Induction of myelodysplasia by myeloid-derived suppressor cells

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Myelodysplastic syndromes (MDS) are age-dependent stem cell malignancies that share biological features of activated adaptive immune response and ineffective hematopoiesis. Here we report that myeloid-derived suppressor cells (MDSC), which are classically linked to immunosuppression, inflammation, and cancer, were markedly expanded in the bone marrow of MDS patients and played a pathogenetic role in the development of ineffective hematopoiesis. These clonally distinct MDSC overproduce hematopoietic suppressive cytokines and function as potent apoptotic effectors targeting autologous hematopoietic progenitors. Using multiple transfected cell models, we found that MDSC expansion is driven by the interaction of the proinflammatory molecule S100A9 with CD33. These 2 proteins formed a functional ligand/receptor pair that recruited components to CD33’s immunoreceptor tyrosine-based inhibition motif (ITIM), inducing secretion of the suppressive cytokines IL-10 and TGF-β by immature myeloid cells. S100A9 transgenic mice displayed bone marrow accumulation of MDSC accompanied by development of progressive multilineage cytopenias and cytological dysplasia. Importantly, early forced maturation of MDSC by either all-trans-retinoic acid treatment or active immunoreceptor tyrosine-based activation motif–bearing (ITAM-bearing) adapter protein (DAP12) interruption of CD33 signaling rescued the hematologic phenotype. These findings indicate that primary bone marrow expansion of MDSC driven by the S100A9/CD33 pathway perturbs hematopoiesis and contributes to the development of MDS.

Introduction

Understanding the selective pressures and mechanisms involved in the initiation of stem cell malignancies is critical to development of effective strategies for prevention and treatment. Inflammatory molecules have long been implicated as regulatory cues driving the proliferation and apoptotic death of hematopoietic progenitors in myelodysplastic syndromes (MDS) (1–3). Chronic immune stimulation, coupled with senescence dependent changes in both hematopoietic stem/progenitor cells (HSPC) and the BM microenvironment, are believed to be critical to the pathogenesis of the disease (4). Increasing evidence implicates the activation of innate immune signaling in both hematopoietic senescence and the pathobiology of MDS (5). TLR4, for instance, is overexpressed in MDS BM HSPC and is implicated in progenitor apoptosis and subsequent development of cytopenias (6). Furthermore, gene expression of the TLR adaptor E3 ubiquitin ligase, TNF receptor-associated factor-6 (TRAF6), is markedly upregulated in MDS CD34+ cells (7) accompanied in some cases by amplification of genomic regions encoding TRAF6 or the Toll IL-1 receptor domain-containing adaptor protein (TIRAP), key intermediates in TLR4 signaling (8, 9). Recent investigations showed that TLR signaling is constitutively activated in MDS cells with chromosome 5q deletion [del(5q)] as a result of allelic deletion of microRNA 145 (miRNA-145) and miRNA-146a and consequent upregulation of their respective targets, TIRAP and TRAF6 (10). Moreover, knockdown of these specific miRNAs or overexpression of Traf6 in murine HSPC recapitulates the hematologic features of del(5q) MDS in a transplantaion model (10), providing convincing evidence that sustained TLR activation is a critical factor driving the malignant phenotype. More recent findings indicate that TRAF6 is essential for survival and proliferation of MDS HSPC (11) and sustained TLR activation skews their commitment toward the myeloid lineage while suppressing osteoblast differentiation (12, 13), analogous to the senescence-dependent changes observed with normal aging (14).

Immature myeloid-derived suppressor cells (MDSC), known to accumulate in tumor-bearing mice and cancer patients, are site-specific inflammatory and T cell immunosuppressive effector cells that contribute to cancer progression (15, 16). Their suppressive activity is in part driven by inflammation-associated signaling molecules, such as the danger-associated molecular pattern (DAMP) heterodimer S100A8/S100A9 (also known as myeloid-related protein 8 [MRP-8] and MRP-14, respectively), which interact with several innate immune receptors that are involved in the biology of MDSC activation (17–20). Murine CD11b+Gr1+ MDSC form the basis of the vast majority of the mechanistic studies; however, much less has been reported on their human counterparts. Human MDSC lack most markers of mature immune cells (LIN–, HLA-DR+) but possess CD33, the prototypical member of sialic acid–binding Ig-like super-family of lectins (Siglec) (15, 21–23). Importantly, while its precise action is unknown, CD33 possesses an immunoreceptor tyrosine-based inhibition motif (ITIM) that is associated with immune suppression (23).
Here, we show that LIN–HLA-DR–CD33+ MDSC specifically accumulate in the BM of MDS patients (herein referred to as MDS-MDSC) and impair hematopoiesis through a mechanism that involves S100A9 as an endogenous ligand for CD33-initiated signaling. Importantly, using S100A9 transgenic (S100A9Tg) mice, we show that sustained activation of this inflammatory pathway leads to the development of MDS and that this hematologic phenotype is rescued by strategies that suppress CD33 ITIM signaling. Our finding that S100A9 ligates CD33 to induce MDSC expansion suggests that targeting this pathway may provide a therapeutic approach for the treatment of MDS. Finally, the discovery of this signaling pathway verifies the role of S100A9 as an important initiator of immune suppression. S100A9Tg mice may therefore serve as a useful model for the study of MDS pathogenesis, treatment, and the overall role of MDSC in cancer.

Results

Lin–HLA-DR–CD33+ MDSC are expanded in MDS primary BM specimens and direct the suppression of autologous erythroid precursors. BM mononuclear cells (BM-MNC) were isolated from MDS BM aspirates (n = 12), age-matched healthy BM (n = 8), or non-MDS cancer patients (4 breast and 4 lymphoma) and analyzed for the presence of LIN–HLA-DR–CD33+ MDSC by flow cytometry. MDS patients exhibited markedly higher numbers of MDSC (median 35.5%, P < 0.0001) compared with healthy donors or non-MDS cancer patients (less than 5%, Figure 1A). To determine whether MDS-MDSC are derived from the malignant MDS clone, LIN–HLA-DR–CD33+ MDSC were sorted from MDS specimens with chromosome 7 FISH of sorted MDSC or non-MDSC from MDS BM-MNC (n = 5, CEP7 is green and 7q31 orange), β-thymidine incorporation (C) and IFN-γ ELISA (D) of stimulated autologous T cells cocultured with sorted MDS-MDSC at 1:0.25 and 1:0.5 ratios (T cells:MDSC). Error bars denote SD of 3 separate patient samples tested in triplicate. (E) BrdU incorporation of stimulated T cells after admixing with autologous unsorted, MDSC depleted (–MDSC), or remixed (+MDSC) BM-MNC. Sorted MDSC from MDS or healthy donor BM tested for IL-10 (F), TGF-β (G), NO (H), and arginase (I) production after 24 hours of culture. (J) MDSC/erythroid precursor contact zone of admixed sorted MDS-MDSC and autologous erythroid precursors at a ratio of 1:3 (MDSC/erythroid precursor) by microscopy at 0 and 30 minutes. Cells were stained for CD71 (pink), CD235a (blue), CD33 (red), and granzyme B (green). (K) Sorted MDSC from MDS or healthy donors labeled with CD33 (red) and granzyme B (green) coincubated with purified autologous erythroid precursors (0 or 30 minutes) and monitored by microscopy. (L) Counts of MDSC-HPC conjugate mobilized granules. Original magnification, ×400 (B); ×600 (J and K). (M) Annexin V exposure on erythroid precursors (CD71–CD235a+)) incubated with or without sorted autologous MDS-MDSC. (N) Colony-forming ability of unsorted, –MDSC, or remixed (+MDSC) MDS BM-MNC (ratio of 1:3); *P < 0.005; **P < 0.001; ***P < 0.05.

MDS-MDSC-mediated suppressive and cytotoxic effector functions require direct contact with target cells. One mechanism utilized by cytotoxic effectors is the mobilization of pore-forming granules and the release of caspase-activating effector proteases, such as granzyme B, to the site of effector/target contact, which induces apoptosis of the target cell (25). Since MDSC reside in close proximity with hematopoietic progenitor cells (HPC), which in MDS display an increased apoptotic rate, we tested whether MDS-MDSC-mediated cytostatic activity may contribute to HPC death. To address this, we examined MDS-MDSC granule mobilization and release of granzyme B using 4-color immunofluorescence staining. MDS-MDSC exhibited strong granzyme B polarization at the site of cell contact with CD235a+ (glycophorin A)/CD71+ autologous erythroid precursors (Figure 1J). After 30 minutes incubation, the frequency of such effector/target conjugates in MDS patient specimens was significantly higher (34%) than in samples from healthy donors (5% P < 0.001, Figure 1, K and L). These cellular interactions resulted in apoptosis of targeted erythroid precursors (Figure 1M), demonstrating that in addition to known MDSC-mediated immune suppressive functions, there is an unrecognized MDSC-mediated hematopoietic suppressive capacity. To corroborate this finding, we examined the effects of MDSC on the proliferative capacity of HSPC in MDS-MDSC–depleted, HSPC-enriched BM patient specimens in a methylcellulose colony formation assay. Burst-forming unit–erythroid (BFU-E) and GM-CFU colony formation were significantly higher in MDS-depleted specimens compared with both MDSC-supplemented and unsorted samples (Figure 1N), demonstrating that MDSC have a direct suppressive role on erythroid and myeloid progenitor cell development.
Increased CD33 expression and signaling contribute to MDSC suppressive functions and hematopoietic impairment. MDSC in humans characteristically express the surface transmembrane glycoprotein CD33, a Siglec 3 receptor that along with other members of this family, has prominent roles in inflammation (23, 26–35). However, the involvement of CD33 in myelopoiesis remains unexplored. Hence, we investigated the relationship between CD33 membrane expression density on MDS-MDSC and its activation and maintenance in MDS BM. We found that these cells robustly express CD33 at levels higher than LIN–HLA-DR–CD33+ cells isolated from non–MDS-associated cancer patients and healthy donor BM cells (Figure 2A). To explore the functional consequences of CD33 engagement, we crosslinked CD33 in U937 cells (a human monocytic cell line with high CD33 expression), which triggered IL-10, TGF-β, and VEGF secretion (Figure 2B). To examine whether CD33 can promote MDSC accumulation and/or activation, we overexpressed CD33 with an adenovirus vector in BM-MNC from healthy donors, which...
significantly suppressed myeloid cell development, as evidenced by reduced expression of the maturation markers CD11c, CD80, and CCR7 (Figure 2C). To further establish the role of CD33 in MDSC-mediated BM suppression, we knocked down CD33 in MDS-MDSC using a lentiviral vector (LV) containing CD33-specific shRNA. Coculture of autologous HSPC with CD33 shRNA–treated MDS-MDSC resulted in a 2- to 3-fold increase in BFU-E and GM-CFU colony recovery compared with those cultured with scrambled shRNA-treated and nontransduced MDS-MDSC and healthy donor BM MDSC (Figure 2, D and E). Moreover, production of IL-10, TGF-β, and arginase was reduced in CD33 shRNA–treated MDS-MDSC compared with control cells (Figure 2, F–H). Collectively, these data delineate a role for CD33 in MDSC activation and expansion in MDS and directing hematopoietic impairment.

S100A9 is a native ligand for CD33. Although our investigations show that CD33/Siglec 3 is a key receptor involved in functional activation of MDSC, its native ligand remains unknown. Therefore, we attempted to identify potential ligand(s) for this receptor by creating a chimeric fusion protein with the ectodomain of CD33 and the Fc portion of human IgG (hereafter referred to as CD33-fusion). BM cell lysates from MDS patients were immunoprecipitated with the CD33-fusion followed by high-throughput mass-spectrometric analysis on associated peptides. S100A9, an inflammatory signaling molecule known to activate MDSC, was among the most prominent protein bands identified (Figure 3A). To confirm the specificity of the binding of this DAMP to CD33, we prepared both S100A8 and S100A9 transfectants in the rhabdomyosarcoma cell line SJCRH30, which lacks detectable expression of endogenous CD33 or S100A9. The CD33-fusion (APC) stained only S100A9 transfected cells, but not S100A8 transfected cells, confirming binding specificity (Figure 3B); transfectants were stained with FITC, and specific binding by the CD33-fusion was stained with APC, shown in red; nuclei were stained with DAPI). Direct binding of S100A9 to CD33 was confirmed further in a sandwich ELISA where the capture antibody was anti-S100A9 (Figure 3C) as well as by dot blot analysis of transfected cell lysates with CD33-fusion (Figure 3D), demonstrating the specificity of the interaction. To fully corroborate the binding of this pair, we set out to perform a reverse immunoprecipitation on CD33 transfected cells showing that S100A9 coprecipitated with CD33 only in recombinant human S100A9–treated (rhS100A9-treated) cells (Figure 3E). To validate clinically the ligand specificity in MDS patients, we compared the pull-down with CD33-fusion (Figure 3D), demonstrating the specificity of this ligation pair, we explored the role of this interaction in replicating the functional responses observed with receptor crosslinking. We again induced CD33 overexpression in healthy BM cells through adenovirus transfection and studied their immunosuppressive properties with or without the addition of rhS100A9. Forced expression of CD33 induced a parallel increase in gene expression and secretion of the suppressive cytokines IL-10 and TGF-β (Figure 4, A–D), which was greatly increased by the addition of rhS100A9. Secretion of the suppressive cytokine TGF-β was only observed after rhS100A9 treatment of CD33 transfected cells (Figure 4D), accompanied by an increase in the expression of NO2 and ARG2, consistent with the suppressive cytokine profile (Figure 4, E and F). Figure 4, G and H, demonstrates the transfection efficiency of the CD33 adenovirus at both the gene and protein expression levels measured by qPCR and GFP by flow cytometry, respectively.

To further delineate the specific role of S100A9’s ligation with CD33 compared with its other ligands, we blocked RAGE, TLR4, and CD33 as well as their combination with antibodies before treating healthy BM cells with rhS100A9. As expected, rhS100A9 treatment is able to induce the generation of suppressive cytokines as compared with untreated cells. Importantly, our data show that blockade of CD33 reduced both IL-10 and TGF-β production (Figure 4, I and J), while treatment with anti-RAGE and anti-TLR4 had no significant effects on IL-10 production but displayed modest suppression of TGF-β secretion and expression (Figure 4, I and J). These findings suggest that although CD33 plays a critical role in the secretion of both cytokines, we cannot completely exclude the contribution of other receptors associated with local BM inflammation. To confirm that S100A9 expression contributes to inflammation in the BM microenvironment, we knocked down S100A8 and S100A9 in MDS-MDSC using gene-specific shRNAs (Figure 4K). Given that S100A9 usually pairs with S100A8 as a heterodimer and both are concomitantly regulated, we were not surprised to find reciprocal changes in gene expression after downregulation in the expression of the alternate protein (36). Importantly, reduction of S100A8/S100A9 expression profoundly attenuated IL-10 and TGF-β production (Figure 4, L and M) and rescued autologous BFU-E and GM-CFU colony formation (Figure 4N). These data show that inflammation-associated S100A8/S100A9 signaling plays a critical role in the activation of MDS-MDSC and suppresses normal hematopoiesis.
Identification of S100A9 as a native ligand for CD33. (A) Coomassie blue staining of BM lysates precipitated with either control IgG or CD33-fusion. (B) Transfected SJCRH30 cells (S100A9 on top left and S100A9 lower left) stained with CD33-fusion (APC, red). (C) S100A9 capture-ELISA of lysates from untransfected (negative) or S100A9 transfected cells. Secondary antibody was either anti-S100A9 (positive control) or CD33-fusion. (D) Serial dilution of both AD293 and SJCRH30 cytoytic cells, either untransfected or transfected with vector or S100A9, onto a PVDF membrane and blotted with CD33-fusion. Coomassie blue staining serves as loading control. (E) S100A9 immunoprecipitation of SJCRH30 CD33/S100A9 cotransfected cell lysate blotted against CD33. (F) PBMC and BM-MNC from healthy (H) and MDS (M) samples immunoprecipitated with CD33-fusion and blotted for S100A9. (G) Immunofluorescence staining of rhS100A9-DDK incubated with either CD33-transfected (top) or vector-transfected (bottom) SJCRH30 cells at indicated time points. DAPI = nuclei, APC-DDK = rhS100A9. Treatment of SJCRH30-CD33 cells with rhS100A9 induced IL-10 (H) and TGF-β expression (I). Treatment of U937 cells (high CD33 expression myeloid cell line) with rhS100A9 also induced IL-10 (J) and TGF-β expression (K). S100A9 protein concentration in the plasma of MDS patients (n = 6) measured by ELISA (L). MDS-MDCS treated with 1 μg of rhS100A9 were stained for CD33-FITC and anti-DDK-APC (M) and immunoprecipitated with anti-CD33 antibody followed by blotting with anti–SHP-1 (N). Original magnification, ×400 (B); ×200 (G); ×600 (M). (O) BM plasma from either healthy donors (n = 3) or MDS patients (n = 3) was used to assay SHP-1 recruitment. In all experiments, error bars represent the SEM of 3 separate experiments.
yield a comparable impairment of hematopoietic integrity. These findings were supported by sequential analyses of peripheral blood counts in which mixed source transplant recipients had cytopenias that were intermediate in severity relative to those in mice receiving the S100A9Tg or WT donor cells (Figure 6, D–F). Interestingly, while mixed donor recipients had the same proportion of HSPC after engraftment as Tg transplanted mice, their WBC counts were higher than those of mice transplanted with S100A9Tg HSPC and lower than the levels found in WT HSPC recipients. Similarly, onset of anemia was delayed in recipients of the mixed versus S100A9Tg donor cells, eventually matching the proportion of KLS HSPC in mixed versus transgenic mice at the end of the study (8 weeks, as shown in Figure 6C). These findings suggest that normal HSPC from WT mice are able to partially rescue hematopoiesis but, with time, it is suppressed by accumulated MDSC derived from S100A9Tg donor cells.

To address whether S100A9 alone has direct effects on HSPC, we treated BM CD34+ (MDSC depleted, MDSC−) from MDS patient specimens with rhS100A9 for 24 and 48 hours and assessed apoptosis by flow cytometry. We observed a decrease in the number of CD34+ HSPC after treatment compared with controls accompanied by a corresponding increase in the apoptotic fraction among surviving cells after 48 hours of exposure (Figure 6, G and H, and refs. 41–43). In order to corroborate these findings, we cultured healthy BM-derived CD34+ cells (Lanza) with rhS100A9 and again observed a decrease in viable cells after treatment (50.7% viability in control cells versus 24.7% in rhS100A9-treated cells, Figure 6I). These findings suggest that S100A9 has a direct apoptotic effect in human HSPC.

**Forced maturation of MDSC restores hematopoiesis.** To confirm the effector role of MDSC and investigate the potential benefit of targeted suppression of MDSC, we treated S100A9Tg mice with all-trans-retinoic acid (ATRA). ATRA induces MDSC differentiation into mature myeloid cells and neutralizes ROS production, thereby extinguishing MDSC through forced terminal differentiation (44, 45). To this end, we examined whether ATRA would induce MDSC differentiation in S100A9Tg mice and improve hematopoiesis. S100A9Tg and WT mice were treated with ATRA (250 µg/200 µl) or vehicle control orally for 5 consecutive days. Two days after completion of the treatment, we found that ATRA reduced the total number of MDSC, while numbers in WT mice remained at basal levels (Figure 7A). Reductions in MDSC numbers in S100A9Tg mice were coupled to an increase in mature cells following ATRA treatment (Figure 7B). Treatment of primary MDS BM specimens with ATRA also reduced in vitro MDSC accumulation (data not shown). Importantly, BM progenitor cultures from ATRA-treated S100A9Tg mice showed significantly improved BFU-E recovery compared with vehicle-treated controls (Figure 7C). Analysis of changes in peripheral blood counts showed that rbc, wbc, and platelet counts significantly increased compared with vehicle-treated controls (Figure 7D), indicating that terminal differentiation of MDSC can restore effective hematopoiesis.

Our investigations showed that MDSC employ CD33-associated ITIMs to inhibit their own cellular maturation (Figure 3, N and O). However, ITIM signals can be overridden by stimulatory immunoreceptor tyrosine–based activation motif–mediated (ITAM-mediated) signals (46, 47). Some CD33-related receptors, such as certain Siglecs, lack ITIMs and instead function as activating receptors. Mouse Siglec-H and human Siglec-14 have been shown to interact with DAP12 (26, 27, 48, 49), an ITAM-containing adaptor that can promote myeloid cell maturation (Supplemental Figure 3) and inhibit TLR activation (50). We compared the DAP12 gene expression of purified MDS-MDSC to age-matched healthy donor MDSC (n = 5). DAP12 mRNA was significantly lower in MDS-MDSC in all specimens tested (Figure 7E). We reasoned that overriding CD33-ITIM signaling via DAP12 would induce the differentiation of these immature myeloid cells and improve hematopoiesis. To test this hypothesis, we created a constitutively active form of DAP12, named P23, and transfected AD293 cells with GFP, WT-DAP12, or active-DAP12 P23 viral vectors. Our results show that P23 binds Syk kinase and activates downstream signaling in transduced AD293 cells without external stimulation (Figure 7F). Furthermore, P23 promotes primary human DC maturation as demonstrated by upregulation of CD80, CD83, and CCR7 antigens (Supplemental Figure 4 and refs. 26, 27). Based on these findings, we examined whether P23 could induce the maturation of MDS-MDSC and thereby prevent or disrupt hematopoietic suppression. We analyzed expression of the human monocyte and granulocytic surface markers CD14 and CD15 after transfection with these constructs and compared the effects of DAP12 and DAP12 containing adaptor that can promote myeloid cell maturation (Supplemental Figure 3) and inhibit TLR activation (50). We found that while WT-DAP12 transfection alone was sufficient to induce upregulation of CD14 and CD15, P23 transfection induced even greater expression of both maturation markers (Figure 7G). Moreover, P23 promoted the maturation of primary MDS-MDSC, as demonstrated by upregulation of CD80, CD83, and CCR7 markers (Figure 7H). Finally, we tested whether DAP12-mediated MDSC maturation relieved suppression of erythropoiesis. We purified MDS-MDSC from 7 MDS patients and infected them with control, WT-DAP12, or P23 LV constructs. To assess the suppressive function of the mature MDS-MDSC on hematopoiesis, colony-forming capacity was assessed after culture of infected cells with autologous, MDSC-depleted BM cells. P23-infected MDS-MDSC cocultures yielded significantly higher colony numbers than control viral vector or WT-DAP12–infected MDS-MDSC cocultures (Figure 7I). These findings indicate that DAP12 overrules CD33-associated ITIM signaling to stimulate MDSC maturation and reverses the suppressive effects on HPC colony-forming capacity.
Figure 5
S100A9Tg mice have increased accumulation and activation of MDSC and display dysplastic features that recapitulate human MDS pathology. (A) Gr1+CD11b+ MDSC accumulation in BM-MNC isolated from S100A9Tg mice at 6, 18, or 24 weeks and S100A9KO or WT mice at both 6 and 24 weeks. (B) Percentage of MDSC from BM, spleen, and PBMC of S100A9Tg mice at 6, 18, and 24 weeks of age by flow cytometry. Spleen cells from B were also assayed against the maturation markers F4/80+Gr1– (C) and I-Ad+ (D). (E) FACS-sorted Gr-1+CD11b+ cells (+MDSC) from the BM of mice described in A remixed back with autologous 1 × 10^5 MDSC-negative population (containing HSPC, –MDSC) at 1:1 ratio for 14 days before evaluating colony formation. An MDSC-depleted population was used as the control. (F) MDSC from WT, S100A9Tg, and S100A9KO mice were FACS sorted and incubated in a 96-well plate for 24 hours after which IL-10 and TGF-β production were measured by ELISA. Comparison of the hematopathological analysis of WT (G–J) and S100A9Tg mice (K–N) Original magnification, ×200 (G and K); ×600 (H and L); ×1000 (J, I, M, and N). Detailed descriptions is in Methods. MDS-BM primary specimens were tested for the location of S100A9 in CD33+ cells (O) and CD34+ cells (P). Flow figures are representative of triplicate experiments.
Important role in the age-dependent predisposition to MDS (35). The time-dependent changes driving MDSC expansion may play an (e.g., TNF- α) that suppress hematopoiesis, promote T cell tolerance, and serve as a key source of myelosuppressive and inflammatory molecules, such as S100A9, for the induction of MDS-MDSC-mediated suppressor function. Furthermore, our finding that primary human MDS-MDSC lack molecular genetic abnormalities intrinsic to the malignant clone indicate that MDSC derive from nonneoplastic HSPC and that MDSC activation and expansion likely precede emergence of genetically distinct MDS clones.

We show, for what we believe is the first time, that CD33, a Siglec receptor expressed by many immune cells including MDSC, is markedly overexpressed by MDS-MDSC and that this receptor controls the suppressive functions of MDS-MDSC through disruption of ITIM-mediated signaling. Additionally, our findings that rhS100A9 directly triggers apoptosis in human HPC indicate that this ligand exerts dual roles in the promotion of ineffective hematopoiesis that involve both cellular (MDSC) and humoral mechanisms (CD33, TLR4). Moreover, cellular response to CD33 ligand engagement appears cell-type specific, i.e., triggering apoptosis in HPC versus activation and proliferation in MDSC. Compensatory regeneration within the myeloid compartment may account for the increased proliferative index observed in MDS (41) and preferential myeloid skewing that occurs with age. Indeed, we found that myeloid mass significantly increased with age in the S100A9Tg mice, accompanied by a significant rise in the proportion of cycling BM precursors (Supplemental Figure 1). We propose a model in which overexpression of CD33 impairs maturation signals from ITAM-associated receptors that is critical to expansion of immature MDSC (Supplemental Figure 3). Consistent with this, shRNA silencing of CD33 reduced myelosuppressive cytokine elaboration and, importantly, restored hematopoietic progenitor colony-forming capacity. Moreover, constitutively active DAP12 signaling was sufficient to override CD33-ITIM inhibition and induce MDSC differentiation, which restored erythropoiesis upon active-DAP12 transfection into MDS-MDSC. DAP12 activation can inhibit TLR-mediated signaling pathways, which may also play a role in inflammation-mediated BM suppression (50, 53).

### Discussion

Inflammatory stimuli within the BM microenvironment are recognized as important biological signals stimulating progenitor cell proliferation and apoptosis in MDS. A recent population-based study extended this further by demonstrating a strong linkage between chronic immune stimulation and MDS predisposition (4). Definitive evidence that niche intrinsic abnormalities per se can alone account for development of MDS in a cell-nonautonomous fashion are limited. Raaijmakers and colleagues showed that selective osteoprogenitor dysfunction caused by deletion of Dicer1 in the mesenchymal component of the BM microenvironment was sufficient to perturb hematopoiesis and lead to development of myeloid dysplasia, followed by secondary emergence of myeloid-restricted genetic abnormalities (52).

Our studies show that Lin–HLA-DR–CD33+ MDSC accumulate in the BM of MDS patients, derive from a population that is distinct from the neoplastic clone, and serve as cellular effectors that suppress hematopoiesis, promote T cell tolerance, and serve as a key source of myelosuppressive and inflammatory molecules such as IL-10, TGF-β, NO, and arginase. Using multiple biological and biochemical approaches, we show that S100A9 can serve as an endogenous native ligand for CD33/Siglec 3. Furthermore, forced expansion of MDSC by overexpression of S100A9 in transgenic mice initiates development of hematologic features that phenocopy human MDS, specifically progressive age-dependent ineffective and dysplastic hematopoiesis. These findings indicate that expansion of a single cellular constituent of the BM microenvironment is sufficient to foster neoplastic change in heterologous myeloid progenitors through niche-conducive oncogenesis. The time-dependent accumulation of MDSC in the transgenic mouse model parallels data from recent human studies showing that MDSC expand with age, accompanied by rising serum levels of inflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β), providing evidence that such senescence-dependent changes driving MDSC expansion may play an important role in the age-dependent predisposition to MDS (35).

Importantly, the LIN HLA-DR-CD33+ phenotype did not alone confer suppressor cell function, as evidenced by lack of LIN HLA-DR CD33+ MDSC suppression from age-matched healthy donors or non-MDS cancer patients. Our studies demonstrate the necessity for activation of innate immune signaling and generation of proinflammatory molecules, such as S100A9, for the induction of MDS-MDSC-mediated suppressor function. Furthermore, our finding that primary human MDS-MDSC lack molecular genetic abnormalities intrinsic to the malignant clone indicate that MDSC derive from nonneoplastic HSPC and that MDSC activation and expansion likely precede emergence of genetically distinct MDS clones.

#### Table 1

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</tr>
<tr>
<td>RDW</td>
<td>%</td>
<td>16.3 ± 0.7</td>
<td>15.9 ± 0.1</td>
<td>16.3 ± 0.5</td>
<td>15.5 ± 0.5</td>
<td>16.0 ± 0.5</td>
<td>15.4 ± 0.6</td>
</tr>
<tr>
<td>Hgb</td>
<td>g/dl</td>
<td>14.2 ± 0.8</td>
<td>13.0 ± 0.7</td>
<td>13.9 ± 0.3</td>
<td>11.1 ± 0.7B</td>
<td>13.7 ± 0.6</td>
<td>10.3 ± 0.5B</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dl</td>
<td>29.5 ± 1.1</td>
<td>30.6 ± 1.3</td>
<td>30.4 ± 0.4</td>
<td>31.5 ± 0.9</td>
<td>30.3 ± 1.0</td>
<td>32.3 ± 1.1</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>15.1 ± 0.3</td>
<td>15.2 ± 0.1</td>
<td>15.3 ± 0.1</td>
<td>15.8 ± 0.3</td>
<td>15.2 ± 0.2</td>
<td>16.2 ± 0.4</td>
</tr>
<tr>
<td>rbc</td>
<td>10^12/μl</td>
<td>9.4 ± 0.7</td>
<td>8.5 ± 0.4</td>
<td>9.1 ± 0.1</td>
<td>7.1 ± 0.6B</td>
<td>9.1 ± 0.3</td>
<td>6.4 ± 0.2C</td>
</tr>
<tr>
<td>PLT</td>
<td>10^12/μl</td>
<td>555.7 ± 96.6</td>
<td>412.0 ± 124.0</td>
<td>431.3 ± 33.9</td>
<td>95.7 ± 35.0C</td>
<td>437.0 ± 41.9</td>
<td>61.0 ± 23.5C</td>
</tr>
</tbody>
</table>

All data are mean ± SEM (n = 5 mice). Peripheral blood samples were prepared from both S100A9Tg and control (WT) mice at the ages of 6, 18, and 24 weeks and analyzed on a Hema True Hematology Analyzer (Heska). *P < 0.05; **P < 0.01; ***P < 0.001 vs. WT mice. LYM, lymphocytes; MONO, monocytes; GRAN, granulocytes; HCT, hematocrit; MCV, mean corpuscular volume; RDW, rbc distribution with average; RDW, rbc distribution width; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; PLT, platelets.
Mounting evidence implicates activation of innate immune signaling in the pathogenesis and biologic features of human MDS (7–9). In del(5q) MDS, allelic deletion of miRNA-145 and miRNA-146a results in derepression of the respective targets, TIRAP and TRAF6. Of particular importance, Starczynowski and colleagues showed that knockdown of these specific miRNAs or overexpression of TRAF6 in murine HSPC recapitulated hematologic features of del(5q) MDS in a transplant model through both cell-autonomous and nonautonomous mechanisms involving IL-6 (10, 54). A recent study showed that IRAK1, a TLR4 adaptor kinase, is overexpressed and hyperactivated in MDS and that IRAK1 inhibition impaired expansion of MDS HPC, while sparing normal CD34+ cells (55). Our studies show that the heterodimeric DAMP S100A8/S100A9, specifically released at BM inflammatory sites, is not only aberrantly expressed in MDS but serves as a native ligand for CD33 and a critical effector of...
MDSC expansion and HPC apoptosis. This finding provides evidence that S100 proteins contribute directly to MDS pathogenesis through microenvironment-directed, cell-nonautonomous mechanisms involving MDSC (18, 56). Moreover, TLR activation suppresses osteoblast differentiation, while instructing myeloid commitment in HSPC (12, 13). Prolonged activation of innate immune signaling with age, therefore, may disrupt the BM endosteal niche-supporting maintenance of HSPC quiescence and favor translocation of myeloid progenitors to an angiogenic niche characteristic of MDS (57). Our findings of a skewed distribution of cycling precursors along the endosteal region in S100A9Tg mice, coupled with the myeloid lineage expansion with age support this notion. More importantly, the competitive transplant experiments showed that admixture of S100A9Tg with WT donor HSPC delays suppression of hematopoiesis.

Our findings support a model for MDS pathogenesis in which sustained activation of innate immune signaling in the BM microenvironment creates a permissive inflammatory milieu that is sufficient for development of myelodysplasia. Cell-autonomous neoplastic hematopoietic progenitors may emerge following acquisition of secondary genetic abnormalities in the myeloid compartment. S100A9Tg mice simulate human MDS and can serve as an in vivo model for studying MDS pathogenesis and development of novel therapeutics. Nevertheless, our data suggest that therapeutic interventions that promote MDSC maturation may have remitting potential when applied early in the disease course.

Methods

**Mice.** WT FVB/NJ mice were purchased from Jackson Laboratories, and S100A9KO mice and S100A9Tg mice were generated and used as previously described (37, 38). S100A9Tg mice were generated from FVB/NJ homozygous mice and bred for more than 15 generations. Each experiment group included 5 to 10 mice. For the competitive adoptive transplant experiment, 18-week-old FVB/NJ WT female mice were irradiated once in a rotating gamma irradiator for a total dose of 900 cGy. Concurrently, BM-MNC were purified from the same patients who were previously confirmed to have del5q or del7q using a commercially available test (Abbott Laboratory).

**Immunofluorescence.** BM-MNC were purified from MDS patients, diluted to a concentration of 3 × 10^6 cells/ml, cytopinned onto microscope slides and fixed with methanol/acetone (3:1 ratio at –20°C for 30 minutes). Washes were done with Triton X-100 buffer for 5 minutes and 50 mM Tris buffer (pH 7.4) for 10 minutes prior to blocking for nonspecific binding with serum. We stained the slides with the following primary antibodies: rabbit anti-CD33 antibody (1:100 dilution; Santa Cruz Biotechnology Inc.); mouse anti-granzyme B (1:100 dilution; Fitzgerald Industries); and mouse anti-human CD71 (1:100 dilution; BD Biosciences). This was followed by their respective secondary antibodies: Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen); FITC goat anti-mouse (Sigma-Aldrich); and Alexa Fluor 350 goat anti-mouse IgG (Molecular Probes). Rat anti-human glycophorin A was preconjugated to Alexa Fluor 647 using a kit from Molecular Probes before addition to the sample (1:50 dilution; AbD Serotec) followed by mounting the slides with aqueous medium (Molecular Probes). Immunofluorescence was detected using a Zeiss automated upright fluorescence microscope and images captured by a Nikon camera with the capture program AxioVision.

**Immunostaining on S100A9 transfected SJCRH30 cells.** Detailed methods have been published previously (25). Specifically, SJCRH30 cells that lack detectable expression levels of both CD33 and S100A9 were transfected with either S100A9 or S100A8 (negative control) for 48–72 hours. After incubation with CD33-fusion for 30 minutes (2 μg/ml) they were stained with a secondary anti-human IgG1-APC before analysis by immunofluorescence microscopy. Similarly, SJCRH30 cells, stable-transfected with CD33, were incubated with rhS100A9 tagged with DDK for various time points and stained by the same methodology before analysis.

**Suppression assay.** To determine whether MDSC are capable of mediating T cell suppression, MDSC were sorted from full BM of MDS patients by FACS. T cells were isolated by MACS using CD3 microbeads (Miltenyi Biotech) from autologous peripheral blood. 2 × 10^7 T cells were seeded in 96-well round-bottom plates in triplicate in Iscove’s modified Dulbecco medium (IMDM; Invitrogen) supplemented with 10% human serum PAA (PAA Laboratories). Cultures were stimulated with 30 U/ml IL-2 (Chiron) and anti-CD3/anti-CD28–coated beads (Invitrogen) at a 1:2 ratio of T cells to beads. MDSC were admixed with T cell cultures at ratios of 1:2, 1:4, 1:6, and 1:8. After 3 days of coculture, supernatants were harvested to measure IFN-γ production were statistically significant, 1-way ANOVA with Bonferroni’s post-hoc test was used. P < 0.05 was considered significant.

** Colony-forming assay.** Cells isolated from either human BM or from S100A9Tg, S100A9KO, or WT fibroblasts and embryos were seeded into complete methylcellulose medium (MethoCult complete medium with necessary cytokines and growth factors; StemCell Technologies) following the manufacturer’s instructions. After incubation, colonies of BFU-E and GM-CFU were counted using an inverted light microscope.

**Real-time quantitative PCR.** RNA was isolated by Trizol isolation (Invitrogen) followed by iScript cDNA synthesis (Bio-Rad) and amplification using iQ SYBR Green Supermix (Bio-Rad). The relative level of gene expression for each experimental sample was calculated by the ΔΔCt method, where untreated cells were the experimental control and the housekeeping gene GAPDH was the internal control. Error bars represent the SEM of 3 separate experiments.

**Preparation of the CD33/Siglec 3 chimeric fusion protein.** Recombinant soluble fusion of CD33/Siglec 3 ectodomain was constructed as described previously (59–61). Specifically, cDNA fragments encoding CD33/Siglec 3...
ectodomain were amplified by PCR and inserted into a vector that encodes the human Fcγ receptor IIb that enables interaction with human IgG1. The recombinant proteins were expressed in 293T cells after transfection, using Lipofectamine (Invitrogen), with 3 successive harvests of 25 ml OPTI-MEM I serum-free medium. The harvested supernatant was centrifuged at 3000 g for 10 minutes to remove debris, and stored at 4°C in 0.02% sodium azide. Concentrations of CD33-fusions in culture supernatants were determined by Bradford assay (Bio-Rad).

Mass spectrometry. Following in-gel tryptic digestion, peptides were extracted and concentrated under vacuum centrifugation. A nanoflow liquid chromatograph (Easy-nLC; Proxeon) coupled to an electrospray ion trap mass spectrometer (LTQ; Thermo) was used for tandem mass spectrometry peptide sequencing experiments. The sample was first loaded onto a trap column (BioSphere C18 reversed phase resin, 5 μm, 120 Å, 100 μm ID; NanoSeparations) and washed for 3 minutes at 8 ml/min. The sample was then loaded onto a capillary column (ReproSil-Pur C18-AQ 150 mm, 3 μm, 75 μm ID; NanoSeparations) and eluted with a gradient solvent (A: 2% acetonitrile + 0.1% formic acid; solvent B: 90% acetonitrile + 0.1% formic acid) with a flow rate of 300 nl/min. Five tandem mass spectra were acquired per cycle and converted to .mzML files. The data were processed with the OpenMS (version 1.4) software suite, which is freely available. S100A9 polyclonal antibody (positive control) or CD33-fusion as indicated was then extracted and concentrated under vacuum centrifugation. A nanoflow plate (BioSphere C18 reversed phase resin, 5 μm, 120 Å, 100 μm ID; NanoSeparations) was used to plate 10 μl of the sample onto a trap column (ReproSil-Pur C18-AQ 150 mm, 3 μm, 75 μm ID; NanoSeparations) and eluted with a gradient solvent (A: 2% acetonitrile + 0.1% formic acid; solvent B: 90% acetonitrile + 0.1% formic acid) with a flow rate of 300 nl/min. Five tandem mass spectra were acquired per cycle and converted to .mzML files. The data were processed with the OpenMS (version 1.4) software suite, which is freely available.

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Identification of specific binding of S100A9 to CD33/Siglec 3. Ninety-six-well flat-bottom ELISA plates were coated overnight with 1 μg/ml of monoclonal anti-S100A9, as per the manufacturer’s suggestions. After washing with 1× PBS-T, 50 μl of lysates from untransfected cells (negative) or S100A9 transfected cells was added to the wells. Secondary antibody was either a S100A9 polyclonal antibody (positive control) or CD33-fusion as indicated followed by ELISA HRP reaction analysis at 440 nm.

Preparation of adenoviral vector expressing CD33. CD33 plasmid (GeneCopeia) was subcloned into a pShuttle-IRES-hrGFP-1 vector (containing the CMV promoter and hrGFP). The Pmel-digested shuttle vectors were then cotransformed into electrocompetent BJ5183 bacteria with pADeasy-1 (containing the viral backbone) and selected on Kanamycin LB plates. The plasmid in the bacteria was amplified and purified using a plasmid maxiprep system (QIAGEN). The complete adenoviral vector was linearized by PcaI digestion and then transfected into AD293 cells using Lipofectamine (Invitrogen). All recombinant adenoviruses were amplified in AD293 cells. Viral stocks were obtained by amplification of the AD293 cells followed by standard 2-step CsCl gradient ultracentrifugation, dialysis, and storage in a glycerol stock (10% vol/vol) at –80°C. The titer of each viral stock was routinely tested to be 10^{11}–10^{12} PFU by plaque-forming assay using AD293 cells.

Preparation of shRNA LVs. SureSilencing shRNA Plasmid for Human S100A8, S100A9, and CD33 and negative control (nontarget) were purchased from SABiosciences. 293T cells were transfected using Attracene reagent (SABiosciences) according to the manufacturer’s instructions. Viral containing media was collected 24–48 hours later. Plasmids, pcDNA3 WT DAP12 and P23-DAP12 were cut with HindIII and XhoI (Promega), and DNA Polymerase I Large (Klenow) (New England Biolabs Inc.) was used to fill in recessed 3′ ends of DNA fragments. pWPI, which contains a GFP expression cassette (Addgene Organization), was digested using PMEI (New England Biolabs Inc.). After cutting DNA vector and pWPI was purified using Strata Prep PCR purification kit (QIAGEN), DNAs were ligated (Takara DNA Ligation Kit; Fisher) into vector pWPI, and STBL2 competent cells were transformed (Invitrogen). 293T cells were transfected with pWPI lentivirus vector, the packaging plasmid psPAX2, and the envelope plasmid pMD2.G (Addgene Organization) using Lipofectamine 2000 (Invitrogen) at a ratio of 4:3:1 according to standard protocols. Four days after the first infection, transfected cells were isolated by FAC sorting GFP+ cells with greater than 99% purity.

Infection of MDSC from MDS patients. MDSC isolated from MDS patients were infected 3 times using virus-containing infection medium at 24-hour intervals in the presence of 8 μg/ml polybrene. For each infection, cells were plated in 12-well dishes at 1 × 10^{6} cells/well. Four days after the first infection, cells were harvested and used for qPCR, flow cytometry, or colony formation assays.

Flow cytometry. BM-MNC were stained with appropriate specific conjugated antibodies in PBS with 2% BSA buffer. For MDSC sorting, FITC anti-CD33 and FITC anti-HLA-DR were used as positive controls and isotype IgG1 was used for negative control and to detect nonspecific staining. Sorting was performed on a FACSaria cell sorter by Core facility personnel. For antigenic determination, cells were washed in PBS and then stained with PE-conjugated mAbs specific to CD40, CD80, CD83, CD86, CCR7, CD11c, CD14, CD8, HLA-DR, CD8, and relevant isotype controls (eBioscience) for 30 minutes in the dark, on ice. Live cells were gated based on negative staining for 7-AAD. Samples were acquired on a FACScalibur flow cytometer, and the analysis was performed using FlowJo 6.3.4 software.

Arginase activity. Arginase activity was measured in cell lysates as previously described (62). In brief, cells were lysed for 30 minutes with 100 μl of 0.1% Triton X-100. Subsequently, 100 μl of 25 mM Tris-HCl and 10 μl of 10 mM MnCl₂ were added, and the enzyme was activated by heating for 10 minutes at 56°C. Arginine hydrolysis was conducted by incubating the lysate with 100 μl of 0.5 M L-arginine (pH 9.7) at 37°C for 120 minutes. The reaction was stopped with 900 μl of H₂SO₄ (96%)/H₃PO₄ (85%)/H₂O (1/3/7, v/v/v). Urea concentration was measured at 540 nm after addition of 40 μl of α-isonitrosopropiophenone, followed by heating at 95°C for 30 minutes.

NO production. Equal volumes of culture supernatants (100 μl) were mixed with Greiss reagent solution (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethenediamine dihydrochloride) in double-
distilled water and incubated for 10 minutes at room temperature. Absor- 
bance of the mixture was measured at 550 nm using a microplate reader 
(Bio-Rad). Nitrite concentrations were determined by comparing the 
absorbance values for the test samples to a standard curve generated 
by serial dilution of 0.25 mM sodium nitrite.

Western blot analysis. Cell lysates were prepared by resuspending cell 
particles in 1% NP-40, 10 mM Tris, 140 mM NaCl, 0.1 mM PMSF, 10 mM iodo- 
acetamide, 50 mM NaF, 1 mM EDTA, 0.4 mM sodium orthovanadate, 
10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotonin and 
lyzing on ice for 30 minutes. Cell lysates were centrifuged at 12,000 g for 
15 minutes to remove nuclei and cell debris. The protein concentration 
of the soluble extracts was determined by using the Bio-Rad (Bradford) 
protein assay. 50 μg of protein (per lane) was separated on a 10% SDS- 
polyacrylamide gel by electrophoresis, then transferred to a PVDF mem-
brane. Membranes were probed for indicated antibody: anti-S100A8, 
anti-S100A9, or anti-S100A8/A9 (Santa Cruz Biotechnology Inc.); anti-
phospho ERK, anti-total ERK, anti-phospho Syk, and anti-total Syk (Cell 
Signaling). Proteins were detected with the enhanced chemiluminescence 
detection system (ECL; Amersham).

In vitro dot blotting analysis of direct binding of S100A9 to CD33. Lysate 
from S100A9 transfect untransfected, empty vector, and S100A9 transfect SJCRH30 or 
detection system (ECL; Amersham). Signaling. Proteins were detected with the enhanced chemiluminescence 
phospho ERK, anti-total ERK, anti-phospho Syk, and anti-total Syk (Cell 
Signaling). Proteins were detected with the enhanced chemiluminescence 
detection system (ECL; Amersham).

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