# SUPPLEMENTARY INFORMATION

## SUPPLEMENTARY MATERIALS AND METHODS

### Patient Samples

All patients gave informed consent to participate in this study. MDS patient samples (Supplementary Table 1) were obtained from the Vanderbilt-Ingram Cancer Center Hematologic Malignancy Tumor Bank, collected from patients who gave informed consent for sample banking and under a tissue collection protocol approved by the Vanderbilt University Medical Center (VUMC) Institutional Review Board (IRB #081012). Healthy donor BM cells were purchased (STEMCELL Technologies). All experiments using patient samples were conducted in accordance with the Declaration of Helsinki had the approval and guidance of the VUMC Institutional Review Board (IRB #192382).

### 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). Wild-type (WT) C57BL/6NCrl mice were purchased from Charles River Laboratories. *Vsir*$^{-/-}$ (VISTA KO; GenBank gene NM_028732; GenBank protein JN6-01284) mice were originally purchased from the Mutant Mouse Regional Resource Center at the University of California-Davis. NUP98-HOXD13 transgenic hemizygous MDS mice (NHD13$^{Tg}$; originally generated by Dr. Peter Aplan at the National Cancer Institute, National Institutes of Health, Bethesda, MD) were crossed

with WT or VISTA KO mice to obtain age-matched cohorts of WT, VISTA KO, NHD13$^{Tg}$/ VISTA WT, and NHD13$^{Tg}$/VISTA KO mice.

**Computational analyses of bulk RNA sequencing datasets**

*Processing of whole transcriptome data.* Raw sequencing reads in FASTQ format were aligned to the human reference genome (GRCh38, Ensembl release 86) using STAR (version 2.7.11b) [1]. To ensure precise quantification, reads were mapped directly to the transcriptome. Subsequently, gene expression abundances and raw read counts were estimated using RSEM (version 1.3.3) [2].

*Gene expression quantification and analysis.* To evaluate gene expression levels, Transcripts Per Million (TPM) values were log2-transformed after adding a pseudocount of 1 (log2[TPM + 1]). The expression of the *VSIR* gene was compared between MDS patients and healthy controls. Statistical significance was determined using a two-tailed Student's t-test, with $p < 0.05$ considered statistically significant.

*Differential expressed gene analysis.* To identify genes differentially expressed between MDS patients and healthy controls, we utilized the DESeq2 (1.50.2) [3]. The analysis was performed using a generalized linear model based on the negative binomial distribution, which effectively accounts for the overdispersion typical of RNA-seq count data. Raw read counts were normalized using the internal median-of-ratios method to adjust for differences in sequencing depth across samples. Statistical significance was evaluated using the Wald test. Genes were defined as differentially expressed (DEGs) if they exhibited an absolute Log2-fold change (log2FC) > 0.5, < -0.5 and $p < 0.05$.

*Gene-to-gene correlation analysis.* To identify alterations in gene-gene co-expression patterns associated with MDS, differential correlation analysis was performed using the DGCA (version 1.0.3) [4]. Raw read counts were normalized and transformed via variance stabilizing transformation (VST) within the DESeq2 (1.50.2) framework to account for heteroskedasticity. To ensure the robustness of the correlation estimates, low-expressed genes were filtered out based on a median percentile threshold of 0.3. Pearson correlation coefficients were then calculated for gene pairs in both MDS patients and healthy controls. The statistical significance of the correlation differences between the two groups was evaluated using a permutation-based approach ($n$ = 100). Pairs with a $p < 0.05$ in the MDS group were considered statistically significant.

**Analysis of gene expression data from BM CD34$^+$ cells**

*Microarray data processing.* Raw intensity data in CEL format (Human Genome U133 Plus 2.0 array) were processed using the Robust Multi-array Average (RMA) algorithm within the affy package (version 1.88.0) for background correction, normalization, and summarization [5]. Probe identifiers were mapped to official gene symbols using the hgu133plus2.db. To ensure a unique expression value for each gene, multiple probes corresponding to the same gene symbol were aggregated by calculating their mean expression levels.

*Statistical comparison of gene expression.* To evaluate *VSIR* expression within the microarray dataset, RMA-normalized values were compared between MDS patients

and healthy donors. Statistical significance was determined using a two-tailed Student's *t* test, with $p < 0.05$ considered statistically significant.

*Survival and hazard ratio analysis.* To evaluate the prognostic significance of VSIR expression, patients were stratified into "High" and "Low" expression groups based on the mean expression level. Survival curves were estimated using the Kaplan-Meier method, and the statistical significance of the differences between the survival distributions of the two groups was assessed using the log-rank test. The impact of VSIR expression on overall survival was further quantified by calculating the Hazard Ratio (HR) and 95% Confidence Interval (CI) using a univariate Cox proportional hazards regression model. The proportional hazards assumption was verified using Schoenfeld residuals (cox.zph). All clinical analyses were performed using the survival (version 3.8-6), survminer (version 0.5.1), and broom (version 1.0.12) [6–8]. $p < 0.05$ was considered statistically significant.

**Flow cytometric analysis of patient samples**

Cryopreserved human BM samples were thawed in a 37ºC water bath and washed with RPMI 1640 media (Gibco) containing 10% FBS (Corning), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 1% Pen Strep (Gibco), and 25 U/mL Universal Nuclease for Cell Lysis (Pierce). To discriminate live/dead cells, cells were incubated in PBS (Corning) containing LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen) for 15 minutes at 4ºC then washed with FACS buffer (PBS containing 1% BSA, 0.09% sodium azide, and 2 mM EDTA). After blocking FC receptors with TruStain FcX™ (Biolegend) in FACS buffer for 5 minutes at room temperature, cells were divided

and stained with two separate antibody panels targeting cell surface markers and incubated for 1 hour at 4ºC (Supplementary Tables 2 and 3). After cell surface staining, cells were washed with FACS buffer then fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer protocols. Cells were then incubated with or without antibody targeting CD68 for 30 minutes at room temperature to stain for intracellular CD68. After staining, cells were washed with PBS and analyzed using a Cytek Aurora™ spectral flow cytometer (Cytek Bioscience).

Samples were exported in .fcs format and analyzed using FlowJo 9 software (BD). Example gating of cell populations is shown (Supplementary Figure 2). Mean fluorescence intensity (MFI) as measured by geometric mean of VISTA-BV421 was used to compare VISTA expression. Background fluorescence of each sample was removed by subtracting the MFI of an isotype control for each sample from the MFI of VISTA then transformed to a linear scale using an inverse hyperbolic sine transformation (asinh).

**Evaluation of MDS in mice**

Peripheral blood (PB) was obtained from the tail vein using K2 EDTA as an anticoagulant, and complete blood counts (CBCs) were determined using a GenX™ Veterinary Hematology Analyzer (Oxford Science Inc., Oxford, CT). PB with WBC count one standard deviation greater than the mean was further assessed by flow cytometry. To assess for blasts in PB, erythrocytes were first lysed by incubation with RBC lysis buffer (Biolegend). PB cells were then washed and stained with FITC-conjugated anti-

mouse CD45 (clone I3/2.3, Biolegend) and APC-conjugated anti-mouse CD117 (clone 2B8, Biolegend) antibodies before analysis with a FACSCelesta™ (BD, Franklin Lakes, NJ) flow cytometer. Blasts were identified by CD117 (c-kit) positivity or by CD45/side scatter gating ($CD45^{dim}/SSC^{low}$; Supplementary Figure 5). Mice with greater than 20% blasts in the PB, with WBC count three standard deviations greater than the mean, or found to be moribund were euthanized. A subset of mice was also euthanized at a 7-month timepoint for assessment prior to leukemic progression. After euthanization, BM cells were obtained from femurs and tibia by flushing with collection buffer (Hanks' Balanced Salt Solution [Corning] containing 2% FBS [Corning], 1% Pen Strep [Gibco], 2 mM L-Glutamine [Gibco], and 1 mM EDTA [Corning]) then incubation with RBC lysis buffer to remove erythrocytes. BM cytospins were prepared using a SlidePrep Plus™ cytology centrifuge (EKF Diagnostics, Wales, UK). Morphology of peripheral blood smears and BM cytospins was assessed by Wright-Giemsa staining using the Hema 3™ Stain System (Fisher Healthcare) and pathologist review. To assess immune cell populations, BM cells were stained for flow cytometry analysis as previously described with some modifications [9]. Briefly, cells were stained using LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit for viability discrimination, washed, then incubated with Mouse TruStain FcX™ PLUS (Biolegend) to block FC receptors. BM cells were then incubated with fluorescent antibodies targeting cell surface markers (Supplementary Table 4), fixed with PBS containing 1% paraformaldehyde (Thermo Scientific), and analyzed using an LSRFortessa™ (BD) flow cytometer.

**Colony-forming unit assays**

BM cells were collected from mice as described above and lineage cell-depleted using the EasySep™ Mouse Streptavidin RapidSpheres™ Isolation Kit (STEMCELL Technologies) according to manufacturer protocols. Briefly, BM cells were incubated with EasySep™ Mouse FcR Blocker then labeled with biotin-conjugated antibodies targeting mouse B220 (clone RA3-6B2, BD), CD3e (clone 145-2C11, BD), CD4 (clone GK1.5, BD), CD8a (clone 53-6.7, BD), CD11b (clone M1/70, BD), Gr-1 (clone RB6-8C5, BD), and TER-119 (clone TER-119). Labeled cells were combined with EasySep™ Streptavidin RapidSpheres™ and magnetically separated from unlabeled, lineage-negative cells. Lineage-negative BM cells were plated in Methocult™ GF M3434 (STEMCELL Technologies) at concentration of 1-5x$10^3$ cells per mL for CFU assays or in Methocult™ SF M3436 (STEMCELL Technologies) at a concentration of 5x$10^3$ cells per mL for BFU-E assays. Colonies were counted after 12-14 days days using the STEMvision™ instrument and analysis software (STEMCELL Technologies, Vancouver, Canada).

**Statistics**

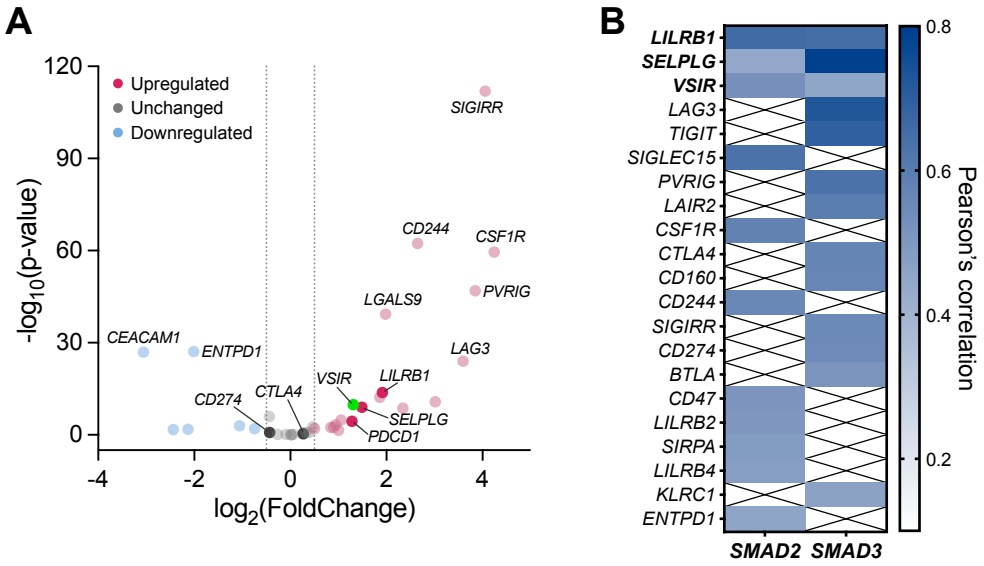
Unless otherwise noted, graphs and statistical analyses were generated using GraphPad Prism version 10.6.1. The Mann-Whitney test with Holm-Šídák's multiple comparisons correction was used to compare cell population frequencies between two groups in statistical analyses of patient samples. Unpaired t tests with Welch correction and Holm-Šídák's multiple comparisons test was performed to compare VISTA expression. To examine relationships between VISTA expression and cell frequencies in MDS patient BM, Spearman's rank-order correlations were calculated. *In vivo* studies

with WT, VISTA KO, NHD13Tg, and NHD13Tg;VISTA KO mice used a mixed-effects model with Holm-Šídák's multiple comparisons test to compare blood counts, and a logrank (Mantel-Cox) test was performed to compare survival trends. The nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test was used to identify differences in cell population frequencies. $p < 0.05$ was considered significant in all statistical analyses.

**Code availability**

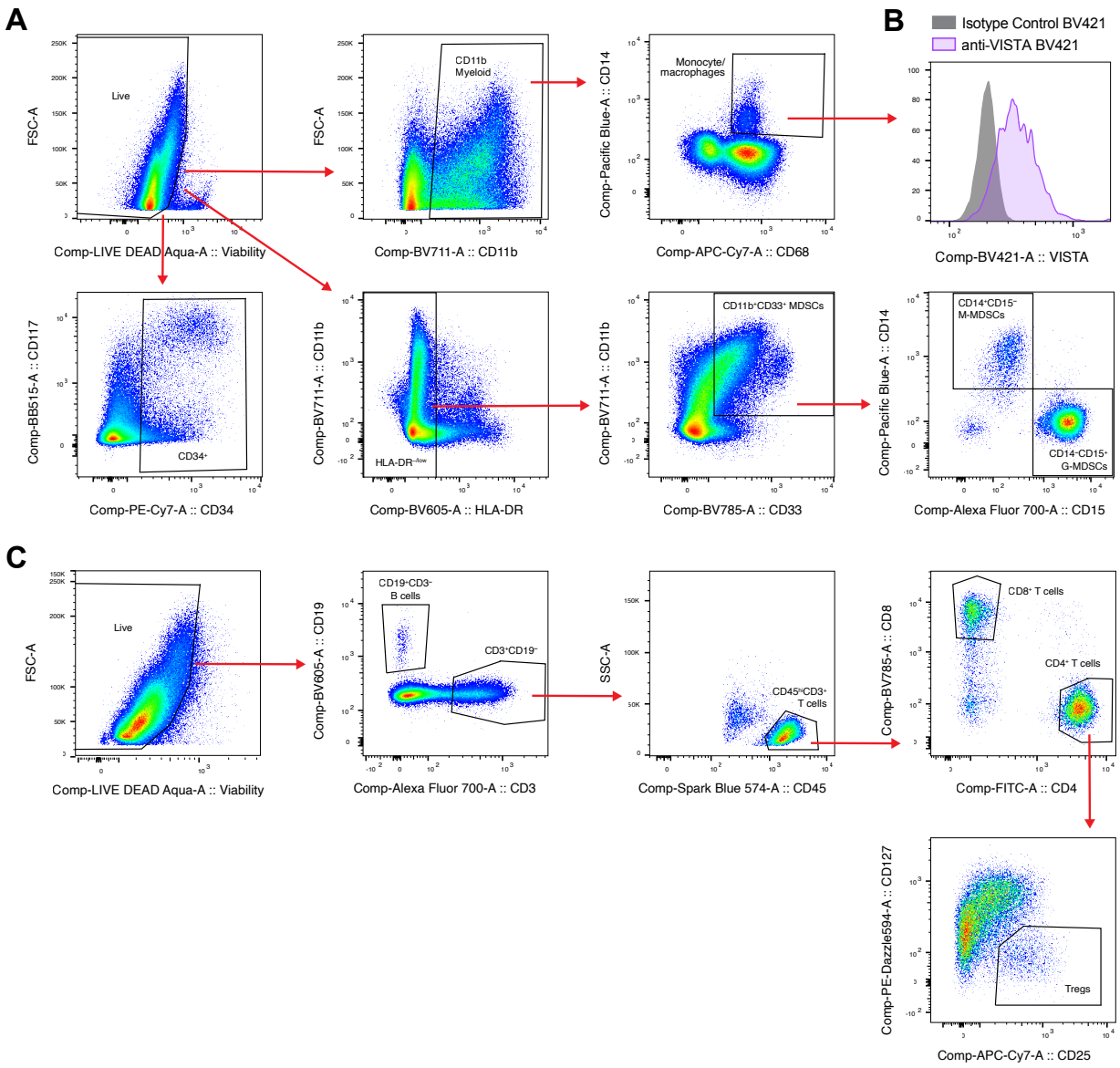
The complete bioinformatics pipeline and custom R scripts used for data processing, statistical analysis, and visualization are available on GitHub at https://github.com/TK-Lab-Vanderbilt-Univeristy/VISTA-mediates-the-progression-of-myelodysplastic-syndrome-Paper-2026/

## SUPPLEMENTARY FIGURES



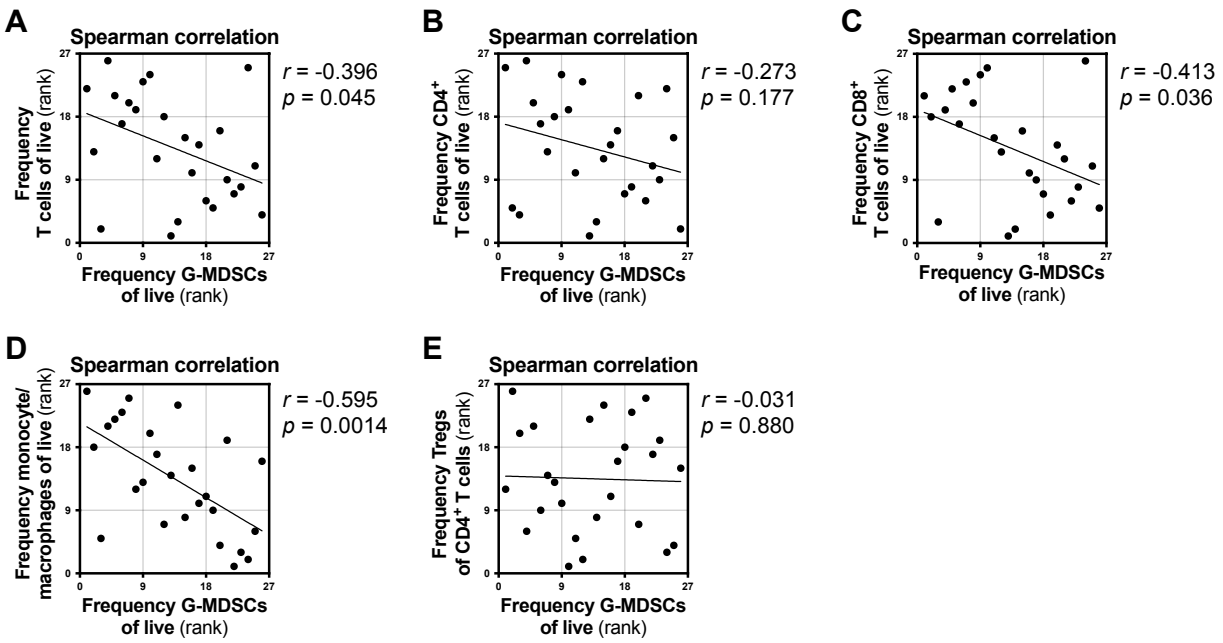

**Supplementary Figure 1. Gene expression analysis of MDS whole BM**

**A** Volcano plot comparing differential expression of immune checkpoint genes in MDS patient vs. healthy donor whole BM. **B** Heat map showing Pearson's correlation coefficients of immune checkpoint gene expression with *SMAD2* or *SMAD3* expression. Non-significant correlations are shown as a crossed-out box. Immune checkpoint genes not significantly correlated with either SMAD2 or SMAD3 are not listed.



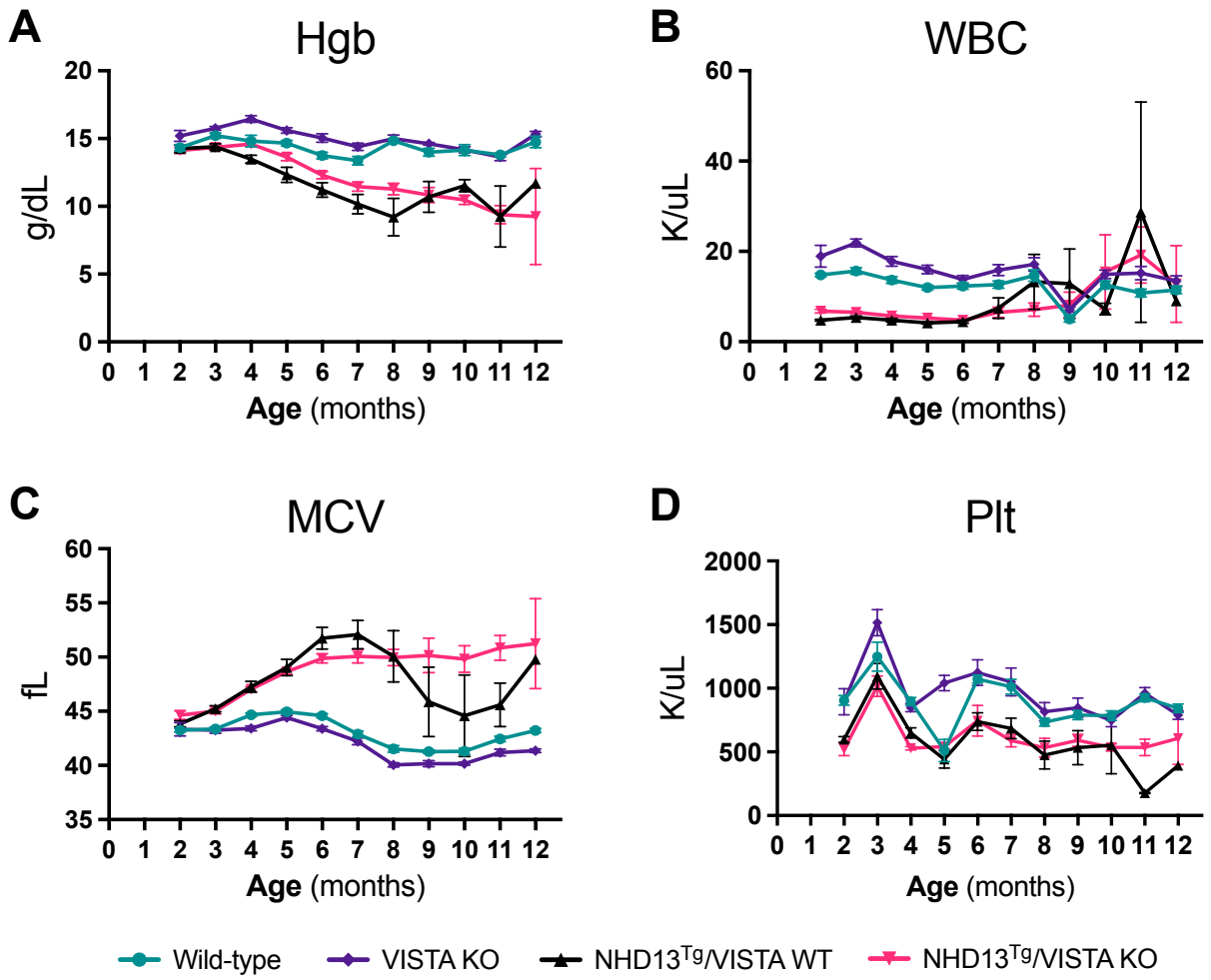

**Supplementary Figure 2. Gating strategy for patient MDS BM flow cytometry**

**A** Representative gating strategy for MDS myeloid panel. **B** Example histogram showing monocyte/macrophage VISTA staining (purple) compared to isotype control (gray). Difference in geometric mean fluorescent intensity was used to calculate VISTA expression. **C** Representative gating strategy for MDS lymphoid panel.



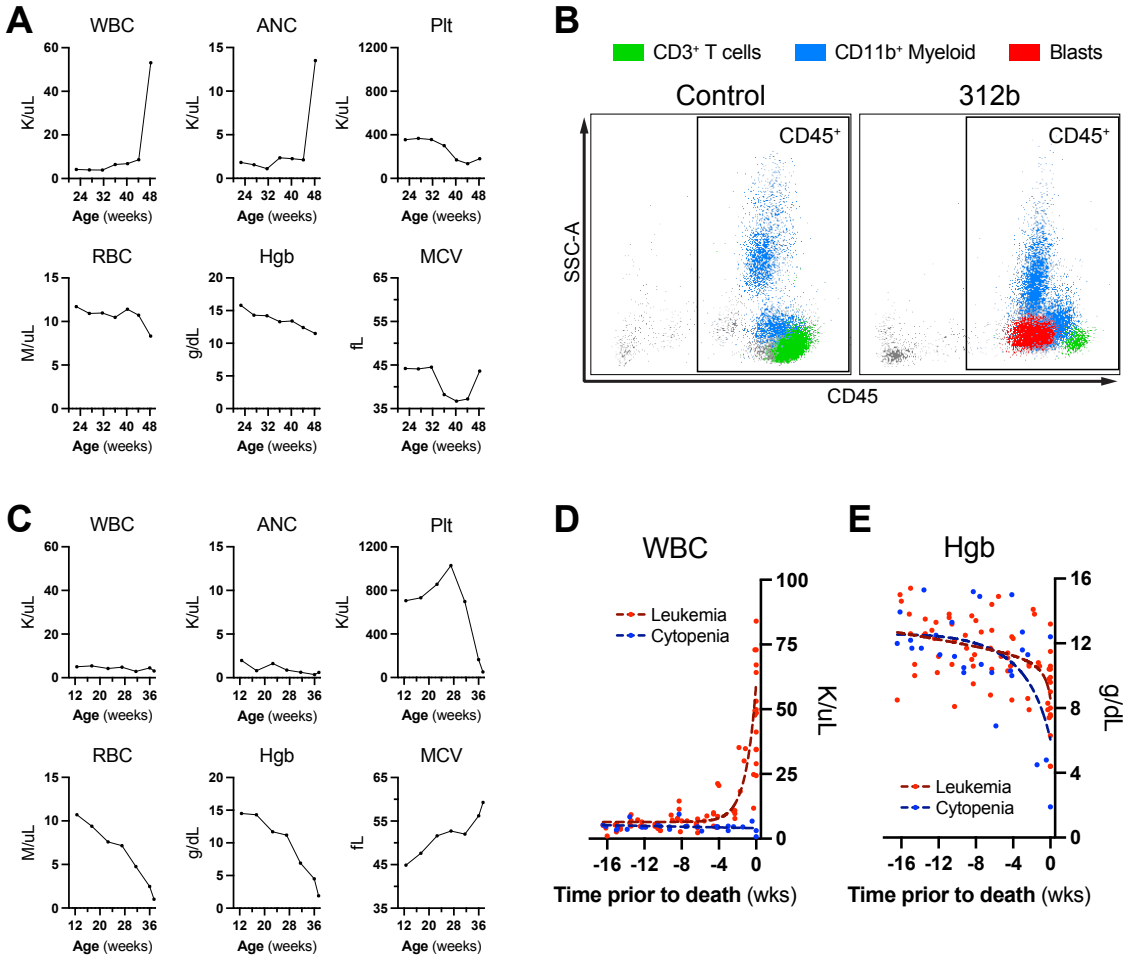

**Supplementary Figure 3. Correlation of G-MDSC frequency with different immune populations**

Correlation between G-MDSC frequency ($CD11b^+CD33^+HLA\text{-}DR^{-/low}CD14^-CD15^+$, % of total live BM cells) and the frequency of **A** T cells ($CD45^{hi}CD3^+$), **B** $CD4^+$ T cells ($CD45^{hi}CD3^+CD4^+$), **C** $CD8^+$ T cells ($CD45^{hi}CD3^+CD8^+$), **D** monocyte/macrophages ($CD11b^+CD14^+CD68^+$), and **E** regulatory T cells ($CD45^{hi}CD3^+CD4^+CD25^{hi}CD127^-$, % of $CD4^+$ T cells). Plots show ranks of each variable. Statistical analyses were performed using Spearman's rank correlation.




**Supplementary Figure 4. Blood counts for experimental animals**

**A** Hemoglobin (Hgb), **B** white blood cell count (WBC), **C** mean corpuscular volume (MCV), and **D** platelet count (Plt) of mice measured at the indicated months of age. Data shown are from the same mice as in Figure 4. Of note, slightly less than half of animals were euthanized at 7-8 months of age for BM analysis. Additional animals were found dead or euthanized due to disease progression past 7 months. Data are shown as mean ± SEM of surviving animals at each time point. Statistical comparisons were performed using a mixed-effects model with Holm-Šídák's multiple comparisons test. Complete results of these statistics are shown in Supplementary Table 5.




202 **Supplementary Figure 5. Monitoring for leukemia progression in NHD13$^{Tg}$ mice**

203 **A** Example of a mouse which was determined to have progressed to leukemia (#312b,

204 NHD13$^{Tg}$). White blood cell count (WBC), absolute neutrophil count (ANC), platelet

205 count (Plt), red blood cell count (RBC), hemoglobin (Hgb), and mean corpuscular

206 volume (MCV) for the individual mouse are shown at the indicated weeks of age. Final

207 measurement was taken immediately prior to euthanization. **B** Analysis of peripheral

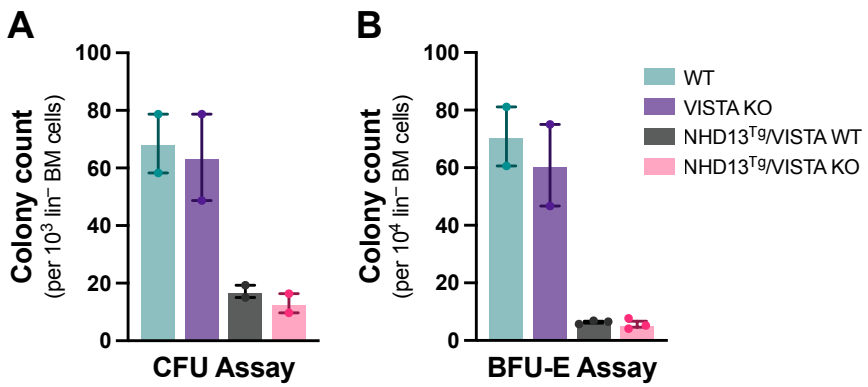
208 blood for blasts gated on single cells. CD117$^{+}$ (c-kit$^{+}$) blasts (red) are shown as a

209 SSC$^{low}$CD45$^{dim}$ population not present in the control sample. **C** Example of a mouse

210 with death associated severe cytopenia without leukemia progression (#318e,

211 NHD13$^{Tg}$/VISTA WT). Blood counts are shown at the indicated weeks of age. Final

measurement taken after the mouse was found moribund and immediately prior to euthanization. **D** WBC and **E** Hgb values of mice either determined to have leukemia progression (red) or death associated with severe cytopenia without leukemia progression (blue). Values are shown at the indicated weeks prior to euthanization or date found dead.




**Supplementary Figure 6. Colony-forming unit assays**

Lineage negative (lin⁻) bone marrow cells were isolated from mice and cultured in methylcellulose for 12-14 days prior to counting. **A** Total colony-forming units (CFU, grown in Methocult™ GF M3434) are shown normalized per 1,000 lin⁻ BM cells. **B** Blast-forming units-erythroid (BFU-E, cultured in Methocult™ SF M3436) are shown normalized per 10,000 plated lin⁻ BM cells. Statistical analyses were performed using Welch's one-way ANOVA with Welch's unpaired *t* multiple comparisons testing (not significant, ns: $p \geq 0.05$).

## SUPPLEMENTARY TABLES

| **Characteristic** | **MDS *(n = 26)*** | **Healthy Donor *(n = 8)*** |
|---|---|---|
| Gender | | |
| Male, n *(%)* | 14 *(53.8%)* | 7 *(87.5%)* |
| Female, n *(%)* | 5 *(19.2%)* | 1 *(12.5%)* |
| Undisclosed, n *(%)* | 7 *(26.9%)* | 0 *(0%)* |
| Age at collection, median yr *(range)* | 72.6 *(39.2 - 87.2)* | 38 *(21 - 47)* |
| No prior treatment, n *(%)* | 25 *(96%)* | n/a |
| WHO diagnosis | | |
| MDS-SLD, n *(%)* | 5 *(19.2%)* | n/a |
| MDS-MLD, n *(%)* | 6 *(23.1%)* | n/a |
| MDS-RS-MLD, n *(%)* | 2 *(7.7%)* | n/a |
| MDS-EB-1, n *(%)* | 7 *(26.9%)* | n/a |
| MDS-EB-2, n *(%)* | 6 *(23.1%)* | n/a |
| IPSS-R risk | | |
| Low/very low, n *(%)* | 6 *(23.1%)* | n/a |
| Int/high/very high, n *(%)* | 17 *(65.4%)* | n/a |
| Unknown, n *(%)* | 3 *(11.5%)* | n/a |
| Karyotype | | |
| Normal, n *(%)* | 11 *(42.3%)* | n/a |
| Complex, n *(%)* | 10 *(38.5%)* | n/a |
| Other abnormality, n *(%)* | 5 *(19.2%)* | n/a |
| Mean hematocrit, % *(±SD)* | 29.9 *(±5.8)* | n/a |
| Mean platelet count, K/uL *(±SD)* | 113 *(±75)* | n/a |
| Mean ANC, K/uL *(±SD)* | 2.37 *(±1.75)* | n/a |

**Supplementary Table 1.** Patient characteristics for samples used in bone marrow flow cytometry analysis. *SLD*: single lineage dysplasia, *MLD*: multilineage dysplasia, *RS-MLD*: ring sideroblasts and multilineage dysplasia, *EB-1*: excess blasts-1, *EB-2*: excess blasts-2, *complex karyotype*: ≥ 3 chromosomal abnormalities, *ANC*: absolute neutrophil count.

| Target | Fluorophore | Clone | Company | Catalog No. |
|---|---|---|---|---|
| CD11b | BV711 | ICRF44 | Biolegend | 301343 |
| CD14 | Pacific Blue | M5E2 | Biolegend | 301816 |
| CD15 | Alexa Fluor 700 | W6D3 | Biolegend | 323026 |
| CD33 | BV785 | WM53 | Biolegend | 303427 |
| CD34 | PE-Cy7 | 8G12 | BD | 348801 |
| CD45 | Spark Blue 574 | 2D1 | Biolegend | 368557 |
| CD68 | APC-Cy7 | Y1/82A | Biolegend | 333821 |
| CD117 | BB515 | 104D2 | BD | 565172 |
| CD163 | PE-Dazzle594 | GHI/61 | Biolegend | 333623 |
| HLA-DR | BV605 | L243 | Biolegend | 307639 |
| VISTA | BV421 | MIH65 | BD | 566751 |
| PSGL-1 | PE | KPL-1 | Biolegend | 328805 |
| PD-1 | PE-Fire810 | A17188B | Biolegend | 621625 |
| PD-L1 | APC | MIH1 | BD | 563741 |
| LAIR-1 | PerCP-Cy5.5 | NKTA255 | Biolegend | 342804 |
| Viability | LIVE/DEAD Aqua | | Invitrogen | L34957 |

**Supplementary Table 2. Human Myeloid Flow Cytometry Panel**

Fluorescent antibodies and stains used in bone marrow flow cytometry study for identification and assessment of myeloid and progenitor cell populations.

| Target | Fluorophore | Clone | Company | Catalog No. |
|---|---|---|---|---|
| CD3 | Alexa Fluor 700 | SK7 | Biolegend | 344821 |
| CD4 | FITC | SK3 | Biolegend | 300506 |
| CD8 | BV785 | SK1 | Biolegend | 344739 |
| CD19 | BV605 | HIB19 | Biolegend | 302243 |
| CD25 | APC-Cy7 | M-A251 | Biolegend | 356121 |
| CD45 | Spark Blue 574 | 2D1 | Biolegend | 368557 |
| CD127 | PE-Dazzle 594 | A019D5 | Biolegend | 351335 |
| VISTA | BV421 | MIH65 | BD | 566751 |
| PSGL-1 | PE | KPL-1 | Biolegend | 328805 |
| PD-1 | PE-Fire810 | A17188B | Biolegend | 621625 |
| PD-L1 | APC | MIH1 | BD | 563741 |
| LAIR-1 | PerCP-Cy5.5 | NKTA255 | Biolegend | 342804 |
| Viability | LIVE/DEAD Aqua | | Invitrogen | L34957 |

**Supplementary Table 3. Human Lymphoid Flow Cytometry Panel**

Fluorescent antibodies and stains used in bone marrow flow cytometry study for identification and assessment of lymphoid cell populations.

| Target | Fluorophore | Clone | Company | Catalog No. |
|---|---|---|---|---|
| CD3 | BV421 | 17A2 | Biolegend | 100228 |
| CD4 | BUV737 | GK1.5 | BD | 612761 |
| CD8 | BUV395 | 53-6.7 | BD | 563786 |
| CD11b | PE | M1/70 | BD | 557397 |
| CD11c | BV570 | N418 | Biolegend | 117331 |
| CD14 | PerCP-Cy5.5 | Sa14-2 | Biolegend | 123314 |
| CD19 | BV605 | 6D5 | Biolegend | 115540 |
| CD45 | APC/Cy7 | 30-F11 | Biolegend | 103116 |
| CD69 | PE/Cy5 | H1.2F3 | Biolegend | 104510 |
| CD206 | AF 700 | C068C2 | Biolegend | 141734 |
| F4/80 | APC | BM8 | Biolegend | 123115 |
| Ly6C | BV785 | HK1.4 | Biolegend | 128041 |
| Ly6G | PE/Cy7 | 1A8 | Biolegend | 127618 |
| MHCII | BV650 | M5/114.15.2 | Biolegend | 107641 |
| NK1.1 | AF 488 | PK136 | Biolegend | 108718 |
| Viability | LIVE/DEAD Aqua | | Invitrogen | L34957 |

**Supplementary Table 4. Mouse Immune Flow Cytometry Panel**

Fluorescent antibodies and stains used for identification and assessment of immune cell populations in the bone marrow of experimental animals after euthanization.

**Supplementary Table 5. Statistics for CBC values** *(Table attached separately)*

Related to Figure 4A-C and Supplementary Figure 4. Animals were followed for 12 months with monthly CBCs. Statistical comparisons were performed using a mixed-effects model with Holm-Šídák's multiple comparisons testing.

## SUPPLEMENTARY REFERENCES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