Published in final edited form as:

Cancer Immunol Immunother. 2013 February ; 62(2): 299–307. doi:10.1007/s00262-012-1332-3.

Comparative analysis of monocytic and granulocytic myeloid-derived suppressor cell subsets in patients with gastrointestinal malignancies

Austin Duffy,

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

Fei Zhao,

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

Lydia Haile,

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

Jaba Gamrekelashvili,

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

Suzanne Fioravanti,

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

Chi Ma,

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

Tamar Kapanadze,

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

Kathryn Compton,

SAIC-Frederick, Inc., National Cancer Institute, Bethesda, MD, USA

William D. Figg, and

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

Tim F. Greten

GI Malignancy Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 9000 Rockville Pike, 10/12N224, Bethesda, MD 20892, USA

tim.greten@nih.gov.

Conflict of interest The authors declare no conflict of interest.

Abstract

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells comprising myeloid progenitor cells and immature myeloid cells, which have the ability to suppress the effector immune response. In humans, MDSC have not been well characterized owing to the lack of specific markers, although it is possible to broadly classify the MDSC phenotypes described in the literature as being predominantly granulocytic (expressing markers such as CD15, CD66, CD33) or monocytic (expressing CD14). In this study, we set out to perform a direct comparative analysis across both granulocytic and monocytic MDSC subsets in terms of their frequency, absolute number, and function in the peripheral blood of patients with advanced GI cancer. We also set out to determine the optimal method of sample processing given that this is an additional source of heterogeneity. Our findings demonstrate consistent changes across sample processing methods for monocytic MDSC, suggesting that reliance upon cryopreserved PBMC is acceptable. Although we did not see an increase in the population of granulocytic MDSC, these cells were found to be more suppressive than their monocytic counterparts.

Keywords

Myeloid-derived suppressor cells; Immune; Suppressor; Cancer

Background

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells comprising myeloid progenitor cells and immature myeloid cells, which have the ability to suppress the effector immune response [1, 10, 12]. In the context of malignancy, they contribute toward a suppressive network that allows evasion of the host's anti-tumor immune response. Targeting MDSC may be important in enhancing both immune and non-immune cancer therapies. A barrier to this, however, is the ability to consistently identify these cells in order to target them and assay their destruction.

In humans, MDSC have not been well characterized owing to the lack of specific markers. The phenotype of these cells has been shown to be mainly CD34⁺, CD33⁺, CD15⁺, and CD14⁺/lin⁻ [1, 2, 16, 19, 21, 22]. We and others have shown that the CD14⁺HLA-DR^{-/low} phenotype also represents MDSC, and this population is increased in HCC and melanoma [9, 14]. It is possible to broadly classify the MDSC phenotypes described in the literature as being predominantly granulocytic (expressing markers such as CD15, CD66, CD33) or monocytic (expressing CD14). This is analogous to the situation in murine MDSC that have been further subdivided into CD11b⁺Gr-1^{high} granulocytic MDSC (which can also be identified as CD11b⁺Ly-6G⁺Ly6C^{low} MDSC) and CD11b⁺Gr-1^{low} monocytic MDSC (which can also be identified as CD11b⁺Ly-6G⁻Ly6C^{high} MDSC). It is not clear, however, whether one subset is predominant or has superior immunosuppressive abilities over the other, or indeed whether this is dependent on the method of sample processing, that is, whether fresh or frozen samples are used or whether the blood is separated across a ficoll gradient or undergoes red cell lysis. There is an obvious logistical advantage to being able to rely upon cryopreserved PBMC samples, particularly in the setting of large multicentre trials where correlative specimens are banked and analyzed at a later date.

In this study, we set out to perform a direct comparative analysis across both granulocytic and monocytic MDSC subsets in terms of their frequency, absolute number and function in the peripheral blood of patients with advanced GI cancer. We also set out to determine the optimal method of sample processing given that this is an additional source of heterogeneity. Our findings demonstrate consistent changes across sample processing methods for monocytic MDSC, suggesting that reliance upon cryopreserved PBMC is acceptable, although with a lower yield. Although we did not see an increase in the population of granulocytic MDSC, these cells were found to be more suppressive than their monocytic counterparts.

Materials and methods

Patients and healthy donors

Blood samples were collected from healthy adults ($N=44$) and patients with GI cancer ($N=41$) who were seen at the GI Malignancies Section, Medical Oncology Branch at the National Cancer Institute, Bethesda, MD. Written consent was obtained from all patients before blood sampling on a research protocol approved by the Institutional Review Board. Table 1 shows the clinical characteristics of all patients in this study.

Cell isolation

Each blood sample underwent both whole blood lysis and PBMC isolation. PBMC were isolated from freshly obtained blood by ficoll density gradient centrifugation as described previously [11]. Whole blood lysis was performed using ACK lysing buffer.

Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) analysis was performed on fresh and cryopreserved PBMC and fresh whole blood. MDSC subsets were enumerated using the following antibodies: 7-AAD; anti-CD14 vioblu (Miltenyi Biotec); anti-CD11b AF488 (FITC); anti-CD33 AF700; anti-CD15 PeCy7; anti-HLADR-APC (all Becton–Dickinson). Multicolor FACS analysis was done on an LSRII flow cytometer (BD Biosciences). Analysis of FACS-data was done using FlowJo software (TreeStar, Inc.). Isotype-matched antibodies were used with all the samples as controls. The gating strategy for both the monocytic and granulocytic MDSC populations is shown in Figs. 1 and 2, respectively. For calculation of the estimated absolute number, we used the method described by Jongbloed et al. [3, 4, 15] among others. The relative frequencies obtained by FACS were applied to the CLIA-certified clinical lab differential count occurred on the same date and time. For the PBMC samples, we calculated the estimated absolute number based on the sum absolute lymphocyte and monocyte count. For whole blood samples, we calculated the estimated absolute number based on the absolute total white cell count. For the frozen PBMC cohort, the manual count pre- and post-freezing and therefore the yield upon thawing were obtained. The same person processed the fresh and frozen samples that were obtained from the same sample and processed together. The threshold for inclusion was viability on trypan blue of 90 %.

Cell sorting

For cell sorting, 100 ml of whole blood was taken from patients with advanced GI cancer. Lysis was performed using ACK lysis buffer. CD14⁺ cells were purified using CD14 Microbeads and AutoMACS separation unit (Miltenyi Biotech) according to the manufacturers' instructions. Remaining CD14⁻ cells were further purified using CD8 Microbeads and AutoMACS. Using the BD FACS Aria cell sorting system (Becton–Dickinson), purified CD14⁺ cells were sorted into CD14⁺HLA-DR^{-/low} and CD14⁺HLA-DR⁺ cells. CD14⁻ cells were sorted into CD15⁺CD11b⁺CD33⁺ and CD15⁺CD11b⁺CD33⁻ populations.

Suppression assay

For functional analysis, CD14⁺HLA-DR^{-/low}, CD14⁺HLA-DR⁺, CD15⁺CD14⁻CD11b⁺CD33⁺, and CD15⁺CD14⁻CD11b⁺CD33⁻ populations were incubated at different ratios with CD3/CD28 (Miltenyi Biotech)-stimulated CD8 T cells. After 72 h, proliferation was measured by incorporation of ³H-thymidine (Amersham) as described. For determination of interferon (IFN)- γ responses, culture supernatants from the suppression assay were removed after 48 h and tested by ELISA (eBioscience, Inc.) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean values $\pm$ standard error of the mean for percentages. Statistical analysis was performed on GraphPad Prism software. Student's *t* test was used to assess the differences between the study groups.

Results

Only CD14⁺HLA-DR^{-/low} MDSC are elevated in patients with GI cancer

Although early reports described the MDSC phenotype as being CD14-negative, a number of subsequent studies have confirmed that the CD14⁺HLA-DR^{-/low} phenotype also describes MDSC and that these cells are elevated in the peripheral blood of patients with advanced cancer [5, 9, 14, 23]. Given that these reports comprised patients with melanoma, hepatocellular carcinoma, myeloma, and prostate cancer, we wanted to confirm the presence of these cells in our population of advanced GI cancer patients, the majority of whom had colorectal cancer (Table 1). In this population, the frequency of CD14⁺HLA-DR^{-/low} cells was increased in the fresh PBMC of patients compared to controls (Fig. 1a). Given that there are a number of potential variables related to how a blood sample is processed, we also compared the CD14⁺HLA-DR^{-/low} phenotype across method of processing, that is, in cryopreserved PBMC and following lysis of the whole blood. We found that the increase in frequency of CD14⁺HLA-DR^{-/low} cells in patients compared to controls was consistent across method of processing as follows: fresh PBMC (5.5 vs 1.2; 95 % CI 2.7–5.8; *p* < 0.0001), cryopreserved PBMC (10.03 vs 4.16; 95 % CI 3.1–8.6; *p* < 0.0001), or whole blood (2.5 vs 0.8; 95 % CI 0.96–2.44; *p* < 0.0001) (Fig. 1a). Of note, the frequency of CD14⁺HLA-DR^{-/low} cells was found to be greater in cryopreserved PBMC samples compared to fresh PBMC (10.03 vs 5.5; 95 % CI 1.6–7.4; *p* = 0.0024), and in fresh PBMC

compared to whole blood (5.5 vs 2.5; 95 % CI 1.3–4.7; $p = 0.0008$). When evaluating across processing method, the relative frequency can be misleading, however, particularly in the case of whole blood analysis where the frequency value will be smaller given the larger denominator. Therefore, we also performed the comparative analysis using estimated absolute numbers—calculated according to lymphocyte and monocyte count in PBMC and total white count in the case of whole blood—and found that the increase in CD14⁺HLA-DR^{-/low} cells in patients compared to controls was consistent whether PBMC or whole blood lysis was employed was also seen when estimated absolute numbers were used (Fig. 1b). Of note, the absolute number of CD14⁺HLA-DR^{-/low} cells was greater in whole blood compared to fresh PBMC (0.17 vs $0.1 \times 10^3/\mu\text{l}$; 95 % CI 0.009–0.13; $p = 0.0248$). Representative dot plots are shown in Fig. 1c and gross appearance on cytospin is shown in Fig. 1d. This analysis confirms previous reports of increased CD14⁺HLA-DR^{-/low} cells in this population of patients with advanced GI cancer.

CD15⁺ CD14⁻ but not CD15⁺ CD14⁻ CD11b⁺CD33⁺ cells are increased in patients with GI cancer

The majority of reports in the literature describe MDSC that bear granulocytic markers including CD15, CD16, or CD33 [6, 16, 18, 26]. Many reports have further defined this cell population as being negative for a panel of lineage markers, including CD14 [1, 2]. Given that these reports comprised patients with heterogeneous cancer types—including breast, head and neck and lung cancer—we wanted to confirm the presence of these cells in our population of patients with advanced GI cancer. Consistent with prior reports, we found that the frequency of CD15⁺CD14⁻ cells was increased in the peripheral blood of cancer patients compared to controls (Fig. 2a), but this was only the case when whole blood lysis was performed (59.7 vs 46.9; 95 % CI 4.5–21; $p = 0.003$). We did not observe an increased frequency of these cells when fresh or cryopreserved PBMC was analyzed (Fig. 2a). The increased frequency of CD15⁺CD14⁻ cells in patients relative to controls was also seen when absolute numbers were analyzed (3.95 vs $3.1 \times 10^3/\mu\text{l}$; 95 % CI 0.04–1.6; $p = 0.0388$) (Fig. 2b). Given that CD15 is a marker for granulocytes, this phenotype by itself does not discriminate for MDSC. In an attempt to further define this subpopulation, we employed two additional markers—CD11b and CD33—which have also been previously reported as defining MDSC in humans [7, 17, 26]. However, when CD15⁺CD14⁻ cells were further gated for CD11b and CD33 positivity, there was no observed increase in either the frequency (Fig. 2c) or absolute number (Fig. 2d) of CD15⁺CD14⁻ CD11b⁺CD33⁺ cells in cancer patients relative to controls irrespective of how the samples were processed. When comparing directly across monocytic and granulocytic MDSC subsets, the absolute number of CD15⁺CD14⁻CD11b⁺CD33^{+/−} cells was greater (in whole blood samples) than for the monocytic CD14⁺HLA-DR^{-/low} cells (0.96 vs $0.17 \times 10^3/\mu\text{l}$). Representative dot plots are shown in Fig. 2e and gross appearance on cyto-spin is shown in Fig. 2f.

Method of processing correlations

In addition to the phenotypic heterogeneity describing MDSC in the literature, there has also been much variation in the processing methods employed. The most common method used in studies has been separation and isolation of PBMC across a ficoll gradient with subsequent analysis being performed on the fresh PBMC or, perhaps more frequently,

following cryopreservation of the PBMC. This latter method has the logistical advantage of facilitating transport and batch analysis and is therefore crucial for immune-monitoring of multicentre studies. It is unclear, however, whether cryopreservation distorts the proportion of MDSC. As shown above in Fig. 1a, b, the observed increase in CD14⁺HLA-DR^{-/low} MDSC was consistent across processing method, and Fig. 3a demonstrates that the increase in CD14⁺HLA-DR^{-/low} cells in fresh PBMC correlated with that seen upon cryopreservation. This finding suggests that it is feasible to cryopreserve PBMC for analysis of CD14⁺HLA-DR^{-/low} MDSC, although the average loss of yield upon subsequent thawing and following manual cell count was 66 %.

After ficoll separation, the cell pellet mainly contains granulocytes that are generally discarded. It is possible that this further distorts the result as potential MDSCs harbored in the pellet will not be analyzed. To evaluate this, we performed red cell lysis of the whole blood sample to look for MDSC in the entire granulocytic and mononuclear populations. As shown in Fig. 1a, b, the increase in CD14⁺HLA-DR^{-/low} MDSC was also seen after analysis of the whole blood fraction following red cell lysis. Likewise this increase was found to correlate with that seen in fresh PBMC (Fig. 3b) and whether absolute numbers or relative frequency was employed (Fig. 3c).

CD14⁺HLA-DR^{-/low} cells inhibit autologous T cell proliferation

Next, we tested whether CD14⁺HLA-DR^{-/low} monocytes from cancer patients can suppress autologous T cell responses, a facility attributed to MDSC. To evaluate this, we sorted both CD14⁺HLA-DR^{-/low} and CD14⁺HLA-DR⁺ cells from the red cell-lysed whole blood of patients with cancer. The suppressive potential of both populations was evaluated by adding each cell type at different ratios to autologous anti-CD3/CD28-stimulated CD8 cells. IFN- γ release and cell proliferation (following the incorporation of thymidine) were analyzed. As shown in Fig. 4a, b, CD14⁺HLA-DR^{-/low} cells suppressed the proliferation and IFN- γ production of autologous CD8 in a dose-dependent manner. In contrast, CD14⁺HLA-DR⁺ monocytes failed to suppress proliferation or IFN- γ secretion. This experiment confirms previous reports that CD14⁺HLA-DR^{-/low} cells are capable of suppressing CD8 cell activity, consistent with MDSC activity.

CD15⁺CD14⁻CD11b⁺CD33⁺ cells inhibit autologous T cell proliferation

Next, we tested whether CD15⁺CD14⁻CD11b⁺CD33⁺ cells from cancer patients can suppress autologous T cell responses. To evaluate this, we sorted CD15⁺CD14⁻CD11b⁺CD33⁺ cells from the red cell-lysed whole blood of patients with cancer. As above for CD14⁺HLA-DR⁺ MDSC, the suppressive potential of this population was evaluated by adding each cell type at different ratios to autologous anti-CD3/CD28-stimulated CD8 cells. IFN- γ release and cell proliferation (following the incorporation of thymidine) were analyzed. As shown in Fig. 4c, d, CD15⁺CD14⁻CD11b⁺CD33⁺ cells were found to suppress the proliferation and IFN- γ production of autologous CD8 cells in a dose-dependent manner and the degree of suppression was greater than that seen with CD14⁺HLA-DR^{-/low} cells. These data suggest that granulocytic MDSC are more immunosuppressive than monocytic MDSC.

Discussion

The presence of natural suppressor cells in patients with cancer was first described more than 25 years ago but over the past decade interest in these cells—now termed MDSC—has greatly increased [25]. In contrast to murine MDSC, which are clearly defined by the expression of Gr-1 and CD11b, the corresponding cells in humans are inadequately characterized due to the lack of uniform markers. Given the increasing consensus that immune evasion and suppression of anti-tumor immunity plays a key role in cancer progression, it is assumed that eradicating these cells will enhance immune and also non-immune therapies [13]. To do this, we need to be able to consistently identify these cells. In this study, we compared two of the most commonly described human MDSC subsets in patients with advanced GI cancer. In addition, we analyzed whether it made a difference how the samples were processed—fresh versus cryopreserved PBMC, and PBMC versus whole blood lysis—as this has important implications for the conduct of correlative studies in multicenter clinical trials.

We selected the two subsets based on the literature where, until relatively recently, the majority of reports described MDSC as being CD14-negative, often including this marker in an exclusionary lineage panel. An increased frequency of CD15⁺ granulocytes with immune suppressor function was initially described in the peripheral blood of patients with breast, colon, and pancreatic cancer [21]. Zea et al. [26] subsequently defined MDSC as CD11b⁺CD14[−]CD15⁺ cells and found that these cells expressed arginase-1 and were elevated in the blood of patients with renal cancer. Similarly, Ko et al. [16] found two overlapping populations of MDSC—CD33⁺HLA-DR[−] and CD15⁺CD14[−]—elevated in renal cancer, both of which decreased in response to treatment with sunitinib. Other investigators have described similar populations of CD15⁺CD11b⁺CD14[−] suppressor cells in patients with advanced lung cancer [17]. Over the past 5 years, a number of reports have shown that the CD14⁺HLA-DR^{−/low} phenotype also represents MDSC and that this population is increased in HCC, melanoma, prostate cancer, and multiple myeloma [5, 9, 14, 23].

In our present analysis, CD14⁺HLA-DR^{−/low} MDSC were found to be increased in cancer patients, and this increase could be appreciated whether PBMC or whole blood was used and whether the PBMC were analyzed freshly or after cryopreservation. Interestingly, the proportion of CD14⁺HLA-DR^{−/low} MDSC in PBMC increased upon cryopreservation which may reflect increased durability of these transformed cells. While the relative frequency of CD14⁺HLA-DR^{−/low} MDSC was lower in whole blood relative to PBMC, the absolute number was increased and this is to be expected as this number represents the proportion of the whole blood as opposed to just the lymphocyte and monocyte populations. The increase in CD14⁺HLA-DR^{−/low} MDSC was found to correlate whether the fresh or frozen PBMC or whole blood was analyzed. This has important implications given that the majority of MDSC analyses in the literature have been performed on PBMC and given also the reliance upon cryopreserved PBMC in the performance of immunomonitoring of clinical trials, particularly in the multicentre setting.

While CD15⁺ cells were found to be increased in the whole blood of cancer patients relative to controls, there was no observed difference when these cells were gated for CD33 and

CD11b positivity. It has been previously documented that neutrophils are increased in patients with cancer, and while some reports have shown these to be suppressive, this is not universally agreed [10, 20, 24]. We did not see an elevation of CD15⁺CD14⁻ cells in either fresh or cryopreserved PBMCs, a finding which is perhaps unsurprising given that ficoll density gradient separation should exclude granulocytes. However, Choi et al. [6] have described a CD15⁺CD16^{low} cell population of activated granulocytes that act as MDSC and were elevated in patients with advanced cancer. Interestingly, in that study, the granulocytes were isolated following both ficoll gradient separation to remove the PBMC and dextran sedimentation and red cell lysis of the pellet, on the basis that MDSC which appear in the PBMC layer are in reality activated granulocytes of altered density. The fact that in our analysis, we did not see a difference when samples were further gated for CD33 and CD11b positivity may reflect that this phenotype does not discriminate granulocytic MDSC from granulocytes. Consistent with this, we found that both CD15⁺CD14⁻CD11b⁺CD33⁺ and CD15⁺CD14⁻CD11b⁺CD33⁻ (data not shown) populations were equally suppressive of CD8 cell proliferation and IFN- γ production, suggesting that in this patient population CD33 is not a discriminatory marker for MDSC. It is possible that this is a specific finding in gastrointestinal malignancies. It has been previously shown that CD11b⁺CD33⁺ MDSC were increased in the peripheral blood of patients with non-small cell lung cancer and head and neck cancer [7]. In contrast, and similar to our findings, Eruslanov et al. [8] described a CD11b⁺CD15^{high}CD33^{low} cell population in the PBMC of patients with bladder cancer which inhibited T cell proliferation through induction of CD4⁺Foxp3⁺ T regulatory cells. They also describe a 'monocyte-type' CD15^{low}CD33^{high} population, although the frequency of these cells was not increased in cancer patients compared to controls. It has also been described that Lin⁻HLA-DR⁻ expression describes the MDSC phenotype [1]. We analyzed the relative frequency of this phenotype in a small cohort of patients ($N=7$, data not shown) and found that while this population were decreased relative to CD15⁺CD14⁻CD11b⁺CD33⁺ cells in both fresh and frozen PBMC, this was not the case in whole blood. This is possibly because the antibody cocktail does not contain CD15, whose degree of expression would be expected to be greater in cells isolated following whole blood lysis given that this is a granulocytic marker. It was also noteworthy that, in our study, the CD15⁺CD14⁻CD11b⁺CD33⁺ populations were more numerous than CD14⁺HLA-DR^{-/low} cells in the peripheral blood of this patient cohort and the immune suppression that they exhibited in terms of reduced CD8 T cell proliferation, and IFN- γ production was more profound.

In conclusion, we confirmed previous reports that CD14⁺HLA-DR^{-/low} MDSC are elevated in the peripheral blood of patients with advanced GI cancer, and this increase was evident whether fresh or cryopreserved PBMC or whole blood was analyzed. We also found that CD15⁺ cells were increased in the whole blood of cancer patients relative to controls; however, there was no observed difference when these cells were gated for CD33 and CD11b positivity, and both CD33 positive and negative CD15⁺ populations were capable of suppressing CD8 T cell proliferation, questioning whether CD33 is a reliable discriminatory marker for MDSC.

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

References

1. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC et al. (2001) Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* 166(1):678–689 [PubMed: 11123353]
2. Almand B, Resser JR, Lindman B, Nadaf S, Clark JI, Kwon ED et al. (2000) Clinical significance of defective dendritic cell differentiation in cancer. *Clin Cancer Res* 6(5):1755–1766 [PubMed: 10815894]
3. Baumgart DC, Metzke D, Schmitz J, Scheffold A, Sturm A, Wiedenmann B et al. (2005) Patients with active inflammatory bowel disease lack immature peripheral blood plasmacytoid and myeloid dendritic cells. *Gut* 54(2):228–236 [PubMed: 15647187]
4. Beckebaum S, Zhang X, Chen X, Yu Z, Frilling A, Dworacki G et al. (2004) Increased levels of interleukin-10 in serum from patients with hepatocellular carcinoma correlate with profound numerical deficiencies and immature phenotype of circulating dendritic cell subsets. *Clin Cancer Res* 10(21):7260–7269 [PubMed: 15534100]
5. Brimmes MK, Vangsted AJ, Knudsen LM, Gimsing P, Gang AO, Johnsen HE et al. (2010) Increased level of both CD4+FOXP3+ regulatory T cells and CD14+ HLA-DR/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma. *Scand J Immunol* 72(6):540–547 [PubMed: 21044128]
6. Choi J, Suh B, Ahn YO, Kim TM, Lee JO, Lee SH et al. (2012) CD15+/CD16low human granulocytes from terminal cancer patients: granulocytic myeloid-derived suppressor cells that have suppressive function. *Tumour Biol* 33(1):121–129 [PubMed: 22081309]
7. Corzo CA, Cotter MJ, Cheng P, Cheng F, Kusmartsev S, Soto-mayor E et al. (2009) Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J Immunol* 182(9):5693–5701 [PubMed: 19380816]
8. Eruslanov E, Neuberger M, Daurkin I, Perrin GQ, Algood C, Dahm P et al. (2012) Circulating and tumor-infiltrating myeloid cell subsets in patients with bladder cancer. *Int J Cancer* 130(5):1109–1119 [PubMed: 21480223]
9. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M et al. (2007) Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte–macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol* 25(18):2546–2553 [PubMed: 17577033]
10. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V (2012) Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* 12(4):253–268 [PubMed: 22437938]
11. Greten TF, Korangy F, Neumann G, Wedemeyer H, Schlote K, Heller A et al. (2002) Peptide-beta2-microglobulin-MHC fusion molecules bind antigen-specific T cells and can be used for multivalent MHC-Ig complexes. *J Immunol Methods* 271(1–2):125–135 [PubMed: 12445736]
12. Greten TF, Manns MP, Korangy F (2011) Myeloid derived suppressor cells in human diseases. *Int Immunopharmacol* 11(7):802–807 [PubMed: 21237299]
13. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5):646–674 [PubMed: 21376230]
14. Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Kruger C, Manns MP et al. (2008) A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)/CD25(+)/Foxp3(+) T cells. *Gastroenterology* 135(1): 234–243 [PubMed: 18485901]
15. Jongbloed SL, Lebre MC, Fraser AR, Gracie JA, Sturrock RD, Tak PP et al. (2006) Enumeration and phenotypical analysis of distinct dendritic cell subsets in psoriatic arthritis and rheumatoid arthritis. *Arthritis Res Ther* 8(1):R15 [PubMed: 16507115]

16. Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P et al. (2009) Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin Cancer Res* 15(6):2148–2157 [PubMed: 19276286]
17. Liu CY, Wang YM, Wang CL, Feng PH, Ko HW, Liu YH et al. (2010) Population alterations of L-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14/CD15+/CD33+ myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer. *J Cancer Res Clin Oncol* 136(1):35–45 [PubMed: 19572148]
18. Mirza N, Fishman M, Fricke I, Dunn M, Neuger AM, Frost TJ et al. (2006) All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res* 66(18):9299–9307 [PubMed: 16982775]
19. Pak AS, Wright MA, Matthews JP, Collins SL, Petruzzelli GJ, Young MR (1995) Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34(+) cells which suppress immune functions within cancers that secrete granulocyte–macrophage colony-stimulating factor. *Clin Cancer Res* 1(1):95–103 [PubMed: 9815891]
20. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW et al. (2012) A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest* 122(1):327–336 [PubMed: 22156198]
21. Schmielau J, Finn OJ (2001) Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T cell function in advanced cancer patients. *Cancer Res* 61(12):4756–4760 [PubMed: 11406548]
22. Srivastava MK, Bosch JJ, Thompson JA, Ksander BR, Edelman MJ, Ostrand-Rosenberg S (2008) Lung cancer patients' CD4(+) T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells. *Cancer Immunol Immunother* 57(10):1493–1504 [PubMed: 18322683]
23. Vuk-Pavlovic S, Bulur PA, Lin Y, Qin R, Szumlanski CL, Zhao X et al. (2010) Immunosuppressive CD14+ HLA-DRlow/–monocytes in prostate cancer. *Prostate* 70(4):443–455 [PubMed: 19902470]
24. Youn JI, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI (2012) Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. *J Leukoc Biol* 91(1):167–181 [PubMed: 21954284]
25. Young MR, Newby M, Wepsic HT (1987) Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic Lewis lung carcinoma tumors. *Cancer Res* 47(1):100–105 [PubMed: 2947676]
26. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J et al. (2005) Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res* 65(8):3044–3048 [PubMed: 15833831]

Fig. 1. CD14⁺HLA-DR^{low} MDSC are increased in patients with GI cancer compared to controls, and this increase is consistent across each method of processing. **a** Flow cytometry analysis of both fresh and cryopreserved PBMC and whole blood which has undergone red cell lysis in patients with advanced GI cancer ($n = 41$) and healthy subjects ($n = 44$). Relative frequency is shown in **a** while **b** illustrates the data calculated for estimated absolute number; **c** representative *dot plots* of CD14⁺HLA-DR^{low} MDSC in fresh PBMC, cryopreserved PBMC or whole blood; **d** gating strategy; **e** gross morphology on cytopsin

Fig. 2. CD15⁺CD14⁻ cells but not CD15⁺CD14⁻CD11b⁺CD33⁺ are increased in patients with GI cancer compared to controls. Flow cytometry analysis of both fresh and cryopreserved PBMC and whole blood which has undergone *red* cell lysis in patients with advanced GI cancer ($n = 41$) and healthy subjects ($n = 44$). **a** Relative frequency CD15⁺CD14⁻; **b** estimated absolute number CD15⁺CD14⁻; **c** relative frequency CD15⁺CD14⁻CD11b⁺CD33⁺; **d** estimated absolute number CD15⁺CD14⁻CD11b⁺CD33⁺; **e** representative *dot plots* of CD15⁺CD14⁻CD11b⁺CD33⁺ in fresh PBMC, cryopreserved PBMC or whole blood; **f** gross morphology on cytopspin

Fig. 3. Correlations CD14⁺HLA-DR^{-/low} MDSC across method of processing. **a** Fresh versus cryopreserved PBMC; **b** PBMC versus whole blood lysis; **c** whether absolute number or relative frequency calculated

Fig. 4.

Analysis of CD14⁺HLA-DR^{-/low} MDSC in patients with GI cancer. CD14⁺HLA-DR^{-/low}, CD14⁺HLA-DR⁺, and CD15⁺CD14⁻CD11b⁺CD33⁺ cells were cocultured at different ratios with autologous anti-CD3/CD28⁻ cells. Supernatant was taken from the cultures before addition of 3H-thymidine and measured for IFN- γ by ELISA. Results shown are representative of 3 independent experiments

Table 1

Patients characteristics

Characteristics	Value
Patient number	41
Average age	61
Male/female	29/12
Stage IV	41
Disease type	
Colorectal cancer	22
Pancreatic cancer	8
Hepatocellular cancer	9
Gastric	2