improve MDS risk stratification and guide immunosuppressive therapeutic decisions.

KEYWORDS
myelodysplastic neoplasms, regulatory T cells

INTRODUCTION

Myelodysplastic neoplasms (MDS) are heterogeneous clonal diseases characterized by cytopenia(s) and dysplasia on myeloid cells that predispose the affected patient to acute myeloid leukaemia (AML).[1] Although it is now well known that specific genetic lesions occurring in myeloid progenitors have a key role in the development of MDS,[2] the pathogenesis of these diseases is multifactorial and also involves extrinsic changes in the bone marrow microenvironment (BMM). Indeed, dynamic interplay between mutated haematopoietic cells and both mesenchymal and immune cell populations is believed to be critical to the pathogenesis and the outcome of MDS. Clinical and laboratory findings clearly suggest that immune dysregulation such as expansion of myeloid-derived

[Correction added on 15 June 2026, after first online publication: The subcategory has been changed.]

suppressor cells (MDSCs),[3–5] altered T-cell homeostasis and increased levels of pro-inflammatory cytokines[6–10] and functional impairment of natural killer (NK) cells[11–14] may represent a key driver for genesis and evolution of MDS.[15] The implication of immune deregulation is also attested by the high rate of systemic inflammatory and autoimmune disorders (SIADs) observed in MDS patients (10%–20% of cases) and the clinical benefit of hypomethylating agents (HMAs) on these clinical manifestations.[16–20] In this context, immunosuppressive therapies (ISTs) have been used for treating patients with low-risk MDS (LR-MDS), leading to a durable objective response in 48.8% of cases.[21] However, IST usage is limited due to the absence of reliable predictive biomarkers and concerns about potentially accelerating disease progression by impairing the immune system's capacity to control malignant clones. There is, therefore, growing interest in examining immune BMM at diagnosis to identify biomarkers that can predict immune escape, guide IST decisions and anticipate patient outcomes, especially in LR-MDS.[22]

Regulatory T cells (Tregs) play a critical role as they naturally suppress immune responses.[23] Studies indicate an increase of Tregs in high-risk MDS (HR-MDS),[24–26] and their elevated amounts are linked to poor progression-free and overall survivals in LR-MDS patients.[27] However, our understanding of the contribution of Tregs to LR-MDS development and progression remains limited[25,28] mainly due to insufficient characterization of the different subpopulations of Tregs, especially in the BMM. Indeed, Tregs consist of diverse subsets with varying immunosuppressive functions. This has been illustrated in aplastic anaemia, where a Tregs subset defined by a $CD95^+/CD45RA^-$ phenotype, mainly corresponding to effector Tregs as classically defined by CD45RA and Foxp3 expression, exhibits high immunosuppressive activity and has been shown to correlate with response to IST.[29,30]

Based on this, we conducted a prospective study to analyse the BM distribution of different Treg subsets in MDS patients at diagnosis and during the follow-up of the disease, focusing on the previously reported highly immunosuppressive $CD95^+/CD45RA^-$ subpopulation.[29] We found that the proportion of $CD95^+/CD45RA^-$ Tregs was increased in the BM of MDS patients at diagnosis and closely related to specific MDS features, notably the outcome of these patients. Our findings reveal therefore that analysing Tregs distribution could serve as a new biomarker for immunological escape, aiding clinical decision-making and potentially enhancing the MDS diagnostic workflow.

MATERIALS AND METHODS

Patients

This study prospectively enrolled 113 patients between January 2018 and January 2022 for BM investigations in the context of MDS suspicion which was confirmed by morphological and cytogenetic analyses according to the WHO criteria.[31] MDS prognosis was assessed using the IPSS-R and the IPSS-M and HR-MDS were defined based on an IPSS-R score >3.5 and an IPSS-M score >0.[32–34] All investigations were approved by the institutional review boards and the procedures were in accordance with the Helsinki Declaration. This study also included 53 non-MDS samples used as control (CTL): (1) 11 BM samples were from healthy elderly patients obtained from the bone of the femoral head (FH) after informed consent, during a surgery for hip replacement (non-interventional study approved by the local ethical committee; CLEP Decision No: AAA-2020-08039); (2) 30 BM samples were from patients without cytopenia referred to our institutions for BM aspiration in a context of suspicion of different haematological neoplasms (mastocytosis, lymphoma or myeloma) without excess of abnormal cells detected by morphology and flow cytometry and (3) 12 BM samples were from patients with cytopenia for whom a diagnosis of MDS was excluded based on morphological analysis and on the identification of a non-MDS-related cause (transient neutropenia, $n=5$; inflammation-associated anaemia, $n=4$; vitamin deficiency, chronic kidney disease, blood loss anaemia, $n=1$).

Mass and conventional cytometry

Mass cytometry was performed on frozen samples (fetal bovine serum with 10% dimethyl sulphoxide) as previously described.[35] Staining for conventional flow cytometry (FCM) was performed within 24 h following aspiration on 2 million BM cells after one wash of whole BM in Dulbecco's phosphate-buffered saline (PBS, Eurobio). Cells from femoral heads were obtained as previously described.[36] See Supplemental materials and methods for detailed protocols and Table S1 for the list of markers.

Genomic testing

Genomic deoxyribonucleic acid extracted from BM aspiration was studied by high-throughput sequencing (HTS) of 45 genes recurrently mutated in myeloid malignancies as previously described.[37] IPSS-M mean was calculated with missing data for two main effects genes (*NPM1* and *MLL* PTD) and for four residuals genes (*CEBPA*, *GNB1*, *NF1* and *PRPF8*).[33]

Statistics

Progression-free survival (PFS) defined according to International Working Group 2023 criteria[34] was estimated using the Kaplan–Meier method and then compared using the log-rank test. The follow-up of patients was censored at the time of last contact. PFS was analysed using a standard Cox proportional hazards model. Calculations were performed using R 3.6.1 (cran.rproject.org) and graphs were drawn using Graph Pad Prism software (San Diego, CA). Excepted when mentioned, *p*-values are reported

without adjustment for multiple testing and are intended for exploratory purposes only.

RESULTS

Identification of Treg subsets in BM MDS samples

Using mass cytometry and an unbiased multidimensional analytical approach, we analysed three MDS BM samples to deeply analyse the phenotypic landscape of $CD4^+$ T cells and more specifically of Tregs. For that, BM mononuclear cells were stained with metal-tagged antibodies against surface and intracellular markers including known markers for Tregs (Foxp3, CD25 and CD127), naïve/memory subsets, homing/trafficking receptors and differentiation/activation markers (see panel in Table S1). After initial gating for $CD3^+$/$CD4^+$/$CD8^-$ T cells, the gated cells were clustered using the Uniform Manifold Approximation and Projection (UMAP) algorithm and Tregs were identified based on their $Foxp3^{hi}$/$CD25^{hi}$/$CD127^{lo}$ phenotype (Figure 1A). Interestingly, density plot analysis and marker expression profiling (CD95, CD45RA, CD45RO, ICOS, CD39, CCR4, CTLA-4) revealed a heterogeneous distribution within the Treg cluster, suggesting the presence of two distinct subpopulations (Figure 1A,B; Figure S1). Phenotypic characterization of these subpopulations closely mirrored those described in aplastic anaemia[29]: a naïve-like CCR7+/CD45RA+ Treg subpopulation #1 and a subpopulation Tregs #2 with expression of markers associated with immunosuppressive function and activation, including CD95, CCR4, CD39 and CTLA-4 (Figure 1B,C). To validate these findings, conventional FCM was performed on one of the three MDS BM samples, confirming the presence of the two Treg subpopulations based on their distinct CD95 and CD45RA expression patterns (Figure 1C,D). Collectively, these data strongly support the existence of functional heterogeneity within the BM Treg compartment of MDS patients.

$CD95^+$/$CD45RA^-$ Tregs are increased in the BM of MDS patients

Next, we hypothesized that the relative proportion of these two subpopulations may influence the immunosuppressive activity of the whole Treg population in MDS at diagnosis and/or during disease progression. Thus, we used conventional FCM to prospectively quantify the Treg population among $CD4^+$ T cells and assess the proportion of $CD95^+$/$CD45RA^-$ Tregs within total Tregs in a large cohort of MDS BM samples at the time of diagnosis ($n=113$), including 77 LR-MDS (IPSS-R ≤ 3.5) and 36 HR-MDS (IPSS-R > 3.5) (characteristics in Table 1) (Figure 2A). Different types of non-MDS BM samples ($n=53$) were used as CTL (characteristics in Table S2). As no significant differences were observed among the various non-MDS conditions regarding Treg frequency within $CD4^+$ T cells and the proportion of $CD95^+$/$CD45RA^-$ Tregs (Figure S2A,B), these were collectively analysed as CTL BM samples. In the MDS cohort, we found that the proportion of Tregs among $CD4^+$ T cells was not significantly different compared to CTL (7.8% and 7.7%, respectively; adjusted $p=0.99$), regardless the MDS risk stratification (7.7% in LR-MDS vs. 7.8% in HR-MDS; adjusted $p=0.98$) (Figure 2B). However, the proportion of $CD95^+$/$CD45RA^-$ Tregs among the total Tregs population was significantly higher in the BM from MDS patients (69.7% vs. 60.7% in CTL; adjusted $p=0.0007$), including at early stage of the disease (65% in LR-MDS; adjusted $p=0.0136$) (Figure 2C). The level of $CD95^+$/$CD45RA^-$ Tregs was also significantly higher in HR-MDS (72.4%) than in LR-MDS (adjusted $p=0.0125$), suggesting a progressive accumulation of $CD95^+$/$CD45RA^-$ Tregs in the BM during the course of the disease.

High proportion of $CD95^+$/$CD45RA^-$ Tregs is associated with specific biological and clinical features of MDS

We next investigated whether BM proportions of Tregs and/or $CD95^+$/$CD45RA^-$ Tregs correlated with specific biological and clinical features of MDS. While no statistically significant differences were detected based on the WHO classification, certain trends in the distribution of BM Tregs appeared within specific MDS subtypes with defining genetic abnormalities. Indeed, patients with MDS-del5q were characterized by the lowest level of Tregs (5.9%) and the lowest proportion of $CD95^+$/$CD45RA^-$ Tregs (62.1%), suggesting a globally weakly immunosuppressive BMM (Figure 3A,B). In contrast, patients with MDS-bi*TP53* displayed a highly immunosuppressive profile, as evidenced by the highest frequencies of both Tregs (9.4%) and $CD95^+$/$CD45RA^-$ Tregs (82.2%) (Figure 3A,B).

We also focused on clinical manifestations of SIADs in a subgroup of 92 MDS patients with available clinical data. We identified 19 patients (21%) with a history of SIADs (Table 1), but the global frequency of Tregs was similar in both groups (7.9%; $p=0.8426$; Figure 3C). However, the proportion of $CD95^+$/$CD45RA^-$ Tregs was significantly lower in patients with SIADs (60% vs. 71.6%; $p=0.0064$; Figure 3D). Given the higher proportion of $CD95^+$/$CD45RA^-$ Tregs in HR-MDS (Figure 2C) and that SIADs were mostly observed in LR-MDS patients (15/19), we focused our analysis on LR-MDS patients. We also observed a significantly lower proportion of $CD95^+$/$CD45RA^-$ Tregs in patients with SIADs (58.0% vs. 67.2%; $p=0.0311$; Figure 3D), highlighting the specific link of this highly immunosuppressive subset of Tregs with autoimmune disorders.

Given the global heterogeneity of $CD95^+$/$CD45RA^-$ Tregs frequency in BM MDS samples, the proportion of $CD95^+$/$CD45RA^-$ Tregs was re-coded as categorical variable using the 33th and 67th percentiles (Figure 3E). We next analysed characteristics of MDS patients stratified

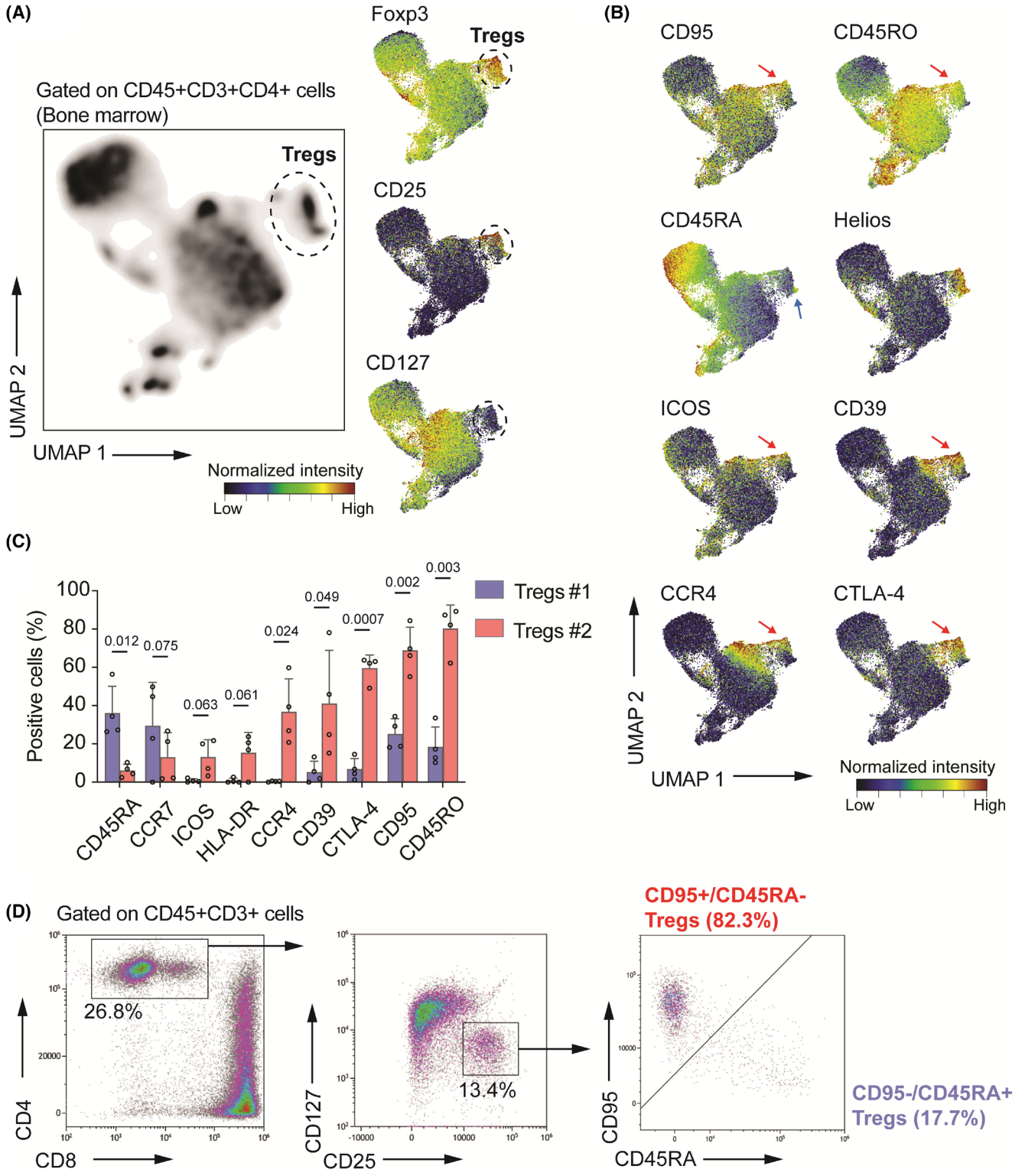


FIGURE 1 Identification of regulatory T-cell (Treg) subsets in bone marrow (BM) MDS samples. (A) Uniform manifold approximation and projection (UMAP) density plot of BM CD4+ T cells analysed by mass cytometry. Merged data from three different MDS patients with low, intermediate and high IPSS-R (see Figure S1 for individual analysis). Tregs were identified using Foxp3, CD25 and CD127. (B) Normalized expression intensities of selected markers were calculated and overlaid on the UMAP plot using mass cytometry data. Blue arrows indicate Treg subpopulation #1 and red arrows indicate Treg subpopulation #2. (C) Expression of markers between the two subpopulation of Tregs measured by mass cytometry in BM from four MDS patients. Means ± standard deviation, statistical significance was determined using paired *t*-test, two-tailed. (D) Gating strategy to identify Treg subsets by conventional flow cytometry (FCM) based on CD95 and CD45RA expression.

TABLE 1 Presenting features of all MDS patients and of patients with LOW, INT or HIGH proportion of $CD95^+/CD45RA^-$ Tregs in the BM.

		$CD95^+/CD45RA^-$ Tregs/Tregs (%)			
Variables	**All ($n=113$)**	**LOW ($n=38$)**	**INT ($n=37$)**	**HIGH ($n=38$)**	***p* value**
Age, years, median (range)	75 (42–94)	75 (42–86)	74 (55–89)	76 (56–94)	0.3443
Males, *n* (%)	69 (61)	20 (53)	25 (68)	24 (63)	0.3936
Hb, g/dL, median (range)	10.5 (5.5–15.4)	10.9 (6.5–14.4)	10.3 (5.5–14.7)	10.4 (6.8–15.4)	0.3775
ANC, $\times 10^9$/L, median (range)	2.13 (0.36–12.24)	1.9 (0.36–7.68)	1.88 (0.52–8.49)	2.76 (0.37–12.24)	0.1968
Plt, $\times 10^9$/L, median (range)	122 (10–584)	183 (10–453)	114 (39–584)	111 (45–560)	0.2156
BM blast %, median (range)	3 (0–17)	3 (0–17)	4 (0–17)	3 (0–11)	0.1732
Abnormal Karyotype, *n* (%)	55 (49)	14 (37)	18 (49)	23 (61)	0.1185
Very good/good	81 (72)	32 (84)	24 (65)	25 (66)	0.1089
Intermediate/poor/very poor	32 (28)	6 (16)	13 (35)	13 (34)	
WHO (2022), *n* (%)					
MDS-LB	56 (50)	21 (55)	19 (51)	16 (42)	**0.0051**
MDS-LB-RS	10 (9)	4 (11)	3 (8)	3 (8)	
MDS-5q	4 (3)	2 (5)	1 (3)	1 (3)	
MDS-IB1	25 (22)	10 (26)	5 (13)	10 (26)	
MDS-IB2	9 (8)	0 (0)	8 (22)	1 (3)	
MDS-TP53	9 (8)	1 (3)	1 (3)	7 (18)	
IPSS-R, *n* (%)[a]					
Low risk (≤3, 5)	77 (68)	32 (84)	21 (57)	24 (63)	**0.0278**
High risk (>3, 5)	36 (32)	6 (16)	16 (43)	14 (37)	
IPSS-M, *n* (%)[b]					
Low risk (<0)	55 (65)	26 (81)	13 (57)	16 (55)	0.0578
High risk (>0)	29 (34)	6 (19)	10 (43)	13 (45)	
SIADs, *n* (%)	19/92 (21)[c]	13/34 (38)	4/27 (15)	2/31 (6)	**0.0045**
Mutated cases, *n* (%)	68/84 (81)[d]	27/32 (84)	17/23 (74)	24/29 (83)	0.5935
Number of mutations, median (range)	2 (0–8)	2 (0–7)	2 (0–8)	2 (0–5)	0.9225

Note: Comparisons between patients with LOW, INT or HIGH proportion of $CD95^+/CD45RA^-$ Tregs (identified based on the 33rd and 67th percentiles of BM $CD95^+/CD45RA^-$ Tregs proportions as illustrated in Figure 3E) were performed using a one-way analysis of variance (ANOVA) for continuous variables and chi-square test for categorical variables (with combination of categories to meet the assumptions of the chi-square test when necessary). For WHO 2022 MDS categories, chi-square *p*-value was estimated via Monte Carlo simulation due to low expected counts; all categories were kept intact. Significant *p*-values are highlighted in bold.

Abbreviations: ANC, absolute neutrophils count; BM, bone marrow; Hb, haemoglobin; MDS-5q, MDS with low blasts and with isolated del(5q); MDS-IB1/2, MDS with increased blasts 1/2; MDS-LB, MDS with low blasts; MDS-LB-RS, MDS with low blasts and ring sideroblasts; NA, not available; Plt, Platelet.

[a]Detailed repartition of all MDS patients according to the IPSS-R: very low, $n=28$ (25%); low, $n=40$ (35%); intermediate, $n=19$ (17%); high, $n=15$ (13%); very high, $n=11$ (10%).

[b]Detailed repartition of all MDS patients according to the IPSS-M: very low, $n=12$ (14%); low, $n=38$ (45%); intermediate low, $n=5$ (6%); intermediate high, $n=12$ (14%); high, $n=5$ (6%); very high, $n=12$ (14%).

[c]Rheumatoid arthritis ($n=6$), sarcoidosis ($n=2$), hypothyroidism ($n=2$), systemic sclerodermia ($n=1$), polychondritis ($n=1$), pyoderma gangrenosum ($n=1$), giant cell arteritis ($n=1$), inflammatory bowel disease ($n=1$), scleroderma ($n=1$), polymyalgia rheumatica ($n=1$), cryoglobulinaemia ($n=1$), sweet syndrome ($n=1$).

[d]Cases with high-throughput sequencing (HTS) data, $n=84$.

according to LOW, intermediate (INT) or HIGH level of $CD95^+/CD45RA^-$ Tregs. Stratification based on the proportion of $CD95^+/CD45RA^-$ Tregs was significantly associated with distinct distributions of the WHO 2022 classification ($p=0.0051$), IPSS-R risk categories ($p=0.0278$) and the presence of SIAD ($p=0.0045$) and showed a trend towards significance for IPSS-M ($p=0.0578$) (Table 1). The proportion of mutated cases and the median number of mutations were similar in all groups (Table 1). Nevertheless, a LOW proportion of $CD95^+/CD45RA^-$ Tregs was significantly more frequently associated with somatic mutations in genes encoding for epigenetic factors ($p=0.0198$), among which *TET2/IDH* were the most frequently mutated ($p=0.0142$) (Figure 3F,G). In contrast, patients with HIGH level of $CD95^+/CD45RA^-$ Tregs were characterized by a higher frequency of mutations in genes encoding for transcription factors ($p=0.0184$), such as *TP53* and *RUNX1* (Figure 3F,G). *ASXL1* mutation was also more frequently detected in patients with HIGH level of $CD95^+/CD45RA^-$ Tregs (Figure 3G). We similarly compared the characteristics of MDS patients with LOW or HIGH level of Tregs among total $CD4^+$ T cells (Figure S3A), but no

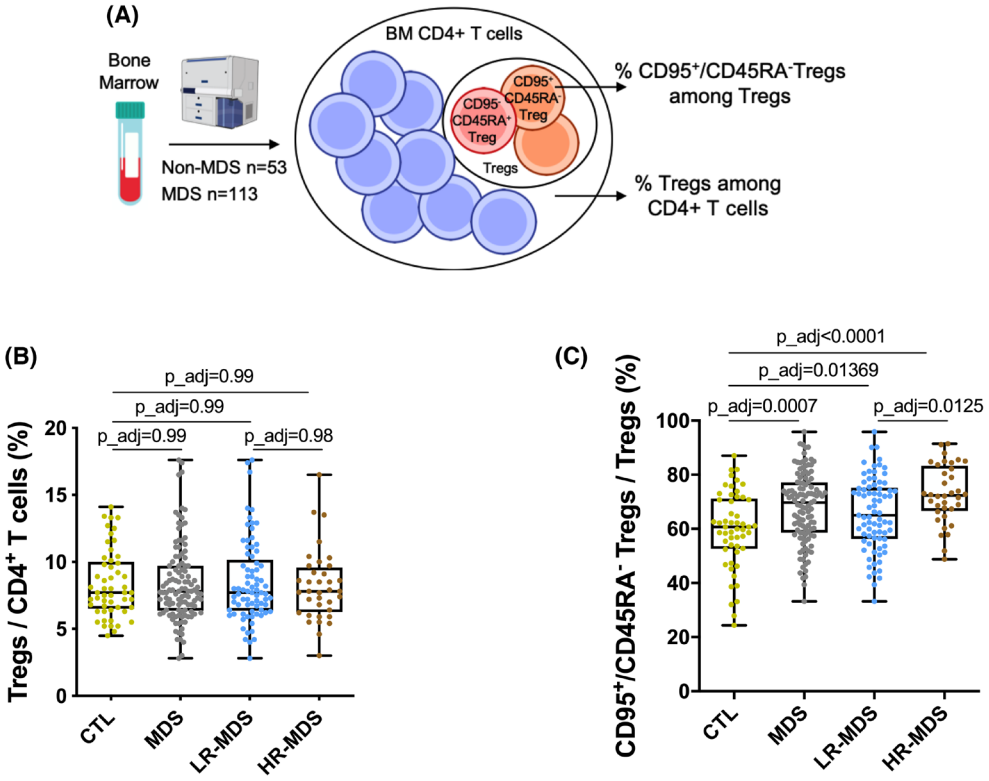


FIGURE 2 MDS BM is characterized by an expansion of $CD95^+/CD45RA^-$ regulatory T cells (Tregs). (A) Schematic overview of flow cytometry data acquisition. Proportion of Tregs among total $CD4^+$ T cells (B) and of $CD95^+/CD45RA^-$ Tregs among the total population of Tregs (C), in control (CTL) samples ($n = 53$), in all MDS samples ($n = 113$) and in low-risk MDS (LR-MDS) ($n = 77$)/high-risk MDS (HR-MDS) ($n = 36$) defined as IPSS-R ≤ or >3.5 respectively. Multiple comparisons were performed using one-way analysis of variance (ANOVA) test and p-values were adjusted for multiple testing using the Holm–Sidak method.

obvious correlations were seen between the level of Tregs and blood counts, the cytogenetic profile, the IPSS-R and the genetic profile (Table S3 and Figure S3B,C). Altogether, these results confirmed therefore the specific relevance of clinical and biological associations with the frequency of BM $CD95^+/CD45RA^-$ Tregs.

MDS patients with elevated frequency of $CD95^+/CD45RA^-$ Tregs have a higher risk of MDS progression

We next followed the level of both Tregs and $CD95^+/CD45RA^-$ Tregs during the course of the disease. We observed in 17 untreated patients with stable LR-MDS (attested by a stable BM blast cell count <5%) but with worsening cytopenias, that unlike the level of Tregs, the proportion of $CD95^+/CD45RA^-$ Tregs significantly increased during follow-up (Figure 4A). However, this increase was no longer observed in patients with hypomethylating agent-mediated stabilization of the disease (Figure 4B). We also investigated the PFS of 90 MDS patients with available follow-up data stratified according to their Tregs and $CD95^+/CD45RA^-$ Tregs proportion at diagnosis. The global log-rank test revealed a significant difference in PFS among the three groups of patients stratified by their $CD95^+/CD45RA^-$ Treg proportion ($p = 0.0375$). However, no significant trend across ordered categories was observed ($p = 0.2913$). The overall difference appeared to be driven by specific group comparisons, as patients with a HIGH baseline proportion of $CD95^+/CD45RA^-$ Tregs had a significantly shorter PFS compared with those with a LOW proportion (26 months vs. not reached; $p = 0.0075$) (Figure 4C). For descriptive and exploratory purposes, subgroup analyses of patients classified according the IPPS-R and the IPSS-M were also performed. Similar trends were observed in both LR- and HR-MDS defined by the IPSS-R ($p = 0.1868$ and $p = 0.0164$, respectively) as well as in the subgroup of LR- and HR-MDS defined by the IPSS-M ($p = 0.0985$ and $p = 0.0034$, respectively) (Figure S4A,B). In contrast, stratification of MDS patients based on the proportion of Tregs among $CD4^+$ T cells in the BM at diagnosis did not reveal any significant difference in the risk of progression (Figure S5A), including when patients were categorized as LR- or HR-MDS according to the IPSS-R or IPSS-M (Figure S5B,C). Finally, in multivariate Cox analysis, HIGH frequency of $CD95^+/CD45RA^-$ Tregs, but not of Tregs, was found to be associated with a higher risk of progression, independently of the IPSS-R (HR = 2.131; 95% confidence interval (CI): 1.2–3.79, $p = 0.00992$) and the IPSS-M (HR = 2.58; 95% CI: 1.33–5.03, $p = 0.0053$) (Figure 4D,E). Altogether,

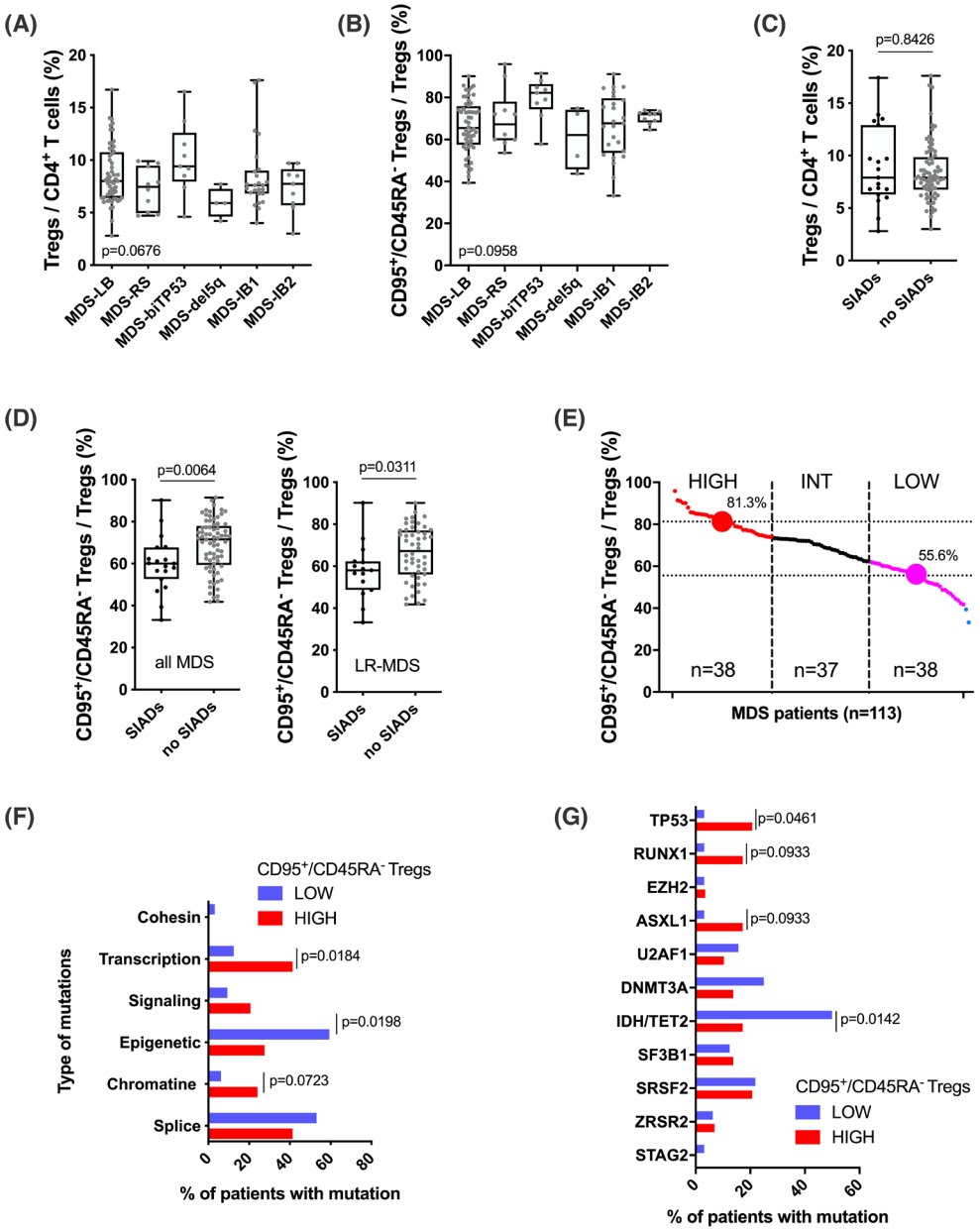
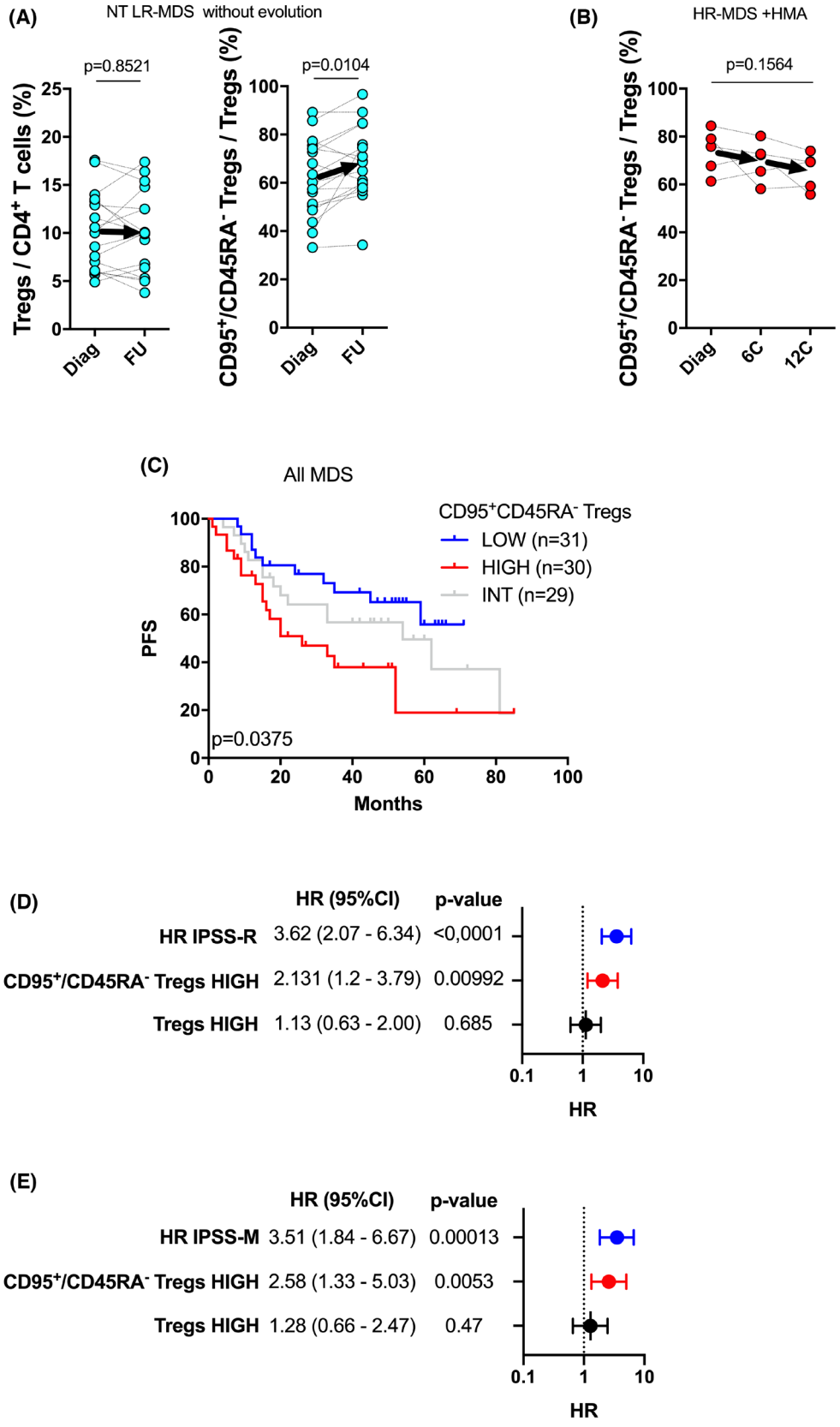


FIGURE 3 Specific patterns of CD95$^+$/CD45RA$^-$ regulatory T-cell (Treg) repartition are associated with specific entities of MDS and with clinical manifestations of systemic inflammatory and autoimmune disorders (SIADs). Proportion of Tregs among total CD4$^+$ T cells (A) and of CD95$^+$/CD45RA$^-$ Tregs among the total population of Tregs (B), in MDS samples classified according the World Health Organization (WHO) 2022 classification (MDS with low blasts (MDS-LB), $n = 56$; MDS with ring sideroblasts (MDS-RS, $n = 10$; MDS-bi*TP53*, $n = 9$; MDS-5q, $n = 4$; MDS-IB1, $n = 25$; MDS-IB2, $n = 9$). Statistical significance was calculated using a Kruskal–Wallis test. (C) Proportion of Tregs among total CD4$^+$ T cells in MDS samples with ($n = 19$) or without ($n = 73$) clinical manifestations of SIADs. Statistical significance was determined using unpaired two-tailed Mann–Whitney tests. (D) Proportion of CD95$^+$/CD45RA$^-$ Tregs among the total population of Tregs in MDS samples with ($n = 19$) or without ($n = 73$) clinical manifestations of SIADs (left panel), in low-risk MDS (LR-MDS) samples with ($n = 15$) or without ($n = 50$) clinical manifestations of SIADs (right panel). Statistical significance was determined using unpaired two-tailed Mann–Whitney tests. (E) Proportion of BM CD95$^+$/CD45RA$^-$ Tregs stratified into three groups of MDS patients based on the 33rd and 67th percentiles. Median proportion of the group of MDS patients with HIGH or LOW proportion of CD95$^+$/CD45RA$^-$ Tregs (81.3% and 55.6% respectively) is highlighted. The prevalence of somatic mutations in mains groups of genes (F) and in oncogenes and leukaemia-relevant genes (G) in BM from MDS patients with LOW ($n = 32$) or HIGH ($n = 29$) level of CD95$^+$/CD45RA$^-$ Tregs. Statistical significance was calculated using Fisher's exact tests.

these results indicated that the detection of HIGH proportion of CD95$^+$/CD45RA$^-$ Tregs but not of Tregs is an attractive biomarker for predicting the outcome of MDS patients, including from early stage of the disease and for monitoring their follow-up.

DISCUSSION

Growing evidence suggests that the immune system plays a crucial role in the pathophysiology of MDS, influencing both disease progression and patient outcomes. Indeed,



dysregulations of the immune system are frequent in MDS patients and can initially contribute to cytopenias but also thereafter increase the likelihood of disease progression to AML. This can be explained by the progressive modulation of the immune system from an initial activated protective state targeting both normal and aberrant haematopoietic

FIGURE 4 CD95$^+$/CD45RA$^-$ regulatory T cells (Tregs) accumulate during evolution of MDS and a high proportion of CD95$^+$/CD45RA$^-$ Tregs at diagnosis is associated with a higher risk of progression. (A) Dynamic of Tregs proportion among total CD4$^+$ T cells (left panel) and of CD95$^+$/CD45RA$^-$ Tregs proportion among the total population of Tregs (right panel), during the follow-up (FU) of non-treated (NT) low-risk MDS patients with stable disease (attested by a stable bone marrow [BM] blast cell count) ($n = 17$; median FU of 17 months; range: 4–48 months). Black arrows indicate the evolution of the mean value between diagnosis (Diag) and FU. Statistical significance was determined using Wilcoxon matched-pairs signed rank tests. (B) Dynamic of Tregs proportion among total CD4$^+$ T cells (left panel) and of CD95$^+$/CD45RA$^-$ Tregs proportion among the total population of Tregs (right panel), during the FU of high-risk MDS (HR-MDS) patients treated by hypomethylating agents (HMA) (after 6 and 12 cures) leading to stabilization of the disease based on the BM blast cell count ($n = 5$).Black arrows indicate the evolution of the mean value between diagnosis (Diag) and FU. Statistical significance was determined using Wilcoxon matched-pairs signed rank tests. (C) Progression-free survival (PFS) curves of MDS patients with a median follow-up of 35 months (range: 5–85 months) classified according to their LOW, INT or HIGH baseline proportion of CD95$^+$/CD45RA$^-$ Tregs. The *p*-value shown corresponds to the comparison using a global log-rank test across the three groups of patients. The pairwise comparison between the HIGH and LOW groups showed a significant difference ($p = 0.0075$). (D) Multivariable Cox analysis for PFS including IPSS-R risk status (high vs. non-high risk), high versus low proportion of CD95$^+$/CD45RA$^-$ Tregs and high versus low proportion of Tregs ($n = 95$). (E) Multivariable Cox analysis for PFS including IPSS-M risk status (high vs. non-high risk), high versus low proportion of CD95$^+$/CD45RA$^-$ Tregs and high versus low proportion of Tregs ($n = 77$). HR, hazard ratios.

stem cells, to a more immunosuppressive response allowing the expansion of malignant clones. Given their crucial role in maintaining immune tolerance, the analysis of Tregs has garnered significant interest due to their potential involvement in disease pathogenesis, progression and their implications for therapeutic strategies. In this study, we identified by mass cytometry two distinct Treg subpopulations in the BM of MDS patients, including the highly activated and immunosuppressive CD95$^+$/CD45RA$^-$ Tregs subset previously described in aplastic anaemia.[29] These results confirmed therefore the relevance of not only assessing overall Treg levels but also investigating the distribution of their subpopulations in the MDS BMM. Indeed, we clearly demonstrated in a large prospective cohort of MDS that the global frequency of Tregs among CD4$^+$ T cells was not significantly different in the BM of MDS patients compared to CTL, including in patients with HR-MDS. Nevertheless, we observed a significant increase of the proportion of the highly immunosuppressive CD95$^+$/CD45RA$^-$ Tregs subpopulation, and this, not only in HR-MDS but also in LR-MDS, suggesting therefore that these cells progressively accumulate in the BM of MDS patients during the course of the disease. Interestingly, this was confirmed in a subgroup of LR-MDS with worsening cytopenias, in whom we observed an increase of the proportion of CD95$^+$/CD45RA$^-$ Tregs during the FU despite a global stability of the disease.

Furthermore, exploratory analyses suggested that BM frequencies of CD95$^+$/CD45RA$^-$ Tregs may vary according to certain clinical and biological characteristics of MDS. Although based on a relatively low number of cases, we observed opposite patterns of Tregs repartition in two specific subtypes of MDS genetically defined. Indeed, BM from patients with bi-*TP53* MDS was characterized by the highest frequency of both Tregs and of CD95$^+$/CD45RA$^-$ Tregs. This trend towards an immunosuppressive BM profile is consistent with previous reports describing an expansion of Tregs with strong suppressive ability in the BM of MDS and secondary AML with *TP53* mutations.[38] Conversely, MDS-del5q harboured a low frequency of Tregs and a low proportion of CD95$^+$/CD45RA$^-$ Tregs, which we supposed may be restored by the immunomodulatory activity of Lenalidomide as previously reported.[28] In addition, we found that patients with SIADs harboured a low level of CD95$^+$/CD45RA$^-$ Tregs and that low proportion of CD95$^+$/CD45RA$^-$ Tregs was associated with *TET2/IDH* mutations. This makes sense with the previous finding that *TET2/IDH* mutations were more frequent in patients with SIADs.[17] Finally, we demonstrated that a high proportion of CD95$^+$/CD45RA$^-$ Tregs, but not of Tregs, was associated with a higher risk of progression, independently of both the IPSS-R and the IPSS-M. Although less powerful than the IPSS-R or IPSS-M, quantification of CD95$^+$/CD45RA$^-$ Tregs may still provide valuable complementary information for patient stratification by capturing features of a globally immunosuppressive BMM. This approach may be particularly relevant in LR-MDS, a subgroup in which a subset of patients can experience rapid and unexpected disease progression despite favourable baseline risk classification. In addition, we can hypothesize that the quantification of BM CD95$^+$/CD45RA$^-$ Treg may guide the use of IST. Indeed, the detection of a globally immunosuppressive BMM could indicate a reduced likelihood of benefit from IST. Moreover, further immune suppression in this setting could theoretically favour disease progression by limiting residual immune control. However, this hypothesis warrants further investigation.

Obviously, one inherent limitation of our study is that, although we focused on the BM to directly assess the immune microenvironment at the site of disease initiation and progression, this approach only allowed for a relative analysis of Treg proportions without providing information on their absolute counts. In line with recent proposals from the i4MDS consortium,[22] future studies should investigate whether peripheral blood analysis could enhance and/or complement BM-based immune monitoring in MDS. In addition, given the genetic complexity of these diseases and the critical contribution of various immune cell populations beyond Tregs to the maintenance of immune homeostasis, it is expected that a larger immune profiling should be necessary to improve patient stratification, particularly for patients with intermediate proportion of CD95$^+$/CD45RA$^-$ Tregs, and to identify predictive markers for treatment

responsiveness. Nevertheless, based on our results, we anticipate that the quantification of $CD95^+/CD45RA^-$ Tregs may represent an initial framework for implementing a broader strategy, thereby refining the understanding of immune dysregulation and disease progression in MDS.

AUTHOR CONTRIBUTIONS

Romain Vazquez, Pierre Boncoeur, Camille Knosp, Amandine Houvert and Nicolas Deredec performed flow cytometry experiments. Nicolas Deredec and Yannick Simoni performed mass cytometry experiments and data analysis and interpretation. Romain Vazquez, Loria Zalmai, Chloe Friedrich, Carole Almire, Charles Dussiau, Ismael Boussaid and Olivier Kosmider collected biological data. Lise Willems, Rudy Birsen, Justine Decroocq and Didier Bouscary recruited patients and collected clinical data. Shahram Kordasti, Michaela Fontenay and Yannick Simoni interpreted data and revised the manuscript. Nicolas Chapuis designed the study, collected biological and clinical data, analysed and interpreted data and wrote the manuscript. All authors edited and approved the paper for submission.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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