Clinical Cancer Research

Selective Targeting of Myeloid-Derived Suppressor Cells in Cancer Patients Using DS-8273a, an Agonistic TRAIL-R2 Antibody

George A. Dominguez[1], Thomas Condamine[1], Sridevi Mony[1], Ayumi Hashimoto[1], Fang Wang[1], Qin Liu[1], Andres Forero[2], Johanna Bendell[3], Robert Witt[4], Neil Hockstein[4], Prasanna Kumar[5], and Dmitry I. Gabrilovich[1]

Abstract

Purpose: Myeloid-derived suppressor cells (MDSC) are one of the major contributors to immune suppression in cancer. We recently have demonstrated in preclinical study that MDSCs are sensitive to TRAIL receptor 2 (TRAIL-R2) agonist. The goal of this study was to clinically test the hypothesis that targeting TRAIL-R2 can selectively eliminate MDSCs.

Experimental Design: The TRAIL-R2 agonistic antibody (DS-8273a) has been tested in 16 patients with advanced cancers enrolled in a phase I trial. The antibody (24 mg/kg) was administered intravenously once every 3 weeks till disease progression, unacceptable toxicities, or withdrawal of consent. The safety and the presence of various populations of myeloid and lymphoid cells in peripheral blood and tumor tissues were evaluated.

Results: The treatment was well tolerated with only mild to moderate adverse events attributable to the study drug. Treatment with DS-8273a resulted in reduction of the elevated numbers of MDSCs in the peripheral blood of most patients to the levels observed in healthy volunteers. However, in several patients, MDSCs rebounded back to the pretreatment level by day 42. In contrast, DS-8273a did not affect the number of neutrophils, monocytes, and other populations of myeloid and lymphoid cells. Decrease in MDSCs inversely correlated with the length of progression-free survival. In tumors, DS-8273a treatment resulted in a decrease of MDSCs in 50% of the patients who were able to provide pre- and on-treatment biopsies.

Conclusions: Targeting TRAIL-R2 resulted in elimination of different populations of MDSCs without affecting mature myeloid or lymphoid cells. These data support the use of this antibody in combination immmunotherapy of cancer. *Clin Cancer Res; 23(12); 2942–50. ©2016 AACR.*

Introduction

It is now well established that the myeloid cells play an important role in regulation of tumor progression and metastases as well as in limiting the effects of cancer immunotherapy (1). Consequently, combination of immune checkpoint blockade, or T-cell–based immunotherapy including treatment with chimeric antigen receptor T (CAR-T) cells, T-cell receptor–transduced T (TCR-T) cells or tumor-infiltrating lymphocytes (TIL) with therapy that targets the immunosuppressive microenvironment holds great promise (2, 3). Myeloid-derived suppressor cells (MDSC) represent one of the major immunosuppressive populations in cancer patients. They consist of populations of polymorphonuclear (PMN-MDSC), monocytic (M-MDSC), and early precursors (eMDSC; ref. 4). In addition to their suppressive abilities, MDSCs can also promote tumor survival, angiogenesis, and metastasis (5). MDSCs accumulation has recently been correlated with tumor burden, as well as overall survival, disease-free survival, and recurrence-free survival in different tumor types (6, 7). Importantly, MDSC accumulation has been reported to correlate with resistance to ipilimumab or nivolumab treatment in melanoma (8–13). Several strategies to target MDSCs have been proposed and are based on inducing depletion or inhibiting their suppressive activity, differentiation, or accumulation (14). However, none of these strategies are specific to MDSCs and many of them (e.g., chemotherapy) have substantial side effects. We have recently reported that MDSCs could be selectively targeted in preclinical settings using TRAIL-R2 agonistic antibody (15).

TNF-related apoptosis induced ligand-receptors (TRAIL-R) are members of the TNF receptor superfamily and consist of two death receptors, TRAIL-R1 (DR4/CD261) and TRAIL-R2 (DR5/CD262), two decoy receptors (DcR1/CD263 and DcR2/CD264), and one soluble receptor (osteoprotegerin, OPG; refs. 16, 17). Ligation of TRAIL with DR4 or DR5 induces trimerization of the receptor, which activates an apoptotic pathway (18). DcR1 is a glycosylphosphatidylinositol (GPI)-linked protein lacking an intracellular domain, and DcR2 contains a truncated death domain. These two receptors can prevent TRAIL-induced apoptosis by competing with DR4 and DR5 for binding to TRAIL or by inhibiting apoptosis via formation of ligand-independent complexes between DR5 and DcR2 (19). In mice, agonistic DR5

[1]The Wistar Institute, Philadelphia, Pennsylvania. [2]University of Alabama at Birmingham Comprehensive Cancer Center, Birmingham, Alabama. [3]Sarah Cannon Research Institute Tennessee Oncology, Nashville, Tennessee. [4]Helen F. Graham Cancer Center & Research Institute, Christiana Care Health System, Newark, Delaware. [5]Daiichi-Sankyo Inc., Parsippany, New Jersey.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

G.A. Dominguez and T. Condamine contributed equally to this article.

Clinical Trial registration ID: NCT02076451.

Corresponding Author: Dmitry I. Gabrilovich, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104. Phone: 215-495-6955; Fax: 215-573-2456; E-mail: dgabrilovich@wistar.org

doi: 10.1158/1078-0432.CCR-16-1784

Translational Relevance

This study demonstrates highly selective targeting myeloid-derived suppressive cells (MDSC) in cancer patients. It is based on recently discovered preferential sensitivity of MDSCs to TRAIL receptor targeting. Treatment with new agonistic TRAIL-R antibody DS-8273a resulted in a temporary reduction of the elevated numbers of MDSCs in the peripheral blood of most patients to the levels observed in healthy volunteers. In contrast, DS-8273a did not affect the number of neutrophils, monocytes, and other populations of myeloid and lymphoid cells. Decrease in MDSCs inversely correlated with the length of progression-free survival. In tumors, DS-8273a treatment resulted in a decrease of MDSCs in 50% of the patients who were able to provide pre- and on-treatment biopsies. It opens an opportunity to regulate tumor microenvironment in patients to enhance the effect of cancer therapeutics.

antibody potentiated the effect of immune checkpoint blockade (CTLA-4 targeting antibody), which resulted in a 5-fold decrease of the tumor growth (15). This suggests that TRAIL-R–targeting antibody can be potentially used in selective elimination of MDSCs in cancer patients. We tested this hypothesis in patients with advanced cancers enrolled in a phase I trial of TRAIL-R2 agonistic antibody DS-8273a.

Materials and Methods

Sample collection and preparation

All samples were collected from patients at Sarah Cannon Research Institute, University of Alabama Birmingham, and University of Chicago who were enrolled in the phase I study registered at clinicaltrials.gov (NCT02076451). Peripheral blood was collected from 16 subjects and pre- and on-treatment tumor tissue from 6 subjects with advanced stage solid tumors. Study was approved by institutional review boards of participating institutions and all patient samples were collected with informed consent. Clinical characteristics are described in Table 1.

Peripheral blood from 6 patients (5 males and 1 females, age 53–82) with stage III head and neck cancer who were not part of the treatment protocol was collected from Helen F. Graham Cancer Center. That portion of the study was approved by the Institutional Review Boards of the Helen F. Graham Cancer Center, and The Wistar Institute. The patient samples were collected with informed consent. Peripheral blood from 12 healthy donors was used as a control and was obtained at the Wistar Institute in accordance to the institutional review board protocol. All samples were analyzed within 24 to 36 hours following collection.

Cell isolation and culture

Human PBMCs were isolated on a Ficoll gradient following the manufacturer's recommendation (Amersham). Cells were then cultured in RPMI (Biosource International) supplemented with 10% FBS, 5 mmol/L glutamine, 25 mmol/L HEPES, 50 μmol/L β-mercaptoethanol, and 1% antibiotics (Invitrogen). For the experiments where myeloid cells were placed in culture, recombinant GM-CSF was added to the media at a concentration of 10 ng/mL (PeproTech). For MDSC isolation, PBMCs and neutrophils were isolated using a histopaque gradient followed by isolation with CD15 antibody and magnetic beads. M-MDSCs were sorted by flow cytometry from the PBMCs depleted of PMN-MDSC after staining with CD14 and HLA-DR antibodies. The purity of all population was >90% following isolation.

Suppression assay

T cells from one healthy donor were purified using a human T-cell enrichment column (R&D) and used as responder cells. Dendritic cells were generated from adherent monocytes in the presence of GM-CSF and IL4 (PeproTech; 20 and 10 ng/mL, respectively) for 6 days as described previously (20) and used as stimulator cells. Responder and stimulator cells were then mixed at a 10:1 ratio and different subsets of MDSCs were added at various ratios (1:1, 1:2, and 1:4 MDSCs to T-cell ratio). T-cell proliferation was assessed after 5 days of culture by thymidine incorporation.

In vitro killing assay

Human PBMCs and PMNs were isolated by centrifugation over a double density Histopaque (Sigma Aldrich) gradient (1.077 to collect PBMC and 1.119 to collect PMN) from cancer patients.

Table 1. Main clinical characteristics of patients

	Age	Gender	Cancer type	Histology	Stage	Prior chemotherapy
Pt 0002-0014	37	F	Breast	Adenocarcinoma	IV	Capecitabine/Taxol/MM-121/Tamoxifen/Docetaxel
Pt 0003-0012	51	M	Colorectal	Adenocarcinoma	IV	Oxaliplatin/Bevacizumab/Irinotecan/5-FU/Mitomycin C/Panitumumab
Pt 0002-0015	63	F	Breast	Invasive ductal carcinoma	IV	Faslodex/Halaven/Tamoxifen/Everolimus
Pt 0003-0016	67	M	Pancreatic	Adenocarcinoma	IV	Gemcitabine/5-FU/Irinotecan/Abraxane/Oxaliplatin/Leucovorin
Pt 0002-0018	65	F	Ovarian	Serous carcinoma	IIIC	Topotecan/Filgrastim/Tamoxifen/Paclitaxel/Gemcitabine
Pt 0002-0019	63	F	Endometrial	Adenocarcinoma	IIIB	Taxotere/Carboplatin
Pt 0001-0021	50	M	Appendiceal	Mucinous adenocarcinoma	IV	
Pt 0001-0026	45	F	Liposarcoma	Mixed subtypes	IV	MDM2/MDMX inhibitor
Pt 0002-0023	51	F	Colorectal	Metastatic adenocarcinoma	IV	Leucovorin/Fluorouracil/Avastin/Oxaliplatin/Irinotecan/Zaltrap
Pt 0002-0025	58	M	Colorectal	Adenocarcinoma	IV	Zaltrap/Avastin/Xeloda/leucovorin/Irinotecan/Fluorouracil
Pt 0003-0028	76	F	Melanoma	Acral malignant	IV	Nivolumab/Ipilimumab/Temozolomide/Lirilumab
Pt 0002-0027	58	F	Colorectal	Metastatic adenocarcinoma	IIIC	Irinotecan/Fluorouracil/Avastin/Granisetron/Oxaliplatin/ Bevacizumab/leucovorin/Regorafnib/Panitumumab
Pt 0003-0031	25	F	Osteosarcoma	High-grade osteosarcoma	IV	Docetaxel/Gemcitabine
Pt 0002-0033	57	F	Ovarian	Papillary serous carcinoma	IV	Avastin/Gemzar/Carboplatin/Taxotere
Pt 0003-0034	39	M	Leiomyosarcoma	Leiomyosarcoma	IV	Sirolimus/Gemcitabine/Pazopanib/TH-302/Doxorubicin/Docetaxel/ Dacarzabine
Pt 0002-0035	62	M	Liver	Hepatocellular carcinoma	IV	Nexavar/Doxorubicin

Cells were then culture in RPMI supplemented with 10% FBS, 5 mmol/L glutamine, 25 mmol/L HEPES, 50 μmol/L β-mercaptoethanol, and 1% antibiotics with 10 ng/mL of rhGM-CSF. DS-8273a was added to culture for overnight incubation followed by flow cytometry analysis of M-MDSC or PMN-MDSC levels for cell recovery. In some studies, healthy donor PMN cells were cultured with or without 1 μmol/L thapsigargin (THG; Sigma Aldrich) for 4 and 24 hours. Samples were analyzed by flow cytometry for TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and DR5 (CD262) expression at each time point. After 4 hours, DS-8273a was added to wells at a concentration of 4 μg/mL with or without 2.5 μg/mL of mouse anti-human IgG_1 (Thermo Fisher) to facilitate cross-linking. Cell recovery was assessed using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific).

Flow cytometry

The antibody panels used in this study are described in Supplementary Table S1. FoxP3 staining was performed using the human FoxP3 buffer set according to the manufacturer's recommendations (BD Biosciences). All flow cytometry data were acquired using a BD LSR II flow cytometer and analyzed using FlowJo software (Tree Star).

Immunofluorescence/immunohistochemistry

Following deparaffinization and rehydration, heat-induced antigen retrieval was performed using Tris-EDTA buffer pH 9. Tissues were stained for S100A9 (Novus Biologicals), CD33 (Novocastra), CD8, Neutrophil Elastase, and CD163 (Abcam). PD-1 antibody was obtained from R&D Systems. The following fluorescently conjugated secondary mAbs (Life Technologies) were used: anti-rabbit IgG AF594 for S100A9 and neutrophil Elastase, anti-mouse IgG AF647 for CD33, and anti-mouse IgG AF594 for CD163. Alexa-594 donkey anti-mouse IgG was used for CD8 and Alexa-647 donkey anti-goat antibody for PD1. Cell nuclei were stained using DAPI (Life technologies). Images were obtained using Leica TCS SP5 Confocal microscope. Sixteen frames at 63× magnification were used to calculate the cell count/mm^2.

IHC staining was performed on a Bond Max automated staining system (Leica Microsystems). The Bond Refine Polymer Staining Kit (Leica Microsystems) was used. FoxP3 mAb (Biolegend), CD4 mAb (Leica Microsystems), and CD8 mAb (DAKO) were used and antigen retrieval was performed with ER2 and ER1 (Leica Microsystems) retrieval solutions. Slides were rinsed, dehydrated through a series of ascending concentrations of ethanol and xylene, and then mounted. Images were obtained using Nikon E600 Upright Microscope. Twelve frames at 40× magnification were used to calculate the cell count/mm^2.

Anti-drug antibody detection

Anti-drug antibody (ADA) detection against DS-8273a in the patient plasma was assayed using an electrochemiluminescent (ECL) immunoassay in a bridging assay format. Biotin-labeled (B) and ruthenium-labeled (Ru) DS-8273a were used as capture and detection reagents for anti-DS-8273a antibodies, and the ECL signal generated in the assay in the presence of the Ru-DS-8273a/anti-DS-8273a/B-DS-8273a immune complex was measured using the Meso-Scale discovery (MSD) PRTM 6000 Plate Reader. If a sample was tested ADA-positive, a confirmatory test was performed and considered final.

Statistical analysis

Statistical analysis was performed using a two-tailed Student *t* test (unless otherwise stated), correlation analysis using Spearman rank test. GraphPad Prism 5 software (GraphPad Software Inc.) and StataMP13 (StataCorp LP) were used and significance was determined at $P < 0.05$.

Results

Effect of TRAIL-R2 agonistic antibody on MDSC *in vitro*

To evaluate the populations of MDSC in PBMCs, we used previously described criteria (4, 6, 21). MDSCs are comprised of a mixed population of early-stage precursors and PMN-MDSCs (eMDSCs) defined as Lin (CD3, 14, 19, 56)$^-$HLA-DR$^-$CD33$^+$, PMN-MDSCs defined as CD11b$^+$CD14$^-$CD33$^+$CD15$^+$ cells, and M-MDSCs defined as CD14$^+$HLA-DR$^{-/lo}$ cells (Fig. 1A). Other mononuclear cells including various populations of lymphocytes, monocytes, and dendritic cells were also evaluated as described in Supplementary Fig. S1. In this study, all CD15$^+$ cells present in the PBMC layer were considered as PMN-MDSCs. There are currently no fully effective methods or specific markers to distinguish PMN-MDSCs from regular neutrophils within the PBMC layer. Because of this fact, we wanted to verify the suppressive activity all CD15$^+$ cells found in the PBMC layer from those found in the high-density PMN fraction. This was confirmed in a direct T-cell–suppressive assay using a three-way allogeneic mixed leukocyte reaction using samples from patients with advanced head and neck cancer that were not on the treatment protocol. Figure 1B demonstrates this comparison of suppressive activity of low-density PMN-MDSCs (PBMC fraction) and high-density neutrophils isolated from peripheral blood of the same patients.

To target MDSCs *in vitro*, we used TRAIL-R2 agonistic antibody, DS-8273a developed by Daiichi Sankyo Inc. Cells were isolated from patients with advanced head and neck cancer that were not on the treatment protocol. DS-8273a at concentration 100 μg/mL and higher caused significant killing of PMN-MDSCs but not neutrophils from the same patients. Similar results were observed with M-MDSCs and monocytes (Fig. 1C).

We have previously implicated ER stress in changes in TRAIL-Rs expression observed in myeloid cells in cancer (15). We asked whether ER stress can make control neutrophils sensitive to DS-8273a. PMNs isolated from healthy donors were cultured with GM-CSF and endoplasmic reticulum (ER) stress inducer THG. THG induced downregulation of DcR1 and DcR2 on PMN after 4 hours of incubation. The effect became stronger after 24 hours of incubation (Fig. 1D). Importantly, THG had minimal effects on the expression of DR5 consistent with changes observed in human MDSCs in a previous study (15). Incubation of PMN for 24 hours with GM-CSF did not sensitize these cells to DS-8273a. However, PMN became sensitive to agonistic TRAIL-R2 antibody after incubation with THG (Fig. 1E). These results indicate that ER stress–inducible downregulation of DcR1 and DcR2 on MDSCs make these cells sensitive to the agonistic TRAIL-R2 antibody DS8273a.

Design of the trial and characterization of MDSCs targeted by DS-8273a

The effect of DS-8273a was tested in a multicenter two-part phase I clinical trial NCT02076451 in patients with advanced solid tumors. The subjects were treated at escalating doses of

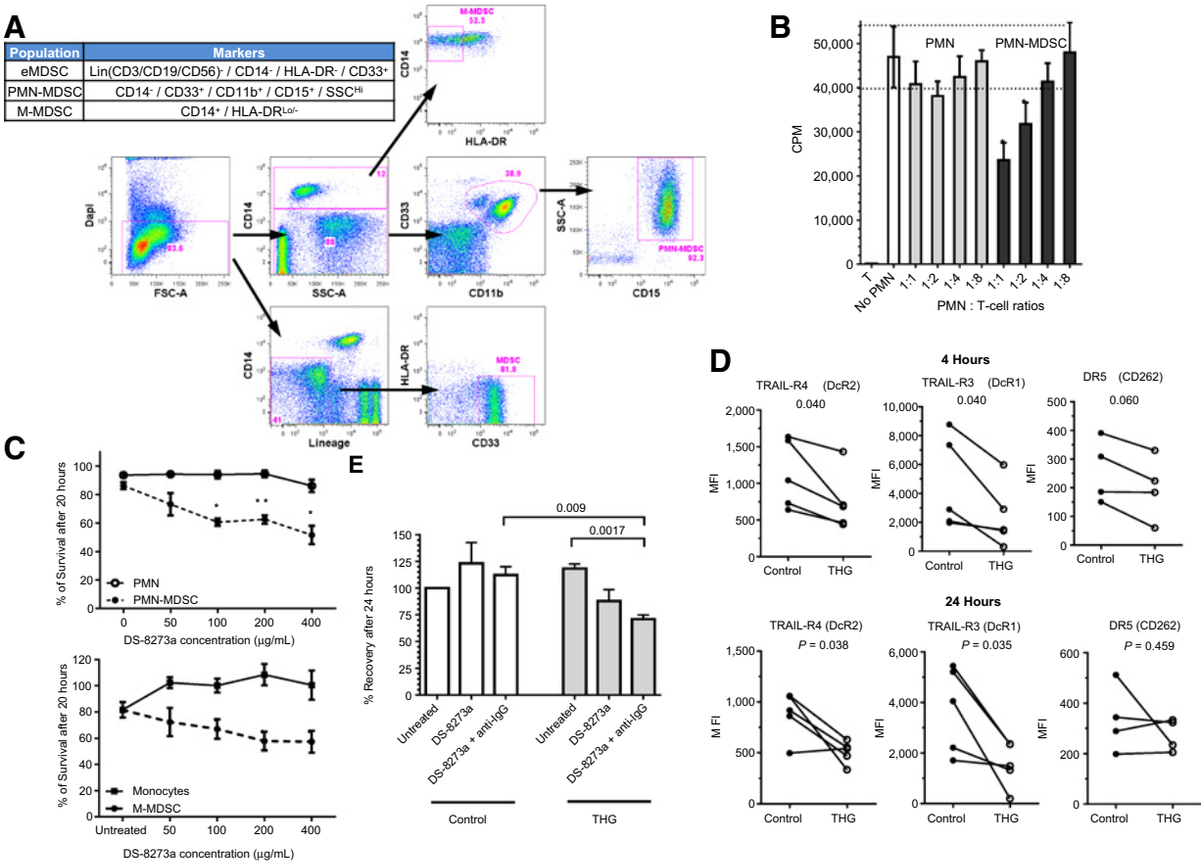

Figure 1.

Characterization of MDSCs. **A,** Phenotype of populations of MDSC evaluated in the study by flow cytometry. Typical example of the analysis of one patient on trial is shown. **B,** Suppressive activity of PMN-MDSCs. PMN-MDSCs were isolated from PBMCs and neutrophils (PMN) from high-density fraction of hystopaque gradient from the same patient followed by magnetic beads isolation with CD15 antibody. Suppressive activity of PMNs and PMN-MDSCs was evaluated in triplicates in three-way allogeneic MLR. Two patients with HNC cancer (not on trial) were tested and showed similar results. *, $P < 0.05$ from control (no MDSCs or PMNs added). **C,** Effect of DS-8273a on survival of cells. Neutrophils, PMN-MDSCs, monocytes, and M-MDSCs were isolated from four patients with HNC (not on trial). Cells were cultured for 20 hours with indicated concentration of DS-8273a in the presence of 10 ng/mL rhGM-CSF and viability was assessed by 7AAD staining. *, $P < 0.05$. **D,** Effect of ER stress inducer THG on TRAIL-Rs expression in PMNs. PMNs were isolated from four different healthy donors and treated for 24 hours with 20 ng/mL GM-CSF and 1 μmol/L THG. Cells were then collected and analyzed using indicated markers. *P* values between control (untreated) and THG-treated PMN are shown. **E,** Effect of DS-8273a on THG-treated PMNs. PMNs were isolated from healthy donors and treated with or without 1 μmol/L of THG for 4 hours in the presence of 10 ng/mL rhGM-CSF followed by the addition of DS-8273a for 24 hours. Untreated cells incubated with media alone, DS-8273a − 4 μg/mL of antibody, DS-8273a + anti-IgG − 4 μg/mL DS-8273a and 2.5 μg/mL anti-IgG_1 (to enhance cross-linking of primary antibody). *P* values of statistically significant differences are shown.

single-agent DS-8273a starting from 2 mg/kg through 8, 16, and 24 mg/kg in the dose escalation part of the study, followed by a dose expansion part, in which all subjects were treated at the highest dose of 24 mg/kg once every 3 weeks that showed no dose-limiting toxicities. The antibody was administered intravenously every 3 weeks till disease progression, unacceptable toxicities, or withdrawal of consent. The clinical trial demonstrated excellent safety and tolerability of DS-8273a at all the dose levels tested up to 24 mg/kg every 3 weeks (manuscript submitted). Evaluation of the effect of the antibody on MDSCs was performed only in subjects treated with DS-8273a at 24 mg/kg in the dose escalation and the dose expansion cohorts (Supplementary Fig. S2). The mean C_{max} at this dose level after the first infusion of DS-8273a was 621 μg/mL and the C_{min} was 132 μg/mL that were both higher than the minimal concentration observed to be effective in inducing apoptosis in the MDSCs based on *ex vivo* experiments mentioned above.

Blood samples were collected from 18 patients who received DS-8273a at 24 mg/kg every 3 weeks. Pretreatment samples from two patients were lost during transportation. Therefore, 16 patients were evaluated (Supplementary Fig. S3). Most of the patients had stage IV cancer and had failed multiple prior chemotherapies. No concurrent cancer therapy was administrated during the treatment with DS-8273a (Table 1). A risk of ADA formation affecting continued treatment of the antibody was not identified in the study. Fifteen of the 16 subjects were ADA-negative at all time points assessed. One patient who developed ADA positivity discontinued the study due to clinical progression, and without a follow up sample for re-testing ADA. Pharmacokinetic data showed no appreciable difference

in the plasma concentrations of DS-8273a between cycle 1 and cycle 2 (data not shown).

DS-8273a selectively eliminated PMN-MDSCs and eMDSCs in cancer patients

Samples of blood were collected in heparin tubes at baseline (prior to the treatment), on day 14, 21 (end of cycle 1), 28, and 42 (end of cycle 2) and shipped overnight on ice for analysis (Supplementary Fig. S2). Control blood samples from healthy volunteers were stored on ice overnight to create comparable conditions. Prior to the beginning of the treatment, patients had lower number of $CD4^+$ T cells, B cells, natural killer (NK) cells, and dendritic cells (DC) in comparison with healthy donors. In contrast, they had higher numbers of neutrophils, eMDSCs, and PMN-MDSCs (Fig. 2). When various cellular populations were analyzed in all patients during the treatment, no significant effect of DS-8273a was observed on lymphocytes, neutrophils, monocytes, and DCs (Supplementary Fig. S4). However, a clear trend for a decrease in the numbers of MDSCs was observed (Supplementary Fig. S4). To better understand the changes in MDSCs, we separated patients based on their pretreatment levels of eMDSCs and PMN-MDSCs. In 9 of 16 patients whose peripheral blood was available for analysis, the MDSC levels were higher than the range established in healthy individuals, whereas in 7 patients it was within the control range (Fig. 3A). In patients with elevated numbers of eMDSCs and PMN-MDSCs, treatment with DS8273a caused a significant drop in the number of these cells (to a level observed in healthy donors) 14 days after the start of the treatment (Fig. 3B). The presence of these cells remained at the control level on day 21 and started increasing after day 28. A mixed-effects spline model with two knots at 14 and 21 days demonstrated that upward trend was not significant ($P = 0.175$). A similar trend was continued after the second cycle of treatment; however, the change was not significant ($P = 0.965$). Thus, although populations of PMN-MDSCs showed a trend to increase after day 28, it was not statistically significant.

DS-8273a did not affect populations of neutrophils and monocytes (Fig. 3C) nor did it have an impact on other myeloid and lymphoid cells (Supplementary Fig. S5). Treatment with DS-8273a did not affect the number of MDSCs in patients that had pretreatment eMDSCs or PMN-MDSCs numbers within the healthy donor range (Fig. 3D).

We performed a similar analysis with the population of M-MDSCs. In contrast to PMN-MDSCs, only two patients had elevated level of these cells (Fig. 3E). In both patients, DS-8273a dramatically reduced the numbers of M-MDSCs to the control level and remained low for the duration of the

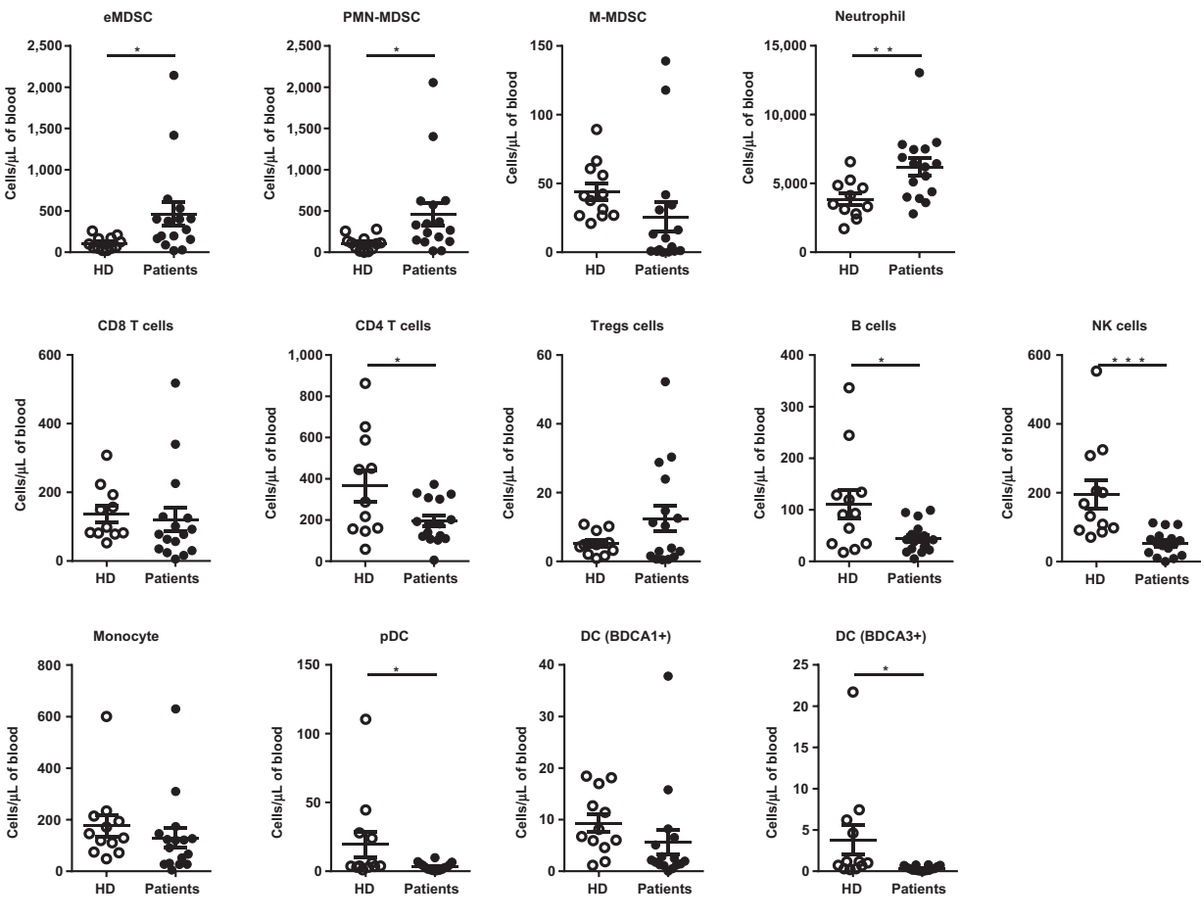

Figure 2.
The presence of different populations of cells in patients before start of the treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ from values in healthy donors (HD).

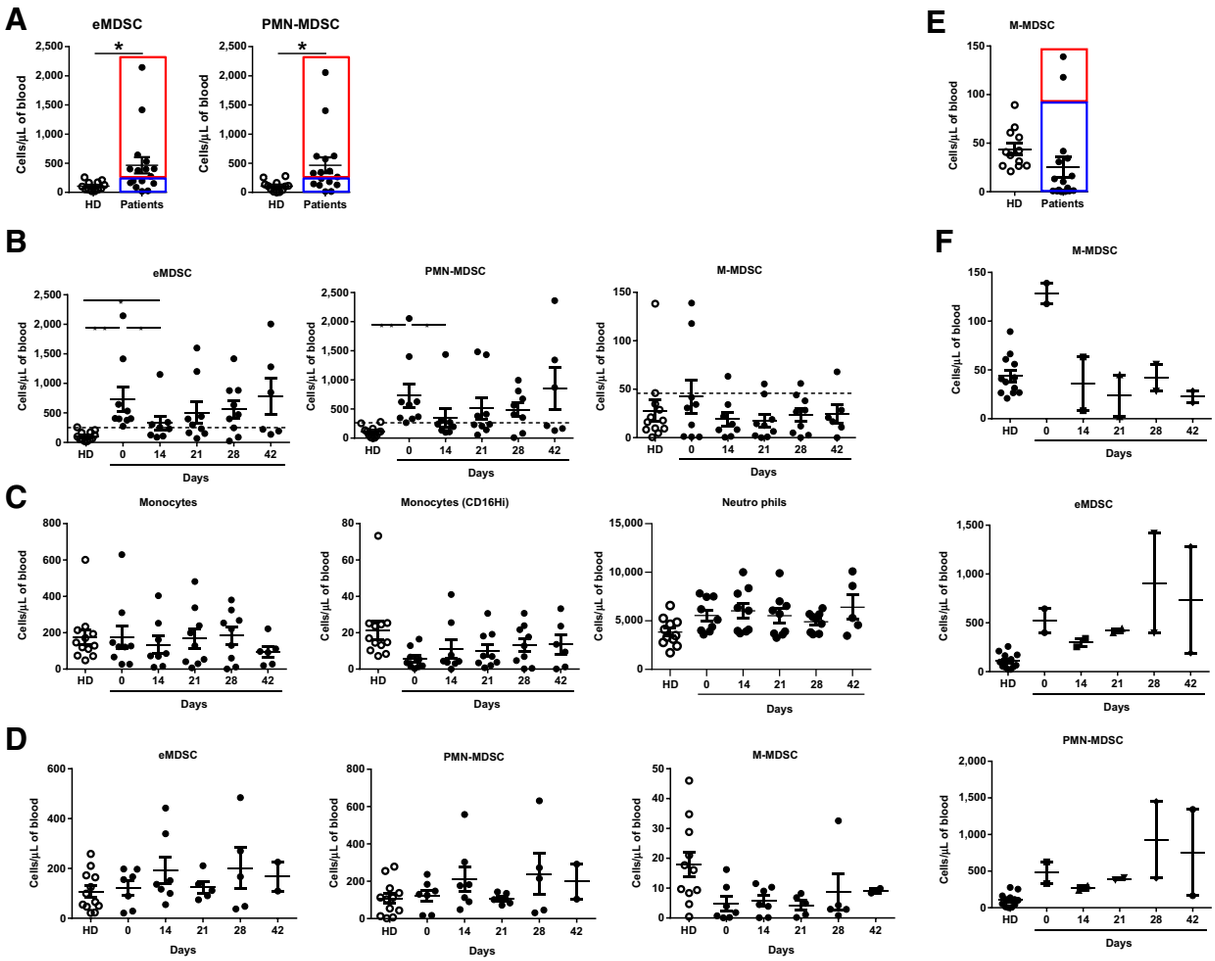

Figure 3.

The effect of DS-8273a on populations of MDSCs. **A,** Selection of patients with elevated and control level of eMDSCs and PMN-MDSCs; **B,** The number of different population of MDSCs during the treatment in patients with elevated pretreatment level of PMN-MDSCs; **C,** The number of monocytes and neutrophils during the treatment in patients with elevated pretreatment level of PMN-MDSCs; **D,** The number of MDSCs in patients with control pretreatment level of MDSCs. **E,** Selection of patients with elevated and control level of M-MDSCs; **F,** The number of different population of MDSCs during the treatment in patients with elevated pretreatment level of M-MDSCs. In this figure, *, $P < 0.05$ and **, $P < 0.01$ from values in healthy donors (HD).

treatment (Fig. 3F). Thus, DS-8273a specifically target elevated population of MDSCs without affecting other myeloid cells and lymphocytes.

Depletion of MDSCs in tumor tissue and association with clinical outcome

Tumor biopsies were collected in 10 patients enrolled to the dose expansion cohort. Six of the patients had paired pre- and posttreatment (day 28) biopsies. We evaluated the populations of $CD163^+$ macrophages, elastase positive neutrophils, putative ($CD33^+S100A9^+$) MDSCs (22) as well as Foxp3-positive regulatory T cells, $CD4^+$, $CD8^+$, and $CD8^+PD1^+$ T cells by immunofluorescence staining and IHC (Fig. 4A; Supplementary Fig. S6). Prior to start of the treatment, no correlations between the presence of various populations of myeloid cells and T cells were observed. Three of 6 patients demonstrated a decrease in intratumoral MDSCs, whereas one patient had decreased macrophages and neutrophils (Fig. 4B). In those 3 patients who had a decrease in MDSC number within the tumor, the ratios of $CD8^+$:MDSC and $CD4^+$:MDSC, as well as proportion of $PD1^+$ cells among $CD8^+$ T cells:MDSC were increased, whereas in patients with unchanged levels of MDSCs, the ratios were either unchanged or decreased (Fig. 4C), indicating that the MDSC decrease was not a reflection of the overall changes in the immune cell infiltration of the tumor and was specific for those cells.

Because we have implicated TRAIL-R expression on MDSC as being associated with their sensitivity to agonist antibody, we evaluated the expression of three TRAIL-Rs (DR5, DcR1, DcR2) in PMN-MDSCs during the treatment. No changes were observed during the treatment in the expression of DR5 and DcR1 (Fig. 4D). However, expression of DcR2 was significantly lower on day 14 ($P = 0.03$) with a trend to an increase on days 21 and 28, which follows the pattern of PMN-MDSC changes. Pretreatment level of DcR2 as well as other TRAIL-Rs did not correlate with subsequent changes in PMN-MDSC numbers.

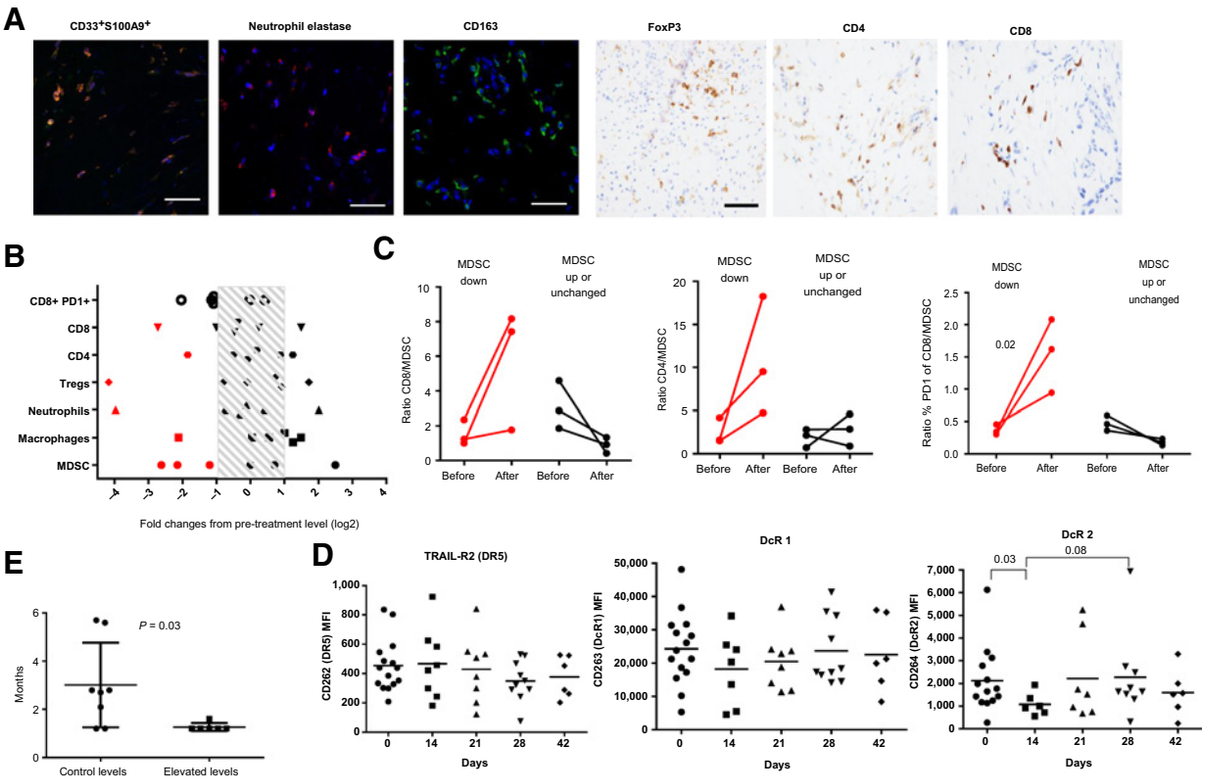

Figure 4.

Effect of the treatment with DS-8273a on tumor MDSC. **A,** Typical examples of staining of tissues. $CD33^+S100A9^+$—immune fluorescent staining (red, S100A9; green, CD33; blue, DAPI). Neutrophil elastase, immunofluorescent staining (red, elastase; blue, DAPI). CD163, immunofluorescent staining (green, CD163; blue, DAPI). FoxP3, CD4, CD8, $PD1^+CD8^+$, immunohistochemical staining as described in Materials and Methods. **B,** Changes in the presence of population of myeloid and lymphoid cells in tumor tissues after the treatment with DS-8273a. **C,** Changes in CD8/MDSCs, CD4/MDSCs, and proportion of $PD1^+$ out of $CD8^+$ cells/MDSC ratio after the treatment. **D,** Expression of TRAIL receptors in PMN-MDSCs during the treatment. *P* values of significant differences from healthy donors control are shown. **E,** TTP (months) in patients treated with DS-8273a. Patients on day 28 after start of the treatment were split into two groups: control and elevated number of PMN-MDSCs. The control values were established on the basis of the results in healthy donors shown in Fig. 3. *P* values were calculated in two-way *t* test.

There were no objective clinical responses (CR and PR) in DS-8273a-treated patients on this trial. We assessed whether the changes in PMN-MDSC during the treatment (ratio between PMN-MDSC numbers during and prior to the treatment) correlated with the time to progression (TTP) or duration of progression-free survival (PFS). Both TTP and PFS were the same in the subjects in whom the MDSCs were analyzed. Changes in PMN-MDSC on day 14 were not associated with PFS. However, on day 21, changes in PMN-MDSC demonstrated a weak but significant inverse correlation with the TTP. This correlation became stronger on days 28 and 42 (Table 2). These results indicate that a decrease in MDSCs during DS-8273a treatment was associated with longer TTP in these patients. As described above, the populations of PMN-MDSCs increased in some patients on day 28 of the treatment so we asked whether those changes were associated with clinical outcome. We calculated the TTP for patients who retained control levels of PMN-MDSCs by day 28 as well as for those who had elevated levels of these cells, and patients who retained control levels of MDSCs had a significantly longer TTP than the patients who had elevated levels of PMN-MDSCs (Fig. 4E).

Discussion

We report here the first successful attempt to selectively eliminate MDSCs in cancer patients. This study is based on previous observations that MDSCs in mice and cancer patients are more sensitive to TRAIL-R2 agonistic antibody or TRAIL than their normal counterparts (15). In mice, this effect was mediated by upregulation of TRAIL-R2, whereas in cancer patients it was mediated mostly by a decrease in DcR2. Targeting of TRAIL-R in mice caused depletion of MDSCs and substantially improved the antitumor effect of a CTLA4-blocking antibody (15). These results suggested that targeting TRAIL-R2 could be potentially useful in targeting MDSCs in humans. The use of TRAIL-R2 agonist antibodies in cancer patients has been previously reported and demonstrated a very good safety profile (23, 24). However, the

Table 2. Correlation between changes in PMN-MDSCs and TTP

	Day 14	Day 21	Day 28	Day 42
Spearman *R*	−0.05	−0.51	−0.64	−0.98
P two-tailed	0.76	0.077	0.024	<0.0001
P one-tailed		0.039		
Number of pairs	11	11	11	7

antitumor activity of this antibody as monotherapy was limited (25). Tigatuzumab in combination with gemcitabine led to eight partial responses in pancreatic cancer patients ($n = 61$) and demonstrated no anticancer activity in lung cancer patients ($n = 91$), both being phase II clinical trials (26, 27). Another TRAIL-R2–targeting antibody, conatumab, was also tested in a randomized phase II clinical trial in combination with chemotherapy and did not demonstrate objective clinical response in patients with colorectal, pancreatic, soft tissue sarcoma, or lung cancer (28–31). Heterogeneous expression of the DR5 receptor on tumor cells, poor penetration of the antibody to solid tumors, and/or the relatively low affinity of the antibody could explain the low clinical efficacy of the treatment. We suggested that TRAIL-R2 antibody could have an effect independent of its ability to directly target solid tumors, by eliminating MDSCs in patients with high levels of these cells suppressing host antitumor immunity.

We observed increases in PMN-MDSCs and eMDSCs in nine of 16 patients (56%). This is somewhat lower than we and others have previously reported (32, 33). Overnight shipment of samples before analysis could contribute to this phenomenon. However, we believe that shipment probably played a relatively minor role due to the fact that control samples were also stored overnight and that in previous studies shipment of samples did not have a significant effect on the function of MDSCs. Other more likely explanation is that the patients in this trial were heavily pretreated with chemotherapy prior to enrollment to the study in addition to being heterogeneous in their tumor types. This may also explain the low number of $CD4^+$ cells, B cells, and NK cells in these patients. Increase of M-MDSCs in only two patients was not surprising as M-MDSC are largely increased in patients with melanoma, multiple myeloma and to some extent prostate cancer, which were very poorly represented in this trial.

The main finding of this study is that DS-8273a caused rapid elimination of MDSCs in patients with elevated levels of these cells at baseline without affecting any other cell populations. It also did not affect MDSCs in patients with control levels of the cells. These results describe the first example of highly selective elimination of MDSCs in patients. The effect was observed until the end of the first cycle (day 21) and was associated with a decrease in DcR2 expression. However, the second cycle of treatment failed to prolong this effect in all patients. A number of patients had elevated number of PMN-MDSCs by day 28 (day 7 of cycle 2) and was associated with an increase in DcR2 expression. Elevated levels of PMN-MDSCs at day 28 were associated with a shorter time to disease progression. Overall, changes in PMN-MDSCs and eMDSCs inversely correlated with clinical outcome supporting the role of these cells in regulation of tumor progression. No direct conclusions could be drawn from these results because of heterogeneous patient population and relatively small number of patients; however, it suggests further that a more detailed study is warranted.

Why did DS-8273a treatment fail to control MDSC levels longer? There are two possible explanations of this phenomenon. Treatment with DS-8273a can cause a compensatory increase in DcR2 expression which could prevent further action of the antibody. However, this explanation is less likely due to the fact that relative upregulation of DcR2 in MDSCs by day 28 just returned it to pretreatment level. That level did not prevent effective elimination of these cells during first cycle of treatment. The most likely explanation is that in the patients with highly advanced cancer elimination of MDSCs by itself was not sufficient to control tumor progression. This phenomenon was previously demonstrated in numerous studies in mice (5). Rapid tumor progression that was observed in the patients on this trial might have resulted in increased production of MDSCs which the antibody was unable to control. The fact that patients with control levels of MDSCs on day 28 had significantly longer TTP than patient with elevated levels of MDSCs support this explanation.

The presented data demonstrate that DS-8273a maintained selective depletion of MDSC for at least 28 days, which may provide a sufficient window of therapeutic activity in combination with immunotherapies using adoptive T cells transfer, or PD-1 antibody. This data provided the first demonstration of selective elimination of MDSCs in patients with advanced cancers that was associated with prolonged TTP. Our observations open an opportunity for the clinical combination of TRAIL-R2–targeting antibody with various immunotherapeutic strategies.

Disclosure of Potential Conflicts of Interest

A. Hashimoto is an employee of Daiichi Sankyo Co., Ltd. D. Gabrilovich reports receiving commercial research grants from Anixa, Biothera, Bristol-Myers Squibb, Daiichi, Galera, Janssen, Peregrin, Reata, and Syndax, and is a consultant/advisory board member for Janssen, Peregrin, and Syndax. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: G.A. Dominguez, T. Condamine, P. Kumar, D.I. Gabrilovich

Development of methodology: G.A. Dominguez, T. Condamine, F. Wang, D.I. Gabrilovich

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G.A. Dominguez, T. Condamine, S. Mony, A. Hashimoto, F. Wang, A. Forero, J. Bendell, R. Witt, N. Hockstein

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.A. Dominguez, T. Condamine, S. Mony, A. Hashimoto, F. Wang, Q. Liu, J. Bendell, D.I. Gabrilovich

Writing, review, and/or revision of the manuscript: G.A. Dominguez, T. Condamine, A. Forero, J. Bendell, N. Hockstein, P. Kumar, D.I. Gabrilovich

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G.A. Dominguez

Study supervision: A. Forero, P. Kumar, D.I. Gabrilovich

Grant Support

This work was supported by Daiichi Sankyo, Inc. and NIH grant CA084488.

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Received July 14, 2016; revised November 11, 2016; accepted December 2, 2016; published OnlineFirst December 13, 2016.

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