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# Mitigating the prevalence and function of myeloid-derived suppressor cells by redirecting myeloid differentiation using a novel immune modulator

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## ABSTRACT

**Background** Immune suppression is common in neoplasia and a major driver is tumor-induced myeloid dysfunction. Yet, overcoming such myeloid cell defects remains an untapped strategy to reverse suppression and improve host defense. Exposure of bone marrow progenitors to heightened levels of myeloid growth factors in cancer or following certain systemic treatments promote abnormal myelopoiesis characterized by the production of myeloid-derived suppressor cells (MDSCs) and a deficiency in antigen-presenting cell function. We previously showed that a novel immune modulator, termed 'very small size particle' (VSSP), attenuates MDSC function in tumor-bearing mice, which was accompanied by an increase in dendritic cells (DCs) suggesting that VSSP exhibits myeloid differentiating properties. Therefore, here, we addressed two unresolved aspects of the mechanism of action of this unique immunomodulatory agent: (1) does VSSP alter myelopoiesis in the bone marrow to redirect MDSC differentiation toward a monocyte/macrophage or DC fate? and (2) does VSSP mitigate the frequency and suppressive function of human tumor-induced MDSCs?

**Methods** To address the first question, we first used a murine model of granulocyte-colony stimulating factor-driven emergency myelopoiesis following chemotherapy-induced myeloablation, which skews myeloid output toward MDSCs, especially the polymorphonuclear (PMN)-MDSC subset. Following VSSP treatment, progenitors and their myeloid progeny were analyzed by immunophenotyping and MDSC function was evaluated by suppression assays. To strengthen rigor, we validated our findings in tumor-bearing mouse models. To address the second question, we conducted a clinical trial in patients with metastatic renal cell carcinoma, wherein 15 patients were treated with VSSP. Endpoints in this study included safety and impact on PMN-MDSC frequency and function.

**Results** We demonstrated that VSSP diminished PMN-MDSCs by shunting granulocyte-monocyte progenitor differentiation toward monocytes/macrophages and DCs with heightened expression of the myeloid-dependent transcription factors interferon regulatory factor-8 and PU.1. This skewing was at the expense of expansion of granulocytic progenitors and rendered the remaining MDSCs less suppressive. Importantly, these effects were also demonstrated in a clinical setting wherein VSSP

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Alterations in myeloid differentiation can lead to immune suppression through the formation of myeloid-derived suppressor cells (MDSCs), which compromise antitumor immunity. One approach to overcome these myeloid defects to enhance therapeutic efficacy has been to deplete such myeloid populations. An alternative underinvestigated concept to cell depletion, which may cause fewer undesirable effects, is to restore myeloid differentiation where MDSCs originate. Our previous work provided the rationale for this study, which showed that a novel innate immune modulator, known as VSSP ('very small size particle') can render MDSCs less suppressive; however, where, and how VSSP did so remained unknown, as well as its potential utility in patients with cancer.

### WHAT THIS STUDY ADDS

⇒ Our findings revealed that VSSP treatment in preclinical models redirected myeloid progenitor fate in the bone marrow from MDSCs to monocytes and dendritic cells (DCs). The ability of VSSP to limit MDSC formation was supported in a phase 0 clinical trial of patients with renal cell carcinoma treated with this single agent. VSSP reduced circulating MDSC levels, which correlated with increased levels of monocytes and DCs and overall survival in this pilot study.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our translational findings support the potential use of VSSP in clinical settings where myeloid dysfunction, including MDSC production is associated with disease progression.

monotherapy significantly reduced circulating PMN-MDSCs, and their suppressive function.

**Conclusions** Altogether, these data revealed VSSP as a novel regulator of myeloid biology that mitigates MDSCs in cancer patients and reinstates a more normal myeloid phenotype that potentially favors immune activation over immune suppression.

## INTRODUCTION

Myelopoiesis during steady-state preserves progenitor pools in the bone marrow (BM) and ensures that the resultant mature myeloid populations home to tissues critical for host defense, repair, and remodeling. Oligopotent granulocyte-monocyte progenitors (GMPs) represent a common early cell type in the BM that can differentiate into either monocytic progenitors (MPs) or granulocytic progenitors (GPs), antecedents to monocytes or neutrophils, respectively.[1] The commitment to a monocytic or granulocytic lineage is tightly regulated by distinct growth and transcription factors. Interferon regulatory factor-8 (IRF8) and PU.1 represent two key transcription factors that are important for proper myeloid differentiation and myeloid lineage specification. IRF8 also regulates differentiation and function across the myelomonocytic lineage, including the generation of dendritic cells (DCs) and other antigen-presenting cells (APCs).[2]

During states of 'emergency' caused by an infection, cancer or chemotherapy-induced leukopenia, myelopoiesis rate and output are adjusted.[3] Such a response is mediated by hyperexposure to growth factors and promotes the expansion of populations that are critical to resolve inflammatory or pathologic insults.[4 5] The transition from physiologic to acute or chronic inflammatory states may also skew myeloid differentiation resulting in functionally impaired monocytes, macrophages or DCs, as well as the pathologic accumulation of myeloid-derived suppressor cells (MDSCs).[6 7] Notably, increased IRF8 expression can blunt this process by reducing MDSC levels, particularly the polymorphonuclear (PMN)-MDSC subset, and promoting monocyte/DC differentiation.[8]

Since it is well recognized that MDSCs or defective myeloid function suppresses antitumor immunity,[6] approaches designed to restore myeloid competence may improve antitumor responses. The rationale to target progenitors in the BM to reverse such myeloid defects is supported by the notion that MDSCs arise from early myeloid progenitors and that BM-derived MDSCs in turn represent a continuous pipeline of immune suppressors to the tumor microenvironment (TME), where MDSCs maximally exert their protumor effects.[8] In addition to the neoplastic process itself, conventional anti-neoplastic agents, including some chemotherapeutics, or supportive care agents, including granulocyte-colony stimulating factor (G-CSF), may contribute to aberrant myelopoiesis in the BM and MDSC expansion.[9] Therefore, maintaining the benefits of these interventions while, at the same time, mitigating their negative influences on myeloid cell biology could improve their therapeutic value.

Previously, we showed that an immune modulator, known as VSSP ('very small sized particle'), can restore immune reactivity through its ability to abrogate MDSC function.[10–12] This novel agent consists of the GM3 ganglioside incorporated into the outer membrane vesicles of *Neisseria meningitidis*[13] that drives DC activation, antigen (Ag) cross-presentation and cytotoxic T lymphocyte induction. Currently, VSSP is being evaluated as a single-agent therapy and as an adjuvant in vaccine platforms for the treatment of advanced cancers,[14–18] including clinical studies presented here in renal cell carcinoma (RCC). Interestingly, in mouse tumor models, VSSP induces an expansion of splenic $CD11b^{+}Gr\text{-}1^{+}$ cells, a canonical phenotype associated with MDSCs. These MDSC-like cells exhibit an increased capacity to differentiate into professional APCs, concomitant with markedly reduced immune suppressive function.[10 11 19 20] This effect of VSSP has also been documented in an experimental model of myeloablation induced by cyclophosphamide (CY). In this context, VSSP accelerates the replenishment of specific leukocytes populations, particularly $CD4^{+}$ T cells, $CD8^{+}$ T cells and $CD11c^{+}$ DCs and also dampens the immune suppressive function of the $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells produced in response to CY-induced leukopenia.[12]

These findings led us to determine if VSSP rescued such myeloid defects in progenitors within the BM, as well as the identity of the progenitor populations that were likely targeted. We posit that agents that restore normal myelopoiesis may have important implications as a therapeutic option as they would hinder the subsequent repopulation of MDSCs or redirect the balance of immune suppressive to immune-activating myeloid cell types at points of origin. To test this hypothesis, we first employed a mouse model of emergency myelopoiesis mediated by G-CSF following CY-induced myeloablation. G-CSF is a myelopoietic cytokine that regulates both steady-state and emergency myelopoiesis[21] and promotes hematopoietic recovery following chemotherapy-induced leukopenia.[22] As a growth factor elevated in certain cancers or as an exogenous therapeutic intervention, G-CSF also shunts myeloid differentiation toward GPs and the formation of PMN-MDSCs, a predominant MDSC subset found in numerous pathologic settings.[8 23] In addition, we strengthened our hypothesis in a tumor-bearing mouse model using the G-CSF-producing 4T1 cell line, which has been associated with the expansion of GPs and the subsequent accumulation of PMN-MDSCs.[8]

Our studies revealed that VSSP mitigated aberrant myelopoiesis induced by G-CSF. VSSP redirected GMP differentiation toward an immune-activating myeloid lineage phenotype. Resultant monocytes, macrophages, and DCs displayed enhanced APC capacity reflected by an increased expression of IRF8 and PU.1. Altogether, our data demonstrated that VSSP is a novel agent, which can 'reprogram' BM-resident myeloid cell output characterized by increased IRF8, PU.1, reduced MDSC levels and function, and a heightened or a more mature myeloid fate, as measured by an enhanced production

of monocyte/macrophages and DCs. Notably, the effects of VSSP on BM progenitors, MDSC differentiation and function were validated in tumor settings. Moreover, the impact of VSSP on MDSCs was also shown in a clinical setting wherein single-agent VSSP significantly lessened circulating PMN-MDSCs and their suppressive function in patients with RCC. Therefore, VSSP has potential therapeutic merit in pathologic settings where myeloid dysfunction, including MDSC accumulation, is associated with disease pathogenesis or progression.

## MATERIALS AND METHODS

### Antibodies and reagents

VSSP[13] and rhG-CSF (G-CSF; LeukoCIM)[24] were produced at the Center of Molecular Immunology (CIM, Havana, Cuba). Concanavalin A (Con A) from *Canavalia ensiformes* and CY were purchased from Sigma-Aldrich (St. Louis, MO); stem cell factor (SCF) and IL-3 were obtained from PeproTech (Rocky Hill, NJ). Functional-grade purified anti-mouse TLR2 (CD282) or TLR4/MD-2 antibodies (Abs) were purchased from eBioscience (San Diego, USA). The antibodies used for flow cytometry are summarized in online supplemental table S1.

### Mice

Female 8 to 12 weeks of age C57BL/6 (H-2$^b$) and BALB/c (H-2$^d$), mice were purchased from the Center for Laboratory Animal Production (Havana, Cuba) or from Charles River Laboratories (Frederick, MD). All mice were maintained in the animal facilities of the CIM or the Roswell Park Comprehensive Cancer Center (Buffalo, NY, USA). All experiments were performed and approved in compliance with Institutional Guidelines and Regulations of the CIM under protocols Res 01/2000 and Res 54/2021 and at Roswell Park by the Institutional Animal Care and Use Committee under protocols 1108M and 1117M.

### Cell lines

The MCA203 sarcoma cell line was derived from C57BL/6 mice (H-2$^b$) and the CT26 colon carcinoma cell line was derived from BALB/c mice (H-2$^d$). The C26GM cell line was obtained from the C26 colon carcinoma (H-2$^d$) genetically modified to release GM-CSF[25] and was propagated in the presence of 0.8 mg/mL of G418 (Invitrogen, Milan, Italy). These cell lines were kindly provided by Dr. Vincenzo Bronte. The 4T1 (H-2$^d$) mammary tumor cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and all cell lines were maintained, as described.[26]

### Flow cytometry

Cell populations were evaluated by flow cytometry using the various Abs listed in online supplemental table S1. Cells were acquired using a FACScan, LSRII or Fortessa B flow cytometers (BD Biosciences, San Jose, CA) and analyzed using FlowJo version 10 (Tree Star, Ashland, OR) and Kaluza 1.2 (Beckman Coulter, Indianapolis, IN) software. FoxP3 Staining Buffer Set (Miltenyi Biotec, BergischGladbach, Germany) was used for intracellular staining for IRF8 and PU.1. Human myeloid DCs (mDCs) were measured using the Blood DC enumeration kit (Miltenyi Biotec).

### Model of emergency myelopoiesis

Leukopenia was induced using CY (200 mg/kg; i.p.). Two days after CY, C57BL/6 mice were injected daily for 2 days with G-CSF (300 µg/kg; s.c). To evaluate the effects of VSSP, groups of mice received one dose of VSSP subcutaneously (200 µg/mouse) either alone or in combination with G-CSF. Control groups received only CY or phosphate-buffered saline (PBS). Mouse BM were collected 4 days after CY administration, and BM cells obtained as described.[8] Progenitor populations were evaluated by flow cytometry using Abs listed in online supplemental table S1 and based on the followed immunophenotypes as defined previously[8]: total GMPs (Lin$^-$Sca-1$^-$c-Kit$^+$CD16/32$^+$CD150$^-$), megakaryocyte-erythroid (MegE) progenitors (Lin$^-$Sca-1$^-$c-Kit$^+$CD16/32$^{lo/-}$), oligopotent GMPs (Lin$^-$Sca-1$^-$c-Kit$^+$CD16/32$^+$CD150$^-$Ly6C$^-$CD115$^{lo/-}$), GPs (Lin$^-$Sca-1$^-$c-Kit$^+$CD16/32$^+$CD150$^-$Ly6C$^+$CD115$^{lo/-}$), and MPs (Lin$^-$Sca-1$^-$c-Kit$^+$CD16/32$^+$CD150$^-$Ly6C$^+$CD115$^+$).

To evaluate the effects of VSSP under conditions of tumor-induced aberrant myelopoiesis, female BALB/c mice were implanted orthotopically with 4T1 cells ($5\times10^4$) and treated with one dose of VSSP (100 µg) when tumors first became measurable (day 5; tumor volume ~0.5 mm$^3$) or when tumor volume reached ~60 mm$^3$ (day 11). Two days later (day 7 or 13, respectively), BM cells were collected, and GPs and MPs were analyzed by flow cytometry.

### In vitro differentiation of BM progenitors

BM cells from CY-treated mice were collected 2 days after CY administration. Lineage positive cells were depleted, as described.[27] Progenitor populations were immunostained as described above and sorted (≥90% purity) using the FACSAria cell sorter (BD Biosciences) and incubated with SCF (50 ng/mL), IL-3 (10 ng/mL), G-CSF (10 ng/mL) or VSSP (10 µg/mL) or G-CSF plus VSSP. Four days later, phenotypes for macrophages (CD11b$^+$F4/80$^+$MHC-II$^+$), DCs (CD11b$^+$CD11c$^+$MHC-II$^+$), monocytes (CD11b$^+$Ly6C$^{hi}$Ly6G$^-$), granulocytes (CD11b$^+$Ly6C$^{lo}$ Ly6G$^+$) and the intracellular expression of IRF8 and PU.1 were analyzed by flow cytometry.

### In vitro differentiation of human monocytes to DCs and allogeneic T cell stimulation assay

Monocytes were obtained from peripheral blood mononuclear cells (PBMCs) that were isolated by Ficoll-Hypaque gradient centrifugation of peripheral blood from healthy donors. PBMCs from healthy donors were obtained after informed consent through IRB-approved protocol I-188310 via the Data Bank and Biorepository at Roswell Park.[28] Monocytes were isolated by negative magnetic-bead selection (StemCell Technology) and cultured

at a density of $5\times10^5$ cells/mL in RPMI 1640 medium containing 10% fetal calf serum (FCS, HyClone), 100 mM L-glutamine (MediaTech), 100 U/mL of penicillin and 100 µg/mL streptomycin (MediaTech). VSSP (10 µg/mL) was added on day 0 along with IL-4 (1000 U/mL; R&D Systems), followed with or without by tumor-conditioned media (TCM) on day one. To stimulate monocyte differentiation into DCs, 8 days later GM-CSF (5 ng/mL; Sigma-Aldrich) was added and the cultures were maintained for 11 days. At day 19, cells were used for allogeneic T cell stimulation assays, as previously described.[29] Briefly, such monocyte-derived DCs were cultured with allogeneic purified $CD3^+$ T cells from normal donors at a DC to T cell ratio of 1:10 for 3 days in 96-well plates. During the final 14 to 18 hours of culture, $^3$H-thymidine (Perkin-Elmer) was added at a concentration of 20 µCi/mL. Cells were then harvested onto a Filtermate Harvester (Perkin-Elmer) and $^3$H-thymidine incorporation was measured using a MicroBeta Trilux (Perkin -Elmer).

For the preparation of TCM, MDA-MB-231 cells from the ATCC were cultured at $2\times10^5$ cells/mL in IMDM, containing 10% FCS, L-glutamine, and penicillin and streptomycin, as above. Cells grew for 3 days, supernatant fluid was collected after centrifugation, and then passed through a 0.2 µm filter to remove any remaining cells or cellular debris. Samples were either frozen at −80°C or added immediately to the culture.

### Induction of MDSCs, in vitro differentiation to APCs, and murine T cell proliferation assays

To evaluate G-CSF-mediated induction of MDSCs and the effects of VSSP in this system, C57BL/6 mice were treated with CY and 2 days later injected with VSSP and/or four daily doses of G-CSF. Splenocytes were isolated 7 days after CY administration and splenic $CD11b^+$ cells were magnetically isolated using CD11b microbeads (Miltenyi Biotec). Phenotype was confirmed by flow cytometry and then the cells were tested for their ability to suppress T cell proliferation. For the differentiation assay, $1\times10^6$ $CD11b^+$ cells from CY±G-CSF-treated mice were further cultured in vitro with VSSP (10 µg/mL) in 6-well plates (BD Falcon, Oxford, UK). Twenty-four hours later, phenotypes were evaluated by flow cytometry.

To assess the in vivo effects of VSSP on tumor-induced MDSCs, female BALB/c mice were implanted s.c. on day 0 with C26GM ($5\times10^5$ cells) or orthotopically with 4T1 ($5\times10^4$ cells). VSSP (100 µg/mouse) was administered s.c. on days 1, 2, and 7. In a third tumor model, MCA203 sarcoma cells ($1\times10^5$ cells) from were inoculated s.c. in female C57BL/6 mice and VSSP (200 µg/mouse; s.c) administered on days 11, 12, and 18. PBS-injected tumor-bearing mice were included as controls. Two days (C26GM and 4T1 models), and 4 days (MCA203 model) after the last VSSP inoculation, splenic $CD11b^+$ cells were isolated, as described earlier as a source of MDSCs, and their ability to inhibit T cell proliferation was measured. To determine the contributions of TLR signaling to the modulatory properties of VSSP, $CD11b^+$ cells were magnetically isolated from the spleens of 4T1-bearing mice on day 11 and cultured in vitro with VSSP (10 µg/mL) in presence (or absence) of anti-TLR2, anti-TLR4 or both Abs (each at 10 µg/mL). Twenty-four hours later, their ability to inhibit T cell proliferation was evaluated by flow-based assays.

For T cell proliferation assays, purified splenic $CD11b^+$ cells or those treated in vitro with VSSP were co-cultured at 20% or 10% of total cells with $3\times10^6$ splenocytes recovered from naïve C57BL/6 or BALB/c mice pre-stained with 2 µM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes; Paisley, UK) in 96-well flat bottom plates (BD Falcon, Oxford, UK). Splenocytes were stimulated with Con A (2 µg/mL) for 96 hours, and proliferation was determined by CFSE dilution.

### Study design for the treatment of patients with metastatic RCC

The clinical trial (PL009) was an open physician-led study approved by the CIM Clinical Steering Committee and the Ethics Committee of the Joaquin Albarrán Hospital under Resolution Number VADI N° 4/2000. Before enrolment, an informed written consent was obtained from every patient. Fifteen enrolled patients had been histologically confirmed for metastatic RCC (mRCC) (predominantly clear cell histology). Most of the patients had metastases or disease recurrence and no further treatment/surgical options. VSSP was administered subcutaneously in two phases. In the Induction Phase, weekly injections (400 µg) were given from day 0 to day 21. The Maintenance Phase began on day 51, with injections (400 µg) on days 51, 81, 111, and 141. Blood samples were collected at inclusion (baseline) and on days 21, 58 and 147 (online supplemental figure S1) for the analysis of PMN-MDSC, monocyte and mDC frequency and the suppressive capacity using $CD11b^+$ preparations.

### Human PMN-MDSC and APC frequency evaluation and T cell proliferation assay

PBMCs were obtained from patient blood samples and 18 healthy volunteer donors. Blood (20 mL) was drawn into sodium heparin collection tubes, diluted threefold with PBS and centrifuged to 1800 rpm for 15 min over Ficoll-Hypaque gradients (Ficoll-Hypaque; GE Biosciences). To measure PMN-MDSC frequency, freshly isolated PBMCs were stained for the expression of CD11b, CD66b and CD14 and defined as $CD11b^+CD14^-CD66b^+$. As controls, 18 healthy volunteers were evaluated and percentages of phenotypically similar populations were considered those under the upper 95% CI of controls mean. Total percentages of DC1 ($CD19^-CD14^-CD303^-CD1c^+$) and DC2 ($CD19^-CD14^-CD303^-CD141^+$) subsets were determined, per manufacturer's recommendations, for the assessment of mDC frequency. For monocytes, the $CD11b^+CD14^+$HLA-$DR^+$ population was analyzed.

For the suppression assay, half of the PBMCs was CD11b-depleted ($CD11b^-$ fraction; ≤1% of remaining $CD11b^+$ cells) using CD11b microbeads. The remaining PBMCs

were not depleted ($CD11b^+$ fraction). Both fractions were stained with 4 µM of CFSE (Molecular Probes) and $2\times10^5$ cells/well were activated with 1 µg/mL of immobilized anti-CD3 antibody (eBioscience) in 96-flat bottom plates. After 96 hours, proliferation was determined by flow cytometry within the $CD8^+$ T cell population. CFSE distribution was analyzed as precursor frequency (PF) or percentage of dividing cells, as defined by the fraction of the original population that had divided at least once during the culture period and was calculated as:

$$PF = \left(\sum_{1}^{i} N^i/2^i\right) \Big/ \left(\sum_{0}^{i} N^i/2^i\right)$$

where i: generation number (0 is the undivided population); Ni: number of events in generation i.[30]

### Statistical analysis

Statistical analysis was performed using SPSS V.16.0 software (SPSS), as well as GraphPad Prism (V.7.04). Equality of variances was analyzed with Bartlett's test and Kolmogorov-Smirnov test was used to verify normal distribution of data. Statistical differences were performed with one-way analysis of variance and the Tukey's test for pairwise comparisons. The Kruskal-Wallis and Dunn non-parametric tests were performed as appropriate. The means from two independent samples were compared using the unpaired t-test (two tailed). For the patient studies, MDSC levels were log10-transformed before survival analysis. The association of MDSCs with patient overall survival (OS) was investigated using Cox proportional hazards models with transformed MDSCs as continuous independent variables. The HRs were reported with 95% CIs and two-sided Wald tests were used to determine statistical significance. The association was considered statistically significant with $p<0.05$. Due to the small sample size, we showed the estimated survival functions corresponding to representative MDSC values, which were selected based on MDSC distribution. The MDSC distribution was characterized by its kernel densities estimated using the Gaussian kernel with the default bandwidth by R density function. The analyses were performed using R V.4.1.2.A $p<0.05$ was considered statistically significant and identified with * or ** when $p<0.05$ or 0.01, respectively.

## RESULTS

### VSSP reestablishes myeloid cell frequencies in the BM during emergency myelopoiesis induced by G-CSF

To address the impact of VSSP on myeloid progenitor fate, we utilized a model of emergency myelopoiesis induced by exogenous G-CSF following CY-driven myeloablation. After exposure to CY, mice were treated with or without G-CSF and/or VSSP (figure 1A). As expected, CY administration alone induced myeloablation, based on reduction in BM counts, that was partially recovered 1 day after the last G-CSF administration (figure 1B). In this context, VSSP had no significant effect on restoring BM counts in the absence or presence of G-CSF.

However, this did not preclude the possibility that VSSP affected the composition or fate of the different myeloid progenitors. Therefore, to further analyze the impact of VSSP in the BM during emergency myelopoiesis, we performed comprehensive flow cytometric analysis on distinct myeloid progenitor populations. First, we broadly assessed total GMPs (which includes oligopotent GMPs and unipotent GPs and MPs) and MegE progenitors (29). While G-CSF-induced recovery resulted in an increase in total GMPs, this effect was antagonized by cotreatment with VSSP (figure 1C). Treatment with VSSP in the absence or presence of G-CSF also led to a decrease in MegE progenitors (figure 1C).

To determine the myeloid cell populations that contributed to the changes in the GMP compartment, we then analyzed individual subpopulations, namely: oligopotent GMPs, GPs, and MPs (online supplemental figure S2). We observed that the addition of G-CSF to CY-treated mice induced an increase in oligopotent GMPs and GPs compared with CY-treated or vehicle-treated mice (figure 1D). Consistent with the results on total GMPs, VSSP did not alter the levels of oligopotent GMPs or GPs compared with mice treated with or without CY. Rather, VSSP reduced oligopotent GMP and GP expansion that resulted from G-CSF-mediated recovery (figure 1D). No significant differences in MP numbers were observed with any of the treatments (figure 1D), suggesting preferential effects on reducing myeloid progenitor differentiation toward the granulocyte lineage.

### VSSP redirects G-CSF-driven myelopoiesis toward monocytic cell differentiation

To determine how treatment altered the progeny of the progenitors, we next evaluated GMP differentiation bias into granulocytes or monocytes induced during emergency myelopoiesis. Oligopotent GMPs, GPs, and MPs were sorted from the BM of CY-treated mice and cultured in vitro with the supportive factors SCF and IL-3. To test whether VSSP could alter differentiation, we treated progenitors with G-CSF, VSSP, or G-CSF plus VSSP. After 96 hours, we quantified the percentages of monocytes or granulocytes, defined by the expression profile of $CD11b^+Ly6C^{hi}Ly6G^-$ or $CD11b^+Ly6C^{lo}Ly6G^+$, respectively (online supplemental figure S3). When oligopotent GMPs were cultured with G-CSF, we observed a significant reduction of monocytes and an increase in granulocytes (figure 2A) compared with the controls. In contrast, we found that VSSP treatment induced higher percentages of monocytes from oligopotent GMPs compared with the controls but had no significant effect on the percentages of granulocytes (figure 2A). The combination of G-CSF and VSSP resulted in an intermediate level of monocyte and granulocyte production compared with single-agent treatments (figure 2A). When differentiation from lineage committed MPs and GPs were examined, VSSP treatment increased the proportion of the resulting

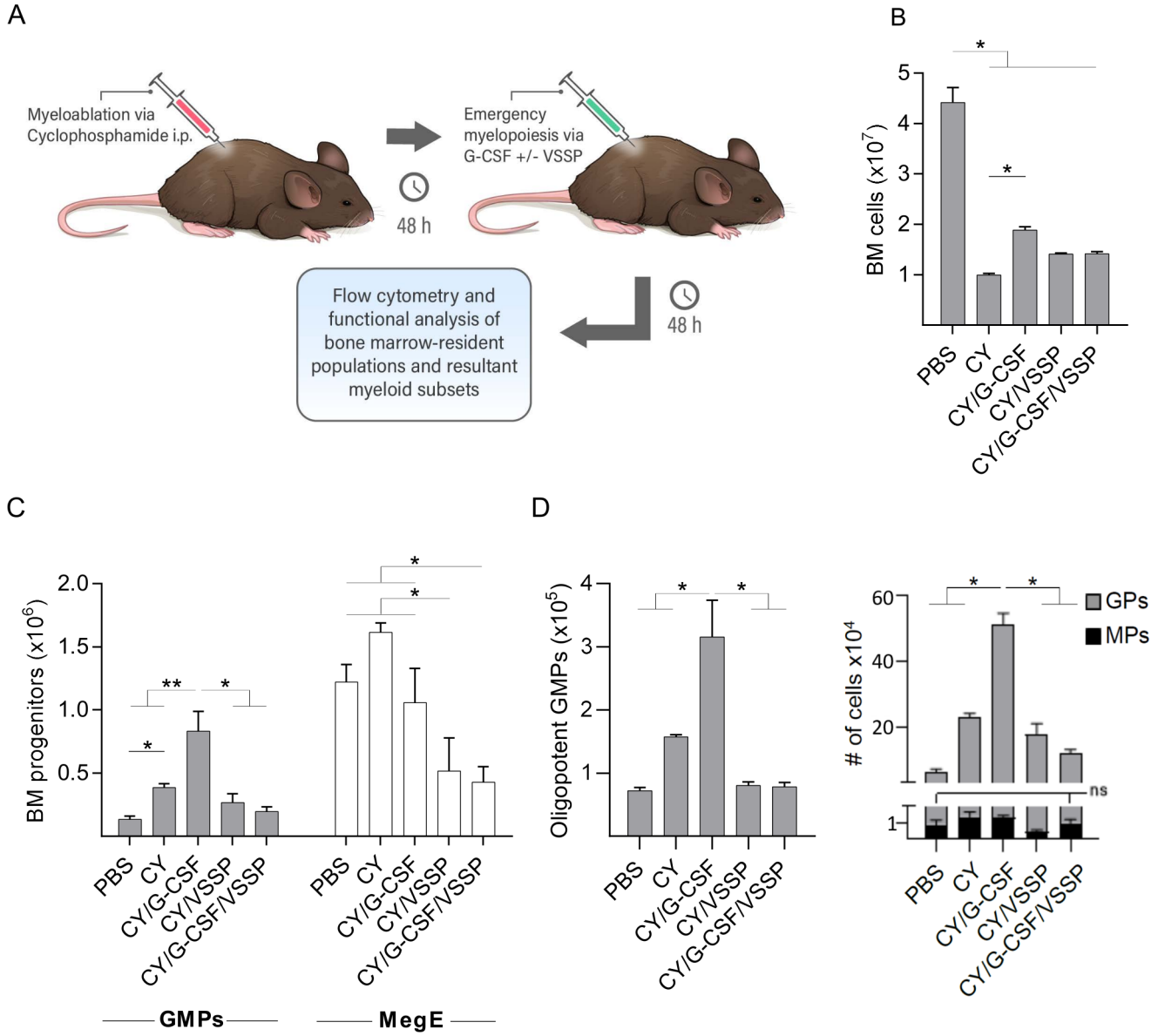


**Figure 1** VSSP restores myeloid cell frequencies following leukopenia. C57BL/6 mice were injected with CY (200 mg/kg body weight, i.p.) and with two consecutive daily doses of G-CSF (300 µg/kg of body weight, s.c.), one dose of VSSP (200 µg, s.c.), or both VSSP and G-CSF starting 2 days after CY treatment (A). Total BM cells (B) and GMP and MegE (C) were determined by cell count and flow analysis of the BM respectively, 4 days after CY administration (n=3). Levels of oligopotent GMPs, GPs and MPs were also determined (D). Data represent the mean of three individual mice±SEM. Results shown are representative of two independent experiments. Asterisks above bars indicate significant differences (* or ** when p<0.05 or 0.01, respectively). Statistical analyses were performed with the Tukey test. BM, bone marrow; G-CSF, granulocyte-colony stimulating factor; GMP, granulocyte-monocyte progenitor; GP, granulocytic progenitor; MPs, monocytic progenitor; VSSP, very small size particle.

monocytic levels and concomitantly decreased the granulocytic levels (figure 2B,C). Under these conditions, G-CSF showed no significant effect on monocyte or granulocyte outcome (figure 2B,C). Interestingly, the combination of G-CSF and VSSP was comparable to VSSP alone (figure 2B,C).

Considering the preferential effect of VSSP toward monocytic commitment, we further examined the differentiation potential of myeloid progenitors into macrophages and total DCs, as defined by the coexpression of CD11b and MHC class II with F4/80 or CD11c, respectively (online supplemental figure S3). Using the same experimental design, we observed a small, but significant increase in the percentages of macrophages and DCs from oligopotent GMPs relative to G-CSF-treated or vehicle-treated controls (figure 2D). Adding G-CSF to VSSP reduced the effect of VSSP, although not significantly, on macrophage or DC production from these progenitors (figure 2D). However, treatment with VSSP alone or in combination with G-CSF led to increased macrophage or DC production from MPs (figure 2E). Additionally, the expression of CD11c and F4/80 (calculated as mean fluorescence intensity (MFI)) on MP-derived macrophages and DCs, respectively, was also enhanced

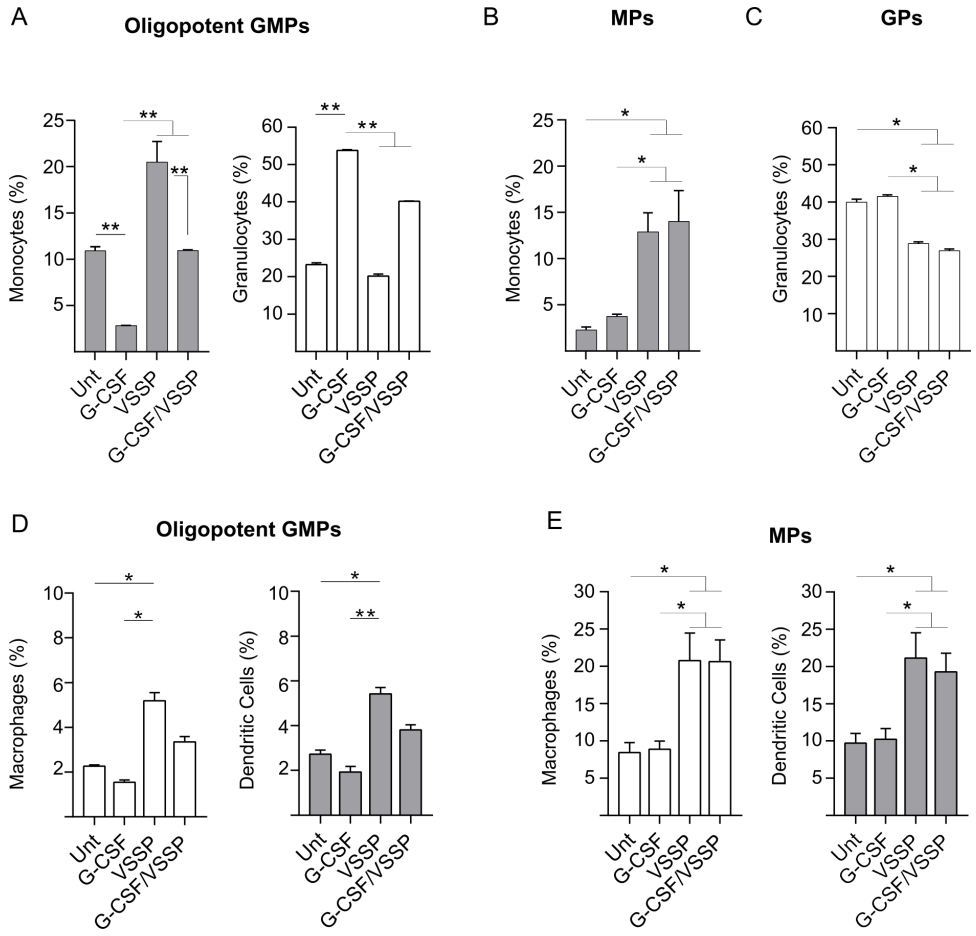


**Figure 2** VSSP promotes progenitor commitment to monocyte and APC differentiation. Oligopotent GMPs (A), MPs (B) and GPs (C) were sorted from the BM of CY-treated mice (200 mg/kg body weight, i.p.) on day 2 (≥90% purity) and cultured in vitro with SCF (50 ng/mL), IL-3 (10 ng/mL), with or without G-CSF (10 ng/mL); or VSSP (10 µg/mL); or both VSSP and G-CSF for 4 days. Frequencies of monocytes (CD11b$^+$Ly6C$^{hi}$Ly6G$^-$) (gray bars) and granulocytic cells (CD11b$^+$Ly6C$^{lo}$Ly6G$^+$) (white bars) were determined after treatment by flow analysis. Oligopotent GMPs (D) and MPs (E) were also cultured in the same above conditions and frequencies of macrophages (CD11b$^+$F4/80$^+$MHC-II$^+$) (white bars) and DCs (CD11b$^+$CD11c$^+$MHC-II$^+$) (gray bars) for each subset (ie, oligopotent GMPs or MPs) are shown. Data represent the mean±SEM from multiple replicates of one of two representative experiments. Asterisks above bars indicate significant differences (* or ** when p<0.05 or 0.01, respectively). Statistical analyses were performed with the Tukey test. APCs, antigen-presenting cells; BM, bone marrow; DCs, dendritic cells; G-CSF, granulocyte-colony stimulating factor; GMPs, granulocyte-monocyte progenitors; GPs, granulocytic progenitors; MPs, monocytic progenitors; SCF, stem cell factor; VSSP, very small size particle.

(online supplemental figures S4A and B). Altogether, these results strengthen the notion that VSSP even when combined with G-CSF can bias differentiation/maturation of progenitors toward the monocytic/DC lineage.

### The frequency of APCs expressing IRF8 and PU.1 increases after treatment with VSSP

The transcription factor IRF8 is required for monopoiesis including the development of monocytes, macrophages and DCs, while it constrains the production of granulocytes/neutrophils. In addition, PU.1 is a common binding partner of IRF8 that forms enhancers on genes required for monocyte lineage commitment and maturation.[6] Considering that VSSP favored monocytic over granulocytic commitment, we examined the expression of both IRF8 and PU.1 in progeny resulting from oligopotent GMPs and MPs cultured in vitro with the different stimuli from CY-treated mice (online supplemental figure S3).

In this setting, VSSP induced a significant increase in IRF8 and PU.1 expression in monocytes, macrophages, and DCs differentiated from oligopotent GMPs (figure 3A). Interestingly, treatment with G-CSF reduced the levels of double-positive IRF8$^+$PU.1$^+$ cells. G-CSF in combination with VSSP also reduced the frequency of IRF8$^+$PU.1$^+$ monocytes, macrophages and DCs compared with VSSP treatment alone (figure 3A). We next examined the expression of IRF8 and PU.1 in monocytes, macrophages, and DCs derived from MP cultures. VSSP induced a significant increase in IRF8$^+$PU.1$^+$ monocytes, macrophages and DCs compared with either untreated or G-CSF culture conditions (figure 3B). G-CSF did not increase the expression of these transcription factors on

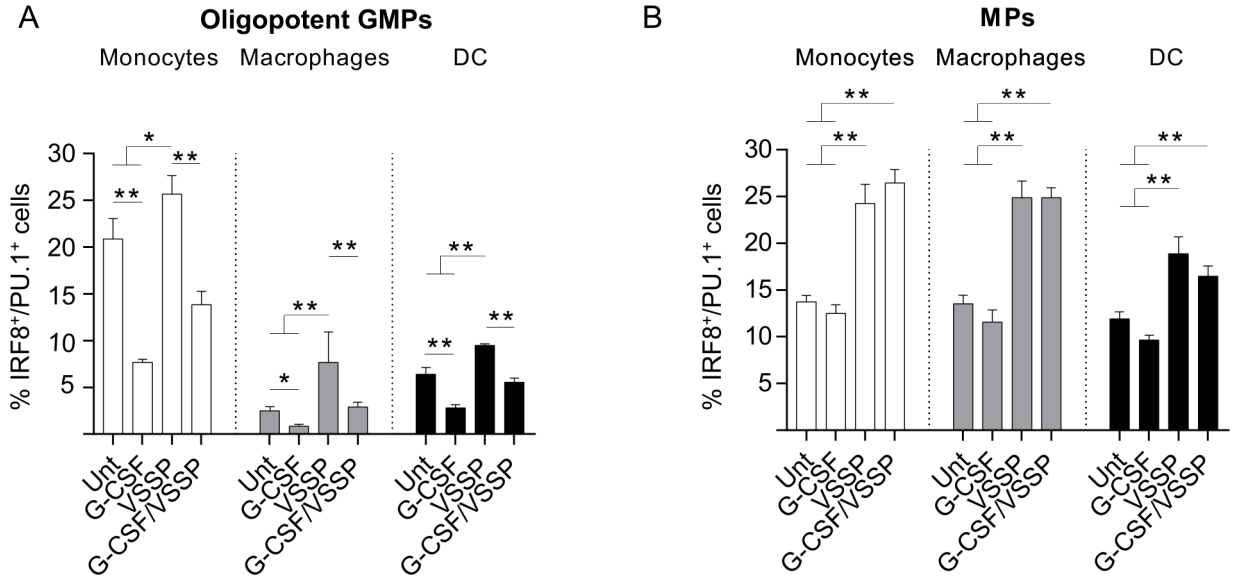


**Figure 3** VSSP enhances the expression of IRF8 and PU.1 on APC differentiated from GMP populations during emergency myelopoiesis. Oligopotent GMPs (A) and MPs (B) were sorted from the BM and treated in vitro as described. Percentage of monocytes (CD11b$^+$Ly6C$^{hi}$Ly6G$^-$) (white bars), macrophages (CD11b$^+$F4/80$^+$MHC-II$^+$) (gray bars) and DCs (CD11b$^+$CD11c$^+$MHC-II$^+$) (black bars) double positive for IRF8 and PU.1 were measured after the indicated treatments by flow analysis. Frequencies for each subset (ie, oligopotent GMPs or MPs) are shown. Data represent the mean±SEM from multiple replicates of one of two representative experiments. Asterisks above bars indicate significant differences (* or ** when p<0.05 or 0.01, respectively). Statistical analyses were performed with the Tukey's test. APC, antigen-presenting cell; BM, bone marrow; DCs, dendritic cells; G-CSF, granulocyte-colony stimulating factor; GMPs, granulocyte-monocyte progenitors; IRF8, interferon regulatory factor-8; MPs, monocytic progenitors; VSSP, very small size particle.

myeloid populations derived from MP cultures relative to the controls (figure 3B). We also analyzed the expression of IRF8 alone and showed that VSSP upregulated IRF8 on all myeloid populations resulting from each progenitor. VSSP also induced a significant increase in IRF8 on monocytes, macrophages, and DCs when differentiated from oligopotent GMPs or MPs even in combination with G-CSF (online supplemental figures S5A and B).

## VSSP dampens the suppressive function of MDSCs generated during emergency myelopoiesis

G-CSF facilitates the myeloid progenitor imbalance and the subsequent production of PMN-MDSCs by mechanisms involving downmodulation of IRF8.[8] VSSP dampens tumor-induced MDSC function[10–12] and our results also show that this agent antagonizes the effect of G-CSF on myeloid progenitors and enhances IRF8 expression. Therefore, we decided to evaluate the effect of VSSP on G-CSF-induced MDSCs in vivo (figure 4). Mice were treated with CY and G-CSF with or without VSSP. MDSC suppressive function is thought to be highest in the periphery outside of the BM. Therefore, to address the suppressive function of MDSCs within the periphery, splenic CD11b$^+$ cells were isolated 7 days after CY exposure (purity of CD11b$^+$Gr-1$^+$ cells > 98%). To assess function, the isolated CD11b$^+$ cells were mixed with Con A-stimulated CFSE prestained lymphocytes (from a naïve syngeneic mouse) and proliferation was determined by dye dilution via flow cytometry as shown on online supplemental figure S6. As expected, MDSCs induced during G-CSF-driven recovery of leukopenia (MDSC$^{CY/G\text{-}CSF}$) suppressed Con A-mediated lymphocyte proliferation, comparable to that of MDSCs from CY-treated mice (MDSC$^{CY}$) (figure 4A). Importantly, when mice were additionally treated with VSSP, the isolated CD11b$^+$ cells (MDSC$^{CY/G\text{-}CSF/VSSP}$) had a significantly reduced suppressive activity compared with MDSC$^{CY}$ and MDSC$^{CY/G\text{-}CSF}$ (figure 4A).

Next, we cultured the splenic CD11b$^+$ cells from CY- or CY/G-CSF-treated mice with VSSP in vitro and analyzed differentiation toward DCs. After 24 hours of culture, some CD11c$^+$ cells were present, particularly when CD11b$^+$ cells were isolated from CY-treated mice (figure 4B). However, adding VSSP to the culture system increased the percentage of cells expressing CD11c from both CY- and CY/G-CSF-treated groups. Moreover, incubation with VSSP increased the percentage and MFI of CD40 on the resulting CD11c$^+$ cells, suggesting a more immune-stimulatory phenotype (figure 4B, lower panel). Functional testing of these cells revealed that VSSP significantly reduced the suppressive activity of MDSC$^{CY/G\text{-}CSF}$ (2.75±1.3% of inhibition) compared with MDSC$^{CY/G\text{-}CSF}$ alone (23.5±0.93% of inhibition) (figure 4C and online supplemental figure S6).

## VSSP modifies BM progenitors and dampens MDSC suppressive function in models of tumor-induced aberrant myelopoiesis

The influence of VSSP in rescuing myeloid defects caused by tumor-induced aberrant myelopoiesis was analyzed by administering one dose of VSSP to 4T1-bearing mice. This

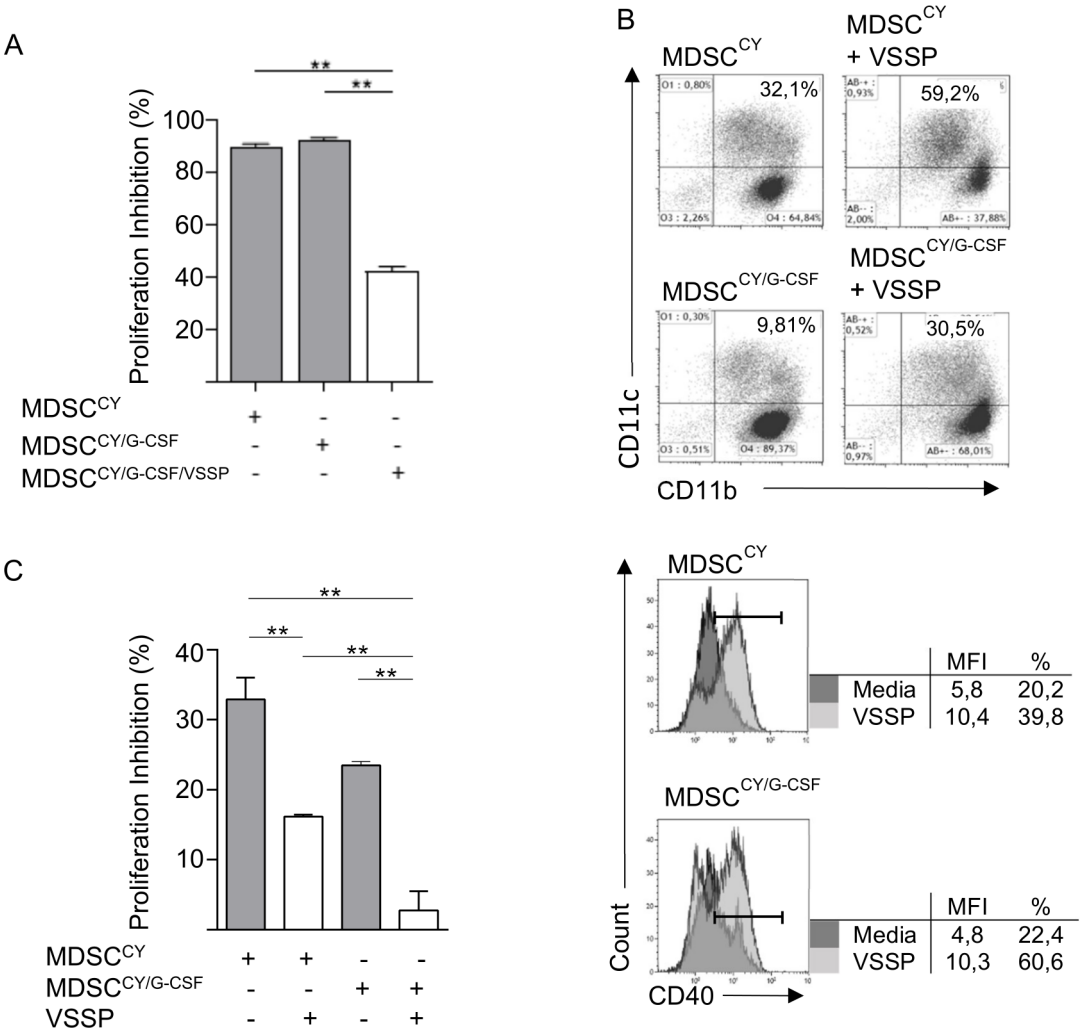


**Figure 4** VSSP renders MDSCs with reduced suppressive capacity and promotes differentiation toward DCs. CY-induced leukopenic mice (n=3) were treated with G-CSF (300 µg/kg of body weight, s.c.), and VSSP (200 µg, s.c.) starting 2 days after CY (200 mg/kg body weight, i.p.). Splenocytes were isolated 5 days later and the suppressive phenotype of the isolated CD11b$^+$ cells was analyzed. Naive splenocytes were stimulated with Con A for 96 hours and inhibition of T cell proliferation by the presence of 20% CD11b$^+$ purified from each treatment group was assessed by dye dilution (A). Data are representative of two experiments. CD11b$^+$ from mice treated with CY and CY and G-CSF were incubated 24 hours in vitro with or without VSSP (10 µg/mL) or media alone and the percentages of differentiated DCs and expression of CD40 were evaluated by flow cytometry within CD11b$^+$ populations. Tables show MFI and percentage of CD40$^+$ within DC population (B). Splenocytes from naive mice were stimulated with Con A and inhibition of T cell proliferation was assessed in cultures with CD11b$^+$ cells (10% of the culture) treated in vitro with or without VSSP (C). Asterisks above bars indicate significant differences)** when p<0.01). DC, dendritic cell; G-CSF, granulocyte-colony stimulating factor; MDSCs, myeloid-derived suppressor cells; MFI, mean fluorescence intensity; VSSP, mean fluorescence intensity.

tumor model secretes G-CSF, expands GPs in the BM, and induces the expansion of MDSCs, mainly PMN-MDSCs.[8] We found that VSSP inhibited tumor-induced GP expansion when VSSP was given once mice had palpable tumors (figure 5A). VSSP significantly increased the level of MPs when administered to mice with larger tumors and normalized the GP/MP ratio in both settings compared with naïve or healthy mice (figure 5A).

To assess the ability of VSSP to differentiate myeloid progenitors into APCs in the presence of tumor-derived factors (TDFs), human monocyte-derived immature DCs were cultured with VSSP in the presence of TCM, and their ability to stimulate T-lymphocyte proliferation was evaluated. Interestingly, cells differentiated from monocytes in the presence of VSSP had an increased ability to stimulate lymphocyte proliferation, even in the absence of GM-CSF in the culture media. Notably, when cultured in TCM, only the addition of VSSP promoted differentiation of DC-like cells with the ability to stimulate lymphocyte proliferation (figure 5B). The in vivo effects of VSSP on the suppressive function of tumor-induced MDSCs was next evaluated in mice bearing 4T1, C26GM, or MCA203 tumors. Consistent with our previous studies, splenic MDSCs derived from tumor-bearing mice treated with multiple doses of VSSP had less suppressive activity, compared with untreated tumor-induced splenic MDSCs (figure 5C,D).

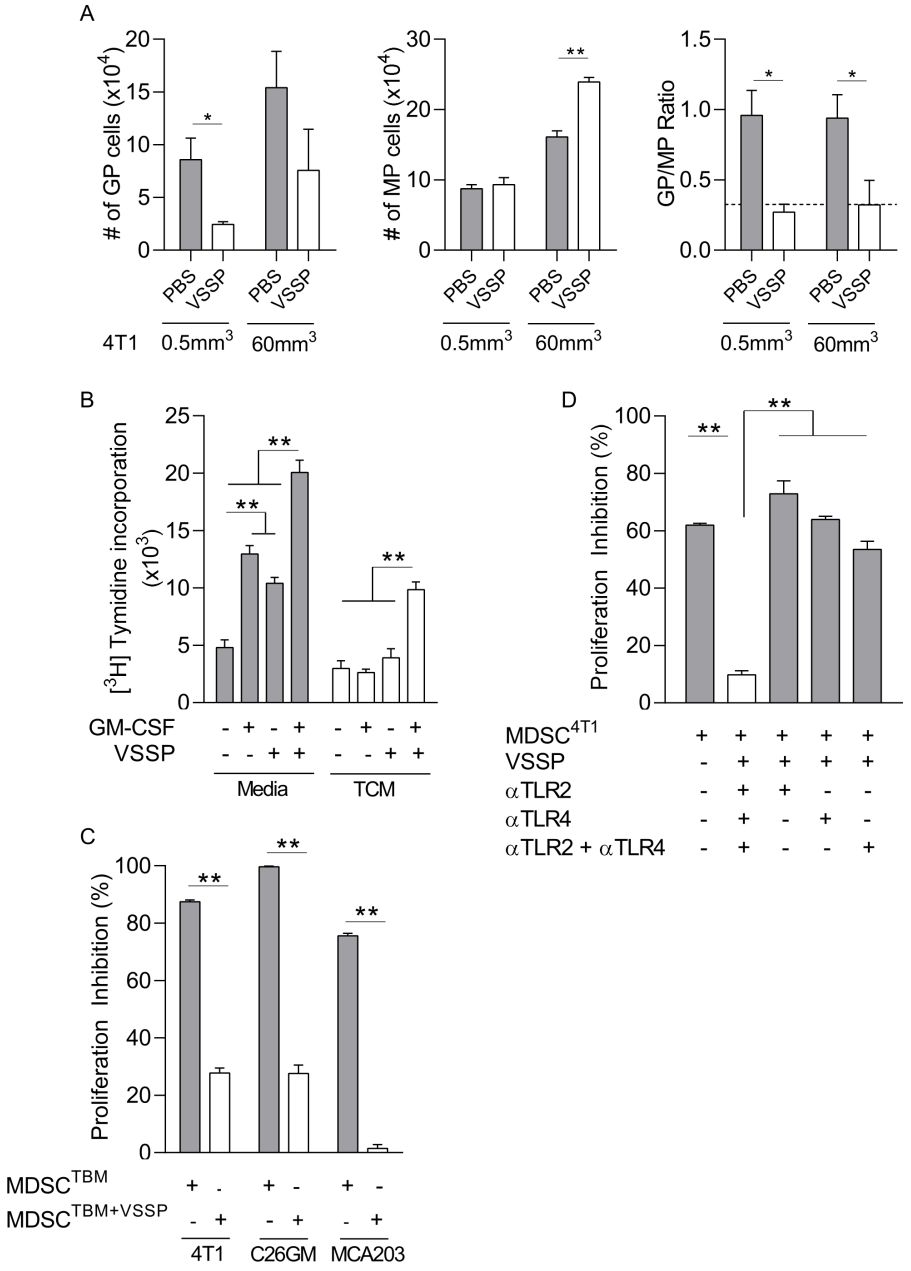


**Figure 5** VSSP modifies BM progenitors and dampens MDSC suppressive function in tumor settings. BALB/c mice were implanted s.c. with 4T1 tumor cells ($5\times10^4$ cells) on day 0 and subsequently received one dose of VSSP (100 µg, s.c.) when tumors were palpable or reached volumes of ~60 $mm^3$. Total GPs and MPs were determined by flow analysis of the BM 2 days after VSSP administration (day 7 or 13) (A). Dashed line represents GP/MP ratio of healthy (naïve) mice. Data represent the mean of three individual mice±SEM. Statistical comparisons between groups were performed with a Student's t test. Human purified monocytes were cultured to differentiate into DCs in the presence of GM-CSF, IL4, TCM and VSSP as described in the Methods. Proliferation of T cells was performed using an allogenic mixed lymphocyte reaction assay and quantified by [$^3$H]-thymidine incorporation (B). Results shown are representative of two independent experiments. Statistical analyses were performed using the Tukey test. MCA203, 4T1, and C26GM tumor cells were injected s.c. into mice, which were then treated with or without three doses of VSSP. Splenic CD11b$^+$ cells were isolated 2 days (C26GM) and 4 days (4T1 and MCA203 tumors) after the last VSSP dose. Naïve splenocytes were then stimulated with Con A for 96 hours and inhibition of T cell proliferation was analyzed in the presence of purified CD11b$^+$ cells (20% of culture), as assessed by dye dilution (C). Data are representative of two experiments. Data represent the mean of three individual mice±SEM. Statistical analyses were performed using the Student's t-test. Splenic purified CD11b$^+$ cells from 4T1-bearing mice were incubated 24 hours in vitro with or without VSSP (10 µg/mL) and anti-TLR2, TLR4 or both antibodies (10 µg/mL). Inhibition of T cell proliferation was assessed by culture of CD11b$^+$ with splenocytes from naïve mice in the presence of Con A (D). Data represent the mean±SEM from multiple replicates of one of two representative experiments. Asterisks above bars indicate significant differences (* or ** when $p<0.05$ or 0.01, respectively). Statistical analyses were performed using the Tukey test. BM, bone marrow; DC, dendritic cells; GM-CSF, granulocyte-colony stimulating factor; GPs, granulocytic progenitors; MDSC, myeloid-derived suppressor cell; MPs, monocytic progenitors; VSSP, very small size particle.

Considering the presence of TLR2 and 4 ligands in VSSP and the high expression of these TLRs in GMPs, we opted to assess the influence of these receptors on the capacity of VSSP to differentiate tumor-induced MDSCs into APCs or less suppressive cells. As shown in figure 5D, the capacity of tumor-derived MDSCs to inhibit T cell proliferation was abrogated when previously cultured with VSSP, and this effect was reversed by the addition of blocking Abs specific for TLR2, 4 or the combination (figure 5D).

### MRCC patients treated with VSSP have reduced frequencies of PMN-MDSCs

Our data show that VSSP biased myelopoiesis toward monocytes, promoted APC differentiation and reduced G-CSF-induced myelopoiesis and MDSC functionality. These results provided the rationale to implement a clinical study to evaluate the effect of VSSP on PMN-MDSC burden and function. Given that patients with advanced mRCC are characterized by a high peripheral blood PMN-MDSC burden,[31–33] we designed a phase 0 clinical trial at the 'Joaquin Albarrán' Hospital in Havana, Cuba in patients with this pathology.[31–33] Fifteen mRCC patients were treated with VSSP as a monotherapy. Endpoints included safety and the impact of VSSP on modulating PMN-MDSC frequency and function (see schedule in online supplemental figure S1). Patient demographic and clinico-pathologic features are shown in table 1. To evaluate PMN-MDSC frequencies, the $CD11b^{+}CD66b^{+}CD14^{-}$ phenotype was analyzed by flow cytometry on freshly isolated PBMCs (online supplemental figure S7). Our data show significantly higher percentages of PMN-MDSCs compared with healthy donors at baseline (figure 6A). However, after 3 weekly doses of VSSP, we observed a marked decrease in the percentage of PMN-MDSCs in treated patients (figure 6A and online supplemental figure S7).

A more detailed analysis showed that at baseline 80% of enrolled patients had PMN-MDSC levels above normal values (figure 6B). Importantly, this percentage was reduced to 47% after three doses of VSSP. In addition, by day 21, more than 80% of the VSSP-treated patients had circulating PMN-MDSC levels lower than the median value calculated at baseline (figure 6B). Although not statistically different, there was also a trend for PMN-MDSC levels to remain low at days 58 and 147 post-VSSP treatment, compared with baseline values (figure 6A). Interestingly, these two evaluations were performed after changing the treatment frequency from weekly to monthly. Considering the impact of VSSP in APC differentiation, we evaluated the percentages of monocytes and DCs in 5 of the enrolled patients. We found increased percentages of monocytes and DCs following VSSP treatment in four of the five evaluated patients (figure 6C).

As shown in table 1, this trial also corroborates previous results related to the safety of VSSP.[9] In this case, we observed that VSSP treatment was well-tolerated at the dose of 400 µg and following the administration schedule described above. Considering that VSSP was well-tolerated, seven patients received a second cycle of treatment commencing between 13 and 33 months after terminating the maintenance phase (online supplemental figure S1). Notably, by day X+21, VSSP reduced PMN-MDSC levels (online supplemental figure S8A), and all patients showed values below the starting median (online supplemental figure S8B).

In addition to frequency and phenotype evaluation, we also examined whether VSSP treatment reduced

**Table 1** Demographic and baseline characteristics of the mRCC patients and treatment related adverse events

| | N (%) | | N (%) |
|---|---|---|---|
| Age, median (range) | 60 (41–78) | Site of Metastasis | |
| | | Lung | 3 (27.2) |
| Gender | | Contralateral kidney | 4 (36.3) |
| Male | 8 (53.3) | Pancreas | 1 (9.09) |
| Female | 7 (46.7) | CNS | 2 (18.1) |
| | | Ovary | 1 (9.09) |
| Race | | | |
| White | 9 (60.0) | Previous nephrectomy | |
| Afro-Caribbean | 5 (33.3) | Yes | 12 (80.0) |
| Other | 1 (6.70) | No | 3 (20.0) |
| Pathology | | Therapy before study entry | |
| Clear cell carcinoma | 15 (100) | Cytokine (IFN-γ) | 3 (27.2) |
| | | RT 50 Gy | 1 (6.70) |
| ECOG PSa | | | |
| 0 | 11 (73.3) | Adverse events‡ | Total (35/100) |
| 1 | 2 (13.3) | Pain at injection site | 11 (31.4) |
| 2 | 2 (13.3) | Asthenia | 2 (5.71) |
| | | Cough | 2 (5.71) |
| Disease stage | | Anemia | 2 (5.71) |
| II | 1 (6.70) | Vomiting | 1 (2.86) |
| III | 2 (13.3) | Fatigue | 1 (2.86) |
| IV | 12 (80.0) | Hypertension | 1 (2.86) |
| | | Local erythema | 1 (2.86) |
| MSKCC risk group† | | Low-back pain | 2 (5.71) |
| Favorable (0 factors) | 5 (33.3) | Lower limb pain | 1 (2.86) |
| Intermediate (1–2 factors) | 7 (46.7) | Elevated ALP | 3 (8.57) |
| Poor (3 or more factors) | 3 (27.2) | Elevated GGT | 3 (8.57) |
| | | Elevated creatinine | 2 (5.71) |
| Metastasis | | Elevated LDH | 2 (5.71) |
| Yes | 11 (73.3) | Elevated eosinophils | 1 (2.86) |
| No | 4 (26.7) | | |

*ECOG indicates (n=15 patients).
†The MSKCC prognostic risk groups was derived using five risk factors: ECOG ≤2 (Karnosfky performance score ≤60), lactate dehydrogenase level (>1.5 times upper limit of normal), hemoglobin level g/L (<lower limit of normal), ≥2 sites of organ metastasis, and time interval from diagnosis to treatment (<1 year).
‡All adverse events were classified as mild or moderate (grade 1 or 2).
ALP, Alkaline phosphatase; CNS, Central Nervous System; ECOG, Eastern Cooperative Oncology Group; GGT, Gamma-glutamyl transferase; LDH, Lactate dehydrogenase; mRCC, metastatic renal cell carcinoma; MSKCC, Memorial Sloan Kettering Cancer Center.

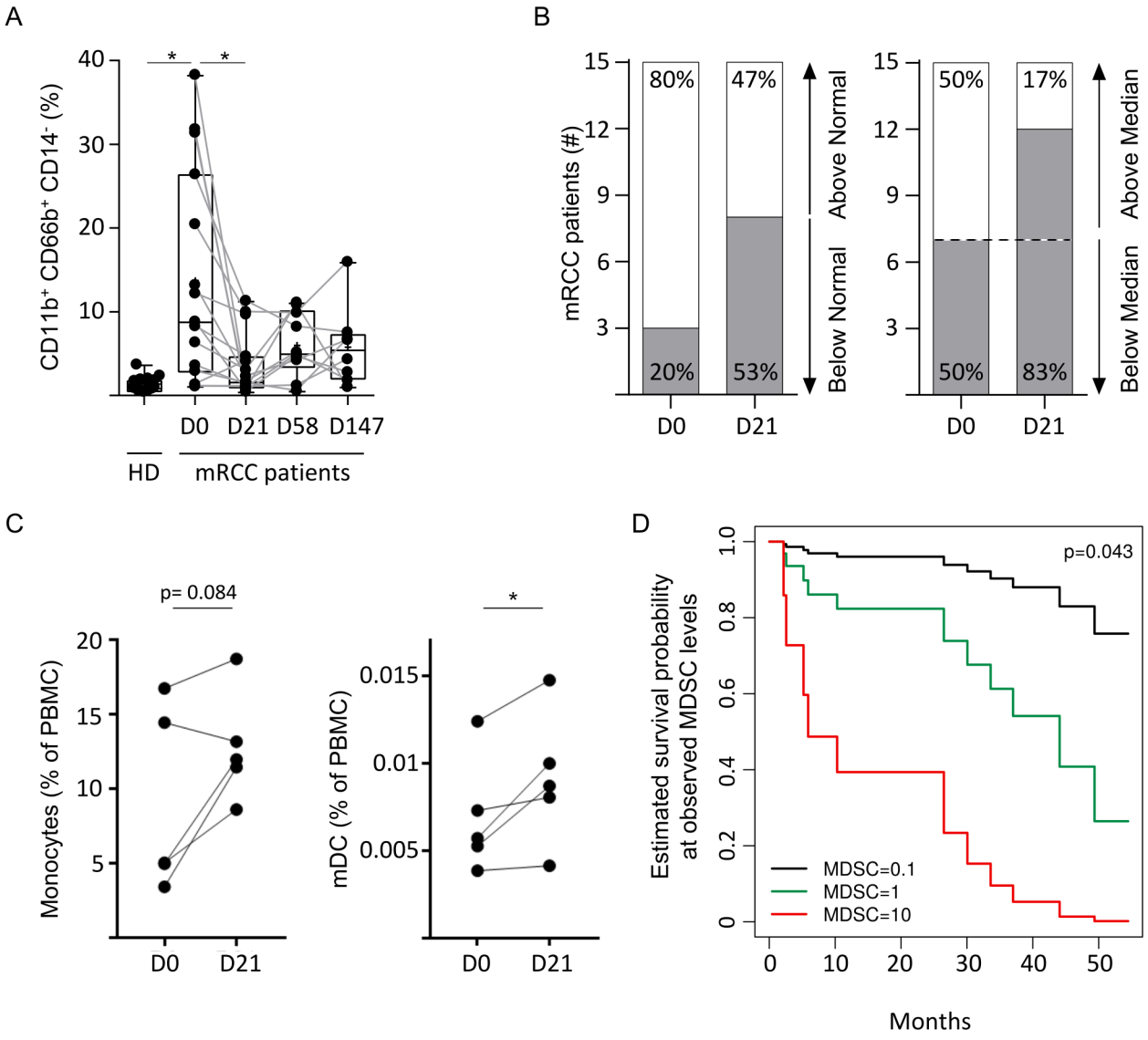


**Figure 6** Metastatic RCC patients treated with VSSP have reduced frequency of circulating PMN-MDSCs. Freshly isolated PBMCs of mRCC patients and healthy donors (HD) were analyzed by flow cytometry to determine the frequency of PMN-MDSCs ($CD11b^+CD66b^+CD14^-$). Statistical analyses were performed via Kruskal-Wallis and Dunn's tests (*p<0.05) (A). Data indicate the number of mRCC patients with PMN-MDSCs on day 0 (D0) and day 21 (D21). Comparisons were performed to those under the upper 95% CI of healthy donors PMN-MDSC values mean (denoted as below and above normal, left panel). Right panel shows median value of patients (dashed line) and proportion of patients at day 0 and day 21 following treatment. (B). Percentage of monocytes ($CD11b^+CD14^+HLA^-DR^+$) and DC ($CD19^-CD14^-CD303^-CD1c^+$) and $CD19^-CD14^-CD303^-CD141^+$) were measured on D0 and D21 by flow cytometry (C). MDSCs at D21 is associated with the survival of patients treated with VSSP. The survival curves show the estimated survival functions for patients with MDSC levels of 0.1, 1 and 10 at D21. The p value is based on Cox regression analysis and two-sided Wald test (D). DC, dendritic cell; MDSC, myeloid-derived suppressor cell; mRCC, metastatic RCC; PBMCs, peripheral blood mononuclear cell; PMN, polymorphonuclear; RCC, renal cell carcinoma; VSSP, very small size particle.

PMN-MDSC suppressive activity. $CD8^+$ T cell proliferation in response to anti-CD3 stimulation was assessed for three patients before and after three doses of VSSP (by day 21). Briefly, blood samples were either depleted of $CD11b^+$ cells (including MDSCs) or not, and the magnitude of $CD8^+$ T cell proliferation was determined for each fraction separately. The flow strategy and a representative histogram are represented in online supplemental figure S9A and B. Before treatment (baseline D0), $CD8^+$ T cell proliferation in the $CD11b^+$ fraction was lower compared with the $CD11b^-$ fraction, validating the suppressive capacity of $CD11b^+$ cells (online supplemental figure S9C). Interestingly, after VSSP treatment, $CD8^+$ T cell proliferation in the $CD11b^-$ fraction decreased compared with the $CD11b^+$ fraction (online supplemental figure S9A) in two out of the three evaluated patients. This result is consistent with the reduced inhibitory capacity of the $CD11b^+$ cells observed in the murine model and the ability of VSSP to induce APC differentiation.

Although survival analysis was not a primary objective of this trial, the median OS was 37.75 months, and the 2-year OS was 66.7%. In accordance with MSKCC/Motzer score,[34] it is important to note that eleven of the enrolled patients at the beginning of the trial had a good to intermediate prognosis. To further evaluate relationships between MDSC and patient outcomes, we analyzed the association between predicted survival and MDSC levels at day 21 following VSSP treatment. The distribution of day 21 MDSC is bimodal (online supplemental figure S9D), so the levels close to the two modes, and the minimum of observed MDSC levels were selected. We estimated the survival probabilities for patients with these three representative levels, which are shown in figure 6D. We found that MDSCs at day 21 are significantly associated with the OS of patients treated with VSSP (figure 6D). The HR is 4.80 (95% CI 1.05 to 21.90, p=0.043), which implies a patient with 10-fold higher MDSCs at day 21 is associated with a 4.8-fold higher risk of death at any given time point after VSSP.

## DISCUSSION

During chronic inflammatory processes, such as cancer, the persistent engagement of inflammatory signals can alter myeloid differentiation, driving the emergence of MDSCs and causing defects in the functionality of the remaining APCs.[9] Strategies to mitigate the impact of such aberrant effects on myeloid biology in cancer, particularly MDSCs, have focused on blocking their recruitment to the TME, limiting their suppressive function or depleting the cells altogether.[35 36] However, since such suppressive myeloid cells are likely to be continuously replenished from early myeloid progenitor sources caused by chronic exposure to TDFs, approaches targeting the intratumoral myeloid populations will need to be complemented with strategies capable of reprogramming myelopoiesis at earlier stages. Interestingly, few studies have attempted to influence and 'reprogram' aberrant myelopoiesis at such early stages.[35 36] In this study, we provide evidence of an immunomodulator, VSSP, that alters this aberrant myeloid path, redirecting bias away from MDSCs and toward monocyte/macrophage and DC differentiation. Altogether, we provide evidence: (1) supporting the broad concept of redirecting myeloid differentiation from MDSCs toward monocyte, macrophage and/or DC production; (2) identifying VSSP as a novel immune modulatory agent capable of promoting myeloid differentiation and dampening MDSC levels or their suppressive activity; and (3) demonstrating the ability of single-agent VSSP to mitigate MDSC levels in mRCC patients, which carries potentially significant clinical implications.

The presence of TLR2/4 on myeloid progenitors have been described previously.[37] These prior findings, together with the presence of TLR2/4 agonists in the VSSP nanoparticles, supported the rationale for examining the effects of VSSP on early myeloid progenitors in the context of emergency myelopoiesis induced by G-CSF. G-CSF has been described as an important TDF in human cancers,[38 39] but at the same time is commonly used to treat chemotherapy-induced neutropenia.[22] Consequently, we and others have hypothesized that exogenous G-CSF administration in cancer settings may unintentionally reinforce the PMN-MDSC-mediated immunosuppressive state.[9] Moreover, although a number of cytotoxic agents, such as gemcitabine, docetaxel, and 5-fluorouracil, can induce apoptosis of MDSCs,[3 9] other agents such as doxorubicin and high-dose CY may promote aberrant myelopoiesis and induce the expansion of MDSCs.[3 9] Thus, efforts to exploit the positive benefits of G-CSF and certain chemotherapies, together with alternative strategies to ameliorate their negative effects, may have important clinical implications. Using this tumor-free model of emergency myelopoiesis, G-CSF induced a preferential increase in oligopotent GMPs and GPs, biasing differentiation toward granulocytic populations. The addition of VSSP shunted differentiation toward IRF8- and PU.1-expressing monocytes, macrophages and DCs, and hampered MDSC suppressive function. Thus, these results may have important implications for the use of VSSP to mitigate the negative effects of G-CSF and CY on MDSC production in such clinical contexts.

The impact of VSSP on IRF8 and PU.1 expression, master regulators of myeloid development and function, is noteworthy. Studies indicate that IRF8 and PU.1 are important for APC function and negatively control MDSC development,[23 40 41] and strategies that target these transcription factors may limit MDSC load and consequently improve immunotherapy response. However, little is known about strategies to alter IRF8 expression to inhibit MDSCs. One study, for example, reported on a pharmacologic approach using a combination of valproic acid and PD-L1 blockade.[42] Our study demonstrates the use of an immune modulatory agent that induces the expression of PU.1 and IRF8, highlighting this effect as a potentially important feature of VSSP that may govern APC or MDSC fate. Future studies are warranted, however, to dissect the precise roles of these transcription factors in the mechanism of action of VSSP.

To extend and strengthen our findings from a tumor-free to a tumor-bearing setting of aberrant myelopoiesis, we selected the 4T1 mammary carcinoma. 4T1 has been well described as a model that secretes multiple tumor factors, including G-CSF, that drives PMN-MDSC production resulting from a robust expansion of GPs in the BM.[8 39 43 44] In this scenario, we found that VSSP recapitulated the effects observed in the tumor-free model of emergency myelopoiesis. A single administration of VSSP to 4T1-bearing mice constrained the expansion of GPs in the BM and maintained the ratio of GPs to MPs relative to that seen in healthy (naïve) mice. Moreover, the PMN-MDSCs recovered from VSSP-treated 4T1-bearing mice exhibited a significantly reduced suppressive activity, compared with the untreated counterparts. However, prior work has shown significant antitumor activity of VSSP when using as many as four biweekly doses in murine models.[45] Repeated VSSP dosing in two additional cancer models (C26GM and MCA203) also reduced the suppressive activity of MDSCs, demonstrating that the effects of VSSP are not tumor-type specific. It is important to emphasize that blocking TLR2 and/or TLR4

(as determined in vitro) was sufficient to inhibit the effects of VSSP on modulating MDSC suppressive activity, supporting the role of these receptors in the mechanism of action. Taken together, these results support the hypothesis that modulation of myeloid progenitor fate and, consequently, the suppressive function of MDSCs are intrinsic properties of VSSP in tumor-free or tumor-bearing settings of pathological or aberrant myelopoiesis.

To further support our hypothesis, we evaluated the impact of VSSP in patients with mRCC, a malignancy accompanied by the accumulation of circulating PMN-MDSCs.[32 46 47] In this clinical context, VSSP reduced circulating PMN-MDSCs in more than 80% of the tested patients. Akin to our preclinical results, VSSP diminished the inhibitory properties of human $CD11b^+$ cells, which include PMN-MDSCs. The change in the proportion of suppressive PMN-MDSCs vs APCs within these $CD11b^+$ cells could explain the differential proliferative responses in two of the three evaluated patients. These findings are aligned with the hypothesis that VSSP interferes with the tumor-induced myeloid imbalance, rendering a more immune-activating myeloid lineage phenotype.

It is tempting to speculate that this result might also explain the 2-year OS rate observed within the enrolled patients. Although preliminary, this small trial showed a similar follow-up median OS compared with the gold-standard first-line therapy for RCC (nivolumab plus ipilimumab). According to the NCCN guidelines in 2018, the 2-year OS rate of nivolumab plus ipilimumab was 67%–69%, with a median follow-up time of 22.3 months.[48] In our study, again although the numbers of patients enrolled were low, it is interesting to point out that the 2-year OS was 66.7%, with a median follow-up time of 37.7 months. Furthermore, we found that MDSCs measured 21 days post-VSSP treatment were significantly associated with increased OS. These results indicate that VSSP could have a significant impact on clinical outcome, but it must be confirmed in more advanced trials, possibly in combination with other immune-activating therapies.

Collectively, our findings demonstrated that VSSP shunts myeloid differentiation toward monocytic and DC populations with improved APC function or immune activation. While these findings demonstrate novel activities in myeloid biology, there remain unresolved questions pertaining to how VSSP may act on additional subsets, including immune suppressive regulatory T cells. For example, prior work has shown that regulatory T cells are decreased in tumor-bearing mice treated with VSSP.[11] Additional mechanistic studies are warranted to delineate the relative contributions of these populations, as well as DC subsets impacted by treatment, toward the antitumor properties of VSSP. Moreover, the status and function of regulatory T cells and other immune suppressive populations in cancer patients treated with VSSP is an open area of investigation. Future studies are planned to comprehensively evaluate such VSSP-induced changes on diverse immune suppressive populations in patient samples by performing spectral flow cytometry analysis.

In summary, we demonstrated that VSSP redirects myeloid progenitor cell differentiation toward monocyte/DC lineages, which could enable such myeloid populations to differentiate into professional APCs. Based on our data, VSSP is likely acting upstream within the myeloid differentiation pathway, such as the GMP stage or perhaps earlier stages (which requires further study), to 'reprogram' aberrant myelopoiesis. This reprogramming is accompanied by inhibition of the suppressive capacity of PMN-MDSCs and their differentiation toward monocyte/DC populations. Future studies should consider evaluating the clinical impact of VSSP alone or in combination with other immunotherapies wherein redirecting myeloid fate away from MDSCs and toward APCs is warranted.

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**Contributors** Performed the experiments: LO, RA, AV, SHC, DYFT, AF, OF-M, LC, MLH, MLR-C; Conception and design: LO, CM, SIA; Clinical study supervision: RD, LP; Analysis and interpretation of data: LO, RA, MJN, HY, KHE, LEF, KL, JBM, CM, SIA; Writing and/or editing of the manuscript: LO, RA, AV, MJN, JBM, CM, SIA. Review, revision, and guarantors of the manuscript: JBM, CM, and SIA.

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**Patient consent for publication** Not applicable.

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