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Novel Therapeutic Approach to Improve Hematopoiesis in low risk MDS by Targeting MDSCs with The Fc-engineered CD33 Antibody BI 836858

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Abstract

We recently reported that the accumulation of myeloid-derived suppressor cells (MDSC), defined as CD33⁺HLA-DR⁻Lin⁻, plays a direct role in the pathogenesis of myelodysplastic syndrome (MDS). In particular, CD33 is strongly expressed in MDSC isolated from patients with MDS where it plays an important role in MDSC-mediated hematopoietic suppressive function through its activation by S100A9. Therefore, we tested whether blocking this interaction with a fully human, Fc-engineered monoclonal antibody against CD33 (BI 836858) suppresses CD33-mediated signal transduction and improves the bone marrow microenvironment in MDS. We observed that BI 836858 can reduce MDSC by antibody-dependent cellular cytotoxicity (ADCC), which correlated with increases in granule mobilization and cell death. BI 836858 can also block CD33 downstream signaling preventing immune-suppressive cytokine secretion, which correlates with a significant increase in the formation of CFU-GM and BFU-E colonies. Activation of the CD33 pathway can cause reactive oxygen species (ROS)-induced genomic instability but BI 836858 reduced both ROS and the levels of double strand breaks and adducts (measured by comet assay and γ H2AX). This work provides the ground for the development of a novel group of therapies for MDS aimed at MDSC and their disease-promoting properties with the goal of improving hematopoiesis in patients.

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Keywords

MDS; BI 836858; CD33; S100A9; Engineered antibody

Introduction

Myelodysplastic syndromes (MDS) are hematologically diverse stem cell malignancies that share features of cytological dysplasia, ineffective hematopoiesis and a propensity for progression to acute myeloid leukemia (AML)¹⁻³. MDS is characterized by a senescence-dependent onset of malignancy^{1, 2, 4, 5}, which provides an important clue as to the force that drives disease pathogenesis. In particular recent work illustrates that MDS is a perfect model to study the pathogenesis of age-induced inflammation, or inflammaging, as a major accelerating factor promoting the escape of malignant cells from immune surveillance through the activation of innate immunity⁶⁻¹⁰. Our work further demonstrates that chronic suppressive inflammation is mediated by the accumulation of myeloid-derived suppressor cells (MDSCs)¹¹, a heterogeneous group of immature myeloid cells¹² in the bone marrow (BM) microenvironment of MDS, which plays a critical role in the pathogenesis of the disease⁷. MDSC, known to accumulate in tumor bearing mice and cancer patients, are site-specific inflammatory and T cell immunosuppressive effectors that contribute to cancer progression^{13, 14}. Moreover, inflammation within the BM microenvironment contributes to myeloid lineage skewing and ineffective hematopoiesis with inflammatory molecules serving as regulatory cues driving the proliferation and death of hematopoietic stem and progenitor cells (HSPC)¹⁵⁻¹⁹. We also reported that MDSC, genetically distinct from the MDS clone, serve as a primary source of inflammatory molecules, such as the danger-associated molecular pattern (DAMP) molecule S100A9, creating a suppressive microenvironment with which it can induce cell death of HSPC^{7, 20-25}.

The most important finding was that CD33, a defining marker of MDSC (CD33⁺HLA-DR⁻Lineage⁻) is highly expressed (receptor density) in MDS MDSC, functions as a receptor for S100A9 and initiates suppressive inflammatory signaling cascades through their ITIM domain^{26, 27} that lead to the secretion of mediators, such as IL-10, TGF β and ROS¹¹. Even more critical is our discovery that this process leads to the development of genomic instability²⁸. We found a strong correlation between cellular ROS/nuclear- β -catenin to DNA damage (γ H2AX⁺ cells), linking S100A9-induced ROS accumulation to genetic instability. This was confirmed by in vitro treatment with S100A9 which led to the induction of double stranded breaks in healthy cells that induced the accumulation of phosphorylated γ H2AX, a main marker of genomic instability²⁹.

Our model that inflammaging and accumulation of CD33⁺ MDSC are a major factor driving MDS clone expansion provides a novel drugable cellular target, other than the malignant clone, that can serve to prevent disease progression. Currently there is no specific chemotherapeutic agent available targeting the CD33 signaling pathway. Anti-CD33 antibodies, in particular antibody drug conjugates, have been tested in clinical trials for the treatment of AML, where CD33 is widely expressed in malignant blast cells. Gemtuzumab ozogamycin (Mylotarg®; GO), an immunoconjugate consisting of a humanized anti-CD33

monoclonal antibody linked to calicheamicin, gained regulatory approval in 2000 but was voluntarily withdrawn from market in 2010 due to safety concerns when given in combination with conventional chemotherapy^{30, 31} Recent phase III trials have shown an improvement in overall survival in patients treated with GO and chemotherapy, resulting in renewed interest in CD33 targeted therapies, and re-confirming CD33 as valid target for AML³².

In the current study we took advantage of the novel, fully human CD33 antibody BI 836858 an unconjugated CD33 antibody, which is Fc-engineered for increased binding to Fc γ receptor IIIa (Fc γ RIIIa). It binds with low nanomolar affinity to human CD33 and displays decelerated internalization kinetics compared to previously developed CD33 mAbs thus making it suitable for exploitation of NK mediated ADCC³³. We demonstrate that BI 836858 can both induce ADCC-mediated reduction of CD33⁺ cells and block signaling through S100A9/CD33. Moreover, BI 836858 not only induced the restoration of normal colony forming capacity in MDS BM primary specimens but also prevented the accumulation of DNA double stranded breaks induced through the S100A9/CD33 axis in HSPC. Considering that BI 836858 is undergoing testing clinical trials (NCT0224070, NCT01690624), this report provides the mechanistic rationale for the potential benefit of this antibody in MDS and highlights the potential of targeting CD33⁺ and MDSC as a novel therapeutic option for MDS.

Materials and Methods

MDS patient sample isolation

All patients were recruited from the Malignant Hematology clinic at the H. Lee Moffitt Cancer Center & Research Institute, after obtaining written informed consent, confirmed by central review and classified in accordance with either the WHO criteria or IPSS. Human healthy BM controls were purchased unprocessed (Lonza-Walkersville), healthy PBMC were obtained from the local blood bank and healthy CD34⁺ isolated cells were purchased (StemExpress). Cells were isolated by Ficoll-Hypaque gradient centrifugation, as previously described³⁴ and cultured in RPMI supplemented with 10% FBS, 1% Penicillin/Streptomycin and 1% L-glutamine (Gibco). The MDS specimens used were classified as shown in Table 1.

Cytotoxicity assays

⁵¹Cr-release assay was performed, as previously described³⁵. Total PBMC, isolated CD8⁺ T cells or isolated NK cells (Stemcell technologies) were treated with either human engineered isotype control (BI 836847), 2 μ g/ml BI 836858 or CD33Ab (clone 6C5/2), 20 μ M Lenalidomide (LEN), 20 μ M ICT or an equal volume of DMSO (vehicle), before labeling with 100 μ Ci of ⁵¹Cr (Amersham Corp, Louisville, CO) in 0.5 mL of medium at 37°C in a 5% CO₂ atmosphere for 1h. After three washes, the labeled cells were incubated with effector cells in triplicate wells of 96-well round-bottomed microplates at 20:1, 10:1, 5:1 and 2.5:1 effector to target (E:T) ratios. After 4 h incubation at 37 °C, supernatants were harvested and counted in a γ -counter. The percentage of specific ⁵¹Cr release was determined as: (experimental cpm – spontaneous cpm)/(total cpm incorporated – spontaneous cpm) \times 100. Alternatively, granule mobilization was measured by pre-staining

PBMC or isolated NK cells with CD107a antibody before admixing with target HL60 cells incubating for 4 hours before fixing and measuring by flow cytometry.

Flow cytometry

MDSC cells were defined and purified by fluorescence activated cell sorting (FACS) of CD33⁺ cells lacking expression of lineage (Lin⁻) markers (CD3, CD14, CD16, CD19, CD20, CD56) and HLA-DR using a FACSAria cell sorter at the Flow Cytometry Core Facility. Cell viability was measured with DAPI. For antigenic determination, cells were washed in 1X PBS in 2% BSA and then stained with the same conjugated monoclonal antibodies described for sorting or relevant isotype controls (eBioscience). Samples were acquired on a FACSCalibur flow cytometer and analyzed using Flowjo 6.3.4. In some cases cells were stained with BI 836858, or its corresponding isotype, conjugated with Alexa 488 (Lifesciences), sorted and labeled with Celtracker Orange before admixing with autologous mononuclear cells. To measure genomic instability cells were stained intracellularly with a γ H2AX antibody.

Real-time Quantitative PCR

RNA was isolated by Trizol isolation (Invitrogen) followed by iScript cDNA synthesis (Biorad) and amplification at 60°C using SYBR Green Supermix (Quantas) as previously done by us with primers against IL-10, TGF β , CD33 and GAPDH¹¹. The relative gene expression was calculated by the Ct method where untreated cells were the experimental control and the housekeeping gene GAPDH was the internal control.

Enzyme-linked Immunosorbent Assay (ELISAs)

96-well plates (Nunc-Immuno Plate) were coated with purified monoclonal antibody against either human IL-10 or TGF β (Pierce-Endogen, Rockford, IL, USA) in 1X PBS, pH 7.4 at room temperature overnight. The plates were then incubated in blocking buffer (Pierce-Endogen, Rockford, IL, USA) for 2 h at room temperature and washed in 0.05% Tween-20. Wells were seeded with serially diluted recombinant human (rh) cytokines (standard, in blocking buffer in duplicates) or with 50 μ L of supernatants in triplicate followed by addition 1 h later of biotin-labelled anti-cytokine antibody. After incubation wells were washed 5 times and coated with streptavidin-horseradish peroxidase for 20 min, washed again 5 times and developed by adding TMB substrate (Pierce-Endogen). The reaction was stopped by the addition of an equal volume of 0.18 M H₂SO₄. The absorbance was read at 450 nm with deletion of background at 650 nm.

Immunoprecipitation

MDS BMMNC lysates were prepared by re-suspending the isolated cells pellets from ficoll-isolation in Mammalian Cell-PE LB buffer (G Bioscience) supplemented with 1:100 protease inhibitor cocktail (G Bioscience) and 0.1mM PMSF. Cleared lysates were then incubated with used to immune-precipitate CD33 (Rabbit polyclonal, Santa Cruz) overnight at 4°C in a rotor and immunoblotted with anti-SHP1 antibody (Santa Cruz) just as we did before¹¹.

ROS production

The oxidation-sensitive dye, DCFDA, (Molecular Probes/Invitrogen) was used to measure ROS production by U937, PBMC or HSPC in MDSBM MDSC. Cells were incubated at room temperature in complete RPMI in the presence of 3 μ M DCFDA for 30 min, in a water jacketed incubator. Further labeling was carried out after washing with 1X PBS and analyzed using flow cytometry.

Colony-forming Assay

Healthy or MDS human BMMNC or healthy isolated CD34⁺ cells (StemExpress) were seeded in duplicate in 35-mm culture dishes (1×10^4 cells/dish) into complete methylcellulose media (MethoCult complete medium with necessary cytokines and growth factors; StemCell Technologies) and incubated at 37°C in 5% CO₂ for approximately 10–14 days at which point BFU-E and CFU-GM colonies were counted using an inverted light microscope.

Comet Assay

Alkaline comet assay was performed with the Trevigen CometAssay kit following the manufacturer's recommended protocol. Briefly, 1×10^5 cells/ml were seeded into low melting agarose at a ratio of 1:10 v/v and pipetted 50 μ L into comet slides followed by lysis in Lysis solution on ice for 4°C for 1h. Afterwards, slides were placed in unwinding solution for 1h at room temperature. Slides were electrophoresed in the Alkaline Electrophoresis Solution at 21 volts for 30 minutes. Slides were then washed in water and 70% ethanol and dried at less than 45°C for 10–15 minutes followed stained with SYBR Green I and measured in a fluorescent microscope.

Statistics

All data was presented as means \pm SEM. Differences between individual groups were analyzed by Student's t-test. P values of <0.05 were considered to be statistically significant.

Results

BI 836858 confers ADCC mediated by NK cells against CD33⁺ cells

We have demonstrated the crucial role that CD33⁺ MDSC play in the initiation and maintenance of MDS including the role of their S100A9/CD33 suppressive signaling¹¹. Therefore, we hypothesized that BI 836858 can induce a targeted reduction of CD33⁺ MDSC by ADCC mediated via NK cells, which have been shown to be functional in MDS³⁶. Healthy human PBMC were able to induce ADCC of opsonized HL60 cells (CD33⁺, ATCC) pre-coated with BI 836858 in an E:T ratio-dependent manner when tested in a Chromium-51 [⁵¹Cr] release assay, but not those pre-coated with isotype control (BI 836847, Figure 1A). Cytotoxicity was highest at an E:T of 20:1 but still observed at an E:T as low as 2.5:1. Similar experiments were carried out with U937 cells as target cells (not shown). Concomitant application of Lenalidomide (Len) to PBMC, a common drug used in MDS known to modulate NK activity, slightly increased cytotoxicity against target cells treated with BI 836858 (Figure 1B). In addition, we confirmed the ADCC-inducing potential

of BI 836858 by monitoring CD107a granule mobilization as a surrogate for the cytotoxic activation of NK cells (Supplemental Figure 1A). We also demonstrated that BI 836858 treatment does not change the amount of NK cells in PBMC from healthy donors (representative experiment Supplemental Figure 1B) or in BMMNC (data not shown). However, the observed cytotoxicity is due only to the effect of NK cells in this system, as it was not induced in isolated CD8⁺ T cells or in NK-depleted PBMC (Supplemental Figure 1C–E). A regular mouse monoclonal anti-human CD33 antibody (CD33Ab, clone 6C5/2) also induced cytotoxicity against HL60 cells, although it was not specific to NK cells. To further confirm the specificity of BI 836858 for CD33⁺ cells we pretreated PBMC with ICT, a compound that reduces MDSC and the S100A9/CD33 axis³⁷, and show that pretreatment with ICT reduced the cytotoxicity induced by BI 836858 in PBMC (Figure 1C).

ADCC mediated by BI 836858 can directly reduce primary MDS MDSC in ex vivo cultures

To further assess the role of BI 836858 in mediating ADCC of MDSC we measured their percentages in an ex vivo culture of MDS BMMNCs treated with BI 836858 or isotype control. We found a significant reduction of MDSC (Lineage⁻HLA-DR⁻CD33⁺) after treatment with BI 836858 (Figure 2A) which was accompanied by a decrease in the level of CD33 surface expression, measured by fluorescence intensity¹¹, suggesting that apart from reduction of MDSC there was a decrease of CD33 receptor density (Figure 2B). To answer whether BI 836858 may mask the measurement of staining CD33 antibodies we conjugated BI 836858 to Alexa 488 and used it to sort MDSCs which we then labeled using a tracking dye (CellTracker Orange) to measure the availability of MDSC, as well as BI 836858, post mixing with autologous mononuclear cells. We observed a decrease in CellTracker⁺BI 836858⁺ cells, after admixing with autologous MNCs (Figure 2C), which was accompanied by a decrease in the immature myeloid population as determined by their scatter properties (Supplemental Figure 2A). These results indicate that BI 836858 mediated reduction of MDSC is not an artifact of the staining properties of the antibodies (i.e. internalization and degradation of the antibody). As for the fate of BI 836858, we found that CellTracker⁺BI 836858⁺ cells declined from 76.4% to 29.6% after addition of autologous MNCs, while the population of cells that displayed low BI 836858 binding, but were CellTracker Orange positive (19.7%), did not show a relevant change (17.2%) after addition of MNCs (Supplemental Figure 2B). This shows that the reduction of CD33⁺ MDSCs is due to the binding of BI 836858 followed by elimination of these cells rather than by CD33 epitope masking.

BI 836858 can directly block CD33 downstream suppressive signaling

BI 836858 causes reduced internalization of CD33-antibody complexes upon binding to CD33, compared to other CD33 antibodies, resulting in prolonged cell surface retention³³. This prolonged retention of BI 836858 on the cell surface may allow for an increased Fc interaction with NK cells but also suggests that it can act as an agonist or antagonist of CD33 receptor-mediated signaling. As an initial indicator we tested the expression profile of suppressive cytokines and found that BI 836858 was able to suppress IL-10 (Figure 3A and Supplemental Figure 3A) and TGFβ (Supplemental Figure 3B and C), at both protein and mRNA expression levels, suggesting that BI 836858 is capable of interrupting CD33 signaling with its corresponding ligand. This antagonistic ability was unique to BI 836858

since CD33Ab did not block IL-10 gene expression in MDS BMMNCs (Figure 3A). We then cross-linked BI 836858 or CD33Ab with a respective anti- Fab antibody on ice for 30 minutes followed by 24 hour culture to mimic ligand binding to the receptor. We found that cross-linking CD33Ab can significantly induce the activation of CD33's downstream signaling as demonstrated by increased gene expression of both IL-10 (Figure 3B) and TGF β (Supplemental Figure 3D) while BI 836858 can slightly, but not significantly, induce IL-10 and CD33 expression (Supplemental Figure 3E). However, it is important to note that CD33Ab significantly induced IL-10 expression compared with that induced by BI 836858 in both MDS BM cells (Figure 3B) and in healthy cells (Figure 3C) after crosslinking, and had the highest recruitment of SHP1 to CD33's ITIM domain (Supplemental Figure 3F) suggesting the importance of the engineered IgG heavy chain in BI 836858 corroborating that blocking CD33-mediated signaling with it may have a beneficial role in preventing downstream signaling of this receptor compared to commercial CD33 antibodies.

Our most recent work demonstrates that downstream of the S100A9/CD33 pathway is the activation of ROS, which plays a critical role in the maintenance of immune suppression and on the initiation of genomic instability characteristic of MDS^{25, 28}. rhS100A9-induced ROS production can be significantly downregulated by BI 836858 in U937 cells (Supplemental Figure 4A and B), healthy human PBMC treated with rhS100A9 (Supplemental Figure 4C) and in MDS HSPC (lineage⁻CD34⁺ cells, Figure 4A) demonstrating BI 836858's ability to suppress S100A9/CD33 downstream induction of ROS. Importantly, other inflammatory stimuli that can induce ROS activation in PBMC, such as lipopolisaccharide (LPS), are not affected by BI836858 (Figure 4B) probably due to the fact that LPS signals through Toll-Like Receptors (TLR), rather than CD33. In MDS BM however, there was no changes in the LPS induction of ROS (data not shown), IL-10 expression (Supplemental Figure 4D), or ROS⁺ percentages compared to controls (Supplemental Figure 4E). However, it is worth noting that the overall percent of ROS⁺ cells were significantly increased in MDS BM compared to healthy PBMC, just as we observed before²⁵, except in MDS BM treated with BI 836858 which was reduced. Similarly, treating healthy PBMC with either BI836858 or CD33Ab increased both the level of ROS although neither this, nor the changes in the percent of positive cells was significant with either treatment (Figure 4C and Supplemental Figure 4F).

ADCC and signaling blockade by BI 836858 can restore hematopoiesis of MDS ex vivo

Targeting CD33 signaling by BI836858 could improve impaired hematopoiesis of MDS, which is the main cause of its characteristic anemia. For this purpose we used whole MDS BM-MNC, which contain both HSPC and MDSCs, treated with BI 836858, CD33Ab or isotype control before culturing in supplemented methylcellulose colony media for 14 days to observe the potential of either antibody to restore hematopoiesis ex vivo. While there were no significant changes in the number of colonies from healthy BM-MNCs, we observed a significant increase of both CFU-GM and BFU-E colonies (Figure 5A) in ex vivo cultured MDS primary specimens treated with BI 836858 (n=10), demonstrating that this antibody has a positive effect on hematopoiesis in MDS ex vivo. This critically beneficial effect was not observed in CD33Ab (n=5) treated cells that were significantly less than the number of colonies in BI836858 treated cells. Importantly, if we treated healthy CD34⁺ cells

with BI836858 or CD33Ab (both n=3) both antibodies affected colony formation when crosslinked, but only cells that were treated with BI835868 had enhanced BFU-E colony formation compared to regular antibody (Figure 5B). We believe that this MDSC-independent effect in the CD34⁺ population may be linked to the fact that certain sub-populations of HSPC also express CD33, which eventually skew their differentiation towards immature myeloid cells becoming a source of MDSC. We have showed recently that the interaction of CD33 with S100A9 leads to the initiation of pyroptosis, or inflammatory cell death, of HSPC which reiterates this isolated effect of BI 836858 on this population²⁵. Inevitably, based on the demonstration here that BI836858 can serve as a blocking antibody for S100A9/CD33 signaling it will affect these cells as well.

Effect of BI 836858 extends to the prevention of S100A9/CD33-mediated genomic instability

Since blockade with BI 836858 can profoundly reduce the S100A9/CD33-mediated activation of ROS, and based on our recent data that S100A9 can directly induce DNA damage in BMMNC²⁸, we investigated whether the blockade initiated by this antibody could prevent the S100A9/CD33-induced DNA damage and genomic instability *ex vivo*. We started by measuring rhS100A9 treated healthy PBMC in the presence or absence of BI 836858 or isotype control in a single cell electrophoretic mobility cell or comet assay. We found that the antibody was capable of significantly reducing the amount of DNA damage induced by rhS100A9 onto these cells (Supplemental Figure 5A, B is a representative figure of tail momentum). Similarly, BI 836858 was able to significantly prevent part of the DNA damage induced by rhS100A9 in MDS BM cells (Figure 6A and Supplemental Figure 5B). This demonstrates the ability of BI 836858 to prevent damage to MDS BM cells which can further contribute to the evolution of malignancy.

ROS-mediated genomic instability is mediated by non-homologous end joining repair markers such as γ H2AX which was completely abrogated with BI 836858 in rhS100A9 treated MDS HSPC (Figure 6B). This suggests that BI 836858 can both reduce immune suppression and prevents further damage to the HSPC compartment in MDS that can lead to the acquisition of further damage and the evolution of the malignant clone. However, CD33Ab did have an increase in γ H2AX after crosslinking and using it or its respective antibody induced an increase in the level of this marker even without crosslinking (Supplemental Figure 5C). In order to corroborate that this effect is specific for CD33, we repeated the experiments with a TLR4 antibody since TLR4 can also bind S100A9 (Figure 6C). We show that only BI 836858 was able to reduce the tail momentum induced by treatment with rhS100A9 while TLR4 blockade had no effect, highlighting not only the importance of CD33 in this process but also the ability of BI 836858 in blocking this process.

Discussion

Development of disease targeted therapeutics for MDS has been slow largely due to a limited understanding of the disease pathobiology, inadequate animal models that can replicate human disease, and a lack of evidence linking molecular abnormalities to disease

pathogenesis. Although three agents are approved for the treatment of MDS in the US, LEN represents the only therapeutic which is effective only in a small fraction of MDS patients with a rare genetic change, a chromosome 5q deletion³⁸. Although treatment with LEN yields sustained red blood cell transfusion independence accompanied by partial or complete cytogenetic remissions in the majority of patients harboring a chromosome 5q deletion (del5q)^{38, 39}, this subtype comprises only 8–12% of the overall MDS population and therefore the majority of the MDS patients do not benefit from it.

Recent studies indicate that the pathogenesis of MDS is complex and likely depends on interaction between hematopoietic cells and their inflammatory microenvironment. Importantly, inflammation within the BM microenvironment contributes to myeloid lineage skewing and ineffective hematopoiesis with inflammatory molecules serving as regulatory cues driving the proliferation and death of HSPC^{15–19}. Mounting evidence implicates activation of innate immune signaling in both hematopoietic senescence and the pathobiology of MDS^{8–10}, with consequent excess generation of inflammatory molecules expansion of regulatory T cells, as well as the up-regulation and activation of TLR. Critical cellular effectors are CD33^{high} MDSCs, a heterogeneous group of immature myeloid cells^{11, 12}. We recently reported that MDSC are markedly expanded in the BM of MDS patients, genetically distinct from the MDS clone, serve as a primary source of inflammatory molecules and directly induce cell death of HSPC¹¹. MDSC, known to accumulate in tumor bearing mice and cancer patients, are site-specific inflammatory and T cell immunosuppressive effectors that contribute to cancer progression^{13, 14}. Their suppressive activity is in part driven by secreted inflammation-associated signaling molecules such as S100A9 that heterodimerizes with its partner S100A8 and interacts with innate immune receptors involved in MDSC activation^{20–24}. Therefore, developing novel approaches that target MDSC for the purpose of improving the tumor microenvironment would be ideal.

In the current study we demonstrate for the first time the potential of targeting CD33-positive MDSC as an alternative approach for therapy in MDS (Figure 7). We show that BI 836858 has the potential to restore ex vivo hematopoietic capability of BM MNC by inducing ADCC of MDSC, which directly insult HSPCs as well as production of CD33's main ligand S100A9, and by direct interruption of S100A9/CD33 signaling. Furthermore, through its blocking ability BI 836858 confirmed our recent observations that CD33 downstream signaling is a major contributor to the genomic instability process that leads to the evolution of the malignant clone. These findings clearly demonstrate that reduction of MDSCs in the BM tumor microenvironment of MDS is a viable and enticing idea as a therapeutic approach for this disease. This is particularly relevant for MDS where, unlike other malignancies, patients rely on non-MDS specific therapies, or transfusions, which have increased risk of secondary effects, including iron overload among other toxicities. Therefore, BI 836858 provides a novel option for the treatment of MDS through the modulation of CD33 signaling in MDSC, improving the local BM microenvironment, and providing the basis for future clinical trials not only in MDS but potentially also for other cancer and inflammation-related diseases. Clinical trials of BI 836858 in AML and MDS are currently ongoing (ClinicalTrials.gov Identifier: NCT01690624, NCT02240706).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Literature cited

1. Nimer SD. Myelodysplastic syndromes. *Blood*. 2008 May 15; 111(10):4841–4851. [PubMed: 18467609]
2. Estey E. Acute myeloid leukemia and myelodysplastic syndromes in older patients. *J Clin Oncol*. 2007 May 10; 25(14):1908–1915. [PubMed: 17488990]
3. Look AT. Molecular Pathogenesis of MDS. *Hematology Am Soc Hematol Educ Program*. 2005:156–160. [PubMed: 16304374]
4. Estey E, Koller C, Tsimberidou AM, O'Brien S, Beran M, Cortes J, et al. Potential curability of newly diagnosed acute promyelocytic leukemia without use of chemotherapy: the example of liposomal all-trans retinoic acid. *Blood*. 2005 Feb 1; 105(3):1366–1367. [PubMed: 15659618]
5. Rothstein G. Disordered hematopoiesis and myelodysplasia in the elderly. *Journal of the American Geriatrics Society*. 2003 Mar; 51(3 Suppl):S22–26. [PubMed: 12588569]
6. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002 Dec 19–26; 420(6917):860–867. [PubMed: 12490959]
7. Chen X, Eksioglu EA, Zhou J, Zhang L, Djeu J, Fortenbery N, et al. Induction of myelodysplasia by myeloid-derived suppressor cells. *The Journal of clinical investigation*. 2013 Oct 15.
8. Kristinsson SY, Bjorkholm M, Hultcrantz M, Derolf AR, Landgren O, Goldin LR. Chronic immune stimulation might act as a trigger for the development of acute myeloid leukemia or myelodysplastic syndromes. *J Clin Oncol*. Jul 20; 29(21):2897–2903.
9. Starczynowski DT, Karsan A. Innate immune signaling in the myelodysplastic syndromes. *Hematology/oncology clinics of North America*. 2010 Apr; 24(2):343–359. [PubMed: 20359630]
10. Starczynowski DT, Karsan A. Deregulation of innate immune signaling in myelodysplastic syndromes is associated with deletion of chromosome arm 5q. *Cell cycle*. 2010 Mar 1; 9(5):855–856. [PubMed: 20160505]
11. Chen X, Eksioglu EA, Zhou J, Zhang L, Djeu J, Fortenbery N, et al. Induction of myelodysplasia by myeloid-derived suppressor cells. *The Journal of clinical investigation*. 2013 Nov; 123(11):4595–4611. [PubMed: 24216507]
12. Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. *Nature reviews Cancer*. 2013 Oct; 13(10):739–752. [PubMed: 24060865]
13. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009 Mar; 9(3):162–174. [PubMed: 19197294]
14. Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer Immunol Immunother*. 2006 Mar; 55(3):237–245. [PubMed: 16047143]
15. Raaijmakers MH. Myelodysplastic syndromes: revisiting the role of the bone marrow microenvironment in disease pathogenesis. *International journal of hematology*. 2012 Jan; 95(1):17–25. [PubMed: 22218882]
16. Della Porta MG. Myelodysplastic syndromes and bone marrow microenvironment. *Leukemia research*. 2011 Nov; 35(11):1442–1443. [PubMed: 21872330]
17. Raaijmakers MH, Mukherjee S, Guo S, Zhang S, Kobayashi T, Schoonmaker JA, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. Apr 8.7290 464:852–857. [PubMed: 20305640]

18. Allampallam K, Shetty V, Mundle S, Dutt D, Kravitz H, Reddy PL, et al. Biological significance of proliferation, apoptosis, cytokines, and monocyte/macrophage cells in bone marrow biopsies of 145 patients with myelodysplastic syndrome. *International journal of hematology*. 2002 Apr; 75(3):289–297. [PubMed: 11999358]
19. Stirewalt DL, Mhyre AJ, Marcondes M, Pogosova-Agadjanya E, Abbasi N, Radich JP, et al. Tumour necrosis factor-induced gene expression in human marrow stroma: clues to the pathophysiology of MDS? *Br J Haematol*. 2008 Feb; 140(4):444–453. [PubMed: 18162123]
20. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med*. 2007 Sep; 13(9):1042–1049. [PubMed: 17767165]
21. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J Leukoc Biol*. 2009 Sep; 86(3):557–566. [PubMed: 19451397]
22. Kapanadze T, Gamrekelashvili J, Ma C, Chan C, Zhao F, Hewitt S, et al. Regulation of accumulation and function of myeloid derived suppressor cells in different murine models of hepatocellular carcinoma. *J Hepatol*. 2013 Jun 21.
23. Sade-Feldman M, Kanterman J, Ish-Shalom E, Elnekave M, Horwitz E, Baniyash M. Tumor necrosis factor-alpha blocks differentiation and enhances suppressive activity of immature myeloid cells during chronic inflammation. *Immunity*. 2013 Mar 21; 38(3):541–554. [PubMed: 23477736]
24. Roth J, Goebeler M, Sorg C. S100A8 and S100A9 in inflammatory diseases. *Lancet*. 2001 Mar 31.357(9261):1041.
25. Basiorka AA, McGraw KL, Eksioglu EA, Chen X, Johnson J, Zhang L, et al. The NLRP3 Inflammasome functions as a driver of the myelodysplastic syndrome phenotype. *Blood*. 2016 Oct 13.
26. Paul SP, Taylor LS, Stansbury EK, McVicar DW. Myeloid specific human CD33 is an inhibitory receptor with differential ITIM function in recruiting the phosphatases SHP-1 and SHP-2. *Blood*. 2000 Jul 15; 96(2):483–490. [PubMed: 10887109]
27. Taylor VC, Buckley CD, Douglas M, Cody AJ, Simmons DL, Freeman SD. The myeloid-specific sialic acid-binding receptor, CD33, associates with the protein-tyrosine phosphatases, SHP-1 and SHP-2. *The Journal of biological chemistry*. 1999 Apr 23; 274(17):11505–11512. [PubMed: 10206955]
28. Wei S, Eksioglu EA, Chen X, Cluzeau T, Basiorka A, Burnette A, et al. Inflammation-Associated Metabolic Alterations Foster Development of the MDS Genotype. *Blood*. 2015; 126(23)
29. Kuo LJ, Yang LX. Gamma-H2AX - a novel biomarker for DNA double-strand breaks. *In vivo*. 2008 May-Jun;22(3):305–309. [PubMed: 18610740]
30. Larson RA, Boogaerts M, Estey E, Karanes C, Stadtmauer EA, Sievers EL, et al. Antibody-targeted chemotherapy of older patients with acute myeloid leukemia in first relapse using Mylotarg (gemtuzumab ozogamicin). *Leukemia*. 2002 Sep; 16(9):1627–1636. [PubMed: 12200674]
31. Rowe JM, Lowenberg B. Gemtuzumab ozogamicin in acute myeloid leukemia: a remarkable saga about an active drug. *Blood*. 2013 Jun 13; 121(24):4838–4841. [PubMed: 23591788]
32. Hills RK, Castaigne S, Appelbaum FR, Delaunay J, Petersdorf S, Othus M, et al. Addition of gemtuzumab ozogamicin to induction chemotherapy in adult patients with acute myeloid leukaemia: a meta-analysis of individual patient data from randomised controlled trials. *The Lancet Oncology*. 2014 Aug; 15(9):986–996. [PubMed: 25008258]
33. Vasu S, He S, Cheney C, Gopalakrishnan B, Mani R, Lozanski G, et al. Decitabine enhances Fc engineered anti-CD33 mAb mediated natural killer antibody dependent cellular cytotoxicity against AML blasts. *Blood*. 2016 Mar 24.
34. Wei S, Chen X, Rocha K, Epling-Burnette PK, Djeu JY, Liu Q, et al. A critical role for phosphatase haploinsufficiency in the selective suppression of deletion 5q MDS by lenalidomide. *Proc Natl Acad Sci U S A*. 2009 Aug 4; 106(31):12974–12979. [PubMed: 19470455]
35. Chen X, Bai F, Sokol L, Zhou J, Ren A, Painter JS, et al. A critical role for DAP10 and DAP12 in CD8+ T cell-mediated tissue damage in large granular lymphocyte leukemia. *Blood*. 2008 Dec 15.

36. Epling-Burnette PK, Bai F, Painter JS, Rollison DE, Salih HR, Krusch M, et al. Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors. *Blood*. 2007 Jun 1; 109(11):4816–4824. [PubMed: 17341666]
37. Zhou J, Wu J, Chen X, Fortenbery N, Eksioglu E, Kodumudi KN, et al. Icarin and its derivative, ICT, exert anti-inflammatory, anti-tumor effects, and modulate myeloid derived suppressive cells (MDSCs) functions. *International immunopharmacology*. 2011 Jul; 11(7):890–898. [PubMed: 21244860]
38. List A, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. *N Engl J Med*. 2006 Oct 5; 355(14): 1456–1465. [PubMed: 17021321]
39. List A, Kurtin S, Roe DJ, Buresh A, Mahadevan D, Fuchs D, et al. Efficacy of lenalidomide in myelodysplastic syndromes. *N Engl J Med*. 2005 Feb 10; 352(6):549–557. [PubMed: 15703420]

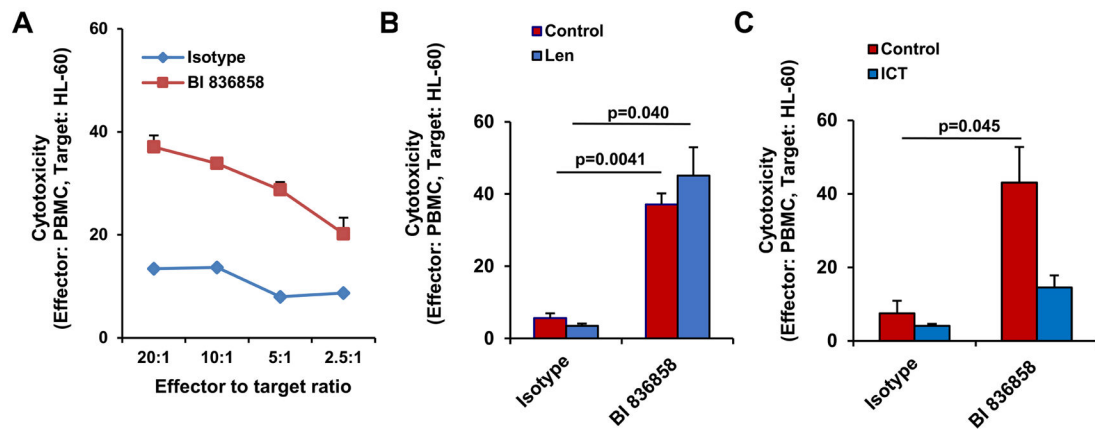


Figure 1. BI 836858 mediates ADCC specific for CD33-positive HL60 cells and does not affect the proportion of NK cells

Chromium (Cr^{51})-labeled HL60 cells (CD33 positive) were incubated for 30 minutes with 2 $\mu\text{g}/\text{ml}$ of BI 836858, or the same concentration of isotype BI 836847, in ice before co-culturing with healthy human PBMC for 4 hours to measure of cytotoxic activity (A). The cytotoxic effect of BI 836858 was not significantly increased by stimulation of NK cells in healthy PBMC with 20 μM of the IMiD Lenalidomide (LEN) (B). C) Healthy human PBMC (n=3) were treated with either DMSO, 20 μM of the anti-inflammatory compound ICT for 4 days prior to addition of BI 836858 [2 $\mu\text{g}/\text{ml}$] or the same concentration of isotype. Error bars represent the SEM of triplicate determinations of three separate donors and the p value was calculated using Student's T-test.

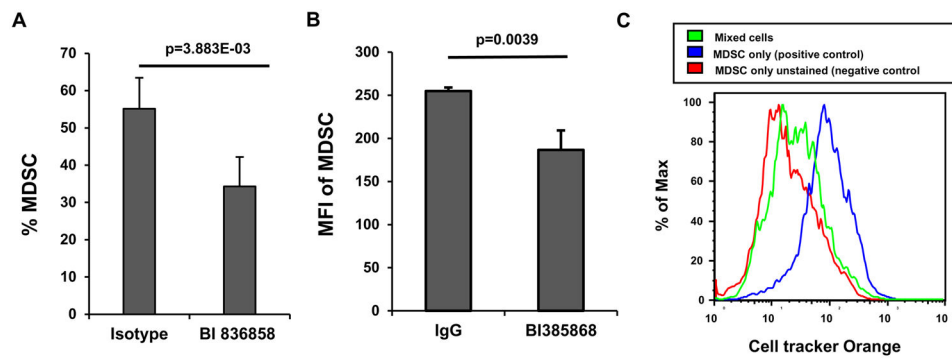


Figure 2. BI 836858 reduces MDSC and CD33⁺ cells in MDS BM specimens

MDS primary BM specimens (n=10) were cultured with 2ug/ml of BI 836858 or isotype control and thereafter were analyzed for changes in the proportion (**A**) or fluorescence intensity (MFI, **B**) of MDSC by flow cytometry (cells defined as HLA-DR⁻Lin⁻CD33⁺). Error bars represent the SEM and the p value was calculated using Two-tailed paired T-test. **C**) Reduction of Cell Tracker Orange sorted population of MDSC after co-culture with autologous MNCs (representative of three separate experiments).

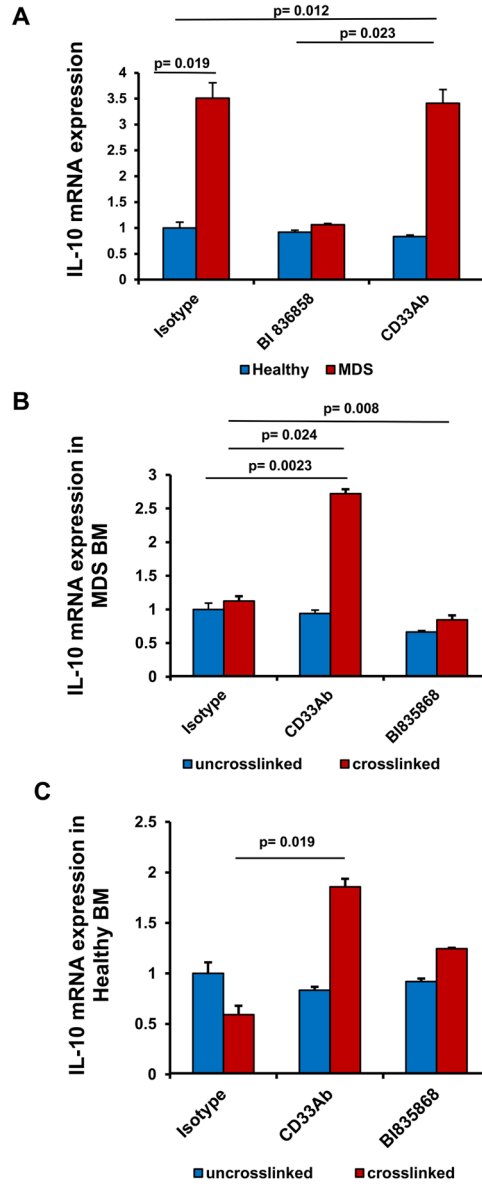


Figure 3. BI 836858 blocks downstream induction of CD33-mediated IL-10 expression
A) Healthy or MDS BM cells co-cultured with 2ug/ml of either BI 836858, CD33Ab or isotype control for 48 hours after which gene expression of the cytokine IL-10 was assayed by qPCR. Similarly MDS BMMNCs **(B)** or healthy BMMNC **(C)** cultured cells were instead cross-linked with an anti-Fc Fab fragment antibody for half hour on ice before culture for 48 hours at which point total RNA was collected for gene expression analysis of IL-10. The qPCR data was normalized against the house keeping gene GAPDH using the Ct methodology. Error bars represent the SEM of three separate experiments measured in duplicates and the p value was calculated using Student’s T-test.

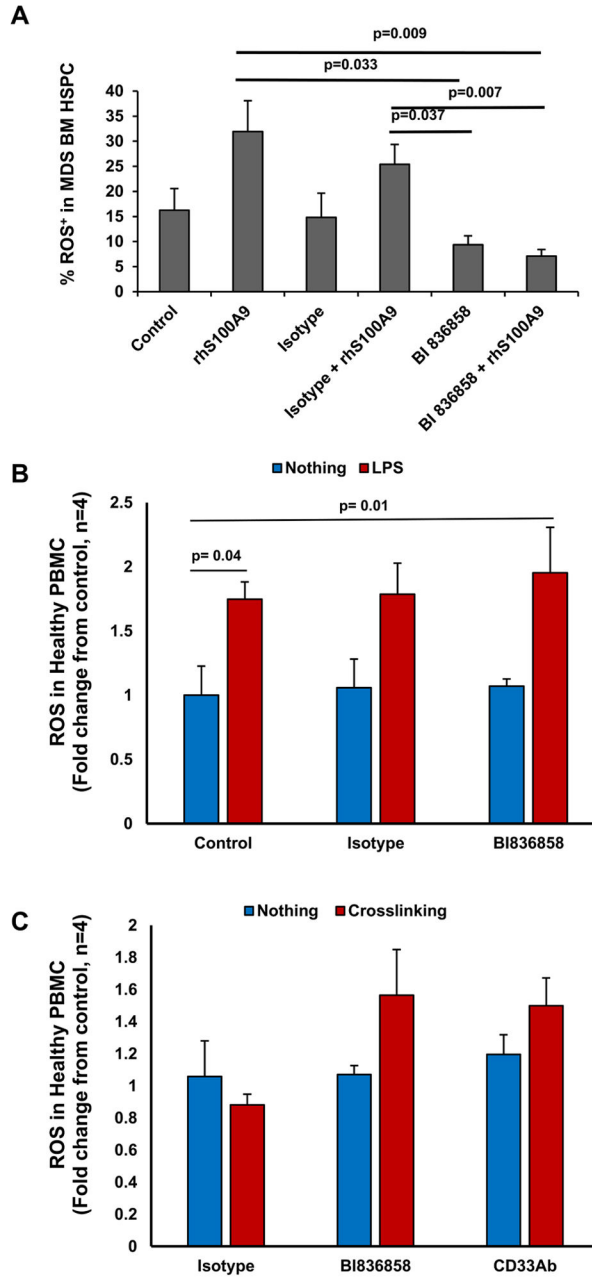


Figure 4. BI 836858 blocks downstream induction of CD33-mediated ROS
A) HSPC (Lineage⁻CD34⁺ cells) from MDS-BM primary specimens (n=4) were cultured ex vivo with BI 836858 or isotype control antibody in the presence or absence of 10ug/mL of rhS100A9 followed by flow cytometric analysis to assess the presence of ROS. Similar experiments as in A were carried out but in the presence or absence of 0.5ug/mL of LPS as stimulant (**B**) or by crosslinking antibody with an anti-Fc Fab fragment for half hour on ice (**C**) in healthy PBMC (n=4) followed by 24 hour culture and ROS analysis. Error bars represent the SEM and the p value was calculated using student T-test.

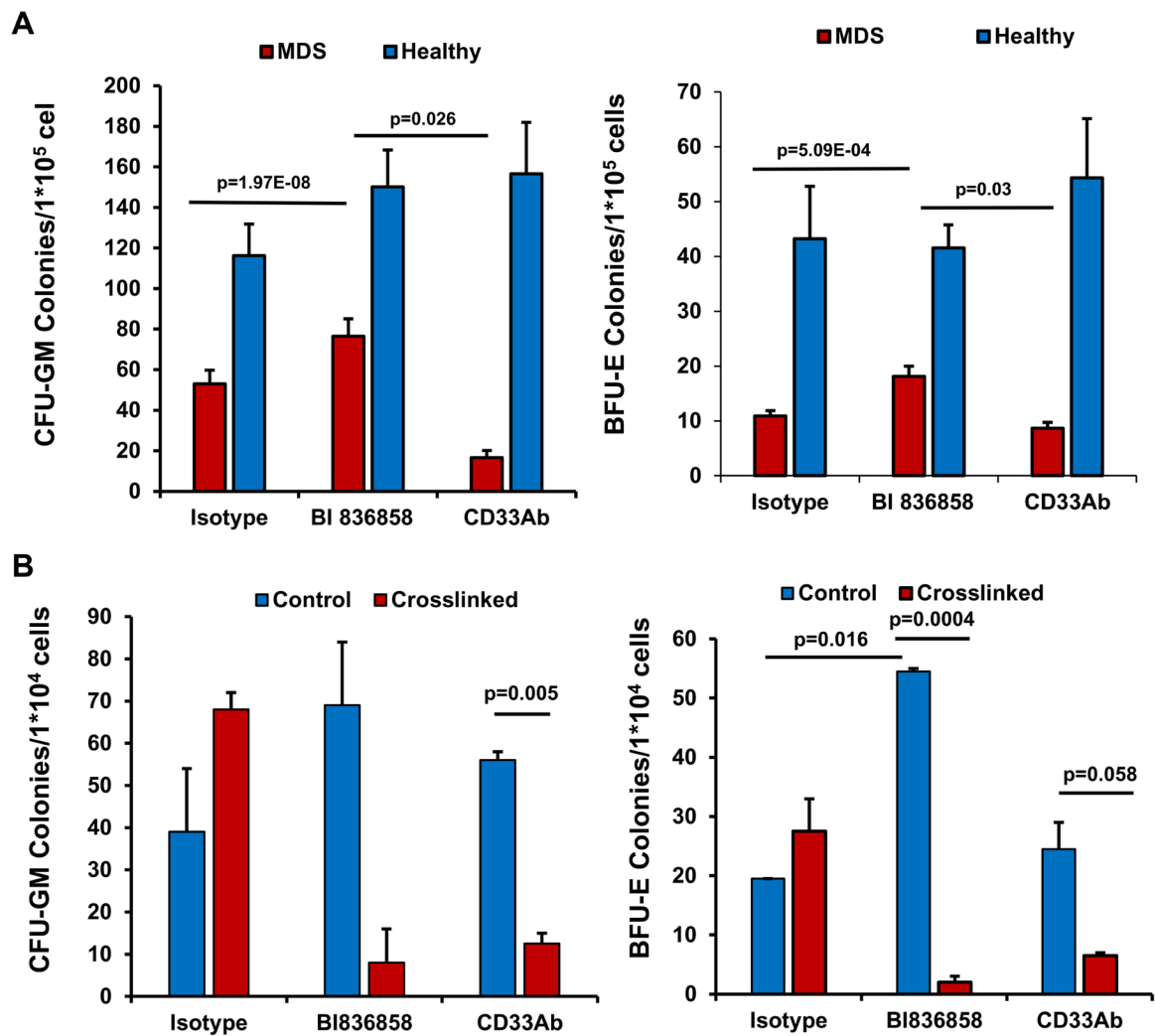


Figure 5. BI 836858 improves colony-forming capacity in MDS BM specimens cultured ex vivo
A) Healthy or MDS primary BM specimens, were cultured with 2ug/ml of BI 836858, CD33Ab or its corresponding isotype control, and then grown in duplicate in complete methylcellulose media for 14 days. N=10 specimens of each were used in isotype and BI836858 and n=4 for CD33Ab. **B)** The same culture analysis as in A but in isolated CD34⁺ cells (StemExpress) including CD33Ab at the same concentration as well as crosslinking with the respective isotypes. CFU-GM and BFU-E colonies were analyzed and microscopically scored. Error bars represent the SEM and the p value was calculated using two-tailed paired T-test.

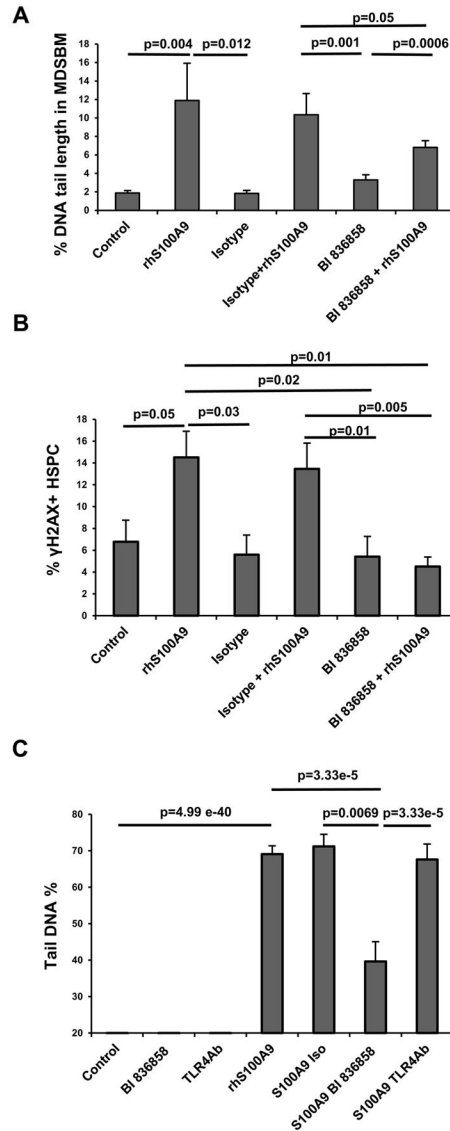


Figure 6. BI 836858 prevents the development of S100A9/CD33-mediated genomic instability
A) HSPC from MDS-BM primary specimens (n=3) were cultured ex vivo with isotype control or BI 836858 antibody in the presence or absence of rhS100A9 followed by comet analysis to assess the protective effect of BI 836858 against S100A9-induced DNA damage. Fifty pictures each from three primary specimens were analyzed. **B)** To confirm the induction of genomic instability the percentage of γ H2AX cells in HSPC from MDS BM was also monitored in the same specimens by flow cytometry. **C)** Healthy BM cells were treated with isotype control, 2ug/ml of BI 836858 or a 2ug/ml blocking TLR4 antibody to assess the specificity of BI 836858 in preventing damage by comet assay. Some cells were also treated with 10ug/ml rhS100A9 to induce damage. Error bars represent the SEM and the p value was calculated using Student's T-test.

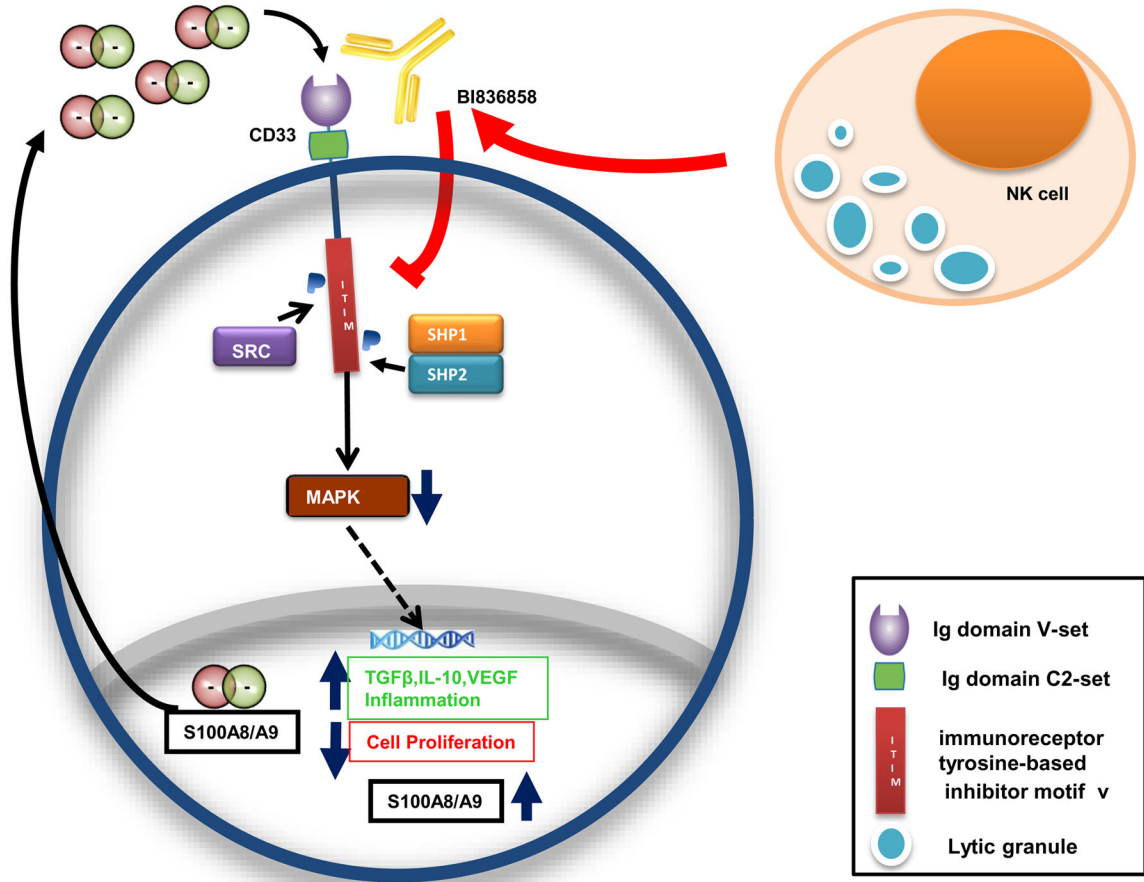


Figure 7.
Schematic representation of BI 836858 functionality in MDS.

Table 1

Primary bone marrow specimens characteristics

Patient	WHO DX	IPSS	GENDER	AGE	CYTOGENETICS
1	RAEB1	INT1	F	72	46XX, del(11)(q13, q23).
2	RCMD	INT1	M	77y	TRISOMY 8
3	RCMD	INT1	M	77y	TRISOMY 8
4	RCMD	INT1	M	77y	TRISOMY 8
5	RARS	LOW	M	82y	46 XY
6	RAEB1	INT2	M	85	46 XY
7	RAEB1	INT2	M	85	46 XY
8	RAEB1	INT2	M	85	46 XY
9	RAEB1	INT2	M	85	46 XY
10	RCMD	INT1	M	68y	del 2, del 13 q
11	RCMD	INT1	M	68y	del 2, del 13 q
12	RCMD	INT2	F	79y	46 XX, -2,-7,+8
13	RCMD	INT1	M	62y	46 XY
14	RCMD	INT1	M	63y	46 XY
15	RCMD	INT1	M	62y	46 XY
16	RCMD	INT1	M	72y	46 XY
17	RCMD	INT1	M	68	46 XY
18	RCMD	INT1	M	68y	46 XY
19	RCMD	INT1	M	68y	46 XY
20	RCMD	LOW	F	62	46 XX, -2,-7,+8
21	RCMD	LOW	F	79	46 XX
22	RCMD	INT1	M	77	47 XY +8 [17], 45 XY -7 [3]
23	RCMD	INT1	F	80y	46 XX
24	RCMD	INT1	F	80y	46 XX
25	RCMD	INT1	F	80y	46 XX
26	RCMD	INT2	F	63	46 XX
27	RAEB2	INT2	F	62y	46 XX
28	RAEB2	INT1	M	73	46 XY

Patient	WHO DX	IPSS	GENDER	AGE	CYTOGENETICS
29	RCMD	UNKNOWN	F	82y	UNKNOWN
30	RCMD	UNKNOWN	F	82y	UNKNOWN
31	RCMD	UNKNOWN	F	82	UNKNOWN
32	RAEB1	INT2	M	70y	TRISOMY 8
33	RAEB1	INT1	M	68	46 XY
34	RCMD	LOW	M	68	46 XY
35	RCMD	INT1	M	75y	46, XY, dup (11)(q21q23)
36	RCMD	INT1	M	75	46, XY, dup (11)(q21q23)
37	RARS	LOW	M	50	46 XY
38	RAEB1	INT2	M	69	TRISOMY 8
39	RAEB2	INT2	M	77	46 XY
40	RARS	LOW	M	50	46 XY

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