Published in final edited form as:
Cancer Immunol Immunother. 2009 January ; 58(1): 49–59. doi:10.1007/s00262-008-0523-4.

Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin–cyclophosphamide chemotherapy

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Abstract

Abnormal accumulation of myeloid-derived suppressor cells (MDSC) is an important mechanism of tumor immune evasion. Cyclophosphamide (CTX) has also been shown in non-tumor bearing animals to cause transient surges in MDSC. Knowledge of MDSC is primarily based on preclinical work, and to date only few published studies have involved cancer patients. The goal of this study was to test the hypothesis that circulating MDSC levels correlate with clinical cancer stage, CTX-based chemotherapy, and metastatic tumor burden. Whole blood was collected from 106 newly diagnosed solid tumor patients (stages I–IV). Percentages of circulating MDSC ($Lin^{-/Lo}$, HLA DR−, $CD33^{+}CD11b^{+}$) were determined prior to initiation of systemic therapy. In 17 early stage breast cancer patients receiving doxorubicin–cyclophosphamide chemotherapy every 14 days (ddAC) blood was collected on day 1 of each cycle. Circulating MDSC were significantly increased in cancer patients of all stages relative to healthy volunteers. A significant correlation between circulating MDSC and clinical cancer stage was also observed. Moreover, among stage IV patients, those with extensive metastatic tumor burden had the highest percent and absolute number of MDSC. Significant increases in circulating MDSC were observed with ddAC

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Conflict of interest statement Authors declare no conflicts of interest.

when compared with pretreatment levels. Circulating MDSC levels correlate with clinical cancer stage, ddAC, and metastatic tumor burden. This information must be incorporated into the design of future trials exploring immune-based therapeutic strategies. Pharmacologic modulation of MDSC should also be tested in future clinical trials.

Keywords

Immature myeloid cells; Myeloid-derived suppressor cells; Breast cancer; Cancer; Cyclophosphamide; Tumor burden; Cytokines; PBMC; T cells

Introduction

Recent research has established that tumor progression, due to tumor-derived factors, can be associated with a progressive accumulation of immature myeloid cells in the blood, lymph nodes, spleen, and primary tumor site [6, 8, 17]. This aberrant balance between immature and mature myeloid cells is a hallmark of cancer and may be one of the central mechanisms of tumor evasion from the immune system, and subsequent tumor progression [15]. In preclinical models, the phenotype of immature myeloid cells or myeloid-derived suppressor cells (MDSC) consists of co-expression of the myeloid lineage differentiation antigens Gr-1 (Ly6G) and CD11b (CR3, Mac-1) [8, 18]. Accumulation of MDSC in both preclinical models and in human samples has been shown to be associated with defective dendritic cell function and inhibition of antigen specific T cell responses [1, 2, 6]. $CD11b^{+}Gr\text{-}1^{+}$ MDSC can also accumulate in non-tumor bearing hosts in response to infection and chemotherapy, e.g. cyclophosphamide [3]. Presently, T cell inhibition by MDSC is thought to be mediated by several mechanisms including increased production of reactive oxygen species and increased metabolism of the amino acid L-arginine by arginase I and nitric oxide synthetase 2 [13, 16].

In contrast to murine models, the phenotype of MDSC in humans is not as well defined. Consequently few published studies to date have investigated the clinical significance of MDSC [1, 11, 19, 23, 30]. To date the most comprehensive work in cancer patients established that MDSC were double negative for the MHC class II molecule (HLA DR) and any other surface markers of mature lymphoid or myeloid cells (CD3, CD19, CD57, CD14) or $Lin^{-/Lo}$ [1, 2]. Subsequent phenotyping of $Lin^{-/Lo}$ HLA DR^{-} cells have revealed that they are also positive for both $CD33^{+}$ and $CD11b^{+}$ [1, 12].

Here, we examined if circulating levels of MDSC in newly diagnosed cancer patients correlated with clinical stage and administration of doxorubicin–cyclophosphamide chemotherapy. Furthermore, we also determined if increased levels of circulating tumor-derived and cyclophosphamide-derived MDSC were associated with T cell immunosuppression. A population of MDSC defined as: $Lin^{-/Lo}$ HLA DR^{-} $CD33^{+}CD11b^{+}$ were readily detected in fresh whole blood from cancer patients without need for ficoll separation. There was a significant correlation between both percentage and absolute number of circulating MDSC and clinical cancer stage. Moreover, in patients with stage IV cancers both percentages and absolute numbers of MDSC directly correlated with metastatic tumor burden. Standard doxorubicin–cyclophosphamide chemotherapy on an every 14-day schedule (ddAC) was associated with a significant increase in MDSC in breast cancer patients.

Patients, materials, and methods

Patients

Peripheral blood specimens were collected from 106 cancer patients (mean age 54; range 27–75) with newly diagnosed and histologically confirmed solid malignancies: stages I/II (n = 31); stage III (n = 19); stage IV (n = 56) in accordance with American Joint Committee on Cancer (AJCC) *Cancer Staging Manual*, sixth edition (2002). Twenty milliliters of venous blood was collected in K_2 EDTA-lavender top tubes (BD, Franklin Lakes, NJ). Patient characteristics are detailed in Table 1. Blood collected from patients with stages I–IV solid malignancies was obtained prior to surgery, radiation, or any systemic chemotherapy. Twenty-one normal healthy volunteers served as controls (mean age 41). The Protocol Review Committee of the Hollings Cancer Center and the Medical University of South Carolina Institutional Review Board approved this study. Written consent was obtained from subjects.

MDSC analysis in breast cancer patients receiving chemotherapy

Patients with early stage breast cancer (n = 17) received four courses of standard ddAC (doxorubicin 60 mg/m^2 plus cyclophosphamide 600 mg/m^2) on day 1, followed by paclitaxel 175 mg/m^2 (ddT) on day 1 for four cycles [9]. Chemotherapy was administered every 14 days. Pegfilgrastim was administered as a subcutaneous injection on day 2 of each cycle of chemotherapy. Whole blood was drawn at baseline (BL) immediately prior to the first cycle of chemotherapy. Subsequent blood draws for MDSC analysis were obtained 14 days *after* the last cycle and immediately prior to receiving the following cycle of chemotherapy.

Antibodies and flow cytometric analysis

All monoclonal antibodies used in the study were purchased from BD Biosciences. Staining was performed on fresh venous blood collected in EDTA-coated vacutainer tubes (BD Biosciences). Briefly 100 μL of blood was mixed with 5 μL of each antibody on a 96-well plate. Plates were incubated at room temperature for 30 min. After incubation, each sample was mixed with 2 mL of 1 × lysing buffer (BD Biosciences) and incubated for 15 min. Samples were washed with FACS buffer (5% BSA in PBS, 0.09% sodium azide). Pellets were resuspended in 300 μL of FACS buffer. Samples were acquired and analyzed by flow cytometry using Cell Quest™ software (BD Biosciences). The absolute number of MDSC was calculated as follows: [total white blood cell count (cells/μL) × percent MDSC]/100.

In vitro assays

Cryopreserved PBMC were thawed and the cell concentration adjusted to 1 × 10^6 cells/mL. Equal cell numbers were incubated in cell culture plates with anti-CD3/CD28-coated beads (Invitrogen) at a 1:1 cell to bead ratio. Plates were incubated at 37°C for 3 days. After culture, total viable cell number was determined using a hemacytometer and cell culture supernatant was assayed for IL-2 and IFN-γ concentration using the Searchlight™ multiplex assay system (Fisher Scientific). Briefly, 50 μL of supernatant sample was added to a 96-well pre-spotted with antibodies that capture either IL-12 or IFN-γ. Standards with known concentrations were also included. After non-bound proteins were washed away, biotinylated antibodies with different specificity within the same cytokines were added. Luminescent signal was generated by adding streptavidin conjugated to horse radish peroxidase followed by addition of a substrate (SuperSignal ELISA Femto Chemiluminescent Substrate). Luminescent signal was detected with the SearchLight Plus CCD Imaging System. The amount of signal produced in each spot was proportional to the amount of cytokine. Concentrations of IL-2 and IFN-γ were extrapolated from a standard

curve. $CD33^+$ (myeloid cells) and $CD3^+$ (T cells) cells were purified from *fresh* whole blood using the Rosettesep™ kit (StemCell Technologies, Inc.) following manufacturer's instructions. Increasing ratios of $CD33^+$ T cells were added to autologous T cells. For T cell activation, anti-CD3 and anti-CD28-coated beads were added to each well at a bead to T cell ratio of 1:1. Plates were cultured for 4 days, then pulsed with 1.0 μCi of (^{3}H)-TdR (NEN, Boston, MA) for 6–8 h and lysed with distilled water. Thymidine incorporation was determined by detecting the amount of radioactivity using a β-counter.

Statistical analysis

For both absolute number of MDSC (cells/μL) and the percent MDSC a log-transform was used to correct for skewness before statistical analysis. Data were summarized using means and 95% confidence intervals assuming normality (Figs. 2, 3). Reported mean estimates were obtained by back-transforming estimates from the log scale. Two sample *t* tests (allowing for differences in variance) were applied to test for differences between groups (e.g., by cancer stages). Random effects models were used to evaluate differences in immunologic measures under different treatment conditions (i.e., pretreatment, ddAC, and ddT) within the same individual (Fig. 5). Fitted regression models were then used to predict mean levels and 95% confidence intervals within groups. Statistical significance for all comparisons was based on two-sided comparisons with alpha of 0.05. To determine increasing percent and absolute MDSC according to the stage distribution, a test for trend using linear regression was performed.

Results

Increased percent and absolute number of circulating MDSC ($Lin^{-/Lo}$ HLA DR^- $CD33^+CD11b^+$) is detected in the peripheral blood of patients with solid tumors

In order to obtain an accurate and unbiased determination of both percentage and absolute numbers of MDSC in cancer patients, whole blood flow cytometry was performed. Patient characteristics are detailed in Table 1. Previous reports in humans have established that MDSC isolated from dendritic cell fractions do not express markers specific for mature T or B lymphocytes, monocytes, NK cells, or granulocytes [1, 2]. Freshly drawn whole blood from patients was therefore labeled with PE-conjugated lineage (Lin)-specific antibodies (anti-CD3, -CD14, -CD19, and -CD57), PE-Cy5 HLA DR Ab, PE-CD 11b, and APC-CD33. After lysis of RBCs, samples were acquired in a flow cytometer. Acquired cells were first gated (R1) based on their expression of lineage markers (Lin) and MHC class II (HLA DR). Thus, R1 was comprised of $Lin^{-/Lo}$ HLA DR^- cells. From this population, the fraction of cells expressing the myeloid marker CD33 and CD11b was determined. Therefore in our study, MDSC were defined as: $Lin^{-/Lo}$ HLA DR^-, $CD33^+CD11b^+$. Percentages of MDSC were calculated as a percentage of total cells. Representative flow cytometric data of a normal healthy volunteer, a patient with stage IV cancer, and a breast cancer patient receiving standard adjuvant chemotherapy with doxorubicin and cyclophosphamide are shown in Fig. 1. Initial analysis of circulating MDSC revealed that there was a significantly higher percentage of circulating MDSC in cancer patients relative to normal volunteers (2.85 vs. 1.26%; $P < 0.0001$) (Fig. 2a).

Frequency and number of MDSC in peripheral blood correlates with clinical cancer stage, metastatic tumor burden, and radiographic response to systemic therapy

Patients were then grouped by clinical cancer stage. Differences between normal volunteers and patients with stages I/II, stage III, and stage IV solid tumors were also statistically significant ($P < 0.0001$). A direct correlation between clinical cancer stage and both percent (Fig. 2b) and total number of circulating MDSC (Fig. 2c) was noted. Although the differences in both percent and absolute number of MDSC between stages I/II and stage III

patients did not reach statistical significance ($P = 0.22$), we performed a test for trend according to stage distribution using linear regression and found the trend to be highly significant both for % MDSC ($P = 3.085 \times 10^{-11}$) and absolute MDSC ($P = 2.48 \times 10^{-05}$) both modeled on the log-scale.

Overall patients with stage IV solid tumors had the greatest mean absolute number (260.04 cells/μL) and the highest percentage (3.77%) of MDSC. The observed differences in mean MDSC between patients with early stages I/II cancer and advanced stage IV cancer in both percentages (1.96 vs. 3.77%; $P < 0.0001$) and absolute numbers (124.1 vs. 260.04 cells/μL; $P < 0.0001$) were significant. Differences in both mean percent (2.46 vs. 3.77%; $P = 0.014$) and absolute number MDSC (163.7 vs. 260.04 cells/μL; $P = 0.031$) between locally advanced cancer patients (stage III) and stage IV were also significant.

Considerable heterogeneity exists even within patients with stage IV metastatic disease. Previously published data in animal models have demonstrated that that MDSC correlate with increased tumor burden [6]. Within our own data we observed that patients with extensive metastatic involvement tended to have the highest numbers of circulating MDSC. Consequently, to address the question whether MDSC levels were highest in patients with widely metastatic disease, patients with advanced clinical stage ($n = 56$), were divided into limited and extensive metastatic tumor burden. Extensive tumor burden was defined as patients presenting with either: diffuse involvement of one organ system or ≥3 distinct organ sites involved. Patients with extensive metastatic tumor burden had both higher mean percentage (Fig. 3a) and absolute number (Fig. 3b) of circulating MDSC, which were highly significant ($P < 0.01$). Moreover, when comparing only those stage IV patients with extensive tumor burden to stage III cancer patients, the differences in both mean percent (4.37 vs. 2.46%; $P = 0.0004$) and absolute number (325.7 vs. 163.7 cells/μL; $P < 0.0001$) of MDSC was even more pronounced than that for all stage IV cancer patients. By contrast, there were no significant differences in circulating MDSC levels between stage III and stage IV patients with limited metastatic tumor burden (163.71 vs. 177.94 cells/μL; $P = 0.75$). Representative radiographic scans of two patients with extensive tumor burden and one patient with limited metastatic disease are shown in Fig. 4a. To explore whether MDSC levels in stage IV patients varied based on radiographic response to systemic therapy, blood was collected serially in six patients with stage IV cancer (Fig. 4b). Interestingly, patients with radiographic disease progression were found to have increased percentages of circulating MDSC. Conversely, patients with radiographic responses were noted to have corresponding decreases in MDSC levels.

ddAC chemotherapy, but not ddT, significantly increases circulating MDSC in breast cancer patients

Transient suppression in T and B cell activity observed in mice during the recovery phase of cyclophosphamide-induced lymphopenia has been recently attributed to the accumulation of MDSC [3]. To address whether administration of cyclophosphamide at standard doses was associated with accumulation of MDSC, blood was collected from 17 newly diagnosed stages II–III breast cancer patients that were uniformly treated with the same chemotherapy regimen either pre- or post-operatively. Administration of standard adjuvant chemotherapy in a dose-dense fashion or every 14 days with the administration of filgrastim (Fig. 5c) has been shown to improve clinical outcome compared to every 21-day administration [9]. Patients receiving this particular regimen were selected as it would enable comparison of MDSC levels during AC and then during paclitaxel; and would eliminate any confounding effect of the use of growth factors in some patients as all patients receive pegfilgrastim. Blood was collected at baseline (BL) and obtained 14 days after the last cycle of chemotherapy and immediately prior to receiving the following cycle of chemotherapy. All patients received four cycles of ddAC followed by ddT. Differences in the total percentage

and absolute levels of circulating MDSC were compared from baseline to during ddAC and ddT chemotherapy. Data within each treatment phase (BL, ddAC, or ddT) were averaged, and then a regression model, accounting for correlation within each patient was applied. Results in Fig. 5 shows a modest but significant increase in *both* the total percentage (2.20 vs. 3.65%; $P = 0.0002$) and absolute number (139.7 vs. 256.8 cells/μL; $P = 0.0006$) of circulating MDSC with paclitaxel chemotherapy compared to BL. By contrast, ddAC was associated with a significant rise in circulating MDSC (11.72%) compared to both BL (2.21%) and paclitaxel (3.65%), which was also statistically significant ($P < 0.00001$). This increase persisted throughout ddAC chemotherapy (data not shown). Mean absolute numbers of MDSC were also significantly increased with ddAC relative to BL (1157.1 vs. 139.7 cells/μL; $P < 0.0001$) and ddT (1157.1 vs. 256.8 cells/μL; $P < 0.0001$), respectively (Fig. 5b). Moreover, mean total white blood counts, during ddAC (10.139×10^3 cells/μL) were significantly higher than either BL (6.54×10^3 cells/μL) or during ddT (7.313×10^3 cells/μL) ($P < 0.0001$).

Immunosuppressive effect of tumor and ddAC-derived MDSC

To determine if greater numbers of circulating MDSC negatively impact the activity of T cells, equal numbers of PBMC from two normal volunteers, two patients with stage IV cancer, and one patient at BL and 14 days after ddAC chemotherapy were cultured with anti-CD3/CD28-coated beads. Figure 6 shows impaired T cell activation in the patients with advanced disease as determined by decreased cell proliferation and secretion of IL-2 and IFN-γ when compared to healthy volunteers. To ascertain whether ddAC-derived MDSC were associated with decreased T cell activity, PBMC from a representative breast cancer patient who received chemotherapy after complete surgical resection were collected at BL and after ddAC chemotherapy. T cell activity in this completely resected patient was comparable to that observed in normal volunteers. After ddAC treatment, levels of MDSC increased from BL. Likewise, T cell function was significantly reduced when compared to BL.

Although impaired T cell activity correlated with increased levels of circulating MDSC, other host-mediated factors could also be playing an immunosuppressive role, e.g. regulatory T cells (Tregs). Moreover, increased levels of MDSC could have an effect on the frequency of T cells. Thus to better understand the impact of MDSC on T cell activity, the $CD33^+$ cell fraction was isolated, and increasing ratios of $CD33^+$ cells were co-cultured with equal numbers of purified autologous T cells. Samples were obtained from two normal volunteers and from three cancer patients. Patients 37 and 38 had stage IV breast cancer. BR1 was a patient with stage III breast cancer undergoing preoperative chemotherapy; blood was collected after cycle 4 of ddAC. The percentages of circulating MDSC for each sample at the time of $CD33^+$ isolation are shown in Fig. 7a. After enrichment, purity of the $CD33^+$ fraction was determined by flow cytometry. Approximately 90% of the recovered cells expressed $CD33^+$ (Fig. 7b). Purified autologous T cells were activated in the presence of increasing ratios of $CD33^+$ cells.

Figure 7c shows that increasing numbers of $CD33^+$ cells isolated from normal volunteers had a minimal impact on the proliferation of their autologous T cells. By contrast, a marked decrease in T cell proliferation was observed when $CD33^+$ cells derived from patients 38 and BR1, and to a lesser extent from patient 37, was added to the culture. These findings corroborate previous reports that direct contact with MDSC results in downregulation of T cell activation [1]. Our results suggest that ddAC-derived MDSC also have T cell immunosuppressive effects.

Discussion

Extensive animal studies have detailed how MDSC in mice can block T cell function through increased nitric oxide and arginase production [5, 7]. However, knowledge of MDSC is primarily based on preclinical work, and to date only few published studies have involved cancer patients [1, 11, 19, 23, 30]. Considering the importance of MDSC as an important mechanism of cancer immune-evasion, more definitive human studies are needed. Previous work established the presence of a Lin^- HLA DR^- immature myeloid cell population in the dendritic cell fraction from cancer patients that suppressed T cell proliferation and antigen activation; subsequent phenotyping of Lin^- HLA DR^- cells revealed that they were also $CD33^+$ and $CD11b^+$ [1]. In this study elevated numbers of MDSC in patients with stages III–IV cancer were found in the dendritic cell fraction of cultured peripheral blood mononuclear cells (PBMC). More recently, the same group recently reported increased levels of $Lin^{-/Lo}$ HLA DR^- $CD33^+$ MDSC in thawed PBMC from 15 patients with refractory solid tumors relative to healthy controls [12]. One limitation of this study, however, was inclusion of relatively small numbers of patients with locally advanced and metastatic disease (clinical stages III and IV) that were grouped together [1]. Absolute numbers of MDSC in this cohort relative to normal controls were not determined, and neither was it discussed whether there were any differences between patients with locally advanced (stage III) or metastatic (stage IV) cancers, or differences between stage IV cancer patients, all clinically relevant questions.

A distinct myeloid suppressor cell population isolated from renal cancer patients has also been reported. This myeloid suppressor population had a polymorphonuclear granulocytic morphology, increased arginase I activity, and were $CD11b^+$, $CD15^+$, and CD14– [30].

More recently, in a much smaller study, a new subset of $CD14^+$HLA $DR^{-/Lo}$ MDSC were identified in stage IV melanoma patients ($n = 16$) which overexpressed TGF-γ [11]. In this study, it was also reported that the frequency of Lin^-, HLA DR^- myeloid cells in PBMC from stage IV melanoma patients ($n = 16$) were low (<1%) and overlapped with healthy controls. Moreover, an increase in the frequency of $CD14^+$HLA $DR^{-/Lo}$ subpopulation was observed after GM-CSF administration. Considerable differences between isolation methods in these two particular studies may explain this discrepancy [1, 11]. HLA DR may serve as a more specific marker than Lin for phenotypic identification of MDSC as only Lin^- HLA DR^- cells suppress T cell proliferation; Lin^- HLA DR^+ do not [1]. One limitation of the study by Fillipazi and colleagues was that they included melanoma patients that had been previously treated and did not report what therapy patients had received. In the context of MDSC this is particularly relevant because chemotherapy, e.g. gemcitabine and bevacizumab, may decrease the proportion of MDSC present in PBMCs [2, 28]. Figure 4b illustrates how MDSC levels in cancer patients can be influenced by prior therapies. A second limitation is that no mention is made of the extent of metastatic disease in the sixteen melanoma patients. Our data shows that metastatic tumor burden significantly affects circulating MDSC levels.

Elimination of additional purification steps such as ficoll separation, cell culture, etc. greatly facilitates clinical investigation of MDSC. Analysis of samples which have undergone manipulations yields mostly qualitative information [4]. Additional purification steps may alter the natural frequency of MDSC in circulation and may unintentionally introduce biases. Previous studies have shown that Ficoll–Paque separation and cryopreservation of mononuclear cells can alter the measurement of lymphocyte subsets by flow cytometry erroneously increasing number of $CD4^+$ and $CD19^+$ cells as well as decreasing the number of $CD8^+$ cells [24]. Consequently, whole blood FACS analysis readily permits quantification

of absolute MDSC numbers, and we consider that this is a more accurate method for enumeration of MDSC in patient samples.

Globally, our results show that MDSC levels in cancer patients of all stages are significantly higher when compared to normal volunteers. One potential shortcoming of our work was that our patient population included a diverse group of patients with many different solid tumors. It is quite possible that MDSC production may be tumor specific and that other mechanisms of tumor-mediated immunosuppression, e.g. regulatory T cells, may play a more central role. However, circulating MDSC were detected in different cancers and percentages and absolute numbers increased with clinical cancer stage and extensive metastatic tumor burden. Therefore, this study provides evidence that MDSC is a clinically important mediator of tumor-mediated immune suppression in patients with solid tumors.

Interestingly, differences between MDSC levels in patients with locally advanced stage III and stage IV cancers were no longer seen when comparing stage III cancer patients only with patients with limited metastatic disease ($P = 0.33$). Two key differences are notable between previous published animal work and our data. First, previous murine studies report markedly elevated numbers of MDSC. In a recent report, the number of MDSC exceeded 50% of all total blood cells [22]. Although we show that MDSC were increased in cancer patients, their absolute numbers in the peripheral blood were much smaller than what has been reported in the animal literature. One explanation is that the overall tumor burden reached in mice may greatly exceed what is typically seen in humans. Second, in humans the relationship between tumor burden and circulating MDSC may be more complex than in mouse models. In patients without distant metastases (stages I–III cancer)— primary tumor size or degree of local invasion (e.g. metastases to adjacent lymph nodes or organs) did not correlate with the number of circulating MDSC ($P = 0.23$). Again, previously published data from animal studies suggests a linear relationship between MDSC and primary tumor size [22].

In non-tumor bearing animals, cyclophosphamide has been shown to lead to transient surges in MDSC [3, 25]. Cyclophosphamide is an active anti-neoplastic agent and remains part of standard chemotherapy for breast cancer patients [9]. To our knowledge, no published study has investigated the influence of cyclophosphamide containing chemotherapy regimens on circulating MDSC in cancer patients. Our data showed that ddAC was associated with significant increases in total WBC counts relative to both BL and ddT. This rise in WBC count was primarily due to significant increases in MDSC. One advantage of evaluating MDSC in breast cancer patients receiving dose-dense therapy is that one can study the contribution of G-CSF in generating MDSC both with and without cyclophosphamide. When looking at the effect of ddT, we can conclude that G-CSF does lead to a very modest increase in MDSC. Additionally, ddAC led to a fivefold average increase in the percentage of circulating MDSC from baseline levels and a threefold increase from ddT. It should be noted that levels of ddAC-induced MDSC were much higher than tumor-derived MDSC, even in patients with increased metastatic tumor burden. Cyclophosphamide has been shown in preclinical studies to enhance cancer vaccine efficiency presumably by decreasing the number of regulatory T cells [20, 21]. Our data, however, suggests that the effects of cyclophosphamide in humans may not be entirely favorable for enhancing the anti-tumor effect of immunotherapy. Indeed, vaccination either at the same time or shortly after cyclophosphamide may have an antagonistic effect. Therefore, as an immunomodulatory agent, cyclophosphamide should be used with caution just as was recently reported with GM-CSF [11].

Consistent with previous reports, we found that increased numbers of circulating MDSC, both tumor derived and ddAC derived, were also associated with decreased T cell activation

[1, 11, 12, 30]. Moreover, we also corroborated previous work that increasing numbers of MDSC in direct contact with T cells was associated with greater inhibition of T cell proliferation. While our data also shows that MDSC is an important mechanism of cancer-related T cell immunosuppression, clearly it is not the only mechanism; proliferation of T cells in cancer patients was significantly lower than those isolated from healthy volunteers even in the absence of MDSC (Fig. 7c).

In conclusion, we demonstrated that circulating $Lin^{-/Lo}$ HLA DR^{-} $CD33^{+}CD11b^{+}$ MDSC correlated with clinical tumor stage, increased metastatic tumor burden, and ddAC chemotherapy. Abnormal accumulation of MDSC is an important mechanism of T cell unresponsiveness in cancer patients. Future cancer vaccine trials should therefore take into account circulating MDSC in patients as their presence may significantly decrease their clinical effectiveness [10, 26]. Pharmacologic strategies to reduce levels of MDSC in patients should therefore be tested in future clinical trials. Promising agents have been studied in mice, including all*trans* retinoic acid, celecoxib, and phosphodiesterase-5 inhibitors, that decrease MDSC numbers or blunt their immunosuppressive properties on T cells [1, 27, 29]. However, it is unknown whether reduction of MDSC in either number or partial reversal of their immunosuppressive properties is possible in cancer patients and whether this would significantly delay tumor progression. This may be a clinically viable strategy to significantly improve the efficacy of immunotherapy in cancer patients [14, 26].

Acknowledgments

The authors would like to thank Amir Al Khami, Tracy Vandenberg, and Steven Hutchens for technical support, and Kristi Vissage for data analysis. Special thanks to Paolo Serafini for critical review of the manuscript. This work was supported in part by a Chain Reaction for the Cure Grant http://www.chainreactionforacure.org/, a seed grant from Hollings Cancer Center and R01CA83672. Funding sources has no involvement in any aspect of conducting this research, data analysis, or manuscript preparation for publication.

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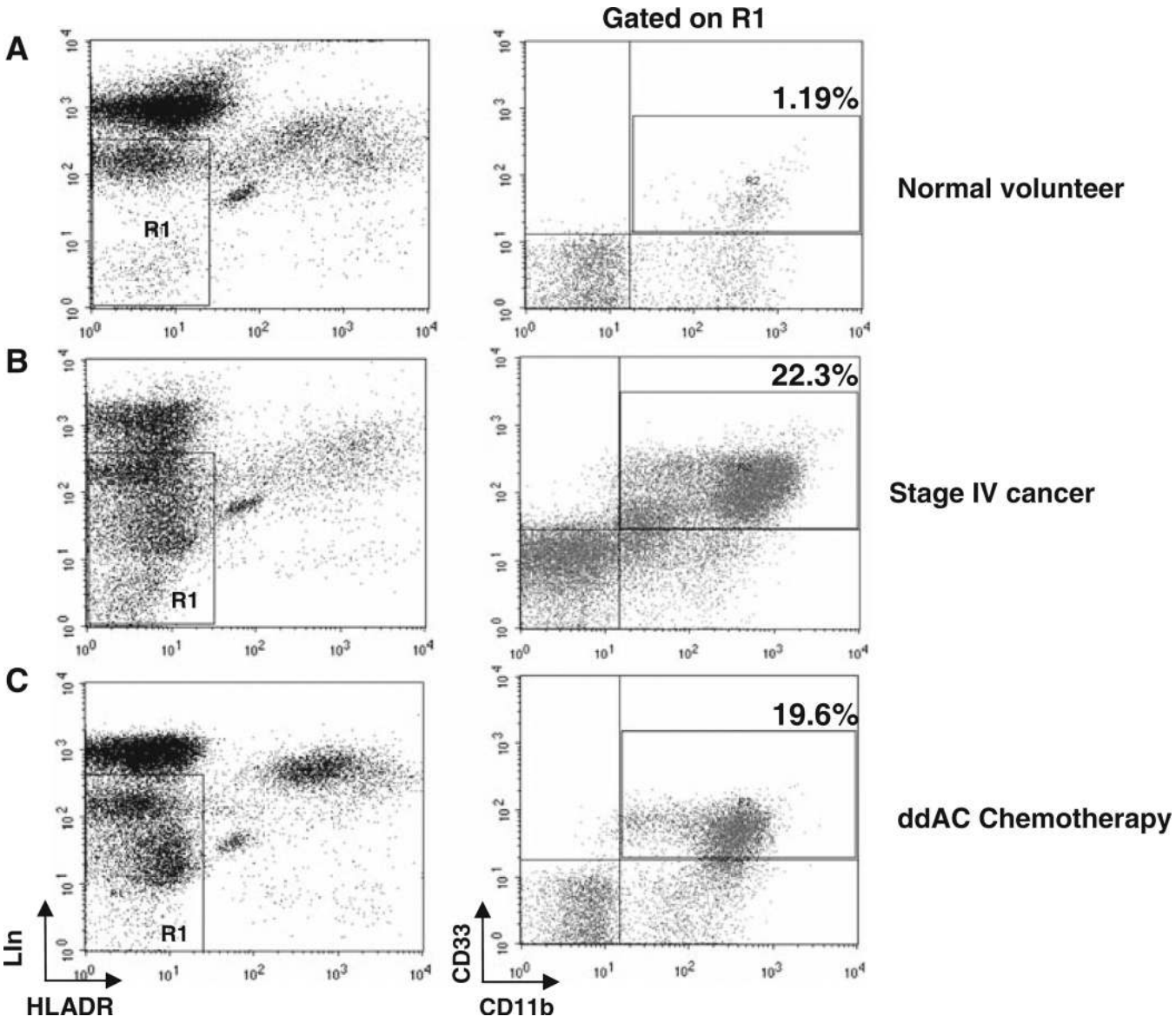

Fig. 1.

Immunophenotyping of MDSC by flow cytometry. To determine the percentage of MDSC in patients, fresh whole blood was incubated with a mixture of anti-Lin, HLA DR, CD33 and CD11b mAbs. Acquired cells were first gated (R1) based on the expression of Lin and HLA DR. R1 was comprised of $Lin^{-/Lo}$ and HLA DR^{-} cells. Within this population the fraction of cells expressing both CD33 and CD11b was determined. Therefore, MDSC were defined as $Lin^{-/Lo}$, HLA DR^{-}, $CD33^{+}$ and $CD11b^{+}$ cells. MDSC percentage was calculated as percentage of total nucleated cells in whole blood samples. Representative flow diagrams of **a** a healthy volunteer, **b** a patient with stage IV breast cancer, and **c** a breast cancer patient receiving ddAC chemotherapy

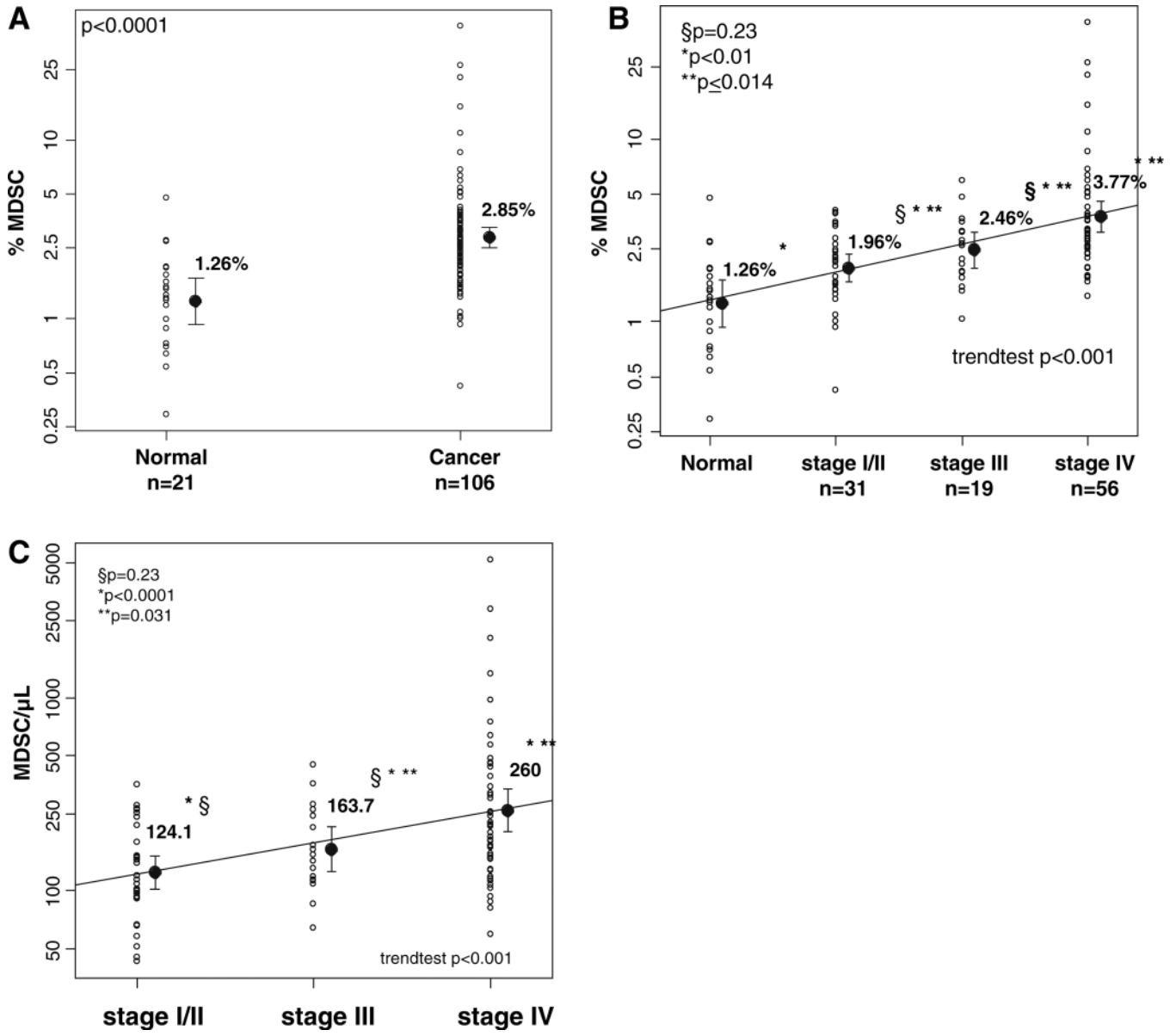

Fig. 2.

Absolute number and total percentage of circulating *MDSC* correlates with clinical tumor stage. Whole blood drawn from patients prior to any therapy was analyzed for the presence of *MDSC*. Results showed a significantly higher percentage of circulating MDSC in cancer patients ($n = 106$) versus normal volunteers ($n = 21$) (2.85 vs. 1.26%; $P < 0.0001$) (**a**). Moreover, both greater percentages (**b**) and absolute numbers (MDSC/µL) (**c**) of *MDSC* were detected in cancer patients with stage IV disease relative to patients with clinical stages I/II and stage III solid tumors. Mean estimates for each stage and 95% confidence intervals for the mean are shown to the right of the data. Differences between normal volunteers (1.26%) and cancer patients of all stages (I/II 1.96%; III 2.46%; IV 3.77%) were statistically significant ($P < 0.01$). Likewise, differences in percent and absolute number of MDSC between stage IV cancer patients and stages I/II and III were statistically significant ($P < 0.03$). Differences in percentage and absolute MDSC between stages I/II and stage III cancer patients were not statistically significant ($P = 0.22$ and 0.23, respectively). However, the

increasing pattern with stage and narrow intervals indicate strong evidence of an increasing trend (trend test $P < 0.001$)

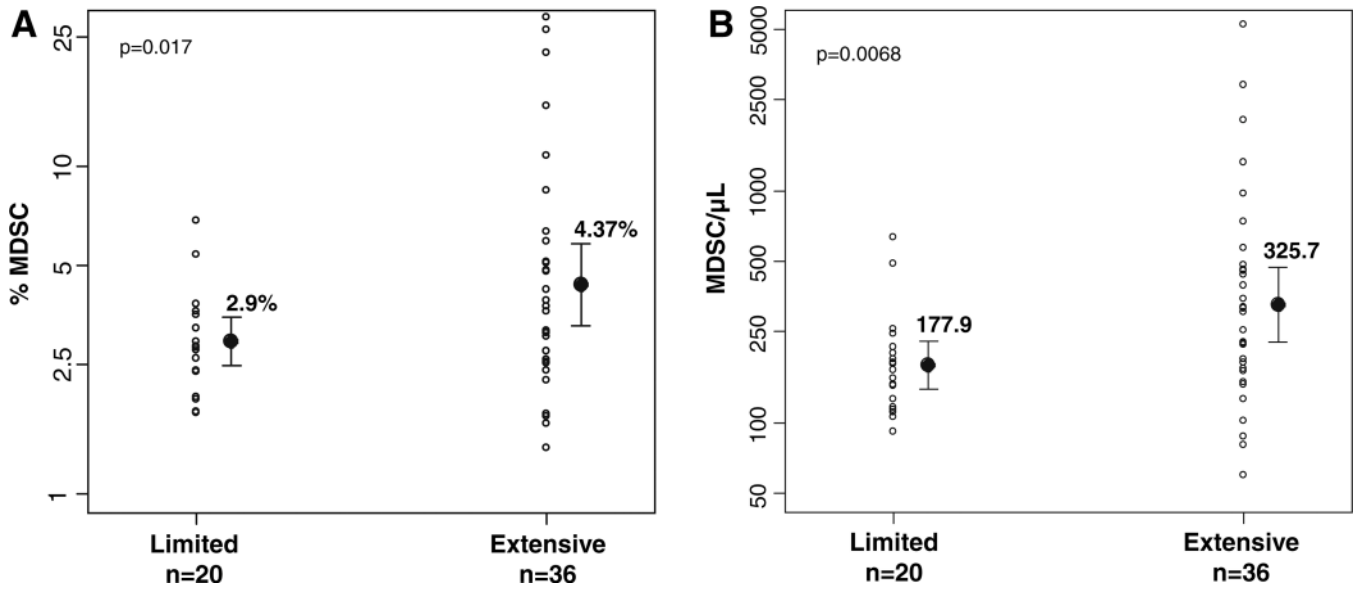

Fig. 3.

Circulating MDSC levels correlate with extensive metastatic tumor burden in patients with advanced stage IV solid tumors. Patients with advanced clinical stage were divided into limited ($n = 20$) and extensive ($n = 36$) tumor burden. Patients with extensive metastatic tumor burden must have had either: diffuse involvement of one organ system or ≥3 or more distinct organ sites involved. Patients with extensive tumor burden had significantly higher percentages (**a**) than patients with limited metastatic tumor burden with 4.37 and 2.9%, respectively ($P = 0.017$) and (**b**) absolute numbers of circulating MDSC (325.7 vs. 177.94 μL^{-1}, respectively; $P \leq 0.01$). Mean estimates and 95% confidence intervals for each group are shown to the right of the data

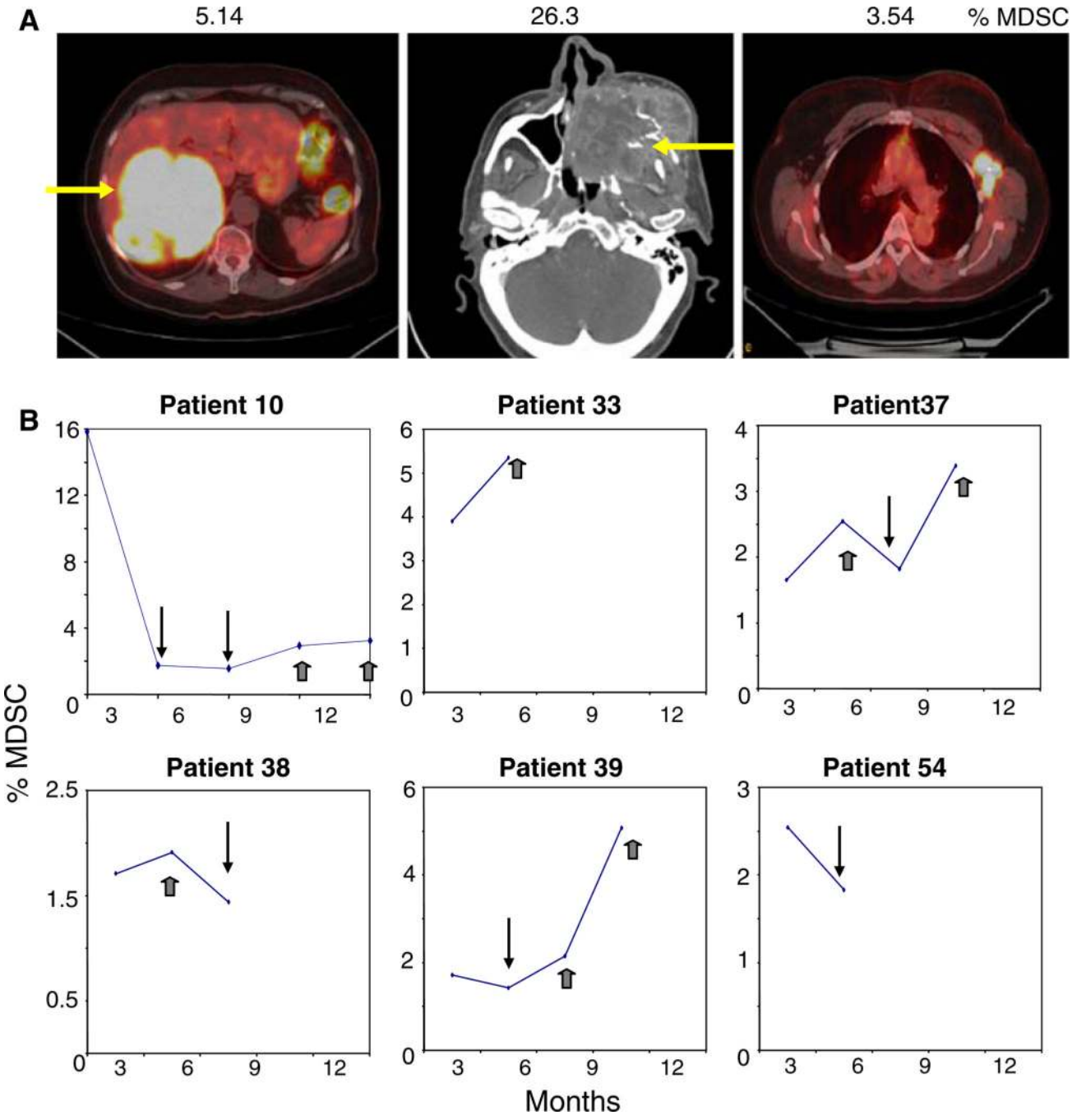

Fig. 4.

Stage IV cancer patients with limited or extensive tumor burden and correlation of MDSC levels with disease progression. **a** PET/CT scan of a patient with stage IV adrenocortical carcinoma with a large 10 × 12 cm adrenal mass (*left arrow*) and extensive bony metastases (not shown); a patient with melanoma of the left sinus (*right arrow*) measuring 15 × 12 cm, and bony, liver, and brain metastases; a patient with prior stage II breast cancer with recurrence in left axillary (*white arrowhead*) and left supraclavicular nodes (not shown). The corresponding % MDSC is shown above. **b** Percentages of circulating MDSC in six patients with stage IV cancer drawn prior to initiation of systemic therapy and again at each time when CT scans were obtained to determine radiographic response. Radiographic responses

(*black arrows*) were associated with corresponding decreases in circulating MDSC levels. Likewise, MDSC levels were found to increase at the time of disease progression (*gray arrows*)

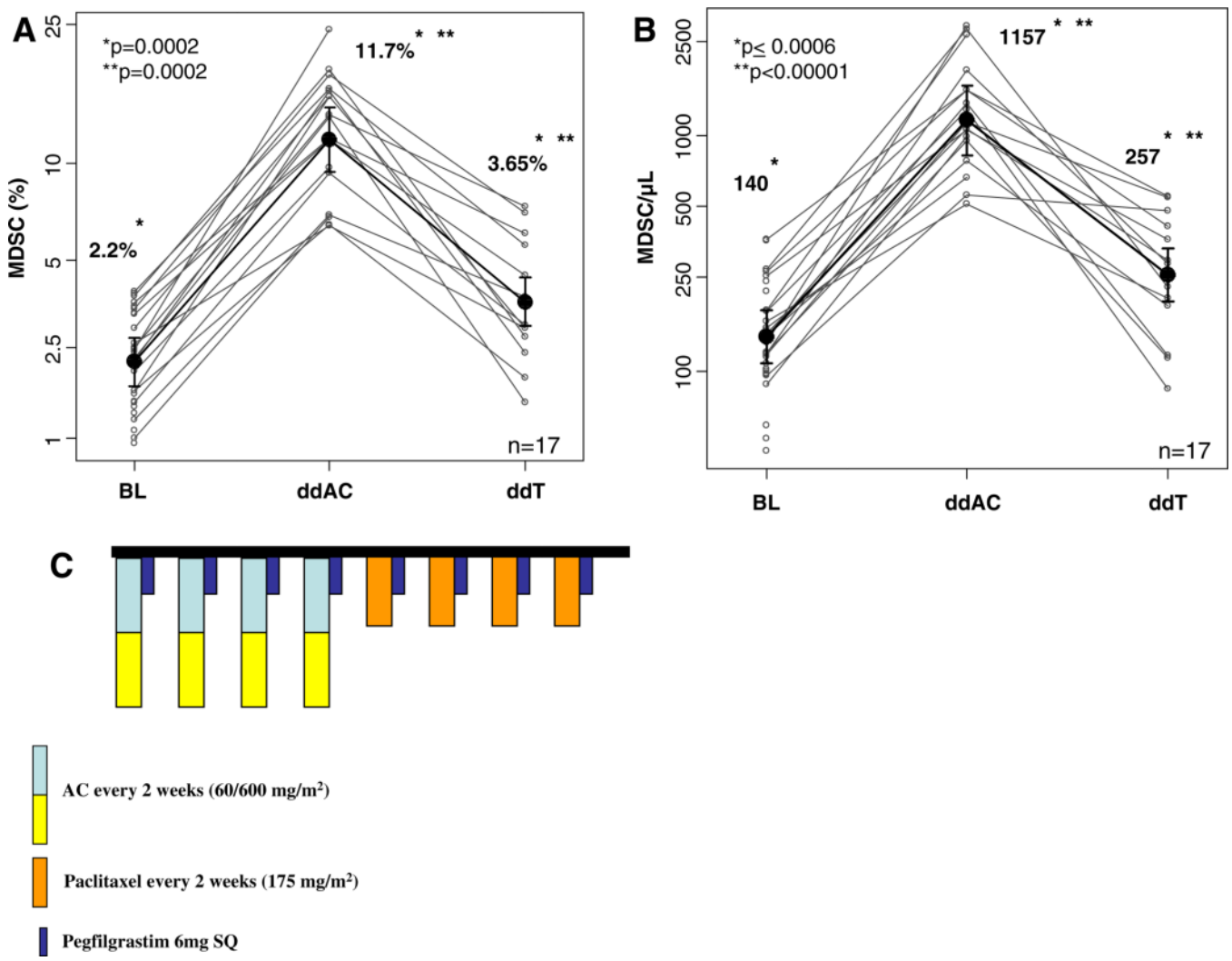

Fig. 5.

ddAC, but not ddT, was associated with significant increases in circulating MDSC in early stage breast cancer patients ($n = 19$). Data from day 1 of all four cycles of ddAC for each individual were averaged, and also for day 1 of all four cycles of ddT. A regression model, accounting for correlation with the patient was applied. Means for individual patients at each treatment phase (BL, ddAC, or ddT) are shown using small circles and observations from within the same patient are connected using thin lines. Based on the regression model, mean estimates at each treatment phase are shown using *black circles* and connected via a *thick line*. 95% confidence intervals (estimated by regression model) are shown for each treatment phase. Results showed significantly greater percentages (**a**) and absolute numbers (**b**) of *MDSC* with ddAC compared to both BL and during paclitaxel. All differences were statistically significant ($P < 0.01$). **c** Schematic representation of ddAC and ddT chemotherapy with growth factor support received by all 19 breast cancer patients

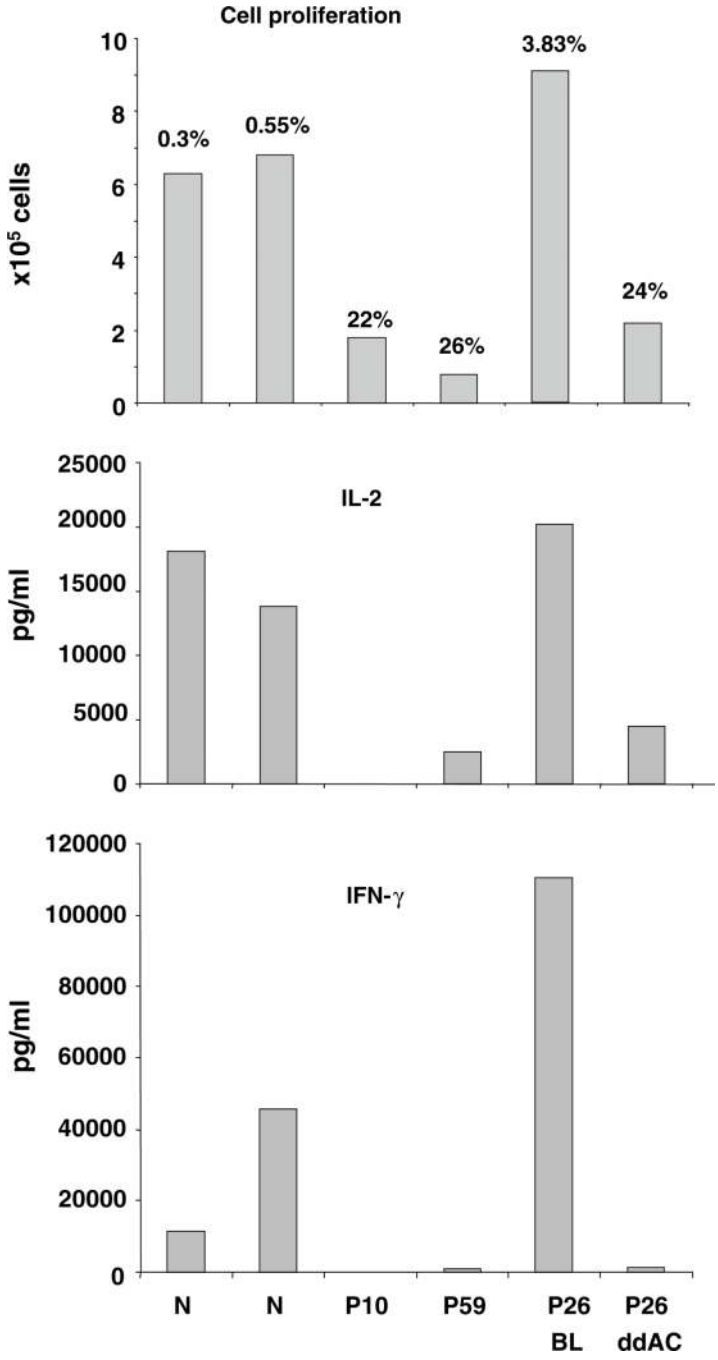

Fig. 6.

Increased levels of circulating MDSC correlate with decreased T cell responses. Equal numbers of PBMC from two normal volunteers (N), two patients with advanced disease (P10 and P59), and a breast cancer patient (P26) before (BL) and 14 days after one course of ddAC chemotherapy were assayed for cell proliferation, IL-2 and IFN-γ secretion in response to activation with anti-CD3/CD28-coated beads. Cell proliferation and cytokine release was determined 3 days after activation. Corresponding percentages of circulating MDSC are shown above in *bold*

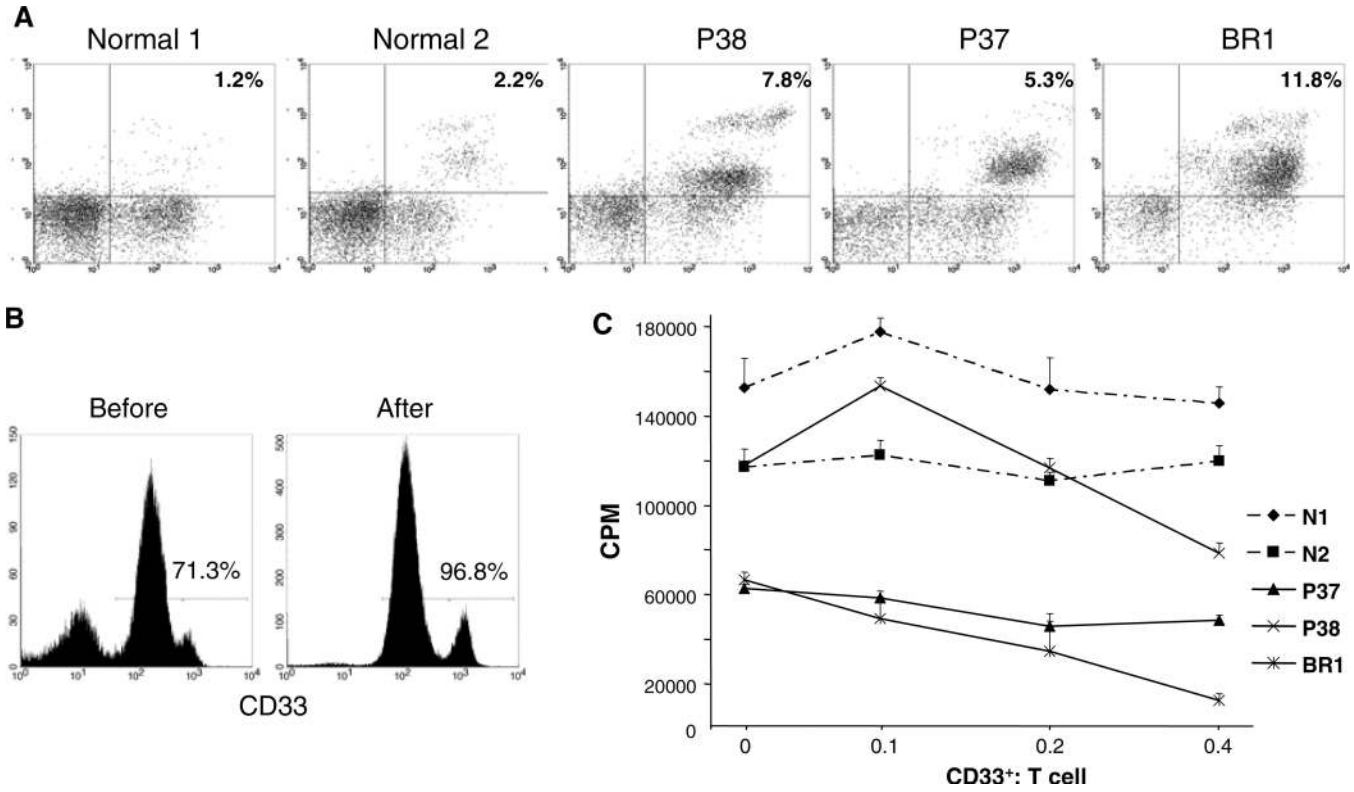

Fig. 7.

Direct contact of T cells with isolated myeloid cells ($CD33^+$) from cancer patients inhibits T cell activation. Myeloid cells ($CD33^+$) were isolated from freshly drawn blood from two normal volunteers (N1 and N2), two patients with stage IV cancer (P37 and P38), and a breast cancer patient receiving ddAC chemotherapy (BR1). **a** Percentage of circulating MDSC at the time of $CD33^+$ isolation. **b** Representative histograms of $CD33^+$ fractions before and after enrichment. **c** Proliferation of isolated autologous T cells in response to CD3/CD28 activation in the presence of the indicated ratios of purified autologous $CD33^+$ cells. Results are the average of triplicate measurements ± SDEV

Table 1

Patient characteristics

Number of patients (*n*)	106
Mean age in years (range)	54 (27–75)
Gender	
Male	36
Female	70
Cancer diagnosis	
Breast	50
Esophageal	5
Colon	10
Pancreatic	15
Non-small cell lung	3
Melanoma	4
Sarcoma	5
Gastric	2
HNSCC	2
Carcinoid	1
Gallbladder	1
Unknown primary	3
Prostate	1
Adrenocortical	1
Appendix	1
Thyroid	1
Hepatocellular	1
AJCC clinical cancer stage	
I/II	31
III	19
IV	56