

VISTA mediates the progression of myelodysplastic syndrome

Raymond J. Zhang

Vanderbilt University <https://orcid.org/0000-0001-6329-7225>

Hyundong Yoon

Vanderbilt University Medical Center

Qianni Hu

University of Manitoba

Carly M. Fielder

Vanderbilt University

Anjali Raman

Vanderbilt University Medical Center

Emily F. Mason

Vanderbilt University Medical Center

Haley E. Ramsey

Department of Internal Medicine, Vanderbilt University School of Medicine, Vanderbilt- Ingram Cancer Center, Nashville, TN <https://orcid.org/0009-0007-1201-250X>

Agnieszka E. Gorska

Vanderbilt University School of Medicine

David Zhang

Vanderbilt University <https://orcid.org/0009-0009-2891-4334>

Carlos H. Serezani

Vanderbilt University Medical Center

Michael R. Savona

Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center

Paul Brent Ferrell

Vanderbilt University Medical Center <https://orcid.org/0000-0003-1140-9154>

Silvia Park

Seoul St. Mary's Hematology Hospital, The Catholic University of Korea College of Medicine

Yoo-jin Kim

Seoul St. Mary's Hematology Hospital, College of Medicine, The Catholic University of Korea

Jeffrey C. Rathmell

University of Chicago <https://orcid.org/0000-0002-4106-3396>

Peter D. Aplan

NIH/NCI <https://orcid.org/0000-0001-5686-9969>

Tae Kon Kim

tae.k.kim@vumc.org

Vanderbilt University Medical Center <https://orcid.org/0000-0002-5182-3870>

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VISTA mediates the progression of myelodysplastic syndrome

Raymond J Zhang^{1,2}, Hyundong Yoon^{1,2}, Qianni Hu^{2,6}, Carly M Fielder^{1,2}, Anjali Raman², Emily F Mason³, Haley E Ramsey^{2,4}, Agnieszka E Gorska², David Zhang², Carlos H Serezani^{3,5}, Michael R Savona^{1,2,4}, Paul Brent Ferrell^{1,2,4}, Silvia Park^{2,7}, Yoo-jin Kim⁷, Jeffrey C Rathmell^{3,5,8}, Peter D Aplan⁹, Tae Kon Kim^{1,2,3,4,5}

¹Program in Cancer Biology, Vanderbilt University, ²Division of Hematology/Oncology, Department of Medicine, ³Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, ⁴Vanderbilt Ingram Cancer Center, and ⁵Vanderbilt Center for Immunobiology, Nashville, TN 37232, USA

⁶Department of Immunology, University of Manitoba, Winnipeg, MB R3E 0T5, Canada

⁷Catholic University College of Medicine, St Mary Hospital, Seoul South Korea

⁸University of Chicago Ben May Department for Cancer Research, Chicago, IL, USA

⁹National Cancer Institute, Center for Cancer Research, Bethesda, MD, USA

Corresponding author: Tae Kon Kim

Email: tae.k.kim@vumc.org

Phone: 615-875-2453

COMPETING INTERESTS

In the past 12 months, TKK has been a consultant for Aimmunelink and a member of advisory board for Arog Pharmaceuticals, but these roles are not relevant to this study. PDA receives royalties from the NIH Technology Transfer Office for the invention of NHD13^{Tg} mice. Other authors have declared that no conflicts of interest exist.

ABSTRACT

Myelodysplastic syndrome (MDS) is characterized by progressive cytopenias and a substantial risk of transformation to acute myeloid leukemia. Immune dysregulation is central to the pathogenesis of MDS, and immune suppression is often associated with disease progression. However, immunotherapies such as anti-programmed cell death protein-1 therapy have shown limited efficacy in patients, indicating an incomplete understanding of immune suppression in MDS. To better characterize the immune features of MDS, we performed extensive immune phenotyping of MDS patient bone marrow (BM). We demonstrate that V-domain Ig suppressor of T cell activation (VISTA), an inhibitory immune checkpoint, is upregulated in MDS patients by CD34⁺ blasts and granulocytic myeloid-derived suppressor cells (G-MDSCs) compared to healthy controls, and G-MDSC VISTA expression positively correlates with T cell suppression. In studies employing the NUP98-HOXD13 transgenic MDS mouse model, genetic deletion of VISTA delays the progression of anemia and decreases the rate of death due to severe cytopenias. Assessment of MDS mice reveals that VISTA deletion reduces BM populations of G-MDSCs and reverses the suppression of T cells and macrophages. Together, our findings suggest that VISTA may augment G-MDSC-mediated immune suppression and significantly contribute to ineffective hematopoiesis in MDS.

INTRODUCTION

Myelodysplastic syndrome (MDS) is a hematologic disorder characterized by ineffective hematopoiesis, cytopenias, and a substantial risk of progression to acute myeloid leukemia (AML) [1]. In order to estimate prognosis and guide management, MDS is stratified into lower- or higher-risk disease. Unfortunately, MDS remains largely incurable for most patients. In high-risk MDS, hypomethylating agents are the only available standard therapy, yet are ineffective in over half of patients [2]. And while recent advancements in MDS therapy have led to better symptomatic management in low-risk disease, including improved cytopenias (e.g. luspatercept), the development of new therapies that can alter the natural history of MDS has likely been hindered by an insufficient understanding of disease biology [3, 4].

Immune dysregulation is seen almost universally in MDS, but how the immune system influences MDS pathogenesis and progression is still poorly understood [5, 6]. While chronic inflammation has often been identified as a key factor in MDS pathogenesis, immune suppressive features are also associated with disease progression, particularly in high-risk MDS [7–10]. Unfortunately, attempts to normalize immune evasion (so called adaptive immune resistance) in MDS using immune checkpoint inhibitors for programmed cell death protein-1 and its ligand (PD-1/PD-L1) or cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) have shown limited efficacy in patients, indicating that other immune suppressive mechanisms may be present in this disease [11–15]. Myeloid-derived suppressor cells (MDSCs), which are a prominent mediator of resistance to anti-PD-1/PD-L1 and anti-CTLA-4 monoclonal antibodies (mAbs) in the tumor microenvironment, have been suggested as one potential

mechanism responsible for the limited efficacy of immunotherapy in MDS [16, 17]. Prior studies have demonstrated that MDSCs are increased in MDS and can contribute to MDS immune evasion or even promote ineffective hematopoiesis, possibly through the secretion of TGF- β or other suppressive cytokines [10, 18]. In fact, one of the few advancements in MDS therapy over the past two decades, luspatercept, targets the TGF- β pathway and inhibits SMAD2/3 signaling, which is overactivated in MDS [6, 19]. However, how these and other immune suppressive mechanisms may be responsible for MDS phenotypes has not yet been fully explored.

V-domain Ig suppressor of T cell activation (VISTA) is a co-inhibitory checkpoint molecule broadly expressed on hematopoietic cells, with highest expression seen in myeloid immune subsets [20, 21]. Besides its role as an inhibitor of T cell activation, VISTA has also been shown to reprogram myeloid cells to anti-inflammatory phenotypes and act as a crucial mediator of immune suppressive function, particularly in MDSCs [22–26]. Myeloid cell VISTA (i.e. macrophages, granulocytes) can mediate AML immune evasion and disease progression *in vivo* while MDSC VISTA overexpression in AML patients may directly contribute to T cell suppression [27–29]. Whether VISTA contributes to immune evasion and disease progression in MDS as well is thus far unknown. Here, we demonstrate that (a) VISTA is significantly upregulated in human MDS BM and associated with worse patient survival; (b) the expression of VISTA by granulocytic MDSCs (G-MDSCs) is associated with T cell suppression; and (c) genetic deletion of VISTA in a murine MDS model can delay MDS progression and reverse immune suppression.

METHODS

Patient samples

All patients gave informed consent to participate in this study, which was performed in accordance with the Declaration of Helsinki and had the approval and guidance of the Institutional Review Board at Vanderbilt University Medical Center (VUMC). MDS patient BM aspirate samples (patient characteristics available in Supplementary Table 1), which were coded and processed by VUMC, were obtained from the VUMC Hematologic Malignancy Tumor Bank. Healthy donor BM cells were purchased (STEMCELL Technologies, Vancouver, Canada).

MDS patient BM immune phenotyping

Cryopreserved MDS patient BM samples (Supplementary Table 1) were assessed by staining with two separate antibody panels targeting myeloid/progenitor or lymphoid cell markers (Supplementary Tables 2 and 3) before analysis using a Cytex Aurora™ spectral flow cytometer (Cytex Bioscience, Fremont, CA). Normalized VISTA expression was calculated by subtracting geometric mean fluorescence intensity (MFI) of an isotype control for each sample from the MFI of VISTA-BV421 then applying an inverse hyperbolic sine transformation (asinh) to transform values to a linear scale.

MDS mouse model

All animal experiments were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center (M1900082-01). NUP98-HOXD13 transgenic hemizygous MDS mice (NHD13^{Tg})

were crossed with *Vsir*^{-/-} mice (VISTA KO) to obtain VISTA-deficient MDS mice. To monitor animals, peripheral blood was obtained from the tail vein and assessed using a GenX™ Veterinary Hematology Analyzer (Oxford Science Inc., Oxford, CT) to measure complete blood counts (CBC) or by flow cytometry to identify leukemic transformation. Mice found moribund or determined to have leukemia were euthanized (Supplementary Figure 5), while a subset of mice were also euthanized at 7-8 months for assessment prior to leukemic transformation. At these endpoints, peripheral blood was collected for CBC and blood smears, and BM was harvested for flow cytometric staining (Supplementary Table 4) and preparation of cytopsins. Blood smears and BM cytopsins were assessed by Wright-Giemsa staining and pathologist review.

Statistics

Statistical analyses were performed using GraphPad Prism software, unless otherwise noted. Briefly, when comparing cell frequencies between two groups, the Mann-Whitney test with Holm-Šídák's multiple comparisons correction was used, or for three or more groups, the nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test was used. Unpaired *t* tests with Welch correction and Holm-Šídák's multiple comparisons test was performed to compare VISTA expression in human MDS samples, and associations were calculated using Spearman's rank-order correlations. A mixed-effects model with Holm-Šídák's multiple comparisons test was used to compare blood counts in mice, and the logrank (Mantel-Cox) test was performed to compare survival trends. *p* < 0.05 was considered significant in all statistical analyses.

134 Detailed descriptions of all experimental procedures are provided in the Supplementary
135 Information.

RESULTS

***VSIR* transcript (VISTA) is upregulated in MDS BM and predicts worse patient survival**

Increased TGF- β signaling is a key pathogenic factor in the progression of MDS disease phenotypes, including immune evasion and ineffective hematopoiesis [6, 30]. We previously reported that *V-set immunoregulatory receptor* (*VSIR*, VISTA gene) is highly expressed in human AML at both transcript and protein expression levels, compared with healthy donor BM, contributing to AML immune evasion and progression [27]. By analyzing previously published RNA sequencing datasets, we found that the expression of several immune checkpoints, including *VSIR*, was upregulated in whole BM of MDS patients compared to healthy donor BM controls (Figure 1A, Supplementary Figure 1A) [31, 32]. SMAD2 and SMAD3 are central signal transducers in the TGF- β pathway, especially in driving ineffective hematopoiesis in MDS [19]. Interestingly, we found that *VSIR* was one of three immune checkpoints positively correlated with increased expression of both *SMAD2* (Pearson $r = 0.528$ [$p = 0.009$]) and *SMAD3* (Pearson $r = 0.454$ [$p = 0.03$]; Figure 1B), in addition to *SELPLG*, a known VISTA binding partner, and *LILRB1* (Supplementary Figure 1B) [33]. Given that both *VSIR* and its binding partner appeared as genes significantly correlated with *SMAD2/3* expression, we believed that the VISTA pathway may play an important role in TGF- β signaling in MDS.

VISTA either on cancer cells as a ligand or on immune cells as a receptor exerts immune suppressive and pro-tumorigenic effects [28, 34–37]. To determine whether *VSIR* is upregulated specifically by MDS progenitor cells, we assessed previously

published mRNA expression data of CD34⁺ cells isolated from MDS patient BM [38]. Our analysis revealed that *VSIR* was significantly upregulated by CD34⁺ progenitors from MDS patients ($n = 159$) compared to healthy donor controls ($n = 17$; Figure 1C). We previously showed that high *VSIR* expression is associated with poor overall survival in AML [27]. By stratifying MDS patients based on CD34⁺ progenitor *VSIR* expression, we found that expression of *VSIR* higher than the mean was predictive of worse overall survival in MDS (median 2.9 yr vs. 5.6 yr for high vs. low *VSIR*, respectively; HR 2.1 [95% CI 1.1-4.0, $p = 0.022$]; Figure 1D). Together, these findings suggest a potential role of *VSIR* expression on MDS disease progression.

VISTA is overexpressed on the surface of progenitors and granulocytic MDSCs in MDS

VISTA on macrophages and granulocytes contributes to immune suppression, including in AML [39, 40, 27]. To validate distinct expression of VISTA protein in cell subsets of MDS BM, we profiled cryopreserved BM aspirate specimens collected from 26 MDS patients and 8 healthy donors by flow cytometry. Increased blast count is often seen in MDS, particularly in high-risk subtypes [41]. Indeed, CD34⁺ progenitors were significantly increased in overall MDS cases compared to healthy donors (Figure 2A), and this difference was primarily due to significantly increased CD34⁺ BM cells (blasts) in high-risk MDS (IPSS-R intermediate, high, and very high risk; median 5.8% of total BM cells, $n = 17$) compared to both healthy donors (median 1.6% [$p = 0.029$], $n = 8$) and low-risk MDS (IPSS-R very low and low risk; median 2.7% [$p = 0.014$], $n = 6$; Figure 2B). CD34⁺ progenitors in MDS BM were not increased in cases of low-risk MDS

compared to healthy donors ($p > 0.99$). MDS BM also displayed a significantly increased frequency of CD11b⁺ myeloid immune cells (median 65% of total live BM cells) compared to healthy donors (median 40% [$p = 0.01$]; Figure 2A). Furthermore, MDSCs (CD11b⁺CD33⁺HLA-DR^{lo/-}) were increased in MDS BM (median 22% vs. 11% in healthy donors [$p = 0.0008$]), and specifically CD14⁻CD15⁺ G-MDSCs (median 18% of live BM cells in MDS vs. 9% in healthy donors [$p = 0.001$]; Figure 2C). In contrast to MDSCs, the frequency of monocyte/macrophages (CD11b⁺CD14⁺CD68⁺) was significantly decreased in MDS compared to healthy donors (median 1.6% vs. 21%, respectively [$p < 0.0001$]). Lymphocyte populations (i.e. CD4⁺ and CD8⁺ T cells and B cells) were decreased in MDS compared to healthy donors as well (median 10% T cells in MDS vs. 22% in healthy donors [$p = 0.012$]; median 1.6% B cells in MDS vs. 7.4% in healthy donors [$p = 0.0007$]; Figure 2A, 2D). These findings show an expansion of immunosuppressive MDSCs with a relative decrease in proinflammatory effectors.

We next assessed the expression of VISTA in these cell populations. In accordance with our analysis of *VSIR* transcript (Figure 1C), surface expression of VISTA protein was significantly upregulated in CD34⁺ MDS progenitors compared to healthy donors ($p = 0.004$, Figure 2E). In addition, VISTA expression was significantly upregulated on G-MDSCs in MDS BM compared to in healthy donors ($p = 0.008$). Other myeloid populations, including monocyte/macrophages and monocytic MDSCs also displayed trends towards increased VISTA expression (Figure 2E). Notably, the expression of VISTA on G-MDSCs was significantly inversely correlated with both the frequency of total T cells (Spearman $r = -0.39$ [$p = 0.023$]; Figure 3A) and CD4⁺ T cells in MDS BM (Spearman $r = -0.47$ [$p = 0.008$]; Figure 3B). Meanwhile, G-MDSC VISTA

expression was significantly positively correlated with the frequency of regulatory T cells (Tregs, CD4⁺CD25⁺CD127⁻; Spearman $r = 0.37$ [$p = 0.03$]; Figure 3D). In addition, the frequency of G-MDSCs in MDS BM was also significantly inversely correlated with the frequency of total T cells ($r = -0.40$ [$p = 0.045$]), CD8⁺ T cells ($r = -0.41$ [$p = 0.036$]), and monocyte/macrophages ($r = -0.59$ [$p = 0.001$]; Supplementary Figure 3A-D). Collectively, these findings suggest that high VISTA expression in MDS BM may be associated with an increased frequency of G-MDSCs, and higher VISTA expression by these G-MDSCs may in turn mediate immune suppression of T cells, leading to MDS immune evasion.

Immune cell VISTA contributes to disease progression in a murine model of MDS

NHD13^{Tg} mice are a well-established MDS mouse model. Starting around 2-3 months of age, NHD13^{Tg} mice develop disease phenotypes resembling human MDS, including progressive cytopenias and myeloid cell dysplasia [42, 43]. A majority of these mice transform from an MDS phenotype to leukemia starting around 8-12 months, succumbing to the disease shortly after leukemic transformation. To determine the role of VISTA on MDS disease progression in NHD13^{Tg} mice, we assessed CBC and leukemia progression in cohorts of wild-type (VISTA WT; $n = 20$), VISTA-deficient (VISTA KO; $n = 17$), NHD13^{Tg}/VISTA WT ($n = 17$), and NHD13^{Tg}/VISTA KO ($n = 23$) mice. As expected, NHD13^{Tg} animals showed progressive cytopenias (i.e. anemia, leukopenia, thrombocytopenia) and increasing mean corpuscular volume (MCV) compared with non-NHD13^{Tg} animals starting around 2-4 months (Figure 4A-C, Supplementary Figure 4, Supplementary Table 5). Histological review of BM cytopins

at 7 months of age also confirmed the presence of marked dysplasia of myeloid (i.e. abnormal nuclear lobation) and erythroid elements (i.e. multinucleation) in both NHD13^{Tg}/VISTA WT (Figure 4D) and NHD13^{Tg}/VISTA KO mice (Figure 4E), all consistent with an MDS phenotype in NHD13^{Tg} animals. Interestingly, NHD13^{Tg}/VISTA KO mice displayed significantly higher hemoglobin (Hgb) than NHD13^{Tg}/VISTA WT mice starting at 4 months of age (mean Hgb 13.4 g/dL [NHD13^{Tg}/VISTA WT] vs. 14.6 g/dL [NHD13^{Tg}/VISTA KO] at 4 months [$p = 0.016$]; Figure 4A) and lower MCV at 6 and 7 months (mean MCV 51.7 fL [NHD13^{Tg}/VISTA WT] vs 49.9 fL [NHD13^{Tg}/VISTA KO] at 6 months [$p = 0.009$] and 52.0 fL vs. 50.0 fL at 7 months [$p = 0.005$]; Figure 4C), representing a delayed progression of MDS-related anemia. In contrast, we did not observe any significant difference in white blood cell count (WBC) and platelets between NHD13^{Tg}/VISTA KO and NHD13^{Tg}/VISTA WT mice (Figure 4B, Supplementary Figure 4D, Supplementary Table 5). These CBC findings suggest that the expression of VISTA in NHD13^{Tg} mice may contribute to the progression of anemia, consistent with dyserythropoiesis in MDS.

To determine the impact of VISTA on survival and leukemic transformation in MDS, we followed these mice for 12 months. Past 7 months of age, average CBC values showed greater variability and wider distributions as some animals succumbed to severe disease or leukemic transformation (Supplementary Figure 4). Progression to leukemia was determined by monitoring for increases in WBC and the presence of circulating blast cells (c-Kit⁺) in the peripheral blood (Supplementary Figure 5A,B). Mice identified to have leukemic transformation or found to be moribund were euthanized. During our study we observed a trend towards shorter leukemia-free survival in

NHD13^{Tg}/VISTA WT mice (median 8.7 months) compared to NHD13^{Tg}/VISTA KO mice (median 10.9 months [$p = 0.09$]), suggesting a possible role for VISTA in mediating MDS progression (Figure 4F). We also noticed that while most mice progressed to leukemia, a subset of mice were found dead or moribund without any apparent signs of leukemia progression (i.e. peripheral blasts, upward trending WBC on CBC) at or in the weeks prior to death. Rather, these mice had severe cytopenias either at time of euthanization, or at the most recent CBC prior to being found dead (Supplementary Figure 5C-E). While there appeared to be no difference in leukemia transformation between NHD13^{Tg}/VISTA WT mice and NHD13^{Tg}/VISTA KO mice (HR 1.01 [95% CI 0.32-3.17, $p = 0.99$]; Figure 4G), we observed that deaths associated with severe cytopenias without leukemia occurred at a significantly higher rate in NHD13^{Tg}/VISTA WT mice compared to NHD13^{Tg}/VISTA KO mice (HR 10.69 [95% CI 2.28-50.0, $p = 0.006$]; Figure 4H). Taken together, these findings suggest that VISTA may not contribute to leukemic transformation in MDS but rather may play a role in the progression of MDS disease phenotypes, including severe anemia.

Immune cell VISTA contributes to immune suppression in MDS mice

Although VISTA primarily acts by modulating immune cell function, including in MDSCs, it is unknown whether VISTA expression by hematopoietic stem and progenitor cells (HSPC) may have a cell-intrinsic influence on hematopoietic function. To determine whether VISTA-mediated anemia in MDS may be due to an undiscovered role of VISTA in HSPCs, we tested the direct impact of VISTA on MDS progenitors by performing colony-forming unit (CFU) and burst-forming unit-erythroid (BFU-E) assays using

lineage-negative BM cells isolated from WT, VISTA KO, NHD13^{Tg}/VISTA WT, and NHD13^{Tg}/VISTA KO mice. We observed no significant difference in CFUs or BFU-Es detected in lineage-negative cells from WT or VISTA KO BM (Supplementary Figure 6A,B). Consistent with prior studies, lineage-negative BM cells from NHD13^{Tg} mice showed approximately 4-fold and 11-fold decreases in CFUs and BFU-Es, respectively, compared to WT BM cells [44]. However, there was no significant difference in CFUs or BFU-Es detected between NHD13^{Tg}/VISTA WT and NHD13^{Tg}/VISTA KO lineage-negative BM cells (Supplementary Figure 6A,B). These data suggest that while the NHD13 transgene appears to have a notable impact on HSPC function, genetic deletion of VISTA does not affect colony-forming capacity in HSPCs. Therefore, impaired erythropoiesis as a result of HSPC-intrinsic VISTA activity is not likely the driver of anemia progression in NHD13^{Tg}/VISTA WT mice.

Our human phenotyping suggests that VISTA may mediate BM immune suppression in MDS (Figures 2-3). To elucidate the impact of VISTA on the BM immune microenvironment in MDS, we evaluated the BM of a subset of mice at around 7 months of age, prior to leukemic transformation. The BM of NHD13^{Tg}/VISTA WT and NHD13^{Tg}/VISTA KO MDS mice displayed increases in CD11b⁺ myeloid cell populations compared to their non-NHD13^{Tg} WT and VISTA KO counterparts (Figure 5A), consistent with myeloid skewing in MDS. G-MDSCs (CD11b⁺MHC-II^{lo}Ly6G⁺Ly6C^{int}) were present at a higher frequency in the BM of NHD13^{Tg}/VISTA WT mice compared to NHD13^{Tg}/VISTA KO mice (median 66% vs 50%, respectively, of live CD45⁺ BM cells [$p = 0.003$]) and trended towards an increase in VISTA WT (non-NHD13^{Tg}) compared to VISTA KO mice (non-NHD13^{Tg}; median 59% vs 44%, respectively [$p = 0.07$]; Figure

297 5B), supporting a role for VISTA in driving G-MDSC expansion. Compared to non-
298 NHD13^{Tg} WT mice, NHD13^{Tg}/VISTA WT MDS mice showed significant reductions in
299 CD11b⁺F4/80⁺ macrophages (median 3.4% vs. 0.2% in non-NHD13^{Tg} WT vs.
300 NHD13^{Tg}/VISTA WT mice, respectively [$p = 0.01$]; Figure 5C) and CD3⁺ T cell
301 populations (median 0.59% vs. 0.11% in non-NHD13^{Tg} WT vs. NHD13^{Tg}/VISTA WT
302 mice, respectively [$p = 0.03$]; Figures 5E,F). Deletion of VISTA in NHD13^{Tg} mice
303 restored frequencies of these cell populations back to normal levels, suggesting that
304 VISTA expression may be in part responsible for the downregulation of these immune
305 cell subsets in murine MDS. Notably, BM macrophages in these mice largely did not
306 display an immunosuppressive M2-like phenotype (CD206⁺MHC-II⁻; median 1.7%
307 [range 1.3-3.0%] of macrophages; Figure 5D), but were primarily CD206⁻MHC-II⁻.
308 Taken together, the immune alterations seen in NHD13^{Tg} murine MDS reflect our
309 findings in human disease, including the association of increased myeloid immune cells
310 and decreased T cells in MDS BM. In addition, the decrease in G-MDSCs in
311 NHD13^{Tg}/VISTA KO mice compared to NHD13^{Tg}/VISTA WT mice coincided with the
312 restoration of CD4⁺ T cell frequency and supports the inverse correlation between G-
313 MDSC VISTA expression and CD4⁺ T cell frequency seen in human MDS.

DISCUSSION

In this report, we provided evidence from human data and an experimental mouse model that upregulation of the immune checkpoint VISTA in G-MDSCs may contribute to immune evasion and disease progression in MDS. In both our analyses of publicly available RNA expression datasets and our flow cytometric evaluation of MDS patient BM samples, we demonstrated that VISTA is upregulated in MDS, especially by CD34⁺ blasts and G-MDSCs. Our study is one of the few showing extensive immune phenotyping of MDS BM, and to our knowledge, this is the first instance demonstrating cell surface VISTA upregulation in MDS. Furthermore, we also reported that VISTA expression inversely correlates with T cell immunity in MDS BM and decreased survival in MDS. These findings were further corroborated by an *in vivo* MDS model, where VISTA expression contributed to the progression of severe cytopenia in MDS.

Immune escape in MDS has been posited as a significant contributing factor to disease progression and leukemic transformation through the uncontrolled expansion of dysplastic or preleukemic clones [45, 46]. Our study demonstrated that MDSCs, particularly G-MDSCs were increased in the BM microenvironment in both MDS patients and NHD13^{Tg} MDS mice, which is in agreement with prior studies of MDS [47, 18, 48]. In addition, VISTA was upregulated by CD34⁺ blasts and G-MDSCs in MDS, and G-MDSC-expressed VISTA was significantly correlated with reduced T cell activity. Our findings were consistent with previous studies that have demonstrated the role of VISTA in both promoting the expansion of MDSCs as well as mediating their T cell suppressive function [35, 23–25, 29]. A similar finding was reported by Kittang and colleagues, who saw that MDSCs and Tregs were positively correlated in MDS, though

they did not thoroughly explore how MDSCs may regulate Treg proliferation [10]. Given that VISTA can enhance Treg differentiation, our study proposes a possible mechanism of MDSC-induced Treg expansion in MDS, whereby G-MDSC-expressed VISTA may either modulate the production of effector cytokines or directly interface with CD4⁺ T cells to suppress T cell immunity and induce Treg differentiation [21, 28, 29, 49].

Our findings in the NHD13^{Tg} model also support a clear role for VISTA in G-MDSC expansion and T cell suppression. We found that VISTA-mediated immune suppression may contribute more meaningfully to MDS phenotypes, such as worse anemia and a greater likelihood of death due to severe cytopenias, than to leukemic transformation, as VISTA expression did not promote leukemic progression in these MDS mice. Moreover, our data support an immune-mediated mechanism of impaired erythropoiesis rather than an HSPC-intrinsic VISTA function, as VISTA deletion in NHD13^{Tg} progenitors had no apparent effect on their *in vitro* colony formation capacity compared with control.

Myelosuppression and ineffective erythropoiesis in MDS has been linked to overactivation of the TGF- β signaling pathway, and MDSCs, by the secretion of TGF- β or other immunosuppressive cytokines, can contribute to progressive cytopenias in MDS [50, 6, 51, 18]. Luspatercept, an inhibitor of the TGF- β pathway, has shown clinical efficacy for the treatment of anemia in low-risk MDS [3, 52]. It promotes erythroid maturation by acting as a ligand trap for TGF- β superfamily ligands and thus reducing downstream SMAD2/3 signaling [19]. In MDS, the expansion and activation of MDSCs or other immunosuppressive populations contributes to upregulated TGF- β signaling [8, 10]. VISTA is known to mediate the production of immunosuppressive cytokines by

myeloid cells and enhance TGF- β -induced Treg differentiation [22, 53]. Although it is not yet clear whether VISTA may specifically promote TGF- β production in MDSCs, VISTA has been shown to facilitate TGF- β expression and secretion in other cell types [54, 55]. Regardless, our data suggest that VISTA may contribute to ineffective erythropoiesis through the enhancement of immune suppression in MDS BM, possibly through the potentiation of MDSC-mediated activation of TGF- β signaling. Given that luspatercept is ineffective in a fraction of MDS patients, our findings advocate for further investigation into whether VISTA blockade may augment luspatercept efficacy via inhibition of the upstream production of TGF- β superfamily ligands.

Our study has a few limitations. First, our cohort of MDS BM samples is relatively modest in number and restricted to patients seen at our institution, which could be a potential source of bias in our data. Furthermore, MDS displays significant heterogeneity in genetic mutations, cytogenetics, and clinical presentation, and we did not fully assess the various subtypes of MDS in this study. Validation and extension of our findings using independent cohorts will be necessary to confirm whether these results are generalizable to MDS patients as a whole, and larger patient cohorts may allow for the detection of more subtle trends. Furthermore, an orthogonal approach that additionally employs single-cell transcriptomics or spatial proteomics may enable more comprehensive analyses of gene expression and cell-cell interactions or allow utilization of a more extensive archive of formalin-fixed paraffin-embedded BM core biopsies. However, these methods are currently challenging due to technical limitations, such as low BM cell viability inherent to MDS pathology or antigen loss due to common decalcification protocols performed prior to BM core biopsy sectioning. Second, our

interrogation of VISTA function in MDS is partially correlative and cannot definitively characterize the molecular and cellular interactions present within the MDS BM microenvironment. In combination with previously published work by other groups, our findings do suggest that G-MDSC-expressed VISTA is most likely one of the primary contributors to BM immune suppression and impaired hematopoiesis in MDS. However, our observations in NHD13^{Tg}/VISTA KO mice do not preclude the possibility that these phenotypes could be mediated by alternative cell populations. Therefore, further studies that exploit the use of conditional VISTA KOs or cell-specific depletion are still needed to better elucidate the immune mechanisms in MDS. Finally, while NHD13^{Tg} mice are a well-established murine model of MDS, the use of genetically modified animals may not fully recapitulate the biology of human MDS. In fact, the *NUP98-HOXD13* fusion gene is only found in a minor subset of MDS cases. Thus, the data we present here should ideally be validated in other MDS mouse models and patient-derived xenografts.

Recent advances in tumor immunology have facilitated understanding the role of immune evasion on cancer progression and treatment resistance. One important mechanism which cancers employ to evade the immune system includes the exploitation of T cell inhibitory pathways involving molecules such as PD-1 and CTLA-4. Immune checkpoint blockades with anti-PD-1/PD-L1 or anti-CTLA-4 mAb normalize immunological deficits and enhance immunological response to overcome immune evasion [56–58]. These approaches have resulted in significant and durable clinical activities in some patients with advanced malignancies especially metastatic melanoma, lung cancer, bladder cancer, Hodgkin lymphoma, and others [15]. However, a clinical trial using anti-PD-1 mAb in MDS after failure of hypomethylating agent treatment

showed a disappointing result (only 4% overall response as a single agent) [59]. The potential mechanisms for this poor response to anti-PD-1 mAb include MDSCs and TGF- β signaling in MDS BM [60–62]. Decreasing MDSCs has been tested clinically and preclinically in MDS, but these did not successfully impact clinical outcome [18, 63]. Our current study can pave way for targeting MDSCs and potentially TGF- β pathway by VISTA blockade for new immunotherapeutic approaches in MDS.

In summary, we demonstrate that VISTA expression by G-MDSCs is positively associated with T cell suppression in MDS BM, and VISTA-mediated immune suppression may promote progressive cytopenias and reduced survival in MDS. Our data underscore the significance of VISTA expression in MDS and suggest that inhibition of VISTA might enable reversal of immune suppression in MDS BM and the improvement of MDS phenotypes.

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AUTHOR CONTRIBUTIONS

RJZ conceptually designed the study, carried out experiments, analyzed, curated, and interpreted data, and wrote the manuscript. HD contributed experiments, performed computational analysis of publicly available sequencing data, contributed writing, and provided scientific input. QH, CMF, and HER contributed experiments, provided technical support and scientific input, and interpreted data. AEG, AR, and DZ contributed experiments and provided technical support. EFM assessed pathologic

slides and provided scientific input. CHS, MRS, PBF, SP, YK, JCR, and PDA interpreted data and provided scientific input. TKK conceived, conceptually designed, and supervised the study, interpreted data, and wrote the manuscript.

COMPETING INTERESTS

In the past 12 months, TKK has been a consultant for Aimmunelink and a member of advisory board for Arog Pharmaceuticals, but these roles are not relevant to this study. PDA receives royalties from the NIH Technology Transfer Office for the invention of NHD13 mice. Other authors have declared that no conflicts of interest exist.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available in public repositories. The MDS RNA-sequencing data were obtained from the Sequence Read Archive (SRA) under accession number PRJNA650236 [31]. Healthy donor RNA-sequencing data are accessible in the Gene Expression Omnibus (GEO) database under accession number GSE249547 [32]. Additionally, the microarray datasets utilized in this study are available in GEO under accession number GSE58831 [38]. All other data generated during this study are present in the published article, in the supplementary materials, or available from the corresponding author upon request.

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FIGURE LEGENDS

Figure 1. VSIR transcript is upregulated in MDS bone marrow (BM) and associated with worse survival

(A) *VSIR* mRNA expression in whole BM from healthy donors ($n = 10$) or MDS patients ($n = 23$). Boxes show median with interquartile range (IQR). Error bars represent $1.5 \times$ IQR. **(B)** Pearson correlations of *VSIR* expression with *SMAD2* and *SMAD3* expression in whole MDS $\times$ BM. **(C)** *VSIR* mRNA expression by CD34⁺ BM cells isolated from healthy donor ($n = 17$) or MDS patient ($n = 159$) BM. **(D)** Overall survival of MDS patients with high *VSIR* expression (above mean, $n = 80$) or low *VSIR* expression (below mean, $n = 79$). Statistical comparisons of *VSIR* expression were performed using a two-tailed Student's *t* test (** $p < 0.01$, **** $p < 0.0001$). Survival analysis was performed using the log-rank test and hazard ratio calculated using a univariate Cox proportional hazard regression model.

Figure 2. Immune phenotyping of MDS bone marrow

BM cells from healthy donors ($n = 8$) and MDS patients ($n = 26$) were assessed by flow cytometry. **(A)** Frequencies of CD34⁺ cells (blasts), myeloid immune cells (CD11b⁺), T cells (CD45^{hi}CD3⁺), and B cells (CD19⁺) after excluding dead cells (% of total live BM cells). **(B)** MDS samples were categorized using the Revised International Prognostic Scoring System (IPSS-R) into low-risk (IPSS-R very low and low risk; $n = 6$) and high-risk (IPSS-R intermediate, high, and very high risk; $n = 17$) groups to compare frequencies of CD34⁺ cells (blasts, % of total live BM cells). **(C)** Frequencies of myeloid

immune cell subsets (myeloid-derived suppressor cells [MDSCs]: CD11b⁺CD33⁺HLA-DR⁻, monocytic MDSCs [M-MDSCs]: CD11b⁺CD33⁺HLA-DR⁻CD14⁺CD15⁻, granulocytic MDSCs [G-MDSCs]: CD11b⁺CD33⁺HLA-DR⁻CD14⁻CD15⁺, and monocyte/macrophages: CD11b⁺CD14⁺CD68⁺; % of total live BM cells). **(D)** Frequencies of CD4⁺ and CD8⁺ T cells (% of total live BM cells). Cell frequency data are shown as median $\pm$ IQR. Statistical analyses were performed using Mann-Whitney tests with Holm-Šídák's multiple comparisons correction or the Kruskal-Wallis test with Dunn's multiple comparisons tests if >2 groups are being compared. (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). **(E)** Normalized VISTA expression for each indicated cell subset calculated from asinh-transformed Δ MFI between VISTA and isotype control. Violin plots show median with IQR. Statistical analyses were performed using unpaired Welch's t tests with Holm-Šídák's multiple comparisons correction (** p < 0.01).

Figure 3. G-MDSC VISTA expression correlates with BM immune suppression in MDS

Correlation between G-MDSC VISTA expression and the frequency of **(A)** T cells (CD45^{hi}CD3⁺, % of total live BM cells), **(B)** CD4⁺ T cells (CD45^{hi}CD3⁺CD4⁺, % of total live BM cells), **(C)** CD8⁺ T cells (CD45^{hi}CD3⁺CD4⁺, % of total live BM cells), and **(D)** regulatory T cells (Tregs [CD45^{hi}CD3⁺CD4⁺CD25^{hi}CD127⁻], % of CD4⁺ T cells). Plots show ranks of each variable. Statistical analyses were performed using Spearman's rank correlation.

Figure 4. VISTA contributes to disease progression in murine MDS

(A) Hemoglobin, (B) white blood cell count, and (C) mean corpuscular volume of mice measured at the indicated months of age (WT, n = 20; VISTA KO, n = 17; NHD13^{Tg}/VISTA WT, n = 17; NHD13^{Tg}/VISTA KO, n = 23). Data are shown as mean ± SEM. Statistical significance was determined by mixed-effects model with Holm-Šídák's multiple comparisons test. Asterisks represent significant difference between NHD13^{Tg}/VISTA WT and NHD13^{Tg}/VISTA KO groups at the indicated timepoint (**p* < 0.05, ***p* < 0.01). Wright-Giemsa-stained BM cytopins from (D) NHD13^{Tg}/VISTA WT and (E) NHD13^{Tg}/VISTA KO mice showing (i, ii) dysplastic myeloid elements with abnormal nuclear lobation and (iii) dysplastic erythroid elements with multinucleation. (F) Kaplan-Meier plot showing leukemia-free survival of animals over a 12-month study period. Tick marks represent censored subjects, which were euthanized at 7 months of age for further analysis. (G) Kaplan-Meier plot showing leukemia progression in NHD13^{Tg}/VISTA WT and NHD13^{Tg}/VISTA KO mice. Tick marks represent censored deaths with no evidence of leukemia progression. (H) Kaplan-Meier plot showing deaths associated with severe cytopenias without evidence of leukemia progression. Tick marks represent censored events where evidence of leukemia progression was present. Statistical analyses of Kaplan-Meier plots were performed using logrank (Mantel-Cox) tests to determine *p*-value.

Figure 5. VISTA contributes to BM immune suppression in murine MDS

BM cells were collected from WT (n = 12), VISTA KO (n = 4), NHD13^{Tg}/VISTA WT (n = 5), and NHD13^{Tg}/VISTA KO (n = 10) mice at 7 months of age and assessed by flow cytometry. Frequencies and representative gating of (A) myeloid cells (CD11b⁺, % of

live CD45⁺ BM cells), **(B)** M-MDSCs (CD11b⁺MHC-II^{-/lo}Ly6C⁺Ly6G⁻) and G-MDSCs
(CD11b⁺MHC-II^{-/lo}Ly6C^{lo}Ly6G⁺, % of live CD45⁺ BM cells), **(C)** macrophages
(CD11b⁺F4/80^{hi}, % of live CD45⁺ BM cells), **(D)** macrophage subsets (M1-like: MHC-
II⁺CD206⁻, M2-like: MHC-II⁻CD206⁺, and resting: MHC-II⁻CD206⁻, % of total
macrophages), **(E)** T cells (CD3⁺) and B cells (CD19⁺, % of live CD45⁺ BM cells), and
(F) T cell subsets (CD4⁺ or CD8⁺, % of live CD45⁺ BM cells). Data are shown as median
± IQR. Statistical analyses were performed using the Kruskal-Wallis test with Dunn's
multiple comparisons testing (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figures

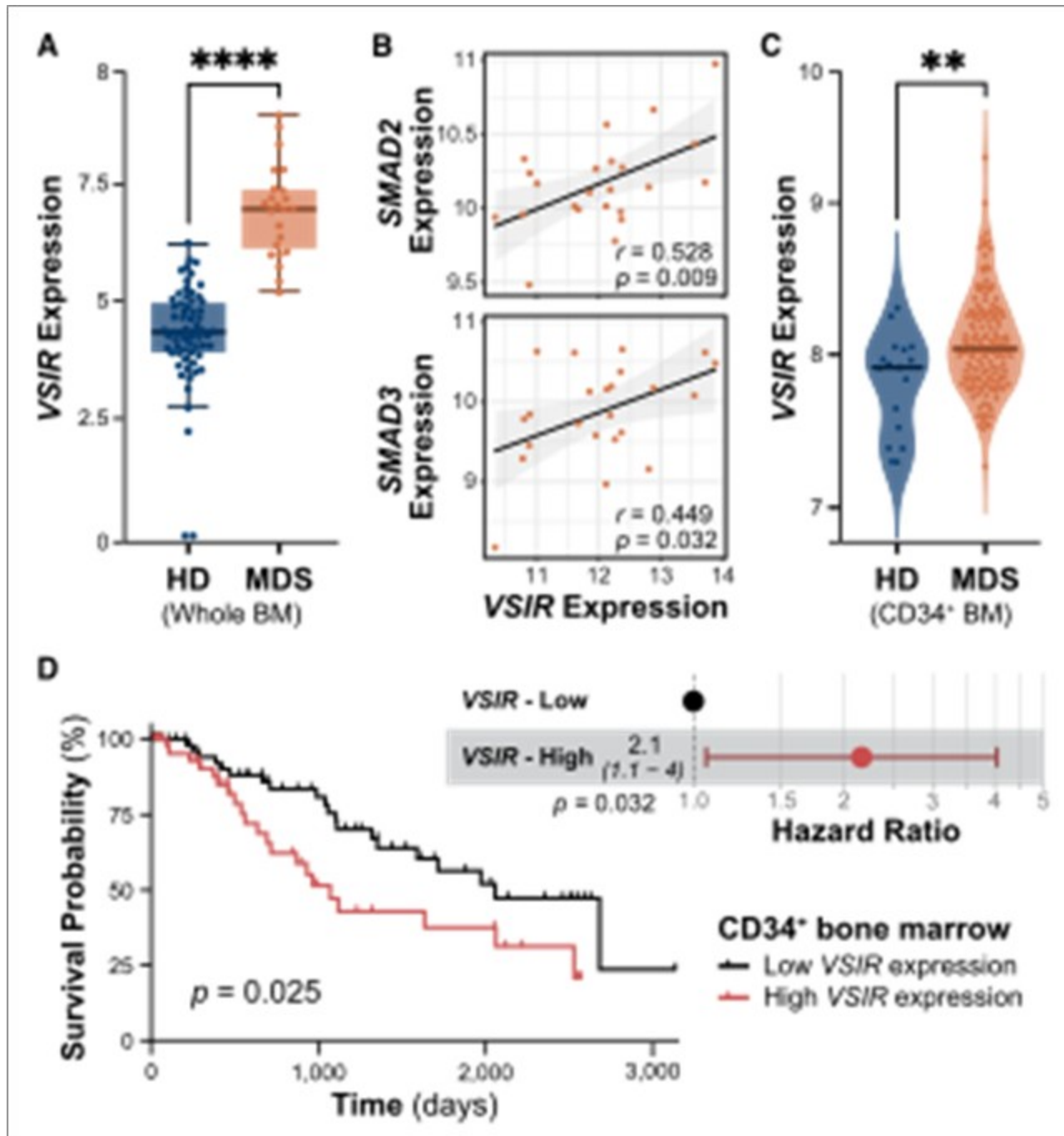


Figure 1

VSIR transcript is upregulated in MDS bone marrow (BM) and associated with worse survival

(A) *VSIR* mRNA expression in whole BM from healthy donors ($n = 10$) or MDS patients ($n = 23$). Boxes show median with interquartile range (IQR). Error bars represent $1.5 \times$ IQR. (B) Pearson correlations of *VSIR* expression with *SMAD2* and *SMAD3* expression in whole MDS xBM. (C) *VSIR* mRNA expression by CD34⁺ BM cells isolated from healthy donor ($n = 17$) or MDS patient ($n = 159$) BM. (D) Overall survival of MDS patients with high *VSIR* expression (above mean, $n = 80$) or low *VSIR* expression (below mean, $n =$

79). Statistical comparisons of *VISTA* expression were performed using a two-tailed Student's *t* test (** $p < 0.01$, **** $p < 0.0001$). Survival analysis was performed using the log-rank test and hazard ratio calculated using a univariate Cox proportional hazard regression model.

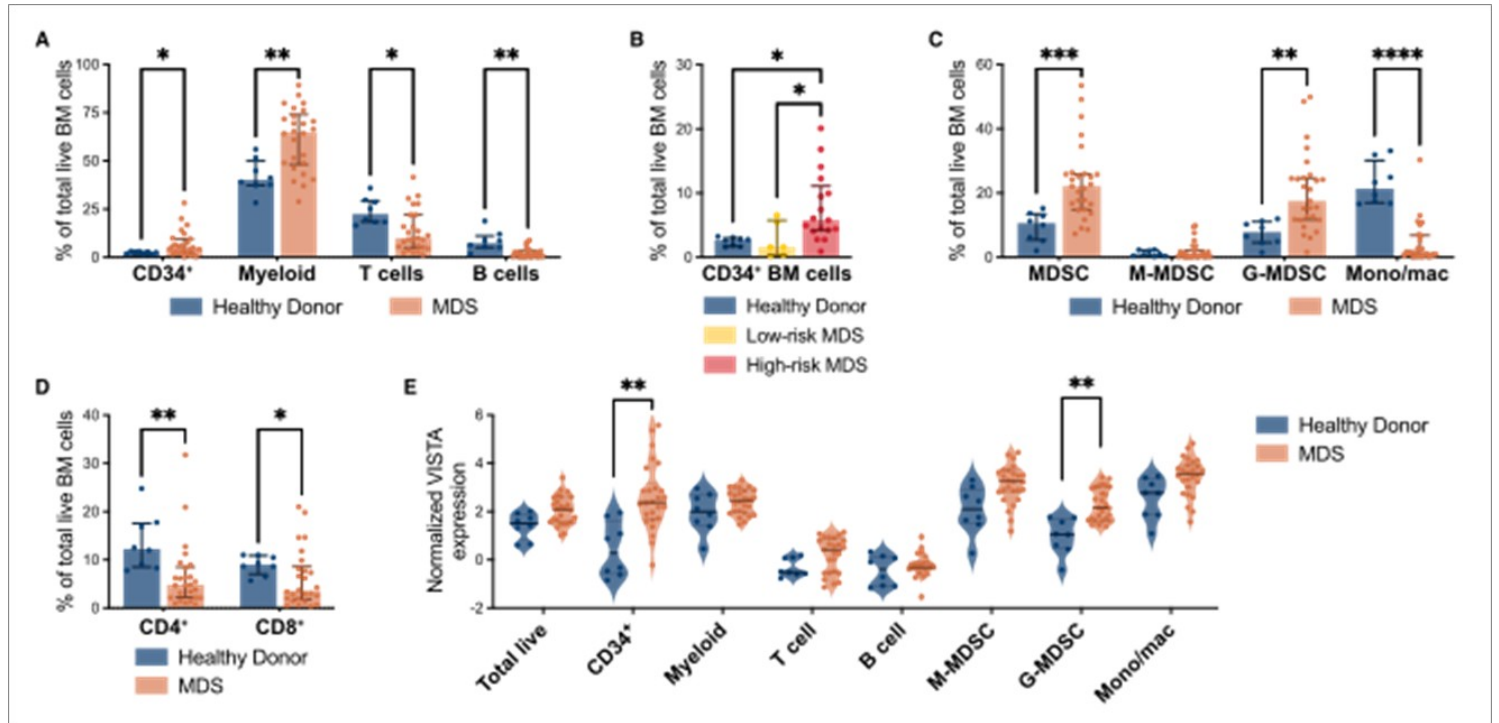


Figure 2

Immune phenotyping of MDS bone marrow

BM cells from healthy donors ($n = 8$) and MDS patients ($n = 26$) were assessed by flow cytometry. **(A)** Frequencies of $CD34^+$ cells (blasts), myeloid immune cells ($CD11b^+$), T cells ($CD45^{hi}CD3^+$), and B cells ($CD19^+$) after excluding dead cells (% of total live BM cells). **(B)** MDS samples were categorized using the Revised International Prognostic Scoring System (IPSS-R) into low-risk (IPSS-R very low and low risk; $n = 6$) and high-risk (IPSS-R intermediate, high, and very high risk; $n = 17$) groups to compare frequencies of $CD34^+$ cells (blasts, % of total live BM cells). **(C)** Frequencies of myeloid immune cell subsets (myeloid-derived suppressor cells [MDSCs]: $CD11b^+CD33^+HLA-DR^-$, monocytic MDSCs [M-MDSCs]: $CD11b^+CD33^+HLA-DR^-CD14^+CD15^-$, granulocytic MDSCs [G-MDSCs]: $CD11b^+CD33^+HLA-DR^-CD14^-CD15^+$, and monocyte/macrophages: $CD11b^+CD14^+CD68^+$; % of total live BM cells). **(D)** Frequencies of $CD4^+$ and $CD8^+$ T cells (% of total live BM cells). Cell frequency data are shown as median $\pm$ IQR. Statistical analyses were performed using Mann-Whitney tests with Holm-Šidák's multiple comparisons correction or the Kruskal-Wallis test with Dunn's multiple comparisons tests if >2 groups are being compared. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). **(E)** Normalized *VISTA* expression for each indicated cell subset calculated from asinh-transformed Δ MFI between *VISTA* and isotype control. Violin plots show median with IQR. Statistical analyses were performed using unpaired Welch's *t* tests with Holm-Šidák's multiple comparisons correction (** $p < 0.01$).

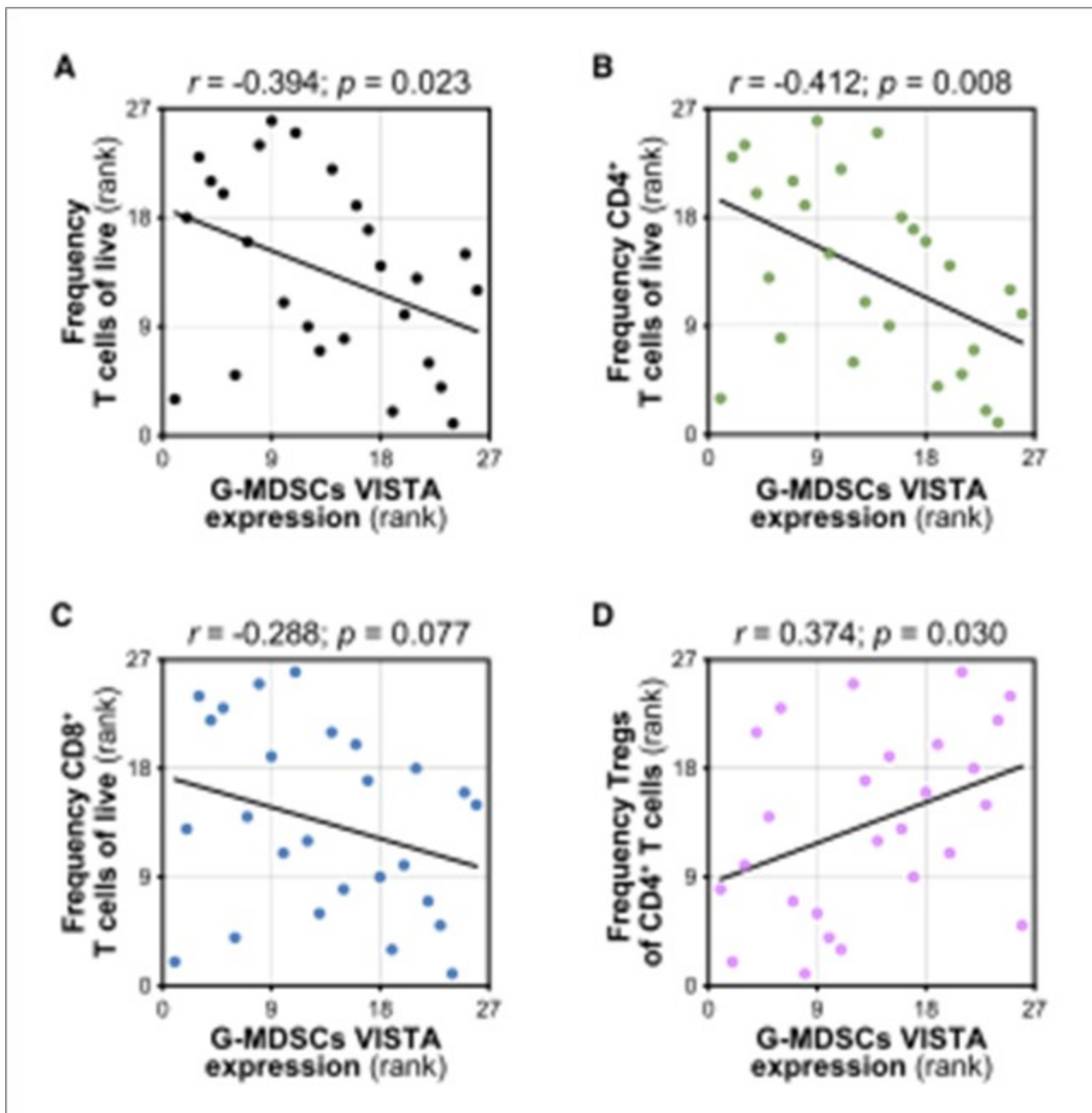


Figure 3

G-MDSC VISTA expression correlates with BM immune suppression in MDS

Correlation between G-MDSC VISTA expression and the frequency of **(A)** T cells (CD45^{hi}CD3⁺, % of total live BM cells), **(B)** CD4⁺ T cells (CD45^{hi}CD3⁺CD4⁺, % of total live BM cells), **(C)** CD8⁺ T cells (CD45^{hi}CD3⁺CD4⁺, % of total live BM cells), and **(D)** regulatory T cells (Tregs [CD45^{hi}CD3⁺CD4⁺CD25^{hi}CD127⁻], % of CD4⁺ T cells). Plots show ranks of each variable. Statistical analyses were performed using Spearman's rank correlation.

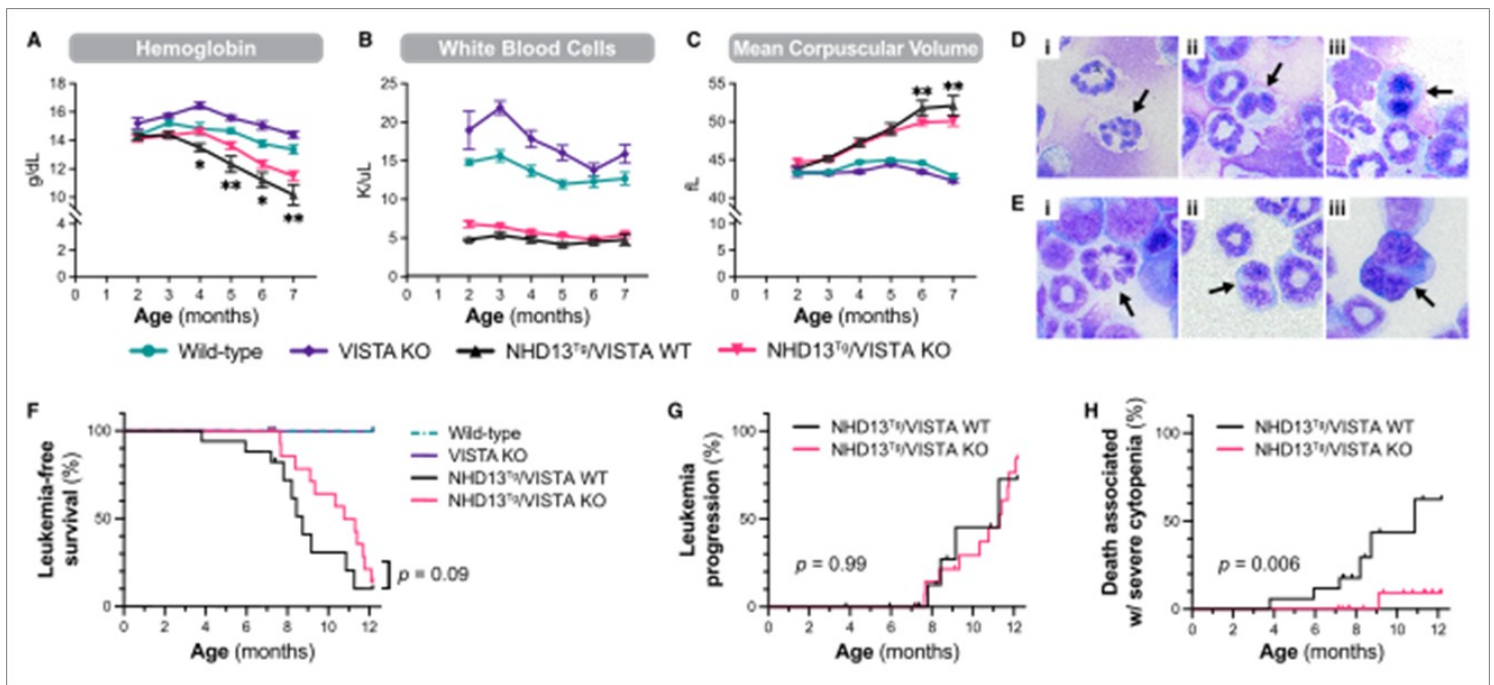


Figure 4

VISTA contributes to disease progression in murine MDS

(A) Hemoglobin, (B) white blood cell count, and (C) mean corpuscular volume of mice measured at the indicated months of age (WT, $n = 20$; VISTA KO, $n = 17$; NHD13^{Tg}/VISTA WT, $n = 17$; NHD13^{Tg}/VISTA KO, $n = 23$). Data are shown as mean $\pm$ SEM. Statistical significance was determined by mixed-effects model with Holm-Šidák's multiple comparisons test. Asterisks represent significant difference between NHD13^{Tg}/VISTA WT and NHD13^{Tg}/VISTA KO groups at the indicated timepoint (* $p < 0.05$, ** $p < 0.01$). Wright-Giemsa-stained BM cytopspins from (D) NHD13^{Tg}/VISTA WT and (E) NHD13^{Tg}/VISTA KO mice showing (i, ii) dysplastic myeloid elements with abnormal nuclear lobation and (iii) dysplastic erythroid elements with multinucleation. (F) Kaplan-Meier plot showing leukemia-free survival of animals over a 12-month study period. Tick marks represent censored subjects, which were euthanized at 7 months of age for further analysis. (G) Kaplan-Meier plot showing leukemia progression in NHD13^{Tg}/VISTA WT and NHD13^{Tg}/VISTA KO mice. Tick marks represent censored deaths with no evidence of leukemia progression. (H) Kaplan-Meier plot showing deaths associated with severe cytopenias without evidence of leukemia progression. Tick marks represent censored events where evidence of leukemia progression was present. Statistical analyses of Kaplan-Meier plots were performed using logrank (Mantel-Cox) tests to determine p -value.

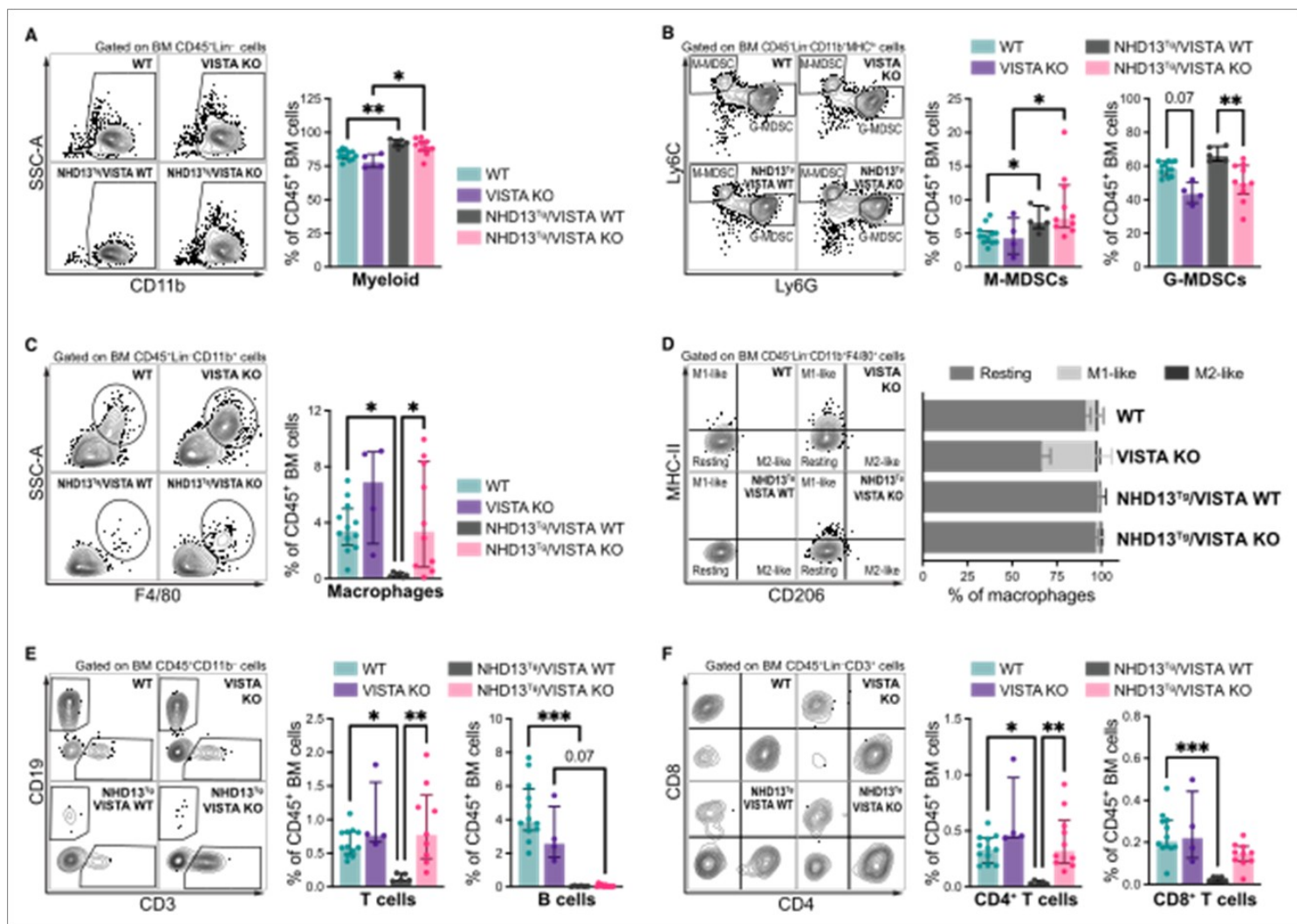


Figure 5

VISTA contributes to BM immune suppression in murine MDS

BM cells were collected from WT (n = 12), VISTA KO (n = 4), NHD13^{Tg}/VISTA WT (n = 5), and NHD13^{Tg}/VISTA KO (n = 10) mice at 7 months of age and assessed by flow cytometry. Frequencies and representative gating of **(A)** myeloid cells (CD11b⁺, % of live CD45⁺ BM cells), **(B)** M-MDSCs (CD11b⁺MHC-II^{-/-}Ly6C⁺Ly6G⁻) and G-MDSCs (CD11b⁺MHC-II^{-/-}Ly6C^{lo}Ly6G⁺, % of live CD45⁺ BM cells), **(C)** macrophages (CD11b⁺F4/80^{hi}, % of live CD45⁺ BM cells), **(D)** macrophage subsets (M1-like: MHC-II⁺CD206⁻, M2-like: MHC-II⁻CD206⁺, and resting: MHC-II⁻CD206⁻, % of total macrophages), **(E)** T cells (CD3⁺) and B cells (CD19⁺, % of live CD45⁺ BM cells), and **(F)** T cell subsets (CD4⁺ or CD8⁺, % of live CD45⁺ BM cells). Data are shown as median ± IQR. Statistical analyses were performed using the Kruskal-Wallis test with Dunn's multiple comparisons testing (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Supplementary Files

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- [SupplementaryTable5FINAL.xlsx](#)
- [SupplementaryInformationFINAL.pdf](#)