A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells

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ABSTRACT

We recently demonstrated that human bone marrow (BM) cells can be treated in vitro with defined growth factors to induce the rapid generation of myeloid-derived suppressor cells (MDSCs), hereafter defined as BM-MDSCs. Indeed, combination of G-CSF + GM-CSF led to the development of a heterogeneous mixture of immature myeloid cells ranging from myeloblasts to band cells that were able to suppress alloantigen- and mitogen-stimulated T lymphocytes. Here we further investigate the mechanism of suppression and define the cell subset that is fully responsible for BM-MDSC-mediated immune suppression. This population, which displays the morphology and markers of promyelocytes, is however distinct from physiological promyelocytes that, instead are devoid of immunosuppressive function. In addition, we demonstrate that promyelocyte-like cells proliferate in the presence of activated lymphocytes and that, when these cells exert suppressive activity, they do not differentiate, but rather maintain their immature phenotype. Finally, we show that promyelocyte-like BM-MDSCs are equivalent to MDSCs present in the blood of breast and colorectal cancer patients and that increased circulating levels of these immunosuppressive myeloid cells correlate with worse prognosis and radiographic progression.
Introduction

One of the mechanisms of immune tolerance induced by cancer is based on the expansion of myeloid-derived suppressor cells (MDSCs), a heterogeneous population of immature myeloid cells, which accumulate in the blood, lymph nodes, bone marrow, and tumour sites in patients and experimental animals with neoplasia, capable of inhibiting both adaptive and innate immunity. 1-2 The heterogeneity of MDSCs has always been a hallmark of this cell population since its original description and many studies advanced that MDSCs might be composed of cells at several stages of differentiation of the myeloid lineage sharing the same functional properties. 2 To explain this heterogeneity, it was advanced that the patterns of cytokines/chemokines that arm myeloid cells with inhibitory function may be tumor dependent. For all these reasons, MDSCs have been shown to express different surface markers, depending both on the stage of myeloid development examined and/or the differentiation context provided by factors secreted by cancer cells.

In this respect, we recently demonstrated that the cytokines GM-CSF, G-CSF, and IL-6 allowed a rapid generation of MDSCs from precursors present in human bone marrow (BM) and that the immuno-regulatory activity of BM-derived MDSCs (BM-MDSCs) was dependent on the C/EBPβ transcription factor 3.

In the present study, we further characterized BM-MDSC mediated-immune suppression. Analogously to tumor-induced MDSCs, BM-MDSCs consist of a heterogeneous population of immature myeloid cells. We thus investigated whether the immune regulatory function of BM-MDSCs could be attributed to different myeloid subsets induced by cytokine treatment or rather to a specific subpopulation. Our results indicate that only one immature cell population, with morphology and phenotype resembling promyelocytes, is responsible for the whole immune suppression mediated by BM-MDSCs and that a cell population with a similar phenotype is expanded in breast and colorectal cancer patients.
MATERIAL AND METHODS

Bone marrow samples, human cohorts, and treatments

Fresh Bone Marrow (BM) aspirate samples with normal cytological characteristics were obtained from patients enrolled in the protocol AIEOP-BFM-ALL 2000, with suspected leukaemia or lymphomas, patients with lymphatic leukaemia after 78 days without recurrences, and patients with lymphatic leukaemia in post-BM transplantation (BMT) as a part of the diagnostic follow-up. Informed consent was obtained from all participating individuals, in compliance with the Declaration of Helsinki, prior to the study that was approved by the ethics committee of the Azienda Ospedaliera of Padova. For more details, see supplemental Methods.

BM aspirates were subjected to lysis to remove red blood cells, with a hypotonic solution of ammonium chloride. Cells were plated (2x10^6 cells/well) into a 24-well tissue culture plate (Becton Dickinson, NJ, US) in IMDM (Iscove's Modified Dulbecco's Medium, Gibco Invitrogen, California, USA) supplemented with 10% FBS (Fetal Bovine Serum, Gibco), 0.01 M HEPES, penicillin/streptomycin and β-mercaptoethanol. Cells were cultured with 40 ng/ml of G-CSF and GM-CSF, for four days at 37°C, 8% CO₂. Human recombinant GM-CSF was a gift from J.F. Parkinson (Bayer Healthcare Pharmaceuticals, Richmond, CA), human recombinant G-CSF was purchased from Sanofi Aventis (Milan, Italy).

Solid tumour patients

Peripheral blood specimens were collected from patients with: stage IV colorectal cancer (n=25) at the University of Miami Sylvester Comprehensive Cancer Center (UMSCCC) and stage IV breast cancer (n=25) at UMSCCC and at the Medical University of South Carolina Hollings Cancer
Center (HCC) starting a new line of therapy. Venous blood was collected in K2 EDTA-lavender top tubes (BD, Franklin Lakes, NJ) prior to initiation of therapy, after every other cycle of therapy and at the time of progression. Protocol Review Committees at HCC and UMSSC and Institutional Review Boards at both institutions approved this study. Written consent was obtained from all subjects.

**Solid tumour patients: statistical**

Random effects linear regression was used to model the association between MDSCs and time in responders and non-responders. The outcome was log (MDSCs) and predictors were time, response status, and an interaction between time and response. Random intercepts were included to account for correlation of repeated measures of MDSCs over time from the same patients. The coefficient on the interaction term was tested to determine if the change in MDSCs over time was the same versus different in responders and non-responders where an alpha level of 0.05 was used. Results were displayed graphically where each patient’s MDSC responses are shown over time, with the estimated regression model shown as solid straight lines. Standard model diagnostics were used to ensure that assumptions regarding residuals were met. Kaplan-Meier analysis was used to estimate survival distributions and differences in survival were tested using log-rank tests. Linear regression was used to determine association between circulating tumour cells (CTCs), Swenerton score (SS), and MDSCs, including estimation of slope, correlation coefficient and statistical significance of the association. Overall survival (OS) was defined as time of study enrolment to date of death.

**Flow cytometric analysis, Abs and reagents**

Cells were harvested and incubated with FcReceptor (FcR) Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) to saturate FcR and then labelled with monoclonal antibodies. For a
detailed description of the antibodies and of the methods used for labelling, see Supplemental Methods. Data acquisition was performed using FACSCalibur or LSRII flow cytometer (Becton Dickinson) and data were analyzed with FlowJo software (Tree Star, Inc. Ashland, OR).

**Confocal microscopy analysis**

Cells were fixed with 4% paraformaldehyde on polylysinated 14 mm round Menzel-Glaser glass in the dark for 15 min at room temperature and permeabilized for 5 min with 0.1% Triton X-100 (Sigma-Aldrich) in PBS, pH 7.4, before incubation for 1 h at room temperature with PBS 2% BSA (Sigma-Aldrich) and 5% normal goat serum (Sigma-Aldrich). Cells were stained with mAb diluted in PBS 0.5% Tween for 1 h at room temperature in the dark. Slides were then washed with the same buffer and incubated with the secondary antibodies for 1 h at room temperature in the dark. For a detailed description of the antibodies and of the methods used for confocal microscopy, see Supplemental Methods.

**Separation of BM-MDSCs subsets**

Lineage negative and positive fractions were separated from BM-MDSCs with Lineage Cell Depletion Kit (Miltenyi), a magnetic labeling system for the depletion of mature hematopoietic cells, following manufacturer’s instructions.

Myeloid fractions were also separated through cell sorting. Briefly, single cell suspensions of *ex vivo* BM or BM-MDSCs were stained with anti-CD11b-PE, anti-CD16-FITC and anti-CD3ε-PC7 and sorted on a MoFlo (DakoCytomation, Fort Collins, CO). CD11b\textsuperscript{low/-}/CD16\textsuperscript{-} cells, CD11b\textsuperscript{+}/CD16\textsuperscript{-} cells and CD11b\textsuperscript{+}/CD16\textsuperscript{+} cells were isolated excluding lymphocytes on the basis of CD3 expression and forward/side scatter parameters. All the fractions were obtained with a purity of at least 90%. Alternatively, myeloid populations were isolated through two consecutive magnetic
sorting; in the first round, BM-MDSCs were depleted of CD3ε+/CD19+/CD56+ lymphocytes, with a cocktail of immuno-magnetic beads obtained by combining anti-human CD3ε, CD19 and CD56 beads (Miltenyi). Subsequently, the CD3ε+/CD19+/CD56- fraction was depleted of CD11b+ cells with immuno-magnetic anti-human CD11b beads (Miltenyi).

**CFSE or CellTrace labelling and proliferation assay**

PBMCs were isolated from the peripheral blood of healthy donors by density centrifugation as described. Cell purity was checked by FACS analysis on forward/side scatter parameters, and viability by Trypan blue dye exclusion.

PBMCs were stained with carboxy-fluorosceindiacetate, succinimidyl ester (CFSE, Invitrogen Molecular Probe), (range 2-4 μM) or with 2 μM CellTrace™ Violet Cell Proliferation Kit (Invitrogen Molecular Probe), according to manufacturer’s instructions. For a detailed description of the methods of the labelling and proliferation assay, see Supplementary Methods.

**Isolation of human monocytes and granulocytes**

Human granulocytes and PBMC used for confocal microscopy analysis were obtained from peripheral blood of healthy donors, as described, while human monocytes were prepared from PBMC by short-term adherence to plastic. Briefly, PBMC were isolated from leukocyte-enriched buffy coats (Transfusion Center, Padova, Italy) and incubated for 1 h at 37°C and 5% CO₂ in flasks (Becton Dickinson, NJ, USA) using RPMI 1640 medium (Life Technologies) supplemented with 3% human serum. Non-adherent cells were removed by washing gently the flask with RPMI 1640 medium and adherent monocytes were removed for successive analysis.
Statistical analysis

The statistical significance to compare parametric groups was determined by the Student’s \( t \) test, while the Mann-Whitney \( U \) test was used to compare non parametric groups. Values were considered statistically significant with \( P<0.05 \). Absence of significance was not reported for brevity.
Results

BM-MDSCs down-regulate the CD3ζ chain expression in CD8+ T cells and require a cell-to-cell contact to inhibit alloantigen-activated T lymphocytes

BM-MDSCs consist of a heterogeneous combination of immature myeloid cells that, based on the combined staining with anti-CD11b and anti-CD16 antibodies, can be qualified as differentiating cells ranging from myeloblasts to band cells, albeit with variable proportions in different cultures. We initially addressed whether some of MDSC functional properties, described both in mice and cancer patients, were also shared by BM-MDSCs. In this regard, one of the mechanisms proposed to explain T-cell dysfunction induced by MDSCs is the proliferative arrest of antigen-activated T cells caused by loss of CD3ζ chain expression, a proximal T cell receptor (TCR)-associated signalling molecule necessary for correct assembly and function of the TCR itself.

To understand whether BM-MDSC-mediated immune suppression induced a decrease in CD3ζ expression, we set-up allogeneic MLRs, with CFSE-labelled PBMCs that were stimulated with a pool of γ-irradiated allogeneic PBMCs and co-cultured with γ-irradiated BM-MDSCs. After 7 days, cell cultures were harvested and CD3ζ chain expression was determined by intracellular staining after gating on CFSE+CD8+CD3ε+ cells. As shown in the representative experiment of Figure 1A, BM-MDSCs induced a marked decrease in T lymphocyte proliferation and this effect was accompanied by a significant reduction in the intracellular levels of CD3ζ chain in CD8+ T cells co-cultured with BM-MDSCs (Figure 1B); this result was also confirmed, by gating on CFSE+CD8+ T cells (data not shown). Moreover the reduction of CD3ζ chain expression was also accompanied by a decrease in the surface expression of CD3ε chain (Supplementary Figure 1A), implying that both chains might be the target of BM-MDSC activity; however, down-regulation of CD3ε chain expression was less evident in comparison to CD3ζ chain.
Several studies have shown that MDSCs inhibit immune responses through cell-to-cell contact.\textsuperscript{6-7} To address this point, we set up cultures with CFSE-labelled PBMCs, which were stimulated with anti-CD3/CD28 and co-incubated with BM-MDSCs, either in the presence or absence of a transwell. The insert ensures the flow of metabolites between the two chambers, so that if the immune suppression of BM-MDSCs is exclusively dependent on the release of soluble molecules, the separation would not prevent the suppressive program of BM-MDSCs. As assessed by the reduction of the CFSE dilutions in PBMC stimulated in the presence of BM-MDSCs compared to the control cultures without BM-MDSCs, an inhibitory effect was evident only in the presence of a cell-to-cell contact between lymphocytes and BM-MDSCs, since separation of BM-MDSCs by the insert did not affect T-cell proliferation (Figure 1C).

We also performed allogeneic MLRs in which the levels of suppression were evaluated through \textsuperscript{3}H\textsuperscript{TdT}R incorporation. MLRs were set up with different combinations of responder and stimulator PBMCs. In these experiments the proliferative rate of responder PBMCs varied, most likely as a result of the different HLA mismatches between effectors and stimulators, which influenced the magnitude of allogeneic response. Interestingly, we observed that in the presence of a high proliferation rate of responder PBMCs (higher than 30x10\textsuperscript{3} cpm), BM-MDSCs could exert a significantly higher suppression of the proliferation, in comparison to a lower proliferation rates of responder lymphocytes (Figure 1D). Indeed, when we evaluated the ability of BM-MDSCs to suppress CD3/CD28-mediated T lymphocyte activation, i.e. a condition in which T lymphocytes are massively activated, suppression was achieved in more than 90\% of the cases, i.e. in a higher proportion of cases compared to alloantigen-specific MLRs (data not shown). These results suggest that MDSCs become fully competent in their suppressive function only in the presence of strongly activated T lymphocytes.

**The Lineage negative fraction of BM-MDSCs is responsible for the immune suppressive activity**
As described above, the gradual increase of CD11b and CD16 expression is used to distinguish among all the differentiation stages of myeloid commitment. CD16 is considered a marker for mature myeloid cells and thus the CD11b+/-CD16+ cell subset represents a more immature myeloid population than CD11b+/-CD16+ cell subset. We observed that the in vitro expansion of BM-MDSCs with the combination of G-CSF + GM-CSF gave rise to a significant increase in immature CD11b+/CD16- elements (Figure 2A, \( P<0.001 \) compared to untreated BM), and that the presence of these cells was correlated with induction of suppressive activity (data not shown).

Interestingly, other groups previously described in cancer patients an expansion of Lineage (Lin) negative population endowed with suppressive activity. We thus decided to explore the function of more immature subsets among BM-MDSCs after cell enrichment by immuno-magnetic sorting with a cocktail of antibodies targeting Lineage antigens, with the aim to deplete mature myeloid populations and B, T, and NK lymphocytes from BM-MDSC cultures. This negative selection procedure yields a population of cells enriched in haematopoietic stem cells and very early myeloid progenitors that are CD11b\(^{low/-}\)/CD16- (Figure 2B). While the cell purity is rather high in the Lin- fraction, the Lin+ fraction was heterogeneous and still contained lymphocytes, mature, and immature myeloid cells. The morphology of the different populations was examined by May-Grünwald-Giemsa staining of cytospin cell preparations and confirmed that unsorted BM-MDSCs were composed of both mononuclear and polymorphonuclear cells, whereas the Lin- fraction was mainly composed of large mononuclear cells (Figure 2B).

To test the suppressive activity of BM-MDSC fractions we measured the proliferation of T cells (allogeneic with respect to BM-MDSCs) by CFSE dilution after CD3/CD28 stimulation in the presence of either unsorted, Lin- or Lin+ BM-MDSC cell subsets. In cultures of T cells stimulated with anti-CD3/CD28, the addition of whole BM-MDSCs caused both a moderate increase in undivided T cell fraction and a strong reduction in the numbers of CFSE+ cells (Figure 2C and 2D). The Lin- fraction was endowed with the highest suppressive activity compared to both the unsorted
population and the Lin\(^+\) subset, which basically had the same suppressive ability of the unsorted BM-MDSCs (Figure 2C and 2D).

We also observed that levels of CD3\(\varepsilon\) chain expression in activated T cells suppressed by the Lin\(^-\) subset of BM-MDSC were constantly reduced in terms of MFI (Figure 2E) in all the experiments performed. This decrease in CD3\(\varepsilon\) expression was also accompanied by a significant reduction in the percentage of CD3\(\varepsilon^+/CFSE^+\) cells (Figure 2F), therefore suggesting that suppression by the Lin\(^-\) subset of myeloid cells was mediated through a profound alteration of signalling machinery associated with a significant reduction in the numbers of CD3\(^+\) T lymphocytes.

**Suppressive activity of BM-MDSCs is entirely contained within the CD11b\(^{low/-}\)/CD16\(^-\) cell subset**

Experiments carried out with the Lin\(^-\) subset of BM-MDSCs highlighted that cells with the strongest suppressive activity were present in this fraction, supporting data from other laboratories showing that MDSCs obtained from cancer patients can be traced among Lin\(^-\) cells.\(^9\) However, this separation protocol does not allow to distinguish various differentiation stages during myeloid commitment. Therefore, to find out whether suppressive activity of BM-MDSCs was either shared by a number of immature subsets or limited to a specific differentiation stage, we separated defined myeloid subsets through cell sorter.

We sorted three different myeloid fractions from fresh BM and cultured BM-MDSCs, based on the expression levels of CD11b and CD16 antigens: the low/negative fraction CD11b\(^{low/-}\)/CD16\(^-\), the intermediate subset CD11b\(^+\)/CD16\(^-\) and the double positive fraction CD11b\(^+\)/CD16\(^+\), as shown in Figure 3. Since BM-MDSCs do not contain mature granulocytes (CD11b\(^+\)/CD16\(^{high}\)), which are instead present in BM cells, we excluded from the analysis the mature granulocyte population (Figure 3A and 3B).
May-Grünwald-Giemsa staining revealed that both unsorted fresh BM cells and cultured BM-MDSCs had a heterogeneous morphology, as confirmed by other phenotypical features; moreover, in our cell cultures we never found contaminating CD14+/CD15- macrophages that could contribute to suppressive activity of BM-MDSCs (data not shown). The CD11b<sup>low/-</sup>/CD16<sup>-</sup> subset isolated from fresh BM cells comprised cell elements with the appearance of myeloid progenitors and promyelocytes, while the corresponding subset isolated from BM-MDSCs contained basophilic cells, resembling promyelocytes (Figure 3A and 3B). The CD11b<sup>+</sup>/CD16<sup>-</sup> subset separated from fresh BM contained myelocytes, metamyelocytes, eosinophils, and monocytes, whereas BM-MDSCs included mainly cells resembling monocytes and eosinophils. At last, metamyelocytes and band cells were present among CD11b<sup>+</sup>/CD16<sup>+</sup> cells isolated from both populations.

When we tested the potential suppressive activity of the sorted subsets, only CD11b<sup>low/-</sup>/CD16<sup>-</sup> cell population isolated from BM-MDSCs was able to suppress the proliferation of activated T cells, whereas the CD11b<sup>+</sup>/CD16<sup>-</sup> and CD11b<sup>+</sup>/CD16<sup>+</sup> cell subsets purified from BM-MDSCs were completely devoid of suppressive activity (Figure 3C). Cumulative data reported in figure 3C demonstrate that the entire suppressive activity of BM-MDSCs is contained within a single subset of cytokine-conditioned promyelocytes, which were able not only to block lymphocyte proliferation, but also to affect IFN-γ production and to induce T cell apoptosis (data not shown).

None of the three corresponding subsets isolated from fresh BM cells was able to interfere with T lymphocyte proliferation (Figure 3A), further highlighting that priming of BM cells with cytokines is mandatory to induce immuno-regulatory MDSCs.

**Cytokine–stimulated CD11b<sup>low/-</sup>/CD16<sup>-</sup> cell subset consists of immature and large mononuclear myeloid cells**

The phenotype of the suppressive CD11b<sup>low/-</sup>/CD16<sup>-</sup> cell subset was further analyzed by flow cytometry. In the course of our attempt to increase the purity and minimize the manipulation of
sorted cells, we observed that the suppressive CD11b\textsuperscript{low/-}/CD16\textsuperscript{-} subset could be also separated through a progressive sorting with magnetic beads in which BM-MDSCs were first depleted of CD3\textsuperscript{+}, CD19\textsuperscript{+}, and CD56\textsuperscript{+} cells, and the resulting population was then depleted of CD11b\textsuperscript{+} cells. The remaining, negatively selected cell population (CD11b\textsuperscript{low/-} BM-MDSCs) had the same phenotypic and suppressive characteristics of the sorted CD11b\textsuperscript{low/-}/CD16\textsuperscript{-} BM-MDSCs (Supplementary Figure 1B).

CD11b\textsuperscript{low/-}/CD16\textsuperscript{-} cells sorted from fresh BM had a peculiar morphology, characterized by a high side scatter, occupying the region of normal granulocytes, but after four days of culture with G-CSF + GM-CSF, these cells gradually reduced their side scatter and increased the forward scatter, thus moving to the monocyte region (Supplementary Figure 1C and Figure 4). However, the surface phenotype of the suppressive CD11b\textsuperscript{low/-}/CD16\textsuperscript{-} cells, separated from BM-MDSCs, indicated that this population lacked the expression of the monocytic marker CD14, and was positive for the CD15 granulocytic antigen (Figure 4A), thus implying that it had characteristics distinct from both mature monocytes and granulocytes.

The suppressive subset was negative for the lineage markers and expressed the myeloid markers CD13 and CD33; IL4R\alpha chain was expressed at low intensity, as previously demonstrated.\textsuperscript{4,10} The expression of CD66b was down-regulated in the cytokine-treated subset, as compared to the same population sorted from fresh BM cells, while CD117 increased its expression after induction with G-CSF + GM-CSF (Figure 4A and Supplementary Figure 1C). Two discrete populations with different expression of HLA-DR molecule (low or negative) were noted. The suppressive cells expressed CD39 but lacked CD73, which are both expressed on the surface of human T regulatory (Tregs) lymphocytes.\textsuperscript{11} Finally, this subset did not express B7-H1, and slightly expressed B7-H2 and B7-H3 (Figure 4A), members of B7 family that are able to regulate immune responses and induce immunological tolerance.\textsuperscript{12}
We also estimated the proliferative rate of the CD11b\textsuperscript{low-}/CD16\textsuperscript{−} cells by intracellular staining of Ki-67\textsuperscript{+} cells and observed that 97% of the cells expressed this antigen, indicating that these cells were actively proliferating in response to cytokine treatment (Figure 4A).

It is known that during the differentiation of polymorphonuclear leukocytes, myeloblasts and promyelocytes proliferate and generate primary granules,\textsuperscript{13} and one of the proteins contained in these granules is the enzyme myeloperoxidase (MPO). CD11b\textsuperscript{low-}/CD16\textsuperscript{−} cells isolated from BM-MDSCs induced from different human samples could assume either one of two main morphological patterns: cells with large nuclei and reduced cytoplasm, without granules, and cells with more abundant cytoplasm and a discrete number of cytoplasmic granules (Figure 4B). By confocal microscopy, the MPO protein appeared prevalently located, as expected, within granules; however, the cytoplasm of the agranular cells showed a diffuse pattern of expression (Figure 4B). Classic, mature granulocytes presented the typical poly-lobated nucleus surrounded by azurophilic granules containing MPO, while the MPO expression in monocytes seemed to be confined within the lysosomal compartment (Figure 4B), as recently described.\textsuperscript{14}

We also used a novel monoclonal antibody against human arginase 1 (ARG1) to evaluate whether the enzyme was present in CD11b\textsuperscript{low-}/CD16\textsuperscript{−} cells and whether it was co-expressed with MPO, as suggested by some studies.\textsuperscript{15} The analysis of ARG1 in this suppressive subset showed cells with different expression pattern: in the majority of cells this enzyme was partially co-localized with MPO, but some cells stained negative for ARG1 (Figure 4B). In contrast, mature granulocytes showed a complete co-localization of the 2 enzymes, while monocytes did not express ARG1, as already described.\textsuperscript{15} The CD11b\textsuperscript{low-}/CD16\textsuperscript{−} cells isolated from fresh BM cells stained positive for ARG1 but showed a decrease of MPO expression, as compared to the same population separated from BM-MDSCs (Figure 4C). In comparison, freshly isolated CD11b\textsuperscript{+}/CD16\textsuperscript{−} and CD11b\textsuperscript{+}/CD16\textsuperscript{+} cells, which represent more advanced maturation stages, presented a progressive increase in the signals for both enzymes (Figure 4C).
Activated T lymphocytes sustain the proliferative rate of the BM-MDSC CD11b<sup>low/-</sup> cells and block their differentiation process

The activation level of T lymphocytes appears to be critical to drive the suppressive activity of BM-MDSCs (Figure 1D). To investigate the relationship between T cell activation and MDSC suppression, we set-up experiments in which either resting or activated T cells, labelled with CellTrace™ fluorescent stain, were co-cultured with BM-MDSC cell subsets, so that we could trace unambiguously the myeloid and lymphoid cell populations in the co-culture and evaluate proliferation after four days. As expected, we observed a high proliferation rate of activated T cells in the presence of CD11b<sup>+</sup> BM-MDSCs, evaluated in terms of CellTrace dilution and, instead, a reduction in the proliferation of T cells co-cultured with the suppressive CD11b<sup>low/-</sup> BM-MDSC fraction (Figure 5A, second line). We also assessed the cell proliferation of the myeloid cell subsets in the cultures by analyzing Ki-67 expression on gated CD3<sup>-</sup>/CellTrace<sup>-</sup> cells. Interestingly, while the CD11b<sup>+</sup> cell subset of BM-MDSCs did not proliferate in culture with either activated or resting T cells (Figure 5A, third lane), the suppressive CD11b<sup>low/-</sup> cell subset maintained a discrete proliferative capacity in presence of resting T cells, which was even increased the presence of activated T cells (geometric mean fluorescent intensity 14.5 vs 29.2, respectively) suggesting that T cell activation supports the proliferation of suppressive cells.

To understand whether suppressive BM-MDSC CD11b<sup>low/-</sup> cells maintain their phenotype or rather differentiate to more mature myeloid subsets when co-cultured with activated T lymphocytes, we analysed the expression of differentiation myeloid markers after cell co-culture. After 4 days of culture we observed that, only in the presence of activated T cells, promyelocyte-like cells maintained their level of immaturity, as demonstrated by the levels of expression in the markers CD11b and CD16; moreover, whereas HLA-DR and CD34 were maintained or even increased, CD66b, a marker of secondary granules, was down-regulated in the presence of activated T cells (Figure 5B). Control cultures of immature promelocyte-like cells in the absence of lymphocytes
showed a differentiation pattern similar to myeloid cells co-cultured with resting T cells (Figure 5B), thus suggesting that only the presence of activated T cells is able to block the default differentiation process of immature promyelocyte-like cells.

Taken together these data indicate that promyelocyte-like cells proliferate, their functional activity is associated with a delay in the differentiation pathway, and both proliferation and block in the differentiation pathway account for their expansion and maintenance of an immature phenotype.

**Increased circulating MDSC levels correlate with progression and worse clinical prognosis**

The suppressive myeloid population of BM-MDSCs was mainly Lin−, CD11b\textsubscript{low/−}, HLA-DR\textsubscript{low/−}, but positive for myeloid markers CD33 (Figure 4), a subset with phenotype similar to MDSCs previously described in tumour-bearing patients.\textsuperscript{9,16} Indeed, in the blood of stage IV breast cancer patients we could clearly identify a Lin−, HLA-DR−, CD33+, CD11b+ MDSC population resembling \textit{in vitro} generated BM-MDSCs (Figure 6A and 6B). We previously showed that this cell subset correlated with clinical tumour stage,\textsuperscript{16} but it is still unknown whether circulating Lin−, HLA-DR−, CD33+, CD11b+ cells also correlate with either metastatic tumour burden or overall survival in cancer patients. We thus evaluated the relationship between MDSCs and CTCs measured by the CellSearch™ assay, a strong, independent predictor of survival in patients with advanced breast cancer.\textsuperscript{17-18} In a cohort of patients with stage IV breast cancer (n=25) CTCs (CellSearch®) and MDSCs were analyzed simultaneously. A generalized estimating equation (GEE) regression model was created and fitted individually for each predictor. There was a significant correlation between circulating MDSCs and CTCs (\(P=0.0001\), Figure 6C). As expected, no significant correlation was observed between CTCs and Swenerton score (SS) (\(P=0.92\)); indeed, CTCs are not a measure of metastatic tumour burden and levels do not correlate with SS in patients with advanced breast cancer.\textsuperscript{17-19} To ascertain whether high circulating MDSC levels were associated with poorer prognosis, survival estimates in breast cancer dataset were calculated using MDSC levels (\%),
drawn either prior to starting a new line of therapy or levels at the last blood draw. Patients with circulating MDSCs > 3.17% (median) at baseline had a poorer overall survival (OS) than patients with circulating MDSCs ≤ 3.17%, with median overall survival times of 5.5 months (95% CI 0.5 to 11.3) and 19.32 months (95% CI 8.7 to INF), respectively (P=0.048, Figure 6D). Likewise, elevated MDSC levels at the last visit were also associated with a significantly poorer OS (P=0.018), with median survival times of 3.8 months (95% CI 0.5 to 7.7) and 16.7 months, respectively (95% CI 3.8 to INF) (Figure 6E).

We then moved to examine another group of cancer patients. In a cohort of patients with stage IV colorectal cancer (n=25) we also found that baseline levels of MDSCs prior to starting chemotherapy also correlated with poorer OS (Figure 6F). Patients with circulating MDSC levels greater than the median value (2.54%) had significantly shorter median OS times than those with levels below (35.6 vs. 14.3 months; P=0.025, Figure 6F). We next investigated whether levels of circulating MDSCs over time in patients with advanced solid tumors receiving systemic chemotherapy correlated with clinical outcomes. MDSC levels in the same cohort of stage IV colorectal cancer patients were drawn every other chemotherapy cycle. Patients underwent routine radiographic assessments as clinically indicated, typically every 2-3 months. Over time MDSC levels were significantly higher in patients who had radiographic evidence of progressive disease compared to levels in patients who achieved either a complete (CR) or partial response (PR) as their best radiographic response (P=0.015, Figure 6G). Taken together, these clinical data suggest, for the first time, that circulating MDSC levels, phenotypically similar to those described in human BM experiments, are clinically relevant and appear to: (i) increase over time in patients with progressive disease; (ii) correlate with an established prognostic marker (CTCs) in advanced breast cancer; and (iii) persistently high or increasing levels following chemotherapy are associated with poorer survival.
Discussion

Aim of our study was to dissect the differentiation stage of the suppressive myeloid cells by taking advantage of the in vitro generation of MDSCs from BM precursor.\textsuperscript{3} The suppressive activity is fully induced in BM-MDSCs only after direct contact with activated T lymphocytes, indicating that suppressive cells are primed by the activation status of target cells and by a cell membrane signal, a result analogous to data obtained with mouse MDSCs.\textsuperscript{10} Moreover, this result is in line with the consideration that myeloid suppressors play a role in turning off potential harmful immune responses carried out by activated T lymphocytes.\textsuperscript{1,20} Our results extend this idea leading to hypothesize that the activity of MDSCs is dictated by the activation level of the T lymphocytes. In fact, suppressive cells are unable to harm resting lymphocytes, and the direct contact between these two cells might ensure that the signals delivered by MDSCs are confined only to target cells and not to bystander cells.

It is known that anti-CD3 and anti-CD28 activated T cells secrete GM-CSF and IL-6,\textsuperscript{21} cytokines that drive the BM-MDSCs development from BM; on the other hand, we observed that also BM-MDSCs are able to produce in vitro IL-6 during the culture with growth factors (data not shown); moreover, it was recently demonstrated that T lymphocytes, upon TCR activation, produce soluble factors that enhance fibroblasts production of IL-6.\textsuperscript{22} An attractive hypothesis is that activated T cells release cytokines that might sustain the induction of the suppressive cells and/or maintain their tolerogenic activity, an auto-regulative loop that has already been demonstrated in a different system.\textsuperscript{10}

Among different cytokine combinations, GM-CSF and IL-6 were the most effective in generating, from healthy donor PBMCs, suppressive CD33\textsuperscript{+} cells that inhibited the proliferation and IFN-\gamma production by autologous human T cells after CD3/CD28 stimulation.\textsuperscript{23} These cells were large mononuclear CD11b\textsuperscript{+} HLA-DR\textsubscript{low} CD66b\textsuperscript{+} cells with basophilic and granular cytoplasm. When molecules and enzymes participating in inhibitory pathways were evaluated by either qRT-
PCR or cytofluorometry, NOS2, TGFβ, VEGF, and NOX2 were found to be up-regulated whereas no significant changes in comparison with the non-suppressive, normal CD33+ cells were detected for B7-H1, B7-H2, and B7-H4. It thus appears that cytokines might induce different cells with immuno-regulatory properties when acting on either BM or blood circulating precursors. Whether these cells represent stages of the same differentiation process is an issue that requires further investigation. It is clear, however, that MDSCs described in the present study are different from either fully differentiated or activated granulocytes and monocytes.

We observed that the reduction of lymphocyte proliferation induced by BM-MDSCs is accompanied by a decreased expression not only of the CD3ζ chain and by a reduction in absolute numbers of T lymphocytes. It is known that the absence or reduction of CD3ζ chain impairs T cell signalling and contributes to immune cell dysfunction and evidence is accumulating that expression of the CD3ζ chain is markedly decreased in both peripheral blood and tumour-infiltrating lymphocytes in patients with different types of tumours. Significantly less is known about the importance of the ε chain of the CD3/TCR complex in tumour immunity, even if some reports have shown down-regulation of CD3ε chain in patients with lung cancer and severe combined immunodeficiency.

In this study we addressed the extent and relevance of cell heterogeneity of MDSCs, generally accepted as a common feature of this cell population, with the aim to define whether immuno-regulatory properties of human BM-MDSCs can be attributed to one or more cell subsets. We observed that the treatment of BM cells with G-CSF + GM-CSF resulted in a significant accumulation of immature myeloid cells (Figure 2A). In this regard, we observed that one of the differences existing between untreated BM and BM-MDSCs was represented by the expansion of immature myeloid cells expressing low levels of CD11b and negative for CD16, a phenotype that is typically associated with myeloblasts and promyelocytes, but not with more differentiated cells (Figure 2A and 3). The accumulation of immature CD11b+/CD16- cells in BM-MDSCs led us to test...
the hypothesis that this myeloid population was entirely responsible for the suppression exerted by BM-MDSCs. Our results clearly indicate that the only subset responsible for the immune suppression exerted by BM-MDSCs is contained within the CD11b<sup>low/-</sup>/CD16<sup>-</sup> cell population of BM-MDSCs, and other immature but more differentiated subsets, such as CD11b<sup>+</sup>/CD16<sup>-</sup> and CD11b<sup>+</sup>/CD16<sup>+</sup> cells, are completely devoid of suppressive activity; accordingly, this subset was able to proliferate in the presence of activated T cells, a feature that is lost by more mature subsets. Interestingly, we also observed that BM-MDSC CD11b<sup>low/-</sup> were able to exert suppressive activity both on CD4<sup>+</sup> and CD8<sup>+</sup> subsets and to induce apoptosis of T cells, an event that was marginal in T cells activated without BM-MDSC CD11b<sup>low/-</sup> (data not shown).

Remarkably, the suppressive cell subset had a phenotype corresponding to promyelocytes and, indeed, these cells morphologically resembled promyelocytes, with a large regular, symmetric nucleus, high nucleus/cytoplasm ratio, and basophilic cytoplasm. We sorted the corresponding subset from fresh BM (CD11b<sup>low/-</sup>/CD16<sup>-</sup>), containing mainly promyelocytes. The two subsets were indistinguishable from a morphological point of view, but they differed completely in terms of ability to suppress activated T lymphocytes, since normal promyelocytes were unable to exert any inhibitory activity (Figure 3A). Here, for the first time, we show that MDSCs proliferate, that this activity is linked to a block in the differentiation pathway, and, interestingly, that both suppression and maturation depend on the activation status of the T lymphocytes (Figure 5).

Since suppressive activity of myeloid cells is limited to a specific subset of promyelocytic-like cells, and the more differentiated population are completely devoid of regulatory activity, this implies that suppressive activity is not a stable trait of MDSCs, but rather a transitory state, possibly ending or being sustained according to local signals coming from the microenvironment where the immature cells migrate. These results, therefore, suggest that MDSCs maintain a plasticity that enable them to differentiate and suspend their tolerogenic program.

We found that ARG1 was expressed in the suppressive CD11b<sup>low/-</sup>/CD16<sup>-</sup> cell subset and partially co-localized with MPO, suggesting its main distribution within primary granules, as
suggested by Munder et al.\textsuperscript{15} However, in comparison with the same population isolated from fresh BM, this subset had an increased expression of MPO, which presented both an expected granular and an uncommon agranular localization. We are currently evaluating whether the altered expression of these enzymes is only a marker of these cells or whether it is related also to their suppressive function.

Finally, one of the challenges in studying MDSCs in humans has been that the phenotype is not as well defined as in mice. We show that MDSCs, defined as Lin\textsuperscript{−}, HLA DR\textsuperscript{−}, CD33\textsuperscript{+}, CD11b\textsuperscript{+}, can be easily traced among blood circulating cells in patients with advanced breast and colorectal cancers, with levels correlating with clinical outcomes.
Acknowledgments

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Authorship contributions

Contributions: S.S. performed research, analyzed and interpreted data and wrote the manuscript; E.F. performed flow cytometry and confocal microscopy studies; C.M.D.M. performed flow cytometry analysis of all data from solid tumour patients, analyzed and discussed results; A.D. performed confocal microscopy study; L.P. performed research; A.R. generated monoclonal antibody against ARG1; S.F. performed bone marrow samples enrolment; G.B. discussed results and provided vital material for the study; P.Z. discussed the results; G.O. & E.G.M. performed statistical analyses of data from breast and colorectal cancer patients; A.J.M. obtained informed consent on all solid tumour patients, helped write results on relevant section, and edited manuscript; V.B. discussed and analyzed the results and wrote the manuscript; S.M. designed the study, analyzed and interpreted data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


FIGURE LEGENDS

Figure 1. Characterization of BM-MDSC-mediated immune suppression.

(A) CFSE-labelled PBMCs were stimulated with allogeneic γ-irradiated PBMCs without (left) or with (right) γ-irradiated BM-MDSCs added at a ratio of 1:1. After seven days, cell cultures were harvested, labelled with anti-CD3ε and analyzed in the CD3ε+/CFSE+ cell gate. The figure shows a representative experiment of cell division analysis out of three performed. The percentages of the undivided cells are indicated.

(B) After seven days of culture, cultures set-up as in (A) were labelled with anti-CD3ε, anti-CD8, fixed, and then labelled with anti-CD3ζ. Mean fluorescence intensity (MFI) of CD3ζ was calculated in the CFSE+/CD3ε+/CD8+ cell gate. On the left panel, black histogram represents the MFI of stimulated PBMCs without BM-MDSCs, while white histogram refers to MFI of stimulated PBMCs in presence of γ-irradiated BM-MDSCs. On the right panel, MFI values of CD3ζ are presented as mean ± SE of three independent experiments P =0.034, Student’s t test.

(C) PBMCs were labelled with CFSE and stimulated with coated anti-CD3 and soluble anti-CD28 (left) and cocultured with BM-MDSCs in the presence (right) or in the absence (center) of a transwell. After four days, cells were harvested, labelled with anti-CD3ε and analyzed in the CD3ε+/CFSE+ gate. The figure shows a representative experiment of three. The percentages of the undivided cells are indicated.

(D) Proliferation of alloactivated PBMCs co-cultured either with or without γ-irradiated BM-MDSCs was assessed by 3H-thymidine incorporation. White dots represent the proliferation of stimulated PBMCs without BM-MDSCs, while gray dots correspond to the proliferation of alloactivated PBMCs in presence of BM-MDSCs. Twenty independent experiments are shown with proliferation of alloactivated PBMCs < 30 x 10³ cpm (column 1 and 2), and 15 experiments with proliferation > 30 x 10³ cpm (column 3 and 4). P=0.01 and P<0.001, Mann–Whitney U test.
Figure 2. The Lineage negative subset contained within BM-MDSCs demonstrates potent suppressive activity.

(A) Flow cytometric analysis of BM cells cultured for four days with G-CSF + GM-CSF (BM-MDSCs) or without growth factors (NT BM). At the end of the culture, cells were harvested and labelled and the percentages of CD11b+/CD16- cells were calculated. The figure represents 22 independent experiments. $P \leq 0.001$, Student’s $t$ test.

(B) Flow cytometry profile of CD16 and CD11b expression and May-Grünwald-Giemsa staining on BM-MDSCs before and after immuno-magnetic depletion with Lineage antibody cocktail.

(C) Flow cytometry analysis of the proliferation of allogenic PBMCs, stained with CFSE and activated with anti-CD3 and anti-CD28 for four days, in the presence of either BM-MDSCs or the fractions Lin+$^+$ or Lin$^-$ sorted from BM-MDSCs. The figure, in which the percentages of undivided CD3$^+$/CFSE$^+$ lymphocytes are shown, represents one out of three independent experiments.

(D) Number of CD3$^+$/CFSE$^+$ events, obtained after activation of PBMCs with anti-CD3/CD28 and co-cultured in the presence of BM-MDSCs or the subsets Lin+$^+$ and Lin$^-$ sorted from BM-MDSCs. The figure, in which the black bars refer to undivided cells and gray bars to divided cells, represent the mean ± SE of six independent experiments. The values of $P$ are indicated in the figure, Mann–Whitney $U$ test.

(E–F) Evaluation of MFI of CD3$^+$ chain expression and percentage of the CD3$^+$/CFSE$^+$ cells in PBMCs stimulated with anti-CD3/CD28 in the presence of BM-MDSCs or the Lin+$^+$ and Lin$^-$ fractions. Mean ± SE of six independent experiments. All comparisons among BM-MDSCs containing cultures versus cultures without BM-MDSCs, $P = 0.041$ and $P = 0.009$ for (E) and (F), respectively, Mann–Whitney $U$ test.
Figure 3. CD11b<sup>low/-</sup>/CD16<sup>-</sup> phenotype defines the subset responsible for the immune suppression in BM-MDSCs.

(A) Flow cytometric evaluation of CD11b and CD16 markers in BM-MDSC or sorted CD11b<sup>low/-</sup>/CD16<sup>-</sup>, CD11b<sup>+</sup>/CD16<sup>-</sup> and CD11b<sup>+</sup>/CD16<sup>+</sup> cell populations from fresh BM samples (left), morphological analysis by May-Grünwald-Giemsa staining (central panel), and CFSE dilution proliferation assay (right panel) in which values reported on histograms represent the percentages of cells in the parental, undivided generation.

(B) Flow cytometric evaluation of CD11b and CD16 markers in BM-MDSCs or sorted CD11b<sup>low/-</sup>/CD16<sup>-</sup>, CD11b<sup>+</sup>/CD16<sup>-</sup> and CD11b<sup>+</sup>/CD16<sup>+</sup> cell populations from BM-MDSCs (left), morphological analysis by May-Grünwald-Giemsa staining (central panel), and CFSE dilution proliferation assay (right panel) in which values reported on histograms represent the percentages of cells in the parental, undivided generation.

(C) Suppression of allogenic CFSE<sup>+</sup> PBMCs activated with anti-CD3 and anti-CD28 and co-cultured in the presence of 1:1 ratio of the different populations sorted from human BM-MDSCs. The suppression was calculated analyzing the number of proliferating cells from generation 3 to generation 10, assumed to be 100% without BM-MDSCs. Mean ± SE of three independent experiments. \( P \leq 0.01 \), Student’s \( t \) test, all comparisons among BM-MDSCs containing cultures versus cultures without BM-MDSCs.

Figure 4. Phenotypic evaluation of the immune-suppressive subset CD11b<sup>low/-</sup>/CD16<sup>-</sup> contained within BM-MDSCs.

(A) Flow cytometry analysis of CD11b<sup>low/-</sup>/CD16<sup>-</sup> cells sorted from BM-MDSCs. The expression of putative MDSCs markers, markers of mature and immature myeloid cells and markers associated to tolerance was evaluated relative to isotype control (black histograms). In the figure is presented one representative out of two independent experiments.
(B) Confocal microscopy localization of MPO and ARG1 in CD11b\textsuperscript{low/-}/CD16\textsuperscript{-} cells, freshly isolated neutrophils and monocytes. Bars, 12 \( \mu \)m.

(C) Localization of MPO and ARG1 in CD11b\textsuperscript{low/-}/CD16\textsuperscript{-}, CD11b\textsuperscript{+}/CD16\textsuperscript{-}, and CD11b\textsuperscript{+}/CD16\textsuperscript{+} cells isolated from fresh BM samples determined by confocal microscopy. Bars, 20 \( \mu \)m.

Figure 5. T lymphocyte activation is driving BM-MDSC proliferation and immune suppression.

(A) CellTrace-labelled PBMCs were stimulated with anti-CD3/CD28 in the presence of BM-MDSC CD11b\textsuperscript{+} and CD11b\textsuperscript{low/-} cell subsets, added at a ratio of 1:1. After three days, cell cultures were harvested, labelled with anti-CD3\textsuperscript{\epsilon} and analyzed in the CD3\textsuperscript{\epsilon}/CellTrace\textsuperscript{-} gate (M) and in the CD3\textsuperscript{\epsilon}/CellTrace\textsuperscript{+} (T) cell gate. The numbers indicated in the upper graphs refer to the percentage of cells gated on either T cells (T) or on myeloid cells (M). The central histograms show the profile of CellTrace dilution of either resting or stimulated T cells (gate T) co-cultured with BM-MDSCs CD11b\textsuperscript{+} and CD11b\textsuperscript{low/-} subsets. Black and grey curves refer to undivided and proliferating cells, respectively. The lower histograms show Ki-67 expression in BM-MDSCs CD11b\textsuperscript{+} and CD11b\textsuperscript{low/-} subsets (gate M) co-cultured with either resting or stimulated T cells. Black histograms, isotype control. The figure shows a representative experiment out of three performed.

(B) Flow cytometry evaluation of CD11b, CD16, HLA-DR, CD34, and CD66b markers in CD11b\textsuperscript{low/-} cell subset sorted from BM-MDSCs either before or after the co-culture with resting or anti-CD3/anti-CD28 activated T cells. The expression of these markers was compared to the autofluorescence signal (black histograms). In the figure one representative out of three independent experiments is presented.

Figure 6. Increase in circulating MDSC levels over time in patients with advanced solid tumors is associated with decreased survival times and radiographic disease progression.
Gating strategy for BM-MDSCs (A) and whole blood MDSCs (B) is shown on a representative flow cytometric plot. (C) Random effects regression model and correlation between MDSCs and CTCs. Flow cytometric analysis was performed on peripheral whole blood in a separate cohort of stage IV breast cancer patients (n=25) prior to initiation of therapy and at defined intervals during therapy. Blood for CTC determination via the CellSearch® was simultaneously drawn. A significant correlation was found between circulating MDSC levels (%) and CTCs ($P=0.0001$). (D) Survival analysis by circulating MDSC levels (%) at first blood draw in patients with stage IV breast cancer starting a new line of systemic chemotherapy (n=26). Survival estimates by median percent MDSCs (≤3.17% and >3.17%) using the first MDSCs observation ($P=0.048$). (E) Survival estimates by median percent MDSCs (≤3.04% and >3.04%) using MDSCs levels drawn at the last visit ($P=0.018$). (F) Survival analysis by circulating MDSC levels at time of study enrollment in patients with stage IV colorectal cancer. Survival estimates by medial percent MDSCs (≤2.54% and >2.54%). (G) Analysis of relationship between changes in circulating MDSC levels over time and best radiographic response in patients receiving systemic chemotherapy (n=25). Plot of MDSCs over time by “best response” defined as patients who achieved complete (CR) or partial radiographic response (PR) while on systemic therapy versus those who did not. MDSCs were drawn prospectively after every other cycle of therapy. Over time circulating MDSCs were significantly higher in non-responders versus patients with CR or PR as best response ($*P= 0.015$ comparing slopes).
Figure 1

A

![Graph showing alloactivated T-cells and BM-MDSC](Image)

B

![Graph showing gated on CD3ε+ CD8+](Image)

C

![Graph showing cells with transwell](Image)

D

![Graph showing [H]-Thymidine Incorporation](Image)
Figure 2
Figure 3

A  fresh BM

B  BM-MDSC

C  Activated T-cells

+BM-MDSC

+CD11b/CD16

+CD11b/CD16

+CD11b/CD16

# of cells

CD16

CD11b

CFSE

P<0.002
Figure 4

A

B

C
Figure 5

A

[Diagram with flow cytometry plots showing CD11b expression in resting and stimulated T cells, and gating on T cells (T) and myeloid cells (M).]

B

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<tr>
<td>4</td>
<td>Resting T cells</td>
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<td>Activated T cells</td>
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[Flow cytometry plots showing CD11b, CD16, HLA-DR, CD34, and CD68 expression over days of co-culture.]
Figure 6

A

B

C

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D

E

F

G

*Responders

Non-responders
A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells

Samantha Solito, Erika Falisi, Claudia Marcela Diaz-Montero, Andrea Doni, Laura Pinton, Antonio Rosato, Samuela Francescato, Giuseppe Basso, Paola Zanovello, Georgiana Onicescu, Elizabeth Garrett-Mayer, Alberto J. Montero, Vincenzo Bronte and Susanna Mandruzzato