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14. ABSTRACT

Development of targeted MDS therapies has been challenged by a limited insight of molecular mechanisms of disease pathogenesis and therefore, current therapeutic approaches remain highly empiric largely ignoring the role of the inflammatory bone marrow (BM) microenvironment. We identified S100A9 as a mediator of myeloid derived suppressor cell (MDSC) activation and as the native ligand to CD33, which is highly expressed in MDS. Therefore, our goals are to develop novel therapeutic strategies that inactivate MDSC to improve survival of HSPC and allow us to provide the basis for more effective, biologically rational MDS therapeutics. Based on our initial hypothesis of hematopoietic improvement through the targeting of the S100A9-CD33 pathway, this funding year we were able show that our proposed approaches can inactivate MDSC in vitro. This is achieved through the development of a specific S100A9 trap, the CD33-chimeric human IgG1 molecule (CD33-fusion). We demonstrated that CD33-fusion is able to neutralize soluble S100A9 and suppresses S100A9 induced pro-inflammatory cytokine production as well as improve hematopoiesis. This focused translational research represents the base to develop novel treatments for MDS providing a

15. SUBJECT TERMS

Nothing listed

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1. INTRODUCTION

Understanding the selective pressures and mechanisms involved in the initiation of stem cell malignancies is critical for development of effective strategies for prevention and treatment. Myelodysplastic syndromes (MDS) are hematologically diverse bone marrow (BM) failure syndromes that share features of cytological dysplasia, ineffective hematopoiesis and a propensity for progression to acute myeloid leukemia (AML). MDS are senescence-dependent myeloid malignancies with a rising prevalence owing to the aging of the American population. Effective disease-altering therapies for patients with MDS are limited due largely to inadequate understanding of the precise pathobiological mechanisms involved in disease initiation and progression. Although innumerable somatic genetic events have been annotated in recent years, many of which are sufficient for disease initiation in murine models, microenvironmental factors conducive for emergence of these genetic events remain to be delineated. In the original proposal we hypothesized that inflammation and aging induce the accumulation of **myeloid-derived suppressor cells (MDSCs),** a heterogeneous group of immature myeloid cells, which play a critical role in MDS pathogenesis. A key finding of our recent studies was the identification of CD33 as a receptor that binds S100A9 to drive expansion and activation of BM MDSCs, and trigger cell death of myeloid progenitors. MDSC-mediated suppressive activity is stimulated by the danger-associated molecular pattern (DAMP) heterodimer S100A9, that heterodimerizes with its partner S100A8, and interacts with innate immune receptors involved in MDSC activation. Therefore **S100A9 is a key factor in MDSC activation.** Based on this preliminary evidence, we have hypothesized that CD33-S100A9 signaling is a critical driver of MDSC activation and MDS pathogenesis that can be therapeutically exploited in a selective fashion. We propose that inactivation of MDSCs through selective interference with S100A9/CD33 signaling offers a novel strategy for MDS therapeutic development. This approach would also further enhance our understanding of mechanisms underlying disease initiation. In the past year, we have made significant research progress toward this goal through the development of novel CD33-chimeric protein (CD33-fusion) to characterize its biochemical and biological activity.

2. KEYWORDS

Myeloid-derived suppressor cells (MDSC); Myelodysplastic syndromes (MDS); S100A9 proteins; inflammation, Genomic instability, bone marrow failure, hematopoiesis; Interleukin 10 (IL10); hematopoietic stem and progenitor cells (HSPC)

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goals of the project are:

Aim 1. To determine if strategies to block S100A9/CD33 signaling in MDSC improves the BM microenvironment and rescues hematopoiesis. We are investigating our recently developed CD33-IgG1 chimeric receptor as an S100A9 ligand trap to neutralize S100A9 and thereby suppress MDSC activation.

Aim 2. To validate a novel MDSC-inactivating compound that disrupts CD33-ITIM signaling. We will interrogate our recently described S100A9-CD33 pathway to characterize a

novel compound, ICTA, to inactivate inflammatory suppression through the maturation of MDSCs as a novel preclinical approach.

Goals for the 2nd 12 months: Following the major objectives of the proposal we have set up two subtasks for the 2nd 12 months: 1) To determine the effect of BI33.1, a humanized CD33 antibody to block its signaling pathway 2) To validate the inhibitory effect of BI33.1. on inactivation of MDSC in BM-MNCs isolated from patients with MDS.

What was accomplished under these goals?

Under these goals, and following the SOW, the major research activities and accomplishments for the 2nd 12 months were:

Based on the evidence that the accumulation of MDSC, defined as $CD33^{High}$ in patients with MDS where it plays an important role in MDSC-mediated hematopoietic suppressive function. In past 12 months, we tested whether blocking this interaction with a fully human, Fc-engineered monoclonal antibody against CD33 (BI836858) 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 γH2AX). 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.

1. **BI 836858 confers ADCC mediated by NK cells against $CD33^{+}$ cells.** Healthy human

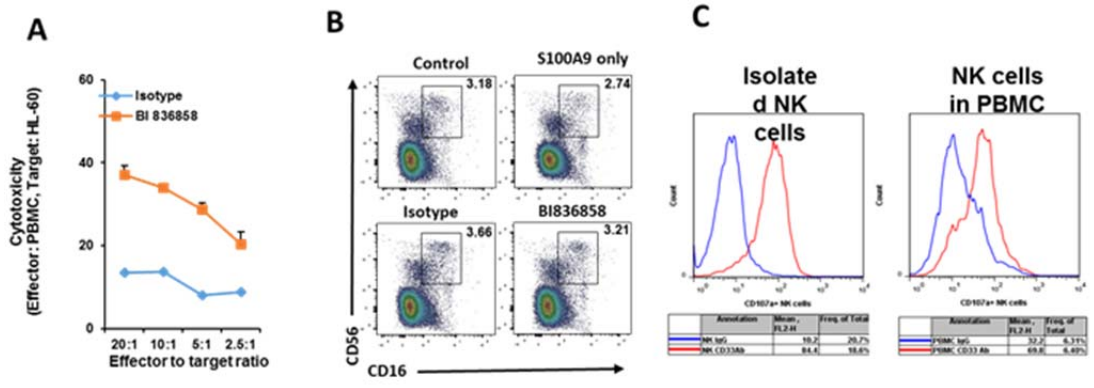

Figure 1. BI 836858 mediates ADCC specific for CD33-positive HL60 cells and does not affect the proportion of NK cells. Chromium (Cr51)-labeled HL60 cells (CD33 positive) were incubated for 30 minutes with 2ug/ml of BI 836858, or the same concentration of isotype BI 836847, in ice before co-culturing with healthy human PBMC for 4 hours to measure of cytotoxic activity (A). **B)** No variation in the proportion of CD56+CD16+ NK cells was observed after treatment of healthy PBMC with 2ug/ml BI 836858 for 72 hours (representative of three separate experiments). Chromium (Cr51)-labeled HL60 cells (CD33 positive) were incubated for 30 minutes with 2ug/ml of BI 836858, an anti-CD33 antibody (CD33Ab, SantaCruz) or the same concentration of isotype BI 836847, in ice before co-culturing with either isolated NK cells. **C)** Granule release was monitored by measuring CD107a by flow cytometry after co-culture of PBMC or isolated NK cells isolated from healthy donors with target HL60 cells (representative figure of three separate determinations). Blue line denotes the CD107a mobilization of cells in co-culture with HL60 cells with isotype antibody while the red line denotes the mobilization with BI 836858.

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 [Cr51] release assay, but not those pre-coated with isotype control (BI 836847, Figure 1A). 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 (Figure 1B). We also demonstrated that BI 836858 treatment does not change the amount of NK cells in PBMC from healthy donors (representative experiment Figure 1C) or in BMMNC (data not shown).

2. **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 ($CD33^{high}$ Lineage-HLA-DR-) after treatment with BI 836858 (Figure 2A) which was accompanied by a decrease in the level of CD33 surface expression, measured by fluorescence intensity, 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 (Cell Tracker Orange) to measure the availability of MDSC, as well as BI 836858, post mixing with autologous mononuclear cells. We observed a decrease in Cell Tracker+ 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. These results indicate that BI 836858 mediated reduction of MDSC is not an artifact of the staining properties of the antibodies.

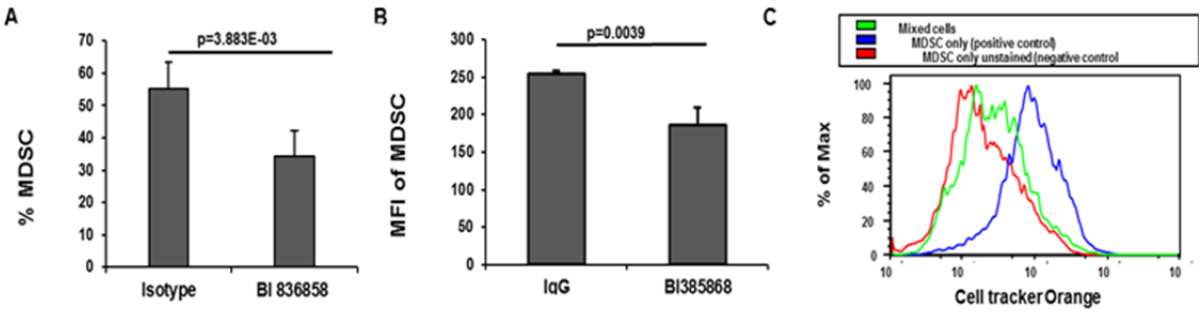

Figure 2. BI 836858 reduces MDSC and CD33+ cells in MDS BM specimens. MDS primary BM specimens (n=10) were cultured with 2ug/ml of BI 836858 or isotype control and thereafter were analyzed for changes in the proportion **(A)** or fluorescence intensity (MFI, **B**) of MDSC by flow cytometry (cells defined 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).

3. **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 retention. 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 Figure 3B**) and TGFβ (**Figure 3Cand D**, 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 TGFβ (**Figure 3F**) while BI 836858 can slightly, but not significantly, induce IL-10 and CD33 expression. 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 and in healthy cells after crosslinking, 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.

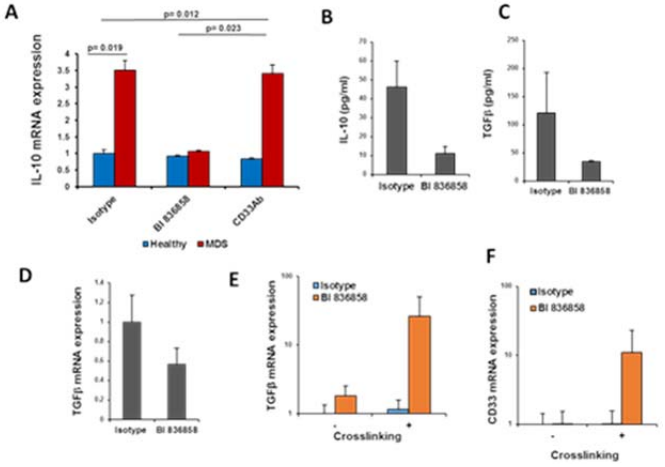

Figure 3. BI 836858 blocks downstream induction of CD33-mediated IL-10 expression. A) Healthy or MDS BM cells co-cultured with 2ug/ml of either BI 836858, CD33Ab or isotype control for 48 hours after which gene expression of the cytokine IL-10 was assayed by qPCR. **B).** MDS BM cells co-cultured with BI 836858, or its respective isotype control, for 96 hours after which expression of the cytokines IL-10. TGFβ **(B and C**) was assayed from either supernatants by sandwich ELISA or qPCR respectively. MDS BM cells were 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 TGFβ **(E**) and CD33 **(F).** Bars represent the SEM of three separate experiments measured in triplicates (ELISA) or duplicates (qPCR).

4. **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 4A**) 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 4B**). 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 population[25]. Inevitably, based on the

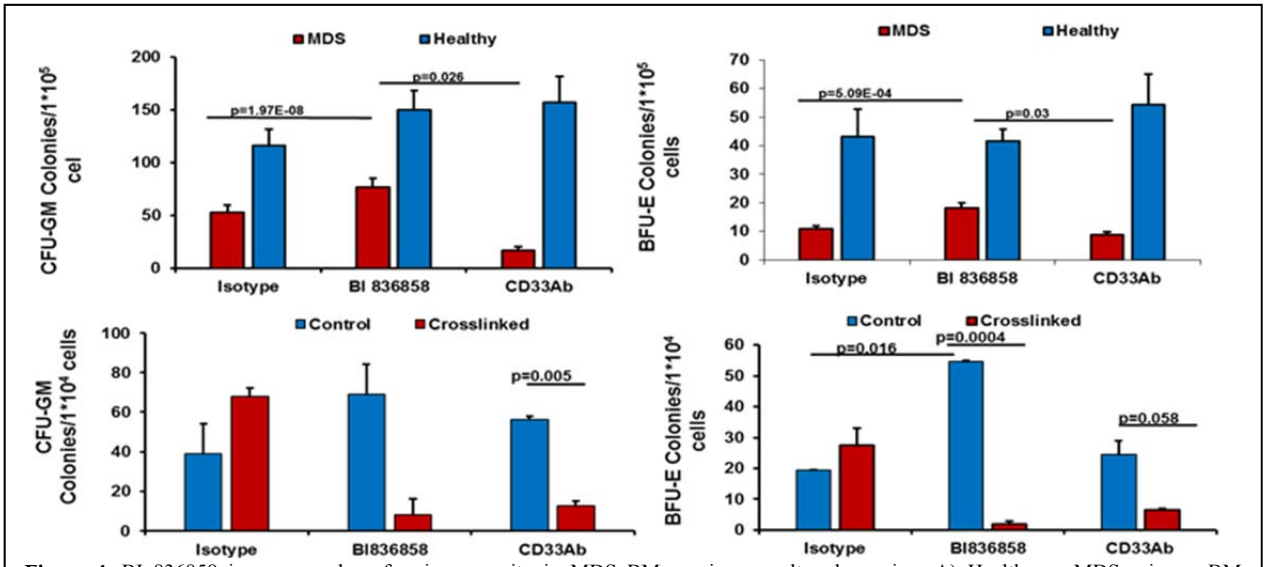

Figure 4. BI 836858 improves colony-forming capacity in MDS BM specimens cultured ex vivo. A) Healthy or MDS primary BM specimens, were cultured with 2ug/ml of BI 836858, CD33Ab or its corresponding isotype control, and then grown in duplicate in complete methylcellulose media for 14 days. N=10 specimens 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.

demonstration here that BI836858 can serve as a blocking antibody for S100A9/CD33 signaling it will affect these cells as well.

In summary, we have made significant research progress under the proposed goals, in line with the SOW, for the 2nd 12 months. The current data has been published in Leukemia this year, a high impact and top tier journal in malignant hematology field. Our published and ongoing research has proved our original hypothesis is innovative and has great clinical potential to benefit patients with MDS. In addition, our research has been validated by other well respected scientists in the field in top journal in the field (*1: Rps14 haploinsufficiency causes a block in erythroid differentiation mediated by S100A8 and S100A9. Schneider RK and Ebert BL. Nat Med. 2016. 22(3):288-97. PMID: 26878232 and 2: Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. Zambetti NA et al. Cell Stem Cell. 2016. 19(5):613-627. PMID: 27666011).* More importantly, based on our study, a phase I trial study using BI836858 antibody has been on going in Moffitt Cancer Center.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

We will follow our original proposal and SOW to further investigate our novel therapeutic approaches targeting S100A9 activated MDSC. 1) In addition to testing the human engineered anti-CD33 antibody, we will test the Tetramer that can trap S100A9 and compare its effect with anti-CD33 blocking antibody 2) We will test the novel MDSC inactivating compound ICTA, developed in our lab, in S100A9 transgenic mice. This will allow us to confirm and validate our novel approaches in an S100A9-associated disease model.

4. **IMPACT:**

MDS are the most common bone marrow failure (BMF) with variable survival ranging from a median of months in patients with high risk disease to years in low-risk patients. More than 50,000 cases are diagnosed annually in the US with the overall disease burden increasing as a result of the aging of the population in a disease characterized by high morbidity and mortality. Currently available treatment options have limited effect and can be associated with severe side-effects and high economical costs. This is mainly due to previous studies of the molecular pathogenesis of MDS focusing primarily on the already genetically altered malignant HSPC responsible for propagation of the malignant clone, rather than selection pressures conducive to the emergence of the clone. As a consequence, current treatments are largely empiric and non-specific. Instead, our hypothesis focuses on targeting S100A9/CD33 pathways to block the activation and accumulation of MDSCs in the local bone marrow microenvironment that suppresses healthy HSPC and induce DNA instability promoting MDS clone expansion. Our major findings obtained in the past year demonstrated that CD-33-fusion has a potential as a therapeutic approach to target MDSC activation and feasibly address a conceptually-novel hypothesis.

What was the impact on the development of the principal discipline(s) of the project?

We propose a paradigm shift that stems from a concept that understanding how the inflammatory microenvironment contributes to HSPC damage and malignant evaluation will have a profound and far-reaching effect on understanding MDS pathophysiology. If successful, this combined in vitro/in vivo approach can lead to immediate clinical application. Therefore, the conditions that these therapeutic approaches address represents an unmet medical need for a disease with few available treatments, thus providing a unique opportunity for success through the development of effective targeted therapeutics, like ours, in this patient population where it would have enormous clinical impact. This will be validated and supported by current ongoing phase I trial (not included in this proposal) using BI836858.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:,

Nothing to Report

6. **PRODUCTS:**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

None

What individuals have worked on the project?

Name:	Sheng Wei
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Dr. Wei is the PI of this project and responsible for the overall direction of the proposed project. He will oversee all molecular validation studies, data analysis and interpretation of experimental results.
Funding Support:	No change

Name:	Xianghong Chen
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	She is responsible for carrying out all the biological and biochemical assays and make all the novel constructs
Funding Support:	No change

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to Report

- **What other organizations were involved as partners?**

  Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES:

One research article published this year.

Title: Novel therapeutic approach to improve hematopoiesis in low risk MDS by targeting MDSCs with the Fc-engineered CD33 antibody BI 836858.

Authors: Eksioglu EA, Chen X, Heider KH, Rueter B, McGraw KL, Basiorka AA, Wei M, Burnette A, Cheng P, Lancet J, Komrokji R, Djeu J, List A, Wei S.

Leukemia. 2017 Feb 10. PMID: 28096534

Leukemia (2017), 1–9

www.nature.com/leu

ORIGINAL ARTICLE

Novel therapeutic approach to improve hematopoiesis in low risk MDS by targeting MDSCs with the Fc-engineered CD33 antibody BI 836858

EA Eksioglu[1,6], X Chen[1,6], K-H Heider[2], B Rueter[3], KL McGraw[4], AA Basiorka[5], M Wei[1], A Burnette[1], P Cheng[1], J Lancet[1], R Komrokji[1], J Djeu[1], A List[1] and S Wei[1]

We recently reported that the accumulation of myeloid-derived suppressor cells (MDSC), defined as $CD33^{+}HLA\text{-}DR^{-}Lin^{-}$, has 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 has 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, 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 γH2AX). 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.

Leukemia advance online publication, 10 February 2017; doi:10.1038/leu.2017.21

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.[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 immunity.[6–10] 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 cells[12] in the bone marrow (BM) microenvironment of MDS, which has a critical role in the pathogenesis of the disease.[7] 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 bone marrow (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 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 ($CD33^{+}HLA\text{-}DR^{-}Lineage^{-}$) 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 interleukin (IL)-10, tumor growth factor (TGF)β and reactive oxygen species (ROS).[11] Even more critical is our discovery that this process leads to the development of genomic instability.[28] We found a strong correlation between cellular ROS/nuclear-β-catenin to DNA damage ($γH2AX^{+}$ 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 γH2AX, a main marker of genomic instability.[29]

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, which can serve to prevent disease progression. At present, 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

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the treatment of acute myeloid leukemia, 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 owing 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 acute myeloid leukemia.[32]

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 Fcγ receptor IIIa. It binds with low nanomolar affinity to human CD33 and displays decelerated internalization kinetics compared with previously developed CD33 mAbs, thus making it suitable for exploitation of NK-mediated antibody-dependent cellular cytotoxicity (ADCC).[33] 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 International Prostate Symptom Score. Human healthy BM controls were purchased unprocessed (Lonza-Walkersville, Walkersville, MD, USA), healthy peripheral blood mononuclear cell (PBMC) were obtained from the local blood bank and healthy $CD34^+$ isolated cells were purchased (StemExpress, Placerville, CA, USA). Cells were isolated by Ficoll-Hypaque gradient centrifugation, as previously described[34] and cultured in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin and 1% L-glutamine (Gibco, Carlsbad, CA, USA). The MDS specimens used were classified as shown in Table 1.

Table 1. Primary bone marrow specimens characteristics

Patient	*WHO DX*	*IPSS*	*Gender*	*Age*	*Cytogenetics*
1	RAEB1	INT1	F	72	46 XX, 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
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

Cytotoxicity assays

^{51}Cr release assay was performed, as previously described.[35] Total PBMC, isolated $CD8^+$ T cells or isolated NK cells (StemCell technologies, Cambridge, MA, USA) were treated with either human engineered isotype control (BI 836847), 2 μg/ml BI 836858 or CD33Ab (clone 6C5/2), 20 μM Lenalidomide, 20 μM ICT or an equal volume of dimethyl sulfoxide (vehicle), before labeling with 100 μCi of ^{51}Cr (Amersham Corp, Louisville, CO, USA) in 0.5 ml of medium at 37 °C in a 5% CO_2 atmosphere for 1 h. 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 ^{51}Cr release was determined as: (experimental cpm—spontaneous cpm)/(total cpm incorporated—spontaneous cpm) × 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 h before fixing and measuring by flow cytometry.

Flow cytometry

MDSC cells were defined and purified by fluorescence activated cell sorting 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 4',6-diamidino-2-phenylindole. For antigenic determination, cells were washed in 1× phosphate-buffered saline in 2% bovine serum albumin and then stained with the same conjugated monoclonal antibodies described for sorting or relevant isotype controls (eBioscience, San Diego, CA, USA). 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, Carlsbad, CA, USA), sorted and labeled with Celtracker Orange before admixing with autologous mononuclear cells. To measure genomic instability cells were stained intracellularly with a γH2AX antibody.

Real-time quantitative PCR

RNA was isolated by Trizol isolation (Invitrogen, Carlsbad, CA, USA) followed by iScript cDNA synthesis (Biorad, Hercules, CA, USA) and amplification at 60 °C using SYBR Green Supermix (Quantas, Beverly, MA, USA) as previously done by us with primers against IL-10, TGFβ, CD33 and glyceraldehyde 3-phosphate dehydrogenase.[11] The relative gene expression was calculated by the ΔΔCt method where untreated cells were the experimental control and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase was the internal control.

Enzyme-linked immunosorbent assays

96-well plates (Nunc-Immuno Plate) were coated with purified monoclonal antibody against either human IL-10 or TGFβ (Pierce-Endogen, Rockford, IL, USA) in 1× phosphate-buffered saline, pH 7.4 at room temperature

overnight. The plates were then incubated in blocking buffer (Pierce-Endogen) for 2 h at room temperature and washed in 0.05% Tween-20. Wells were seeded with serially diluted recombinant human cytokines (standard, in blocking buffer in duplicates) or with 50 µl of supernatants in triplicate followed by addition 1 h later of biotin-labeled anti-cytokine antibody. After incubation wells were washed five times and coated with strepavidin-horseradish peroxidase for 20 min, washed again five times and developed by adding TMB substrate (Pierce-Endogen, Rockford, IL, USA). The reaction was stopped by the addition of an equal volume of 0.18 M H_2SO_4.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 fycoll-isolation in Mammalian Cell-PE LB buffer (G Bioscience, St Louis, MO, USA) supplemented with 1:100 protease inhibitor cocktail (G Bioscience) and 0.1 mM phenylmethane sulfonyl fluoride. Cleared lysates were then incubated with used to immune-precipitate CD33 (Rabbit polyclonal, Santa Cruz, Dallas, TX, USA) overnight at 4 °C in a rotor and immunoblotted with anti-SHP1 antibody (Santa Cruz) just as we did before.[11]

ROS production

The oxidation-sensitive dye, 2',7'-dichlorofluorescin diacetate, (Molecular Probes/Invitrogen, Eugene, OR, USA) was used to measure ROS production by U937, PBMC or HSPC in MDS BM MDSC. Cells were incubated at room temperature in complete Roswell Park Memorial Institute medium in the presence of 3 µM 2',7'-dichlorofluorescin diacetate for 30 min, in a water jacketed incubator. Further labeling was carried out after washing with 1× phosphate-buffered saline 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% CO_2 for ~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 (Gaithersburg, MD, USA) following the manufacturer's recommended protocol. In brief, 1×10^5 cells/ml were seeded into low melting agarose at a ratio of 1:10 v/v and pipetted 50 µl into comet slides followed by lysis in Lysis solution on ice for 4 °C for 1 h. Afterwards, slides were placed in unwinding solution for 1 h at room temperature. Slides were electrophoresed in the Alkaline Electrophoresis Solution at 21 volts for 30 min. Slides were then washed in water and 70% ethanol and dried at < 45 °C for 10–15 min followed stained with SYBR Green I and measured in a fluorescent microscope.

Statistics

All data were presented as means ± s.e.m. 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 have in the initiation and maintenance of MDS including the role of their S100A9/CD33 suppressive signaling.[11] 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 MDS.[36] 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 [Cr^{51}] 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 (Supplementary Figure 1A). We also demonstrated that BI 836858 treatment does not change the amount of NK cells in PBMC from healthy

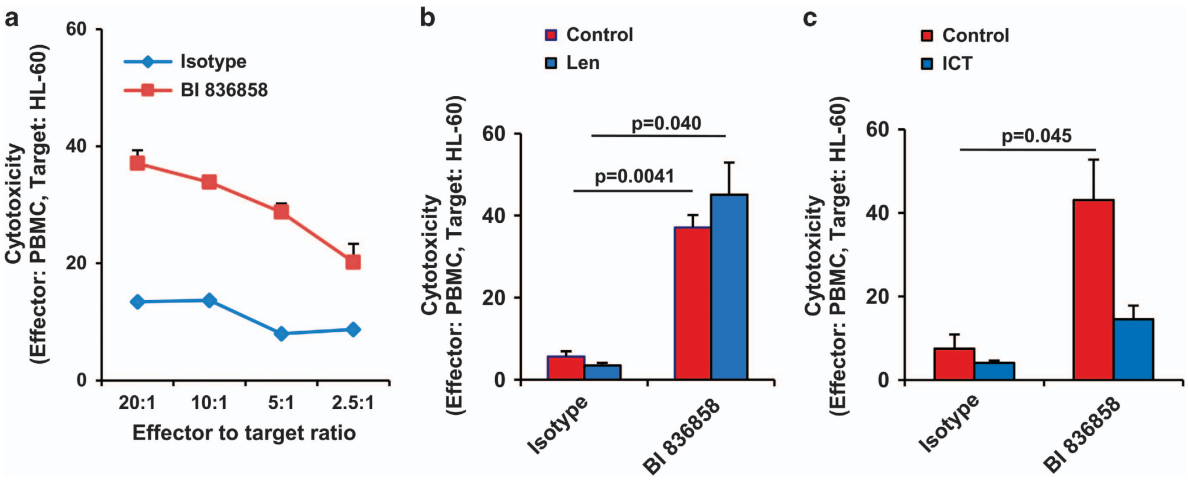

Figure 1. BI 836858 mediates ADCC specific for CD33-positive HL60 cells and does not affect the proportion of NK cells. Chromium (Cr^{51})-labeled HL60 cells (CD33 positive) were incubated for 30 min with 2 µg/ml of BI 836858, or the same concentration of isotype BI 836847, in ice before co-culturing with healthy human PBMC for 4 h 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**) HL60 cells were treated with either DMSO, 20 µM of the anti-inflammatory compound ICT for 4 days prior to addition of BI 836858 (2 µg/ml) or the same concentration of isotype followed by a cytotoxic assay with healthy human PBMC ($n = 3$). Error bars represent the s.e.m. of triplicate determinations of three separate donors and the *P*-value was calculated using Student's *t*-test.

donors (representative experiment Supplementary 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 (Supplementary Figures 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 HL60 with ICT, a compound that reduces MDSC and the S100A9/CD33 axis,[37] 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 ($Lineage^-HLA\text{-}DR^-CD33^+$) after treatment with BI 836858 (Figure 2a), which was accompanied by a decrease in the level of CD33 surface expression, measured by fluorescence intensity,[11] 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 (Supplementary Figure 2A). These results indicate that BI 836858-mediated reduction of MDSC is not an artifact of the staining properties of the antibodies (that is, 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, whereas 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 (Supplementary Figure 2B). This shows that the reduction of $CD33^+$ MDSCs is owing 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 with other CD33 antibodies, resulting in prolonged cell surface retention.[33] 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 Supplementary Figure 3A) and TGFβ (Supplementary Figures 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 as 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 min followed by 24 h 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 TGFβ (Supplementary Figure 3D), whereas BI 836858 can slightly, but not significantly, induce IL-10 and CD33 expression (Supplementary 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 cross-linking, and had the highest recruitment of SHP1 to CD33's ITIM domain (Supplementary 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 with commercial CD33 antibodies.

Our most recent work demonstrates that downstream of the S100A9/CD33 pathway is the activation of ROS, which has 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 (Supplementary Figure 4a and b), healthy

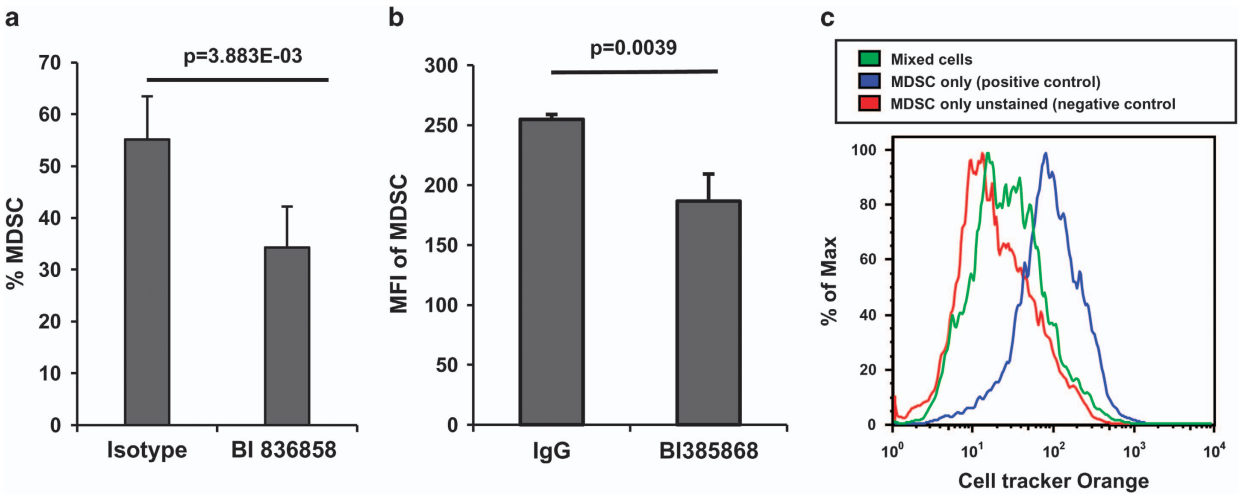

Figure 2. BI 836858 reduces MDSC and $CD33^+$ cells in MDS BM specimens. MDS primary BM specimens ($n = 10$) were cultured with 2 μg/ml of BI 836858 or isotype control and thereafter were analyzed for changes in the proportion (**a**) or fluorescence intensity (MFI, **b**) of MDSC by flow cytometry (cells defined as $HLA\text{-}DR^-Lin^-CD33^+$). Error bars represent the s.e.m. and the *P*-value was calculated using Two-tailed paired *t*-test. (**c**) Reduction of CellTracker Orange sorted population of MDSC after co-culture with autologous MNCs (representative of three separate experiments).

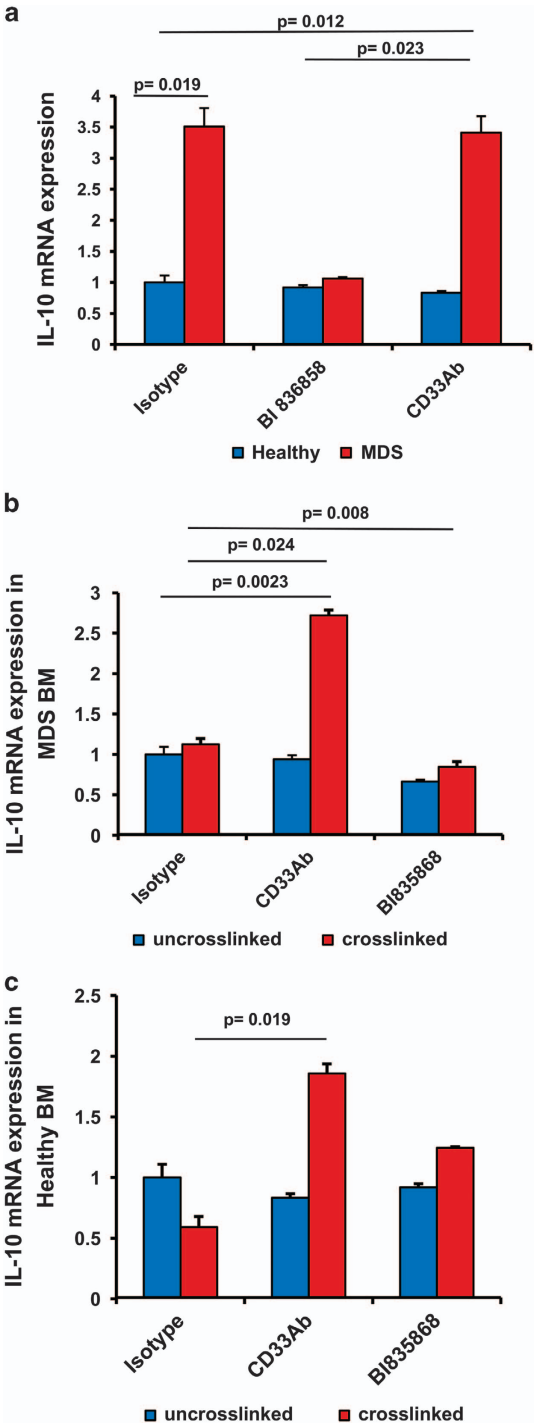

Figure 3. BI 836858 blocks downstream induction of CD33-mediated IL-10 expression. (**a**) Healthy or MDS BM cells co-cultured with 2 μg/ml of either BI 836858, CD33Ab or isotype control for 48 h after which gene expression of the cytokine IL-10 was assayed by qPCR. Similarly MDS BMMNCs (**b**) or healthy BMMNC (**c**) cultured cells were instead cross-linked with an anti-Fc Fab fragment antibody for half hour on ice before culture for 48 h at which point total RNA was collected for gene expression analysis of IL-10. The qPCR data were normalized against the housekeeping gene GAPDH using the ΔΔCt methodology. Error bars represent the s.e.m. of three separate experiments measured in duplicates and the *P*-value was calculated using Student's *t*-test.

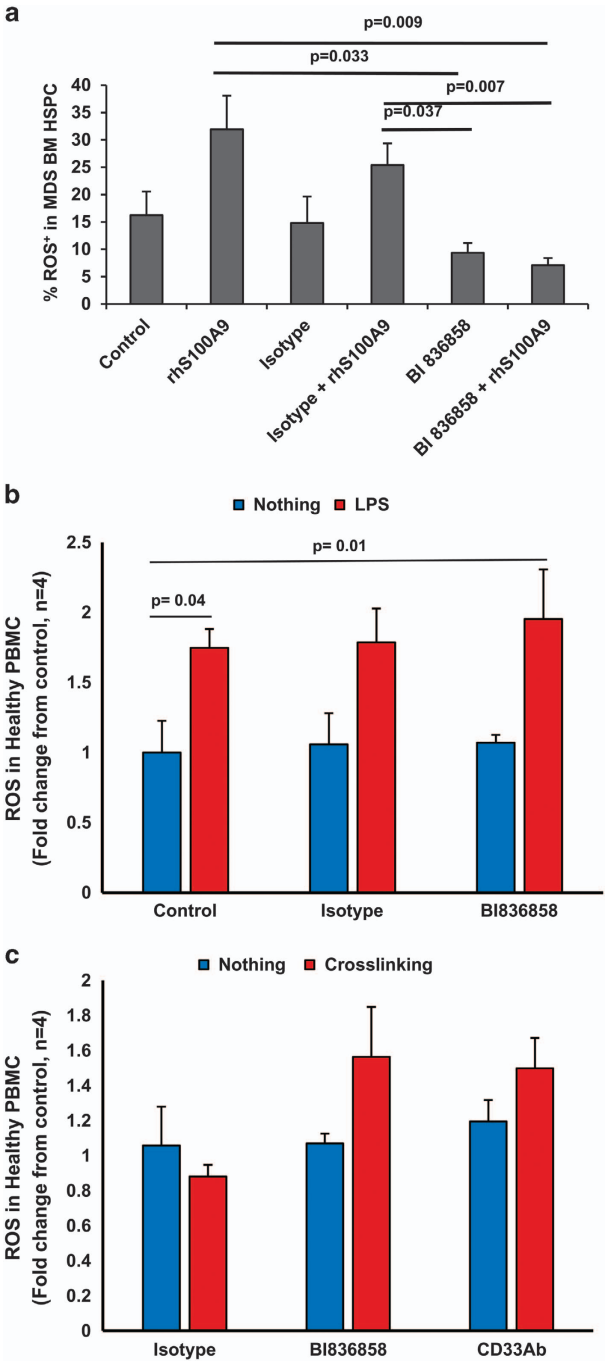

Figure 4. BI 836858 blocks downstream induction of CD33-mediated ROS. (**a**) HSPC (Lineage$^-$CD34$^+$ cells) from MDS BM primary specimens ($n=4$) were cultured *ex vivo* with BI 836858 or isotype control antibody in the presence or absence of 10 μg/ml of rhS100A9 followed by flow cytometric analysis to assess the presence of ROS. Similar experiments as in **a** were carried out but in the presence or absence of 0.5 μg/ml of LPS as stimulant (**b**) or by cross-linking antibody with an anti-Fc Fab fragment for half hour on ice (**c**) in healthy PBMC ($n=4$) followed by 24 h culture and ROS analysis. Error bars represent the s.e.m. and the *P*-value was calculated using student's *t*-test.

human PBMC treated with rhS100A9 (Supplementary 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 lipopolisacharide, are not

affected by BI 836858 (Figure 4b) probably owing to the fact that lipopolisacharide signals through Toll-like receptors (TLR), rather than CD33. In MDS BM however, there was no changes in the lipopolisacharide induction of ROS (data not shown), IL-10 expression (Supplementary Figure 4D), or ROS^+ percentages compared with controls (Supplementary Figure 4E). However, it is worth noting that the overall percent of ROS^+ cells were significantly increased in MDS BM compared with healthy PBMC, just as we observed before,[25] except in MDS BM treated with BI 836858, which was reduced. Similarly, treating healthy PBMC with either BI 836858 or CD33Ab increased the level of ROS although neither this, nor the changes in the percent of positive cells was significant with either treatment (Figure 4c and Supplementary Figure 4F).

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

Targeting CD33 signaling by BI 836858 could improve impaired hematopoiesis of MDS, which is the main cause of its characteristic anemia. For this purpose we used whole MDS BMMNC, 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*. Although there were no significant changes in the number of colonies from healthy BMMNCs, 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 BI 836858-treated cells. Importantly, if we treated healthy $CD34^+$ cells with BI 836858 or CD33Ab (both $n = 3$) both antibodies affected colony formation when cross-linked, but only cells that were treated with BI835868 had enhanced BFU-E colony formation compared with 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 toward 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 population.[25] Inevitably, based on the demonstration here that BI 836858 can serve as a blocking antibody for S100A9/CD33 signaling it will affect these cells as well.

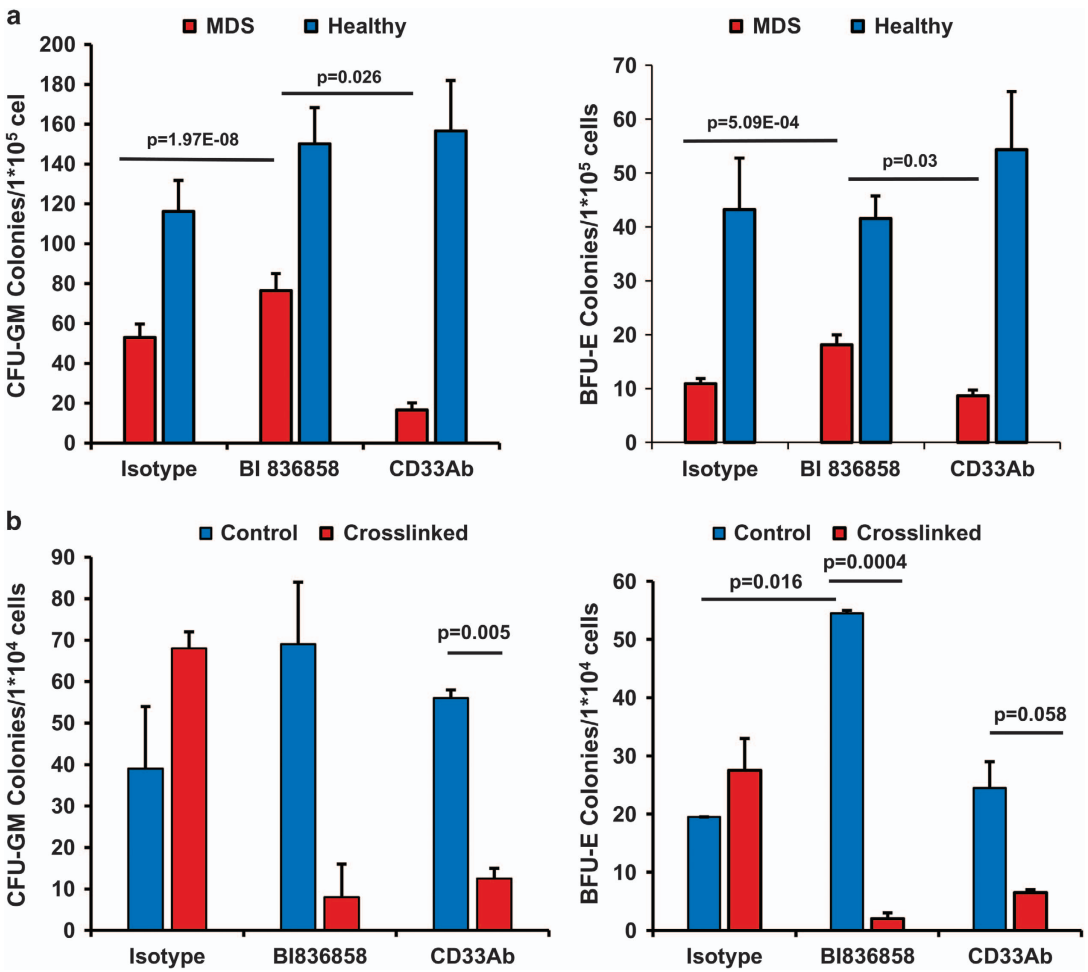

Figure 5. BI 836858 improves colony-forming capacity in MDS BM specimens cultured *ex vivo*. (**a**) Healthy or MDS primary BM specimens were cultured with 2μg/ml of BI 836858, CD33Ab or its corresponding isotype control, and then grown in duplicate in complete methylcellulose media for 14 days. $N = 10$ specimens of each were used in isotype and BI 836858 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 cross-linking with the respective isotypes. CFU-GM and BFU-E colonies were analyzed and microscopically scored. Error bars represent the s.e.m. and the *P*-value was calculated using two-tailed paired *t*-test.

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

As 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 BMMNC,[28] 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 (Supplementary Figures 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 Supplementary 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 γH2AX, 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 γH2AX after cross-linking and using it or its respective antibody induced an increase in the level of this marker even without cross-linking (Supplementary Figure 5C). In order to corroborate that this effect is specific for CD33, we repeated the experiments with a TLR4 antibody as 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.

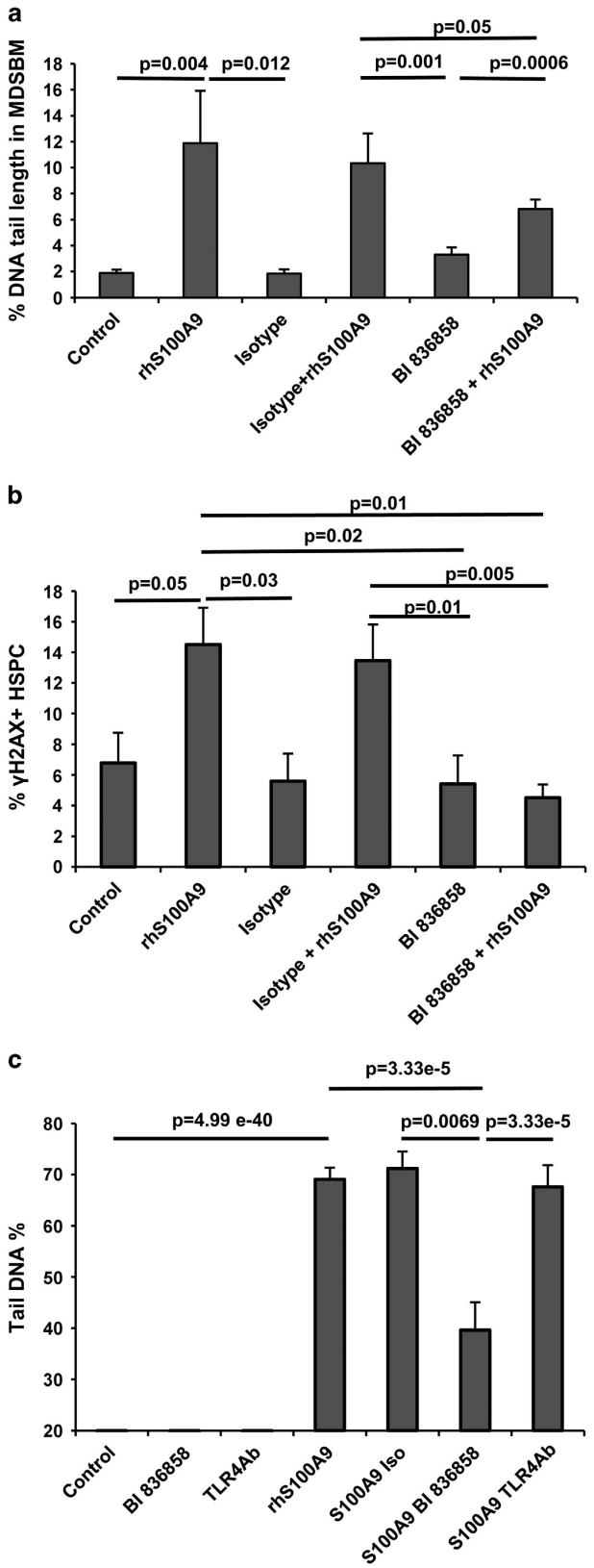

Figure 6. BI 836858 prevents the development of S100A9/CD33-mediated genomic instability. (**a**) HSPC from MDS BM primary specimens ($n = 3$) were cultured *ex vivo* with isotype control or BI 836858 antibody in the presence or absence of rhS100A9 followed by comet analysis to assess the protective 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 γH2AX 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, 2 μg/ml of BI 836858 or a 2 μg/ml blocking TLR4 antibody to assess the specificity of BI 836858 in preventing damage by comet assay. Some cells were also treated with 10 μg/ml rhS100A9 to induce damage. Error bars represent the s.e.m. and the *P*-value was calculated using Student's *t*-test.

DISCUSSION

Development of disease targeted therapeutics for MDS has been slow largely owing 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 United States, Lenalidomide represents the only therapeutic, which is effective only in a small fraction of MDS patients with a rare genetic change, a chromosome 5q deletion.[38] Although treatment with Lenalidomide 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.

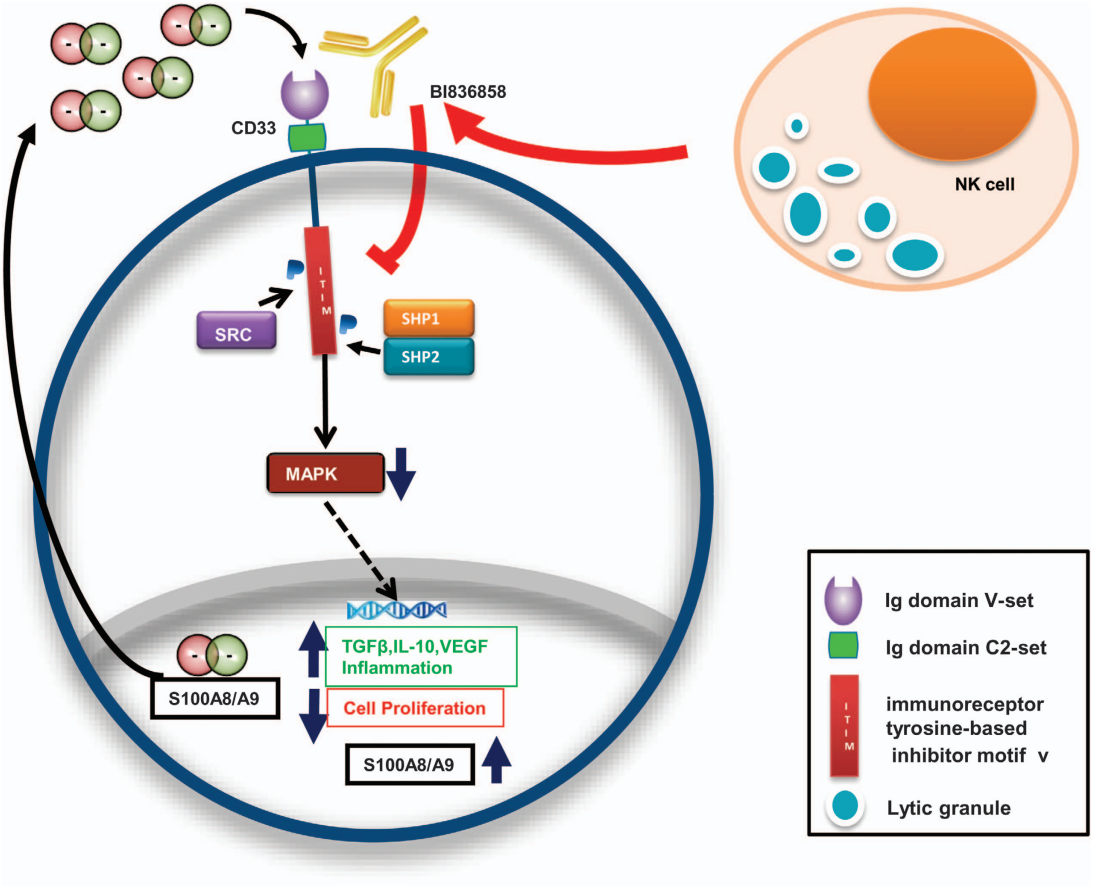

Figure 7. Schematic representation of BI 836858 functionality in MDS.

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 upregulation 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 HSPC.[11] 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 BMMNC 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 acute myeloid leukemia and MDS are currently ongoing (ClinicalTrials.gov Identifier: NCT01690624, NCT02240706).

CONFLICT OF INTEREST

Karl-Heinz Heider and Bjoern Reuter are employees of Boehringer Ingelheim.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)