Supporting Information

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SI Text

Although many diagnostic flow cytometry laboratories are beginning to use six-color flow cytometry into their clinical practice, many laboratories still do not possess the ability to evaluate more than four colors simultaneously. Because identification of myeloid progenitors typically requires at least five colors (Lin, CD34, CD38, CD123, CD45RA), we reevaluated the same flow cytometry data files by using only four colors to identify "pseudo-" or "surrogate" GMP ($CD34^+$, $CD38^+$, $CD123^+$, $CD45RA^+$), CMP, and MEP populations. Unfortunately, using this strategy, attempting to gate on myeloid progenitors without performing a pregate on Lin^- cells did not result in statistically significant differences in low risk MDS and non-MDS samples, possibly due to the variable inclusion of peripheral blood cells retrieved during bone marrow aspiration.

SI Materials and Methods

Human Samples. Human bone marrow samples were collected from the Stanford Department of Pathology, according to an Institutional Review Board-approved protocol, with informed consent, where applicable. Samples were selected from submitted cases solely based on the ability of investigators to obtain and freeze the samples within 72 h after collection. The patient samples represented the full spectrum of World Health Organization (WHO) subtypes of low risk myelodysplastic syndromes (MDS) without excess blasts ($n = 46$; includes refractory anemia, refractory anemia with ringed sideroblasts, refractory cytopenia with multilineage dysplasia, and myelodysplastic syndrome not otherwise specified but with fewer than 5% blasts) and high risk MDS ($n = 7$; refractory anemia with excess blasts, includes both WHO RAEB-1 and RAEB-2 subtypes). Controls included a group of non-MDS related cytopenias ($n = 32$) and young and elderly normal controls ($n = 34$). The group of MDS patients represented both primary diagnostic samples and those with previously known diagnoses. Samples were either processed and frozen as mononuclear cell fractions for future analysis or evaluated immediately. Mononuclear cells were isolated by using Ficoll-Paque Plus (GE Healthcare). Mononuclear cells were cryopreserved in 90% FBS/10% (vol/vol) DMSO. Cryopreserved samples were thawed and washed with IMDM containing 10% (vol/vol) FBS and DNase I.

Flow Cytometry and Cell Sorting. Antibodies used to stain mononuclear cell suspensions for analysis and sorting of hematopoietic stem and progenitor subpopulations included the following: PE-Cy5–conjugated anti-human lineage markers [CD3, S4.1; CD4, S3.5; CD7, CD7-6B7; CD8, 3B5; CD10, 5–1B4; CD14, TU.K.4; CD19, Sj25-C1; CD20, 13.6E12 (Caltag/Invitrogen); CD2, RPA-2.10; CD11b, ICRF44; CD56, B159; GPA, GA-R2 (BD Biosciences)]; V450-conjugated anti-CD45RA, MEM56; PE-Cy7–conjugated anti-CD38, HIT2, APC-conjugated anti-CD34, 8G12, PE-conjugated anti-CD123, FITC-conjugated anti-CD90, 5E10, FITC-conjugated or PE-conjugated anti-CD47, B6H12.2 (BD Biosciences), and FITC-conjugated anti-CRT, clone FMC 75. Cells were analyzed and sorted by using a FACSAriaII cytometer (BD Biosciences). FlowJo Software (Treestar) was used to analyze flow cytometry data.

Mouse Transplantation. NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice obtained originally from The Jackson Laboratory were bred in a specific pathogen-free environment according to a protocol approved by the Stanford Administrative Panel on Laboratory Animal Care. Prenatal day (P)0–P3 newborn pups were preconditioned with 100 rads of gamma irradiation 4–24 h before transplantation. Cells resuspended in PBS containing 2% FCS were transplanted i.v. via the anterior facial vein by using a 30- or 31-gauge needle.

Analysis of Human Chimerism. The following panel of antibodies (Caltag/Invitrogen, and BD Biosciences) was used for analysis of human engraftment/chimerism: PB-conjugated CD45, HI30; APC-conjugated anti-CD34, 8G12; Alexa Fluor 750-conjugated CD3, S4.1; Alexa Fluor 700-conjugated CD19, SJ25-C1; PE-conjugated CD13, TK1; PE-conjugated CD33, P67.6; PE-Cy5–conjugated GPA, GA-R2; FITC-conjugated CD41a, HIP8. The following panel of antibodies (eBiosciences) was used to identify mouse leukocytes and red blood cells, respectively: Alexa488- or PE-Cy7–conjugated CD45.1, A20.1.7; PE-Cy5– or PE-Cy7–conjugated Ter119. Single-cell suspensions were prepared by using standard methods from bone marrow of transplanted mice. Red blood cells were lysed by using hypotonic solution ACK. For analyses and sorting, except otherwise noted below, cells were stained with the appropriate antibody combinations for 30–60 min on ice, and dead cells were excluded by propidium iodide staining.

FISH. Cells were sorted by FACS directly onto a slide for cytogenetic analysis by interphase fluorescence in situ hybridization (FISH). Briefly, slides were fixed in methanol/acetic acid 3:1 for 10–15 min, dehydrated in 70%, 80%, 90%, and 100% ethanol sequentially, and air dried. Slides were fixed in 1% neutral buffered formalin for 5 min, twice treated with microwave heated sodium citrate buffer at pH 6.0 for 5 min, digested with proteinase K (Sigma) for 5 min, fixed again in neutral buffered formalin for 5 min, dehydrated in 70%, 80%, 90%, and 100% ethanol sequentially, and air dried. The Vysis LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen Probe (Abbott Molecular) was used for hybridization, according to manufacturer's instructions. After hybridizations, slides were washed in 1× SSC for 5 min at 72 °C and then 0.05% TBS-Tween for 3 min at room temperature, counterstained with DAPI (Sigma). Nuclei of normal cells contain four distinct signals (two orange and two green). Nuclei from MDS patient bone marrow cells with monosomy 7 contain two distinct signals (one orange and one green).

Macrophage Isolation and Culture. Leukocyte-enriched human peripheral blood was obtained from the Stanford Blood Center. Mononuclear cells were isolated by using Ficoll-Paque (GE Healthcare Learning System) and plated in IMDM + 10% human serum (R&D Systems) with PenStrep and Glutamax for 6-d incubation. Peripheral blood-derived macrophages are an adherent population following 7-d incubation.

In Vitro Phagocytosis Assay. Human peripheral blood-derived macrophages were harvested by incubation in 10% EDTA (GIBCO) for 30 min at 4 °C and gentle scraping. Macrophages were plated at 5×10^4 cells per well in a 24-well tissue culture plate (Falcon) and incubated for 24 h. Fresh, serum-free media were added 2 h before introduction of target cells. To prepare target cells, FACS-sorted normal bone marrow and MDS bone marrow $CD34^+CD38^+$ and $CD34^-$ cells were incubated with 2.5 μM CFSE for 10 min. After incubation, target cells were incubated on ice for 15 min with either PBS, 2 μg/mL CRT-blocking peptide (MBL International), or 10 μg/mL anti-CD47 antibody (clone B6H12; eBiosciences). Target cells (1×10^5) were added to macrophage-containing wells and incubated for 2 h. After coincubation, wells were washed thoroughly with PBS five times and examined under a Leica microscope by using an enhanced green fluorescent protein (GFP) filter

set. Phagocytic index was calculated by using the following formula: phagocytic index = number of ingested cells/number of macrophages × 100. At least 300 macrophages were counted per well.

Statistical Analysis. Statistical analysis by using Student's *t* test, and calculations of specificity and sensitivity were done by using Microsoft Excel and/or GraphPad Prism software.

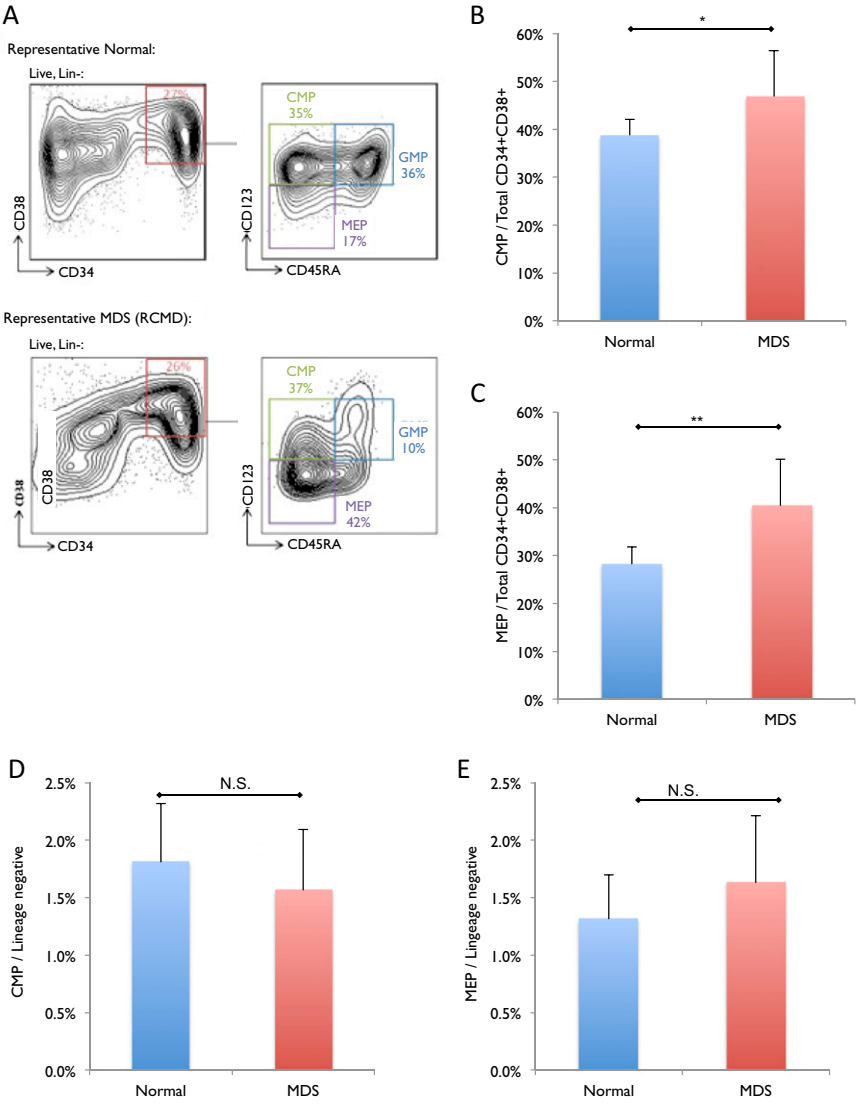

Fig.S1. Frequency of CMPS and MEPs in low risk MDS. (*A*) Representative gating strategy for normal and MDS CMPs ($Lin^-CD34^+CD38^+CD123^+CD45RA^-$), GMPs ($Lin^-CD34^+CD38^+CD123^+CD45RA^+$), and MEPs ($Lin^-CD34^+CD38^+CD123^-CD45RA^-$). (*B*) Frequency of CMPs out of total myeloid progenitors (CMPs + GMPs + MEPs) in normal (n = 34) and low risk MDS (n = 46). *$P < 0.02$ (*C*) Frequency of MEPs out of total myeloid progenitors (CMPs + GMPs + MEPs) in normal (n = 34) and low risk MDS (n = 46). **$P < 0.0003$. (*D*) Frequency of CMPs out of total lineage negative bone marrow mononuclear cells in normal and low risk MDS bone marrow samples. (*E*) Frequency of MEPs out of total lineage negative bone marrow mononuclear cells in normal and low risk MDS bone marrow samples. Error bars represent one standard deviation. "MDS" label represents low risk MDS samples. N.S., no significance.

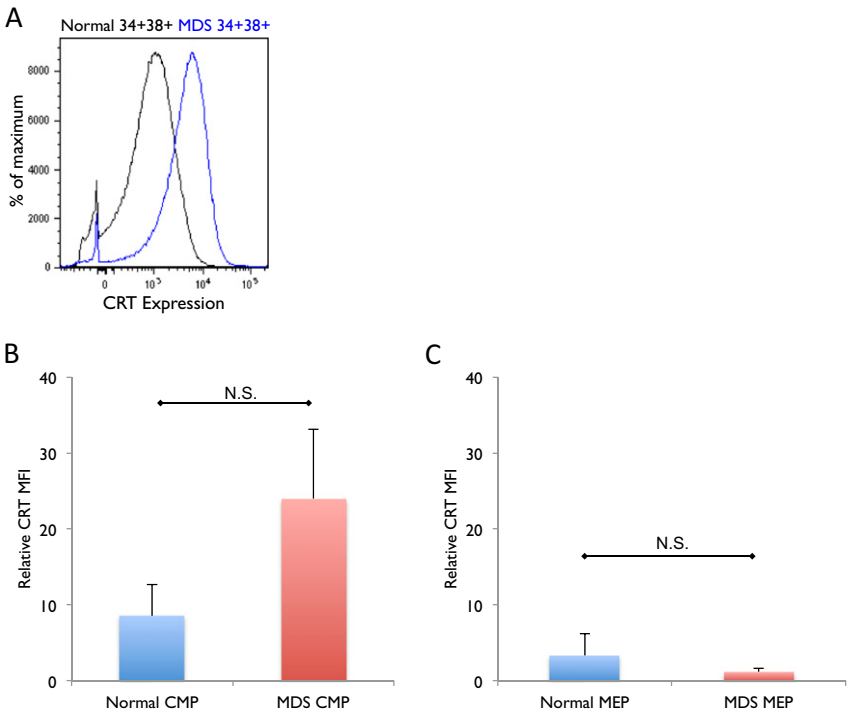

Fig. S2. Cell-surface CRT expression in low risk MDS. (*A*) Representative histogram of cell-surface CRT expression on normal and low risk MDS $CD34^{+}CD38^{+}$ myeloid progenitor cells. (*B*) Cell-surface CRT expression, as expressed by MFI relative to FMO control, on normal (n = 11) and low risk MDS (n = 14) CMPs. (*C*) Cell-surface CRT expression, as expressed by MFI relative to FMO control, on normal and low risk MDS CMPs. Error bars represent one SD. "MDS" label represents low risk MDS samples. N.S., no significance.

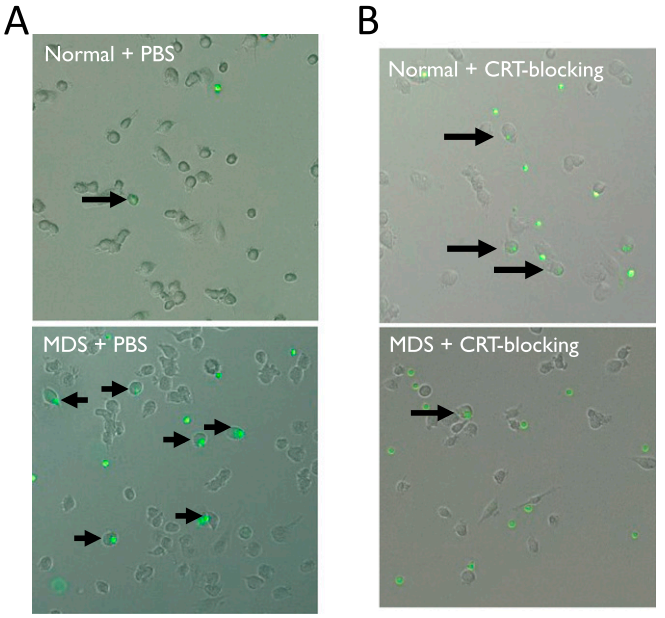

Fig. S3. Representative photomicrographs of phagocytosis of normal and low risk MDS $CD34^{+}CD38^{+}$ myeloid progenitors. Representative photomicrographs of (*A*) normal and low risk MDS $CD34^{+}CD38^{+}$ myeloid progenitors and (*B*) normal and low risk MDS $CD34^{+}CD38^{+}$ myeloid progenitors treated with CRT-blocking peptide.

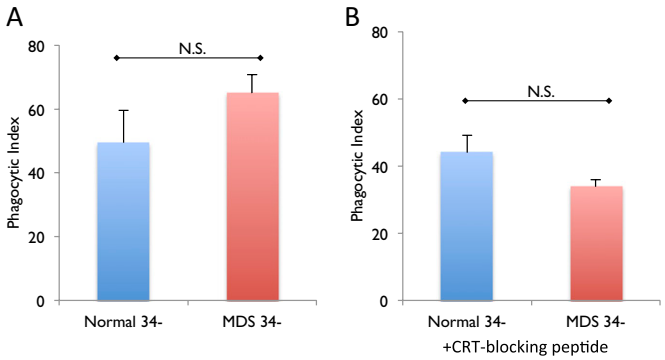

Fig. S4. Phagocytosis of CD34$^-$ cells in low risk MDS. (*A*) Phagocytosis of normal and low risk MDS CD34$^-$ cells. (N.S. no significance) (*B*) Phagocytosis of normal and low risk MDS CD34$^-$ cells treated with CRT-blocking peptide. Error bars represent one SD. "MDS" label represents low risk MDS samples. N.S., no significance.

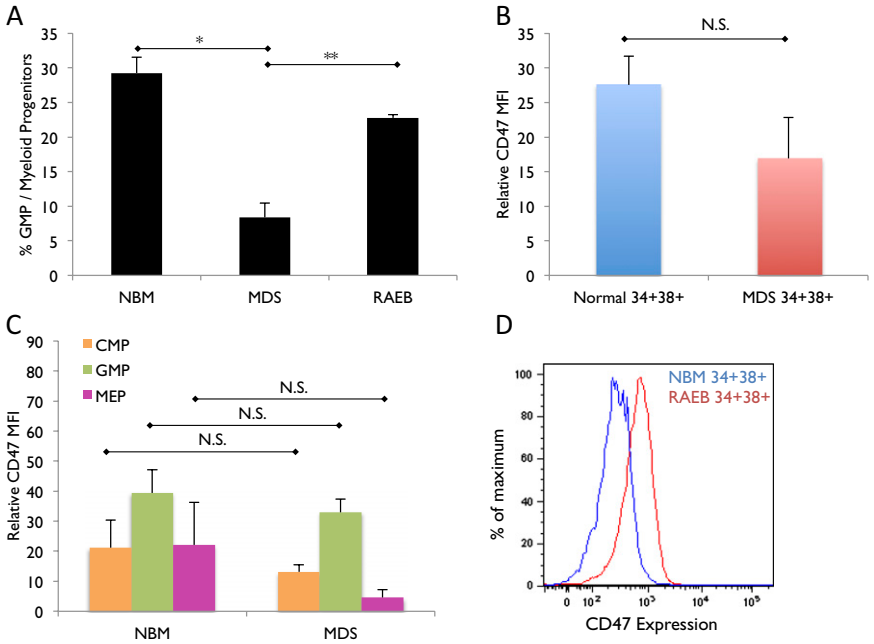

Fig. S5. CD47 expression on myeloid progenitor populations in low risk MDS. (*A*) Frequency of GMPs in normal and low risk MDS, and RAEB CD34$^+$CD38$^+$ populations. *$P < 10^{-13}$; **$P < 0.002$. (*B*) CD47 expression, as expressed by MFI relative to FMO control, on normal and low risk MDS CD34$^+$CD38$^+$ myeloid progenitor populations. (*C*) CD47 expression, as expressed by MFI relative to FMO control, on normal and low risk MDS CMP, GMP, and MEP populations. (*D*) Representative histogram of CD47 expression on normal and RAEB CD34$^+$CD38$^+$ myeloid progenitor cells. Error bars represent one SD. "MDS" label represents low risk MDS samples. N.S., no significance.

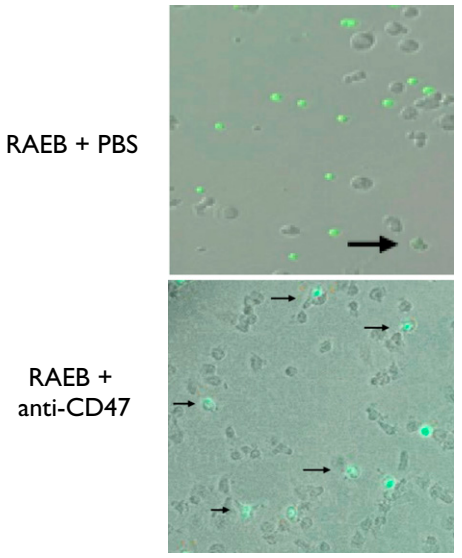

Fig. S6. Representative photomicrographs of phagocytosis of normal and low risk MDS $CD34^{+}CD38^{+}$ myeloid progenitors. Representative photomicrographs of RAEB $CD34^{+}CD38^{+}$ myeloid progenitors treated with PBS control or anti-CD47 antibody (B6H12).