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Bone marrow $CD3^+CD56^+$ regulatory T lymphocytes (T_{R3-56} cells) are inversely associated with activation and expansion of bone marrow cytotoxic T cells in IPSS-R very-low/low risk MDS patients

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Abstract

Background: Emergence of dysplastic haematopoietic precursor/s, cytopenia and variable leukaemia risk characterise myelodysplastic syndromes (MDS). Impaired immune-regulation, preferentially affecting cytotoxic T cells (CTL), has been largely observed in MDS. Recently, we described the T_{R3-56} T cell subset, characterised by the co-expression of CD3 and CD56, as a novel immune-regulatory population, able to modulate cytotoxic functions. Here, we address the involvement of T_{R3-56} cells in MDS pathogenesis/progression.

Objectives: To analyse the relationship between T_{R3-56} and CTL activation/expansion in bone marrow (BM) of very-low/low-risk MDS subjects.

Methods: Peripheral blood and BM specimens, obtained at disease onset in a cohort of 58 subjects, were analysed by immune-fluorescence and flow cytometry, to preserve the complexity of the biological sample.

Results: We observed that a trend-increase of BM T_{R3-56} in high/very-high MDS stage, as compared with very-low/low group, associates with a decreased activation of BM resident CTL; significant correlation of T_{R3-56} with BM blasts has been also revealed. In addition, in very-low/low-risk subjects the T_{R3-56} amount in BM inversely correlates with the presence of activated BM CTL showing a skewed Vβ T-cell repertoire.

Conclusions: These data add T_{R3-56} to the immune-regulatory network involved in MDS pathogenesis/progression. Better knowledge of the immune-mediated processes associated with the disease might improve MDS clinical management.

KEYWORDS
bone marrow, cytotoxic T-lymphocytes, myelodysplastic syndrome, T lymphocytes regulatory

Stefania Leone and Valentina Rubino contributed equally to this study.

1 | INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of acquired haematopoietic clonal disorders characterised by ineffective haematopoiesis with peripheral cytopenia and a variable risk of leukaemia evolution.[1] Compelling evidence indicates that a mutual, dynamic interaction between the genetic alterations of the haematopoietic stem cell, an aberrant pro-inflammatory microenvironment and the immune dysregulation might underlie MDS pathogenesis.[2,3]

The involvement of autoimmune mechanisms and the activity of bystander T cells, potentially recruited by recognised dysplastic antigens and likely related to immunogenic acquired somatic mutations, have been hypothesised to be relevant for the selection/progression of dysplastic clone/s able to escape immune-mediated damaging.[4,5]

Deranged activation and clonal expansion of $CD8^+$ cytotoxic T cells (CTL) in bone marrow (BM) have been described as a key element in MDS pathogenesis.[4,6] In early MDS stages (very-low and low risk),[7] activated CTL and a pro-inflammatory milieu contribute to damage polyclonal haematopoiesis, favouring the selection of dysplastic clone/s able to escape the immune attack. At variance, an immune-suppressive environment might 'turn-off' the CTL functions and foster the dysplastic clone/s expansion/progression in late MDS stages.[8]

The occurrence of haematopoietic stem cell pyroptosis,[3] a deranged inflammatory cytokine profile,[9] an oligoclonal $CD8^+$ and $CD4^+$ T cell repertoire,[10] an ineffective tolerance control[8,11] and the association with autoimmune disorders[12,13] have been largely described in MDS subjects.

Basing on these observations, several immune-modulating strategies have been proposed in MDS clinical management. Immunosuppressive therapies (IST) have been employed in several clinical trials, resulting in a wide variable response rate (0%–66%).[14–17] The efficacy of these treatments, only in a subgroup of MDS subjects, has pointed out the need of valuable selection criteria, that, until now, are lacking.

Innovative therapeutic approaches, targeting molecules involved in immune-mediated pathways, are currently in study in several clinical trials.[18] Encouraging preliminary results are confirming that a better comprehension of the mechanisms underlying immune-dysregulation in MDS might improve not only our knowledge of MDS pathogenesis but also patient clinical management. Moreover, understanding how immune-mediated pathways participate to MDS pathogenesis/progression, will be likely relevant to identify new therapeutic targets.

Fine-tuning of immune response is usually obtained by multiple regulatory processes, all belonging to the immune tolerance network, that are in place to prevent potentially deleterious immune responses against *self*-tissues.[19,20] The key role of regulatory populations in the prevention of autoimmunity and of immune mediated diseases has been largely described.[21]

Regulatory cells represent a heterogeneous group of differentiated T cell subsets including the interleukin-10 producing T_R1,[22] the transforming growth factor-β producing T_H3[23] and the regulatory T (Treg) cells, constitutively expressing the Foxp3 transcription factor.[24]

T cells, characterised by the co-expression of CD3 and CD56, have been described in acute myeloid leukaemia (AML).[25,26] In both studies this T cell population, distinct from NKT subset[27] and expressing a αβ T cell receptor (TCR), was observed to be expanded in patients' peripheral blood (PB), compared to controls. Significant decrease of these cells has been also described in AML remission.[26]

Recently, we found[28] that co-expression of CD3 and CD56 molecules identifies the T_{R3-56} lymphocytes, as a novel human regulatory T cell subset. This cell population preferentially exerts suppressive activity on proliferation, cytotoxicity and IFN-γ production by activated human CTL. Regulatory functions of human T_{R3-56} require cell-to-cell contact and occur in both autologous and allogeneic conditions. T_{R3-56} suppressive activity involves reduction of intracellular reactive oxygen species in CTL. Perturbation in number and function of T_{R3-56} cells has been by us related to the deranged CTL function observed in type 1 diabetes.[28] The involvement of T_{R3-56} in the

TABLE 1 Patient clinical characteristics

Total number of MDS patients	58
Age	
years, median (range)	77 (40–89)
Sex, *n* (%)	
Male	36 (62)
Female	22 (38)
Blood routine, median (range; interquartile range)	
Haemoglobin, g/dl	10.25 (7.8–13.2; 9.1–12.25)
Platelet count, $\times 10^9$/L	140 (30–383; 104–197)
Neutrophil count, $\times 10^6$/L	2061 (320–7588; 1008–3190)
Bone marrow blasts, median (range; interquartile range)	
Bone marrow blasts, %	0.4 (0–18; 0–2)
Cytogenetic by IPSS-R criteria, *n* (%)	
Very good	0 (0)
Good	55 (95)
Intermediate	3 (5)
Poor	0 (0)
Very poor	0 (0)
WHO 2016 classification, *n* (%)	
MDS-SLD	26 (45)
MDS-MLD	14 (24)
MDS-RS-SLD	3 (5)
MDS-RS-MLD	6 (10)
MDS-del(5q)	3 (5)
MDS-EB1	2 (3)
MDS-EB2	4 (7)
IPSS-R classification, *n* (%)	
Very-low risk	14 (24)
Low risk	32 (55)
Intermediate risk	5 (9)
High	3 (5)
Very-high risk	4 (7)

pathophysiology of other immune-mediated disorders needs to be investigated.

Defective cell-mediated immune-regulatory pathways contribute to MDS pathogenesis.[4,10] Indeed, the increase in Treg in advanced stages of MDS, as well as the occurrence of functional defects and altered migration of this cell subset in the first phases of the disease, have been described by us and others.[29–33] Moreover, we previously showed that Treg level in BM identifies a subgroup of low-risk MDS patients characterised by lower Treg percentage and significant BM recruitment of CD8 T lymphocytes with a skewed αβ TCR repertoire.[30]

No data are available on T_{R3-56} involvement in MDS pathogenesis/progression.

This study aims to analyse T_{R3-56} cells in BM of MDS subjects. The possibility that defective control of cytotoxic effectors by T_{R3-56} subset might participate in MDS pathogenesis/evolution has been also addressed.

2 | METHODS AND MATERIALS

2.1 | Patients and controls

Fifty-eight consecutive newly diagnosed MDS patients were enrolled in the study. BM and PB sample collection, haematological investigation, cytogenetic characterisation was performed according to the World Health Organisation (WHO) recommendations. Patients were categorised according to WHO 2016 and Revised-IPSS (IPSS-R) score.[7] Fourteen were classified as very-low risk, 32 as low risk, 5 as intermediate risk, 3 as high risk, 4 as very-high risk. Detailed description of clinical characteristic of our MDS cohort is reported in Table 1. BM and PB samples were obtained during routine diagnostic procedures. For TCR repertoire analysis 10 healthy donors, sex/age matched with MDS subjects, were enrolled in the study.[31] Moreover, to identify the occurrence of BM preferential T cell expansions, BM and PB Vβ TCR repertoire analysis was performed in 5 very-low and 16 low risk MDS patients, as previously described.[31]

Informed consent was obtained from each individual before PB and BM sample collection. Study was approved by the local Ethical Committee. None of the recruited patients was receiving medical treatments that could have an impact on their immune condition. Enrolled patients were not affected by immune-mediated diseases and acute or chronic viral infections.

2.2 | mAb, immunofluorescence and flow cytometry

FITC, PE, PEcy5, PEcy7 and APC labelled mAb against CD3, CD4, CD8, CD56, CD25, CD45, CD54, PE anti-γδ TCR and isotype-matched controls were purchased from BD PharMingen. Anti-Vβ14, anti-Vβ12, anti-Vβ7.2, anti-Vβ20, anti-Vβ18, anti-Vβ7.1, anti-Vβ22, anti-Vβ13.2, anti-Vβ1, anti-Vβ17, anti-Vβ5.3, anti-Vβ5.1, anti-Vβ23, anti-Vβ4, anti-Vβ2, anti-Vβ13.1, anti-Vβ5.2, anti-Vβ8, anti-Vβ9, anti-Vβ11, anti-Vβ3, anti-Vβ13.6, anti-Vβ21.3F, anti-Vβ16, anti-TCR mAbs were from Beckman-Coulter. PE-labelled CD1d tetramer loaded with alpha-galactosyl-ceramide and PE-labelled CD1d negative control tetramer were from ProImmune. T_{R3-56} lymphocytes have been identified by co-staining with anti-human CD3 and anti-human CD56 mAb as described[28]; this T cell subset is distinct from NKT cells and preferentially expresses a heterogeneous TCR αβ repertoire (Figure S1), as previously described.[28] Moreover, in our cohort we observed that T_{R3-56} cells preferentially expressed CD8 co-receptor. Indeed, less than 30% of the T_{R3-56} lymphocytes showed CD4 co-receptor, while less than 3% were negative for both CD4 and CD8 molecules (not shown). All phenotypes referred to flow cytometry analysis of the lymphocyte population gated by using FSC and SSC parameters, as well as CD45 labelling. Flow cytometry and data analysis were performed by a two-laser equipped FACScalibur apparatus and the CellQuest analysis software (Becton Dickinson). For the comparative analysis of CD54 expression on BM CTL, immune-fluorescence data were expressed as ratio of mean intensity fluorescence (MIF) value for the CD8 population and the control MIF value obtained after staining the same cell population with the isotype control mAb, as described.[31] To define CD4 and CD8 TCR skewing, we considered the occurrence of a percentage of expression exceeding of three standard deviation that observed, for each Vβ family analysed, in 10 healthy controls sex/age matched with the MSD cohort, as described.[6,31] Occurrence of a skewed BM CD4 and CD8 repertoire with an expression frequency higher that 20% respect to PB was considered as BM preferential skewing, as described.[31] This approach might be useful in order to identify T cell clone expansions potentially associated with the recognition of BM antigens likely relevant for MDS pathogenesis/progression.

2.3 | Statistical analysis

Statistical evaluation of data, by *InStat 3.0* software (GraphPad Software Inc.), was performed by *Mann–Whitney* or *Spearman's* correlation test. Two-sided *p* values less than .05 were considered significant.

3 | RESULTS

3.1 | T_{R3-56} lymphocytes, activated CTL and blasts in BM of MDS subjects

To address the role of T_{R3-56} subset in MDS pathogenesis/progression, we first analysed their BM level. In addition, we evaluated the activation of BM CTL by their CD54 expression[30]; the correlation of BM T_{R3-56} lymphocytes with BM blast number has been also analysed. As shown in Figure 1 A, an increasing-trend of BM T_{R3-56} percentage was observed from very-low/low risk to high/very-high risk group (5.1 ± 0.39; median 4.65; interquartile range [IQR] 3.43–6.69 in

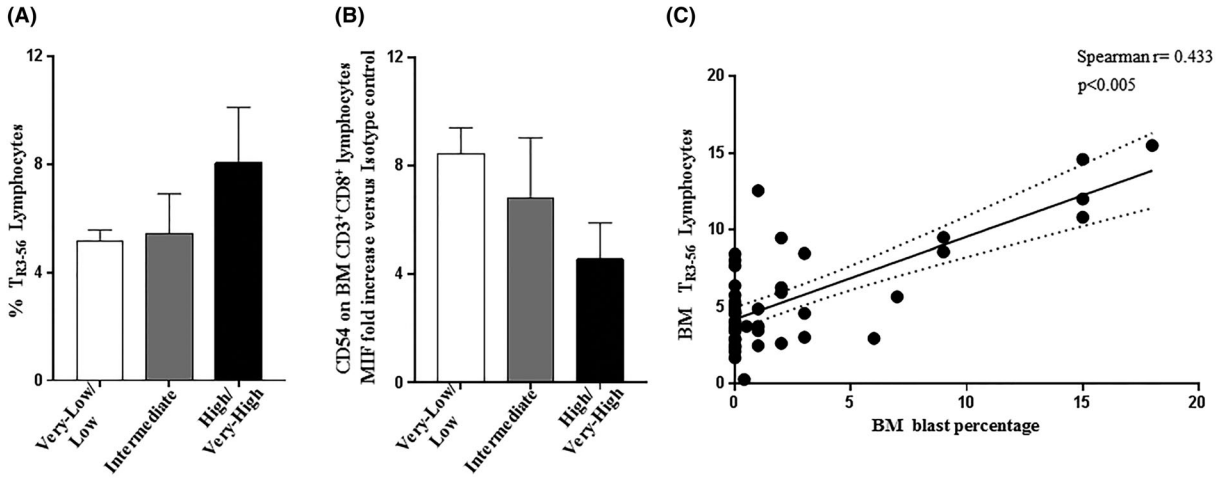
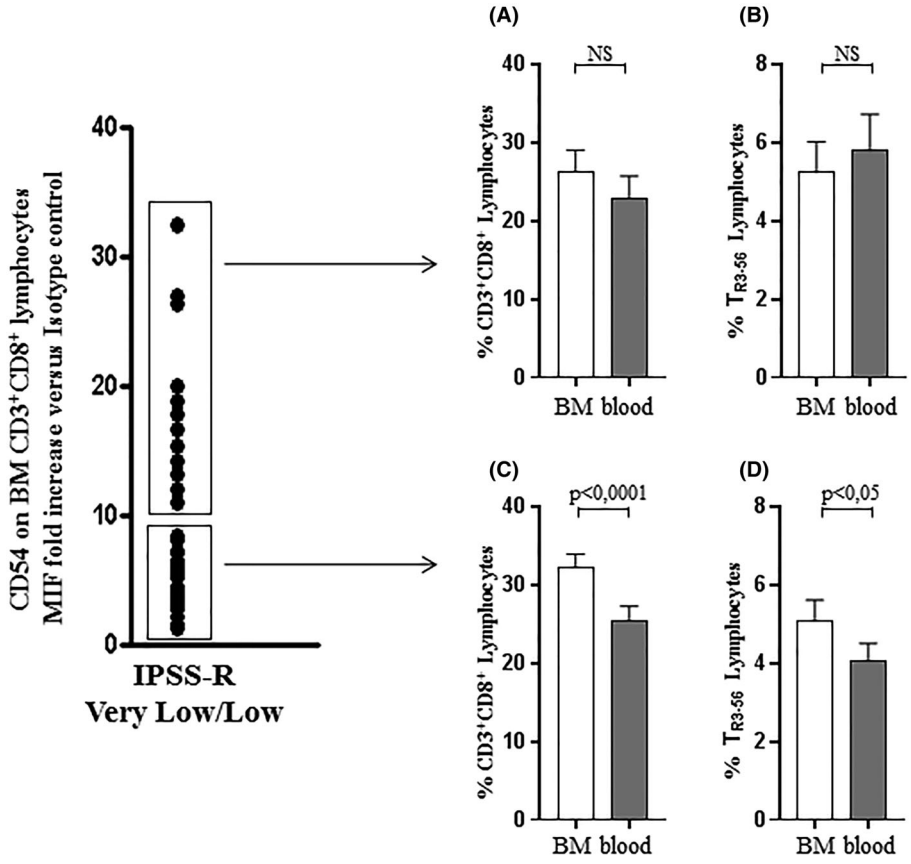


FIGURE 1 BM T_{R3-56} lymphocyte percentage positively correlates with BM blast number and seems to be inversely associated with BM CD54 expression on BM CTL in MDS subjects. (A) White, grey and black columns indicate BM percentage of T_{R3-56} cell subset in very-low/low risk (N 46), intermediate risk (N 5) and high/very-high risk (N 7) MDS patients, respectively. (B) White, grey and black columns indicate CD54 expression on BM CTL in very-low/low risk (N 46), intermediate risk (N 5) and high/very-high risk (N 7) MDS patients, respectively. As shown, an increasing trend of BM T_{R3-56} percentage accompanied by a decreasing trend for CD54 expression on CTL is observed from very-low/low risk to high/very-high risk group. (C) Spearman's evaluation of correlation between BM T_{R3-56} cell subset percentage and BM blast in MDS subjects. As shown, a significant ($p < .005$) positive correlation ($r = .433$) has been revealed

FIGURE 2 T_{R3-56} lymphocyte percentage in BM of very-low/low risk MDS patients inversely correlates with CD54 expression on BM CTL. Left part of the figure shows very-low/low risk MDS patients grouping according to CD54 expression on BM CTL, as previously described.[30] (A–D) Analysis of BM versus blood $CD3^+CD8^+$ and T_{R3-56} in the very-low/low risk MDS patients, grouped according to their CD54 expression on BM CTL. White and grey columns indicate BM and PB percentage, respectively. (A and B) Analysis of BM versus blood CTL distribution in the subjects categorised according their CD54 expression on BM CTL; (C and D) analysis of BM versus blood T_{R3-56} distribution in the subjects categorised according their CD54 expression on BM CTL. As shown, significant BM recruitment of CTL ($p < .0001$) and T_{R3-56} ($p < .05$) lymphocytes has been observed only in the subjects with lower CD54 expression on BM CTL



very-low/low risk; 5.44 ± 1.4; median 3.67; IQR 3.56–9.46 in intermediate risk; 8.22 ± 1.8; median 4.58; IQR 3.69–14.58 in high/very-high risk). In addition, a decreasing-trend has been shown for CD54 expression from very-low/low risk to high/very-high risk group (8.45 ± 0.9 in very-low/low risk; 6.81 ± 2.2 in intermediate risk; 4.65 ± 1.3 in high/very-high risk) (Figure 1B).

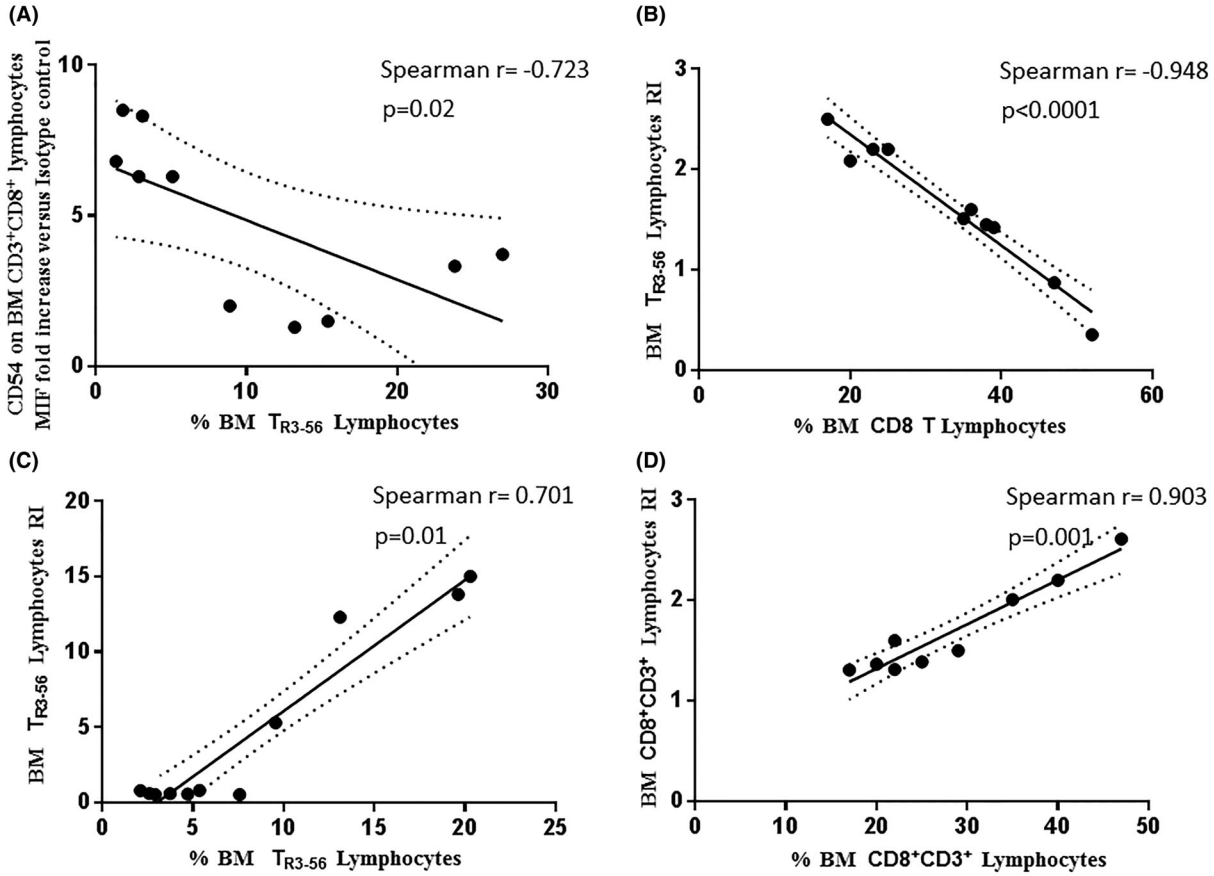


FIGURE 3 Spearman's correlation analysis of BM T_{R3-56} lymphocytes, BM CTL percentage and activation in very-low/low risk MDS patients categorised according to the presence of a preferential BM T cell skewed Vβ repertoire. (A) Spearman correlation analysis of CD54 expression on BM CTL versus BM T_{R3-56} percentage in MDS subjects with ≥2 CTL Vβ skewed clones in BM; significant negative correlation (Spearman $r = -.723$; $p < .05$) is shown. (B) Spearman correlation analysis of BM T_{R3-56} RI (ratio between the percentage of T_{R3-56} lymphocytes in BM vs. their percentage in PB) versus BM CTL percentage in MDS subjects with ≥2 CTL Vβ skewed clones in BM; significant negative correlation (Spearman $r = -.948$; $p < .0001$) is revealed. (C) Spearman correlation analysis of BM T_{R3-56} vs BM T_{R3-56} percentage in MDS subjects with <2 CTL Vβ skewed clones in BM; significant positive correlation (Spearman $r = .701$; $p < .05$) is observed. (D) Spearman correlation analysis of BM CTL RI versus BM CTL percentage in MDS subjects with ≥2 $CD4^+$ Vβ skewed clones in BM; significant positive correlation (Spearman $r = .903$; $p < .005$) is shown

Expression of CD54 on CTL is thinly regulated through the TCR complex and has been largely considered to be directly involved in antigen-dependent CTL activation processes.[32,33] Thus, might represent a valuable marker of antigen dependent CTL activation in BM of MDS patients.

Late stages of MDS have been associated with the occurrence of immune-suppression mechanisms. In order to analyse T_{R3-56} potential role in mediating immune-modulation in BM microenvironment we analysed, by Spearman's assay, the relationship of T_{R3-56} with BM blast number in our cohort. As shown in Figure 1C, a significant positive correlation ($r = .433$; $p < .005$) has been revealed.

Since immune-mediated mechanisms are relevant to the pathogenesis of MDS in the early stages of the disease,[4,6–10] we focused on very-low/low risk MDS patients. We previously described that CD54 expression level on BM CTL may identify two MDS subgroups showing high (≥10 MIF fold increase versus isotype control) or low (<10 MIF fold increase versus isotype control) CD54 expression on BM CTL.[30] Therefore, we analysed BM immune profile in very-low/low risk MDS subjects categorised according to CD54 expression on BM CTL. As shown in Panels B and D of Figure 2, lower CD54 expression in BM CTL associates with increased BM CTL (33.32 ± 2.03% in BM vs. 25.38 ± 1.88% in PB; $p < .0001$) and with higher BM T_{R3-56} (5.07 ± 0.54% in BM vs. 4.05 ± 0.46% in PB; $p < .05$). No significant differences were observed in the BM CTL and T_{R3-56}, nor in PB distribution in subjects showing higher CD54 expression on BM CTL (Panels A and C of Figure 2).

These data add T_{R3-56} subset to the immune-regulatory network potentially involved in MDS pathogenesis/progression mechanisms. Indeed, a T_{R3-56}-dependent control of CTL activation in BM of MDS patients might be hypothesised.

3.2 | T_{R3-56} lymphocytes and CTL skewed T cell repertoire in BM of very-low/low risk MDS subjects

The involvement of Treg subset in the control of T cell expansion in BM of low risk MDS subjects has been by us described.[31] To evaluate

whether T_{R3-56} subset might also participate in the control of BM T cell clonal expansion, we analysed PB and BM TCR repertoire in very-low/low risk MDS patients. This approach allowed the comparative evaluation of TCR repertoire in BM microsite versus PB and the identification of T cell clonal expansions, potentially related to the recognition of BM antigens.

Very-low/low risk MDS individuals were divided in two groups: those with <2 TCR preferential BM Vβ skewing versus those showing ≥2 TCR preferential BM Vβ skewing in CTL repertoire, as previously described.[31] As shown in Figure 3A, a significant inverse correlation (Spearman $r = -.723$; $p < .05$) between BM CTL CD54 expression and BM T_{R3-56} percentage has been observed.

Immune response is a microsite process involving the recognition of specific antigens by resident and/or microsite recruited immune effectors. Thus, the frequency of immune cells, specifically localised in BM respect to PB, might represent a valuable tool to analyse the mechanisms underlying the immune-activation events occurring in BM. With this aim, we analysed the ratio between the percentage of lymphocytes, belonging to the CTL and/or the T_{R3-56} subset, and the percentage of the same cell population in the PB (recruitment index [RI]). In this context, a ratio >1 indicates the preferential BM recruitment from PB of each immune subset.

We show (Figure 3B) a strong negative correlation between BM T_{R3-56} RI and BM CTL percentage (Spearman $r = -.948$; $p < .0001$) in the very-low/low risk MDS subjects showing ≥2 preferential TCR Vβ expansions in BM $CD8^+$ T cells. At variance, (Figure 3C) a significant correlation (Spearman $r = .701$; $p < .05$) between BM T_{R3-56} RI and their BM percentage was observed in the very-low/low risk individuals with <2 preferential BM TCR Vβ skewing in $CD8^+$ T cells.

Subjects with ≥2 preferential BM TCR Vβ expansions in $CD4^+$ T cells (Figure 3D) showed a positive correlation between BM CTL RI and their BM percentage (Spearman $r = .903$; $p < .005$). No significant correlations were observed in the very-low/low risk subjects characterised by the presence of <2 skewed TCR Vβ families in BM $CD4^+$ T cells (not shown).

These data inversely associate BM T_{R3-56} level with BM CTL expansion and activation in our cohort. In addition, a correlation between $CD4^+$ T cell skewing and $CD8^+$ BM percentage, has been also revealed in very-low/low risk MDS subjects with ≥2 TCR Vβ expansions in BM $CD4^+$ T cells.

4 | DISCUSSION

Deranged pro-inflammatory immune response in BM has been largely demonstrated to characterise MDS pathogenesis/progression.[2–5,9,13] The role of a defective immune-tolerance, that preferentially affect CTL response, has been observed to underlie BM failure in MDS, while a BM immunosuppressive milieu is a hallmark of MDS late stages.[4,6,8,10,11,19,20,34]

We recently described T_{R3-56} lymphocytes as a regulatory T cell subset, distinct from NKT and NK population, expressing a αβ TCR and specifically involved in the regulation of CTL effector function.[28] The increase of a T cell population, characterised by the co-expression of CD3 and CD56, has been previously described in AML.[25,26] Notably, significant modulation of these cells has been observed in AML remission.[26]

To address the role of T_{R3-56} in MDS pathogenesis/progression, we analysed their level in PB and BM of 58 MDS patients. An increase-trend for T_{R3-56} was revealed in MDS cohort from very-low/low to high/very-high risk stage of the disease. This scenario is accompanied by a progressively reduced BM CTL activation (analysed by CD54 expression) from very-low/low risk to high/very-high MDS patients. In this context, the absence of statistical significance could be related to the low number of patients in the high/very-high risk stage, compared to the very-low/low risk group. Notably, a significant positive correlation of T_{R3-56} with BM blasts has been revealed.

These findings are conceivable with the involvement of T_{R3-56} cells in MDS pathogenesis/progression, as proposed for the Treg subset[8,29–31,34,35] and it is in line with previous data obtained in AML.[25,26] Indeed, the possible role of T_{R3-56} in favouring dysplastic/leukemic clone immune-escape, might be hypothesised.

Immune-mediated mechanisms have been extensively described as relevant for MDS pathogenesis in the first stages of the disease.[4,6–10,13,29–31] The significant inverse relationship between BM T_{R3-56} amount and BM CTL activation and expansion, observed in very-low/low risk MDS subjects, suggests the possible participation of a defective control of CTL effectors by T_{R3-56} subset to the immune-mediated mechanisms involved in the emergence of dysplastic clones, as proposed for the Treg subset.[29–31]

We previously observed that BM Treg inversely correlates with BM recruitment of CTL showing a skewed TCR Vβ repertoire in low risk MDS subjects.[31] Here, we describe a significant inverse correlation of BM T_{R3-56} and the activation of BM CTL in very-low/low risk individuals. These data add T_{R3-56} lymphocytes to the regulatory cell-mediated network controlling CTL activation, as we recently described in type 1 diabetes.[28] Moreover, we observed a strong inverse correlation between the presence of a BM skewed CD8 T cell repertoire and the amount and BM T_{R3-56} recruitment. Thus, the participation of T_{R3-56} subset to the control of activation and antigen-dependent expansion of CD8 T cells in BM of very-low/low risk MDS subjects might be hypothesised. The lack of significant correlation of T_{R3-56} subset, observed in MDS very-low/low risk subjects showing a skewed $CD4^+$ T cell repertoire, is conceivable with the preferential involvement of this regulatory cell subset in the control of CTL activity, as by us previously described.[28]

The role of deranged CTL in damaging physiological polyclonal haematopoiesis has been largely described.[4,6,10–13] This study adds T_{R3-56} subset to the complex cell-mediated regulatory network involved in the control of adaptive immune response in MDS.

We found[28] that T_{R3-56}/CTL direct contact is able to mediate significant alteration of redox balance in CTL. This effect has been observed to modulate antigen-dependent effector function of human CTL, probably interfering with cytoskeleton rearrangement processes relevant for CTL activity. The strong correlation by us observed between BM T_{R3-56} cells and resident CTL activation and expansion

suggests the relevance of cell to cell contact for T_{R3-56} mediated regulatory function in MDS. These observations support the possibility that the mechanisms, by us previously observed to mediate T_{R3-56} dependent modulation of CTL function, might be also relevant in MDS model.

Further studies are necessary to elucidate the mechanisms below T_{R3-56} contribution to immune dysregulation in MDS. Better knowledge of immune-regulatory pathways in BM microsite may provide new insights into the complex scenario underlying MDS pathogenesis and/or progression, also allowing the proposal of additional molecular targets for innovative therapeutic approaches.

AUTHOR CONTRIBUTIONS

Stefania Leone and Valentina Rubino performed most of the experiments and data analyses and contributed to write the paper. Anna Teresa Palatucci, Angela Giovazzino and Flavia Carriero performed the experiments and data analyses. Stefania Leone, Giuseppe Cerciello and Fabrizio Pane participated in the clinical management of the patients. Giuseppina Ruggiero and Giuseppe Terrazzano designed the research study, analysed the data and wrote the paper.

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CONFLICT OF INTEREST

The author declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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