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IMMUNE ESCAPE MECHANISMS IN HEMATOLOGICAL DISEASES: ROLE OF THE MYELOID DERIVED SUPPRESSOR CELLS AND TUMOR MICROENVIRONMENT

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SUMMARY

BACKGROUND

The interactions between the immune system and the tumor cells occur through complex events that lead to tumor eradication or immune evasion by cancer [1]. Generally, $CD8^+$ cytotoxic T and $CD4^+$ helper T lymphocytes inhibit tumor development by production of interferon (IFN)-γ and cytotoxins [2], but chronic inflammation and immune suppressive factors may promote cancer development [3]. In fact, chronic inflammation within the tumor microenvironment inhibits tumor elimination and enhance transformation of cancer. NFKB signaling in hematopoietic cells has been reported to play a critical pro-carcinogenic role producing various pro-inflammatory cytokines and chemokines (such as TNF, IL1, IL6, and CSF1, type I interferons and IL8) that promote carcinogenesis [4]. Immune system plays a critical role with a dual capacity to both promote and suppress tumor growth. It is well established that tumor cells differ from their normal counterparts in antigenic composition. The immune system is able to recognize and destroy the most vulnerable cancer cells [5], but, on the contrary of not transformed cells which maintain a stable antigenic profile, new antigens are constantly generated in tumor cells as a consequence of genetic instability. Until there is a balance between immune control and tumor growth, the tumor dormancy is maintained [6]. However, tumor cells can evade the immune response through multiple mechanisms, resulting in overt clinical cancer.

Inhibition of tumor antigen presentation

Genetic and epigenetic alterations that are characteristic of all cancers provide a diverse set of antigens that the immune system can use to distinguish tumour cells from their normal counterparts [7]. In addition to antigen loss, downregulation of proteosome subunits transporter associated with antigen presentation (TAP) [8, 9] or mutation or deletion of B2-microglobulin genes [10, 11] can inhibit presentation of MHC-peptide complexes on surface of tumor cells [12].

Immune checkpoints

Immune checkpoints are crucial for the maintenance of self-tolerance to avoid autoimmunity and protect tissues from damage when the immune system is responding to infection [7]. Tumors dysregulate expression of immune-checkpoint proteins which are co-stimulatory and inhibitory signals that regulate T cell receptors (TCR)[13]. Indeed, inhibitory ligands regulating T cell effector functions are commonly overexpressed on tumour cells or on non-transformed cells in the tumour microenvironment (fig1).

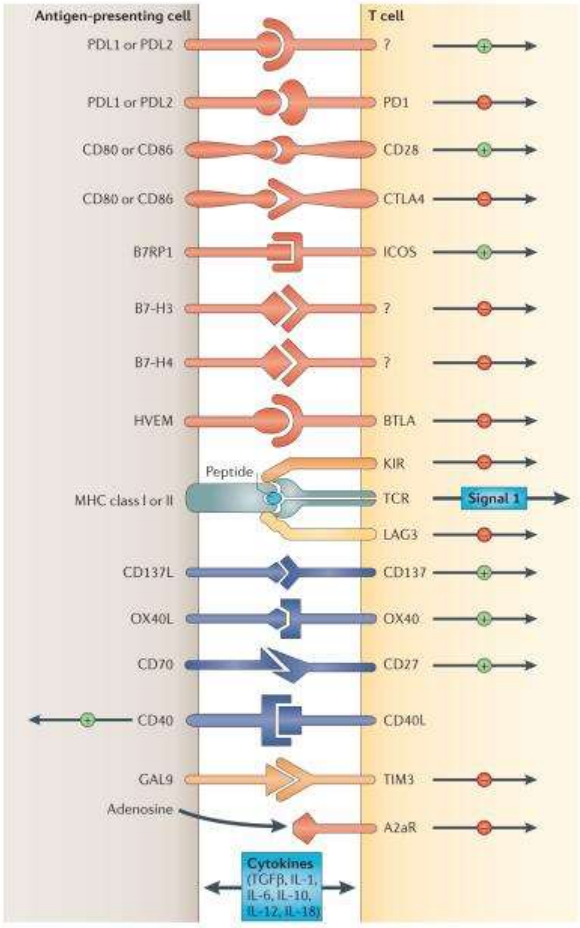

Figure 1. Co-stimulatory and inhibitory interactions which regulate T cell responses. Communication between T cells and antigen presenting cells (APC) is bidirectional. A2aR, adenosine A2a receptor; B7RP1, B7-related protein 1; BTLA, B and T lymphocyte attenuator; GAL9, galectin 9; HVEM, herpes virus entry mediator; ICOS, inducible T cell co-stimulator; IL, interleukin; KIR, killer cell immunoglobulin-like receptor; LAG3, lymphocyte activation gene 3; PD1, programmed cell death protein 1; PDL, PD1 ligand; TGFβ, transforming growth factor-β; TIM3, T cell membrane protein 3.

PD1 is one of the most studied immune checkpoint; it is expressed on a large proportion of tumour-infiltrating lymphocytes. By upregulating ligands for PD1 (PDL1), cancer cells inhibit antitumour immune responses. Several clinical trials of PD-1/PD-L1 signal-blockade agents have exhibited dramatic antitumor efficacy in patients with certain types of solid or hematological malignancies [14].

Enzymes as immune-inhibitory molecules

Another category of immune-inhibitory molecules includes metabolic enzymes, such as arginase 1 and indoleamine 2,3-dioxygenase (IDO). IDO is expressed by both tumour cells and infiltrating myeloid cells and inhibits immune responses through depletion of aminoacids essential for T cells activation and proliferation.

The tumor microenvironment as immunological barrier

The tumor microenvironment, once established, represents a consistently effective barrier to immune cell functions. Some mechanisms responsible for immune suppression are directly mediated by factors produced by tumors, whereas others result from alterations of normal tissue homeostasis occurring in the presence of cancer. Immune cells have also been identified as contributing to the tumor-associated microenvironment via dysregulation of immune-mediated responses. Macrophages, dendritic cells, natural killer (NK) cells, myeloid-derived suppressor cells, and regulatory T cells (T_{regs}) are the first cells attracted and recruited in the site of injury and have all been shown to contribute toward the polarization of a pro-tumorigenic microenvironment [15].

1. Myeloid-derived suppressor cells (MDSC)

MDSCs have myeloid origin, an immature state and are characterized by their potent ability to suppress immune responses, especially T cell proliferation and cytokine production [16]. MDSC were firstly identified in tumor-bearing mice by the expression of CD11b and Gr-1. The mAb used to identify Gr1, however, is able to bind the same epitope of two different molecules belonging to the lymphocyte superfamily (Ly)-6, Ly-6C and Ly-6G, mainly expressed on monocytes and neutrophils respectively, resulting in the identification of two subtypes of murine MDSC: $CD11b^+$ $Ly\text{-}6C^+$ $Ly\text{-}6G^-$ monocytic-like MDSC and $CD11b^+$ $Ly\text{-}6C^-$ $Ly\text{-}6G^+$ granulocytic-like MDSC [17, 18]. As in mice, two main subsets of human MDSC have been identified: $CD14^+HLA\text{-}DR^{low/-}$ M-MDSC and $CD15^+$ $CD14^-$ $CD11b^+$ $CD33^+$ $HLA\text{-}DR^{low/-}$ G-MDSC. However, on the contrary of M-MDSC subset, human G-MDSC represent a more heterogeneous population identified by a set of antigens (CD11b, CD14, CD15, CD33, CD66b, CD16 and HLA-DR) which are well established markers for mature neutrophils or polymorphonuclear neutrophils (PMN) [19, 20]. In contrast to conventional PMN collected from the normal density neutrophil fraction on top of red cells after ficoll separation, G-MDSC are purified from the mononuclear cell fraction (fig.2). Therefore, G-MDSCs are defined as low-density immature cells with neutrophil-like morphology [21, 22].

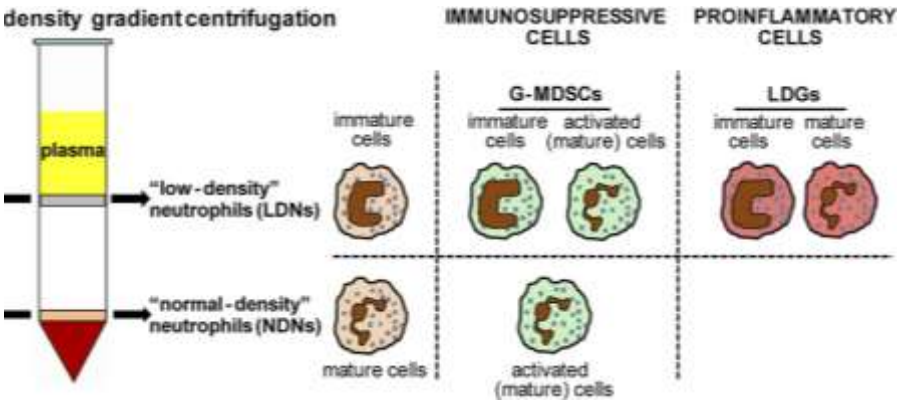

Figure 2. Circulating immature neutrophils (LDNs) and mature neutrophils (NDNs) isolation after ficoll separation.

Both M-MDSC and G-MDSC apply antigen-specific and antigen non-specific mechanisms to regulate immune responses, although these mechanisms are not exclusively used by one of the two subtypes [16, 23]. MDSC-induced immune suppression is mediated primarily by the upregulation of nitric oxide synthase 2 (NOS2), reactive oxygen species (ROS) and overexpression of arginase 1 [24, 25] (fig.3). Moreover, up-regulation of cyclooxigenase-2 and prostaglandin E2 [26], induction of regulatory T cells [27-29], up-regulation of TGF-β [30], depletion of cystein [31], down-regulation of T cell L-selectin expression [32], inhibition of NK function via downregulation of the activating receptor NKG2D [33] have been described. The specific mechanisms used by MDSC are dependent on the context of the microenvironment [34].

Accumulation of MDSC has been described in both solid tumors and hematological malignancies [35]. Tumor progression is frequently associated with their expansion in the peripheral blood (PB), spleen, and tumor [36].

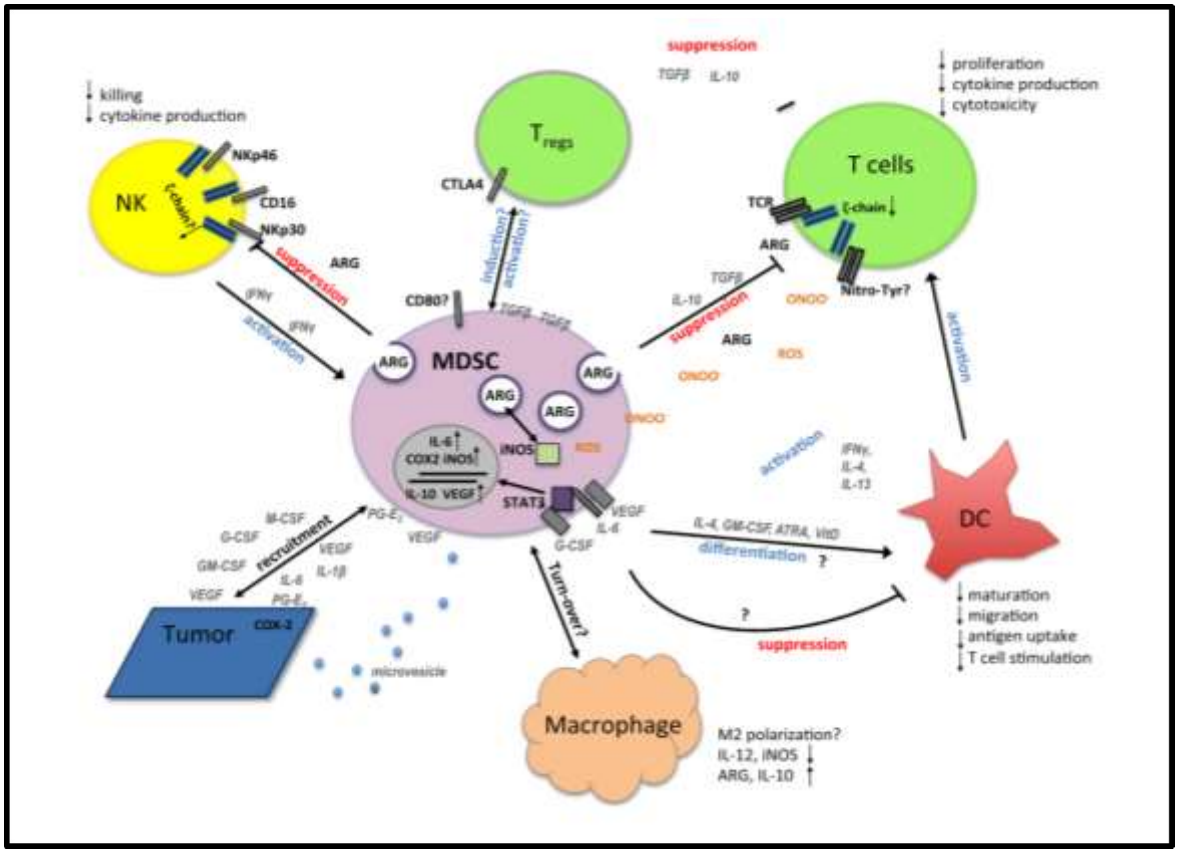

Figure 3. Model of MDSC function in cancer patients. MDSC suppress immune cells by using several mechanisms dependent on the context of the tumor microenvironment.

2. Neutrophils

Neutrophils are the most abundant white blood cells (WBC). They are classically viewed as short-lived effector cells of the innate immune system. They are important in host defense and infectious diseases with intracellular bacteria (such as mycobacteria or *Brucella abortus*)[37, 38], parasites [39, 40] and viruses (such as human immunodeficiency virus-1 or influenza virus) [41, 42].

In the last few years, it has been demonstrated that human neutrophils are extremely dynamic and adaptable cells. They can acquire antigen presenting (APC)-like properties and dendritic cell (DC) characteristics or be reprogrammed into macrophages [43, 44]. It has been demonstrated that human neutrophils, other than interacting with nonimmune cell types such as platelets [45] and mesenchimal stem cells [43], can establish, in vitro and in vivo, cross-

talk with innate immune cells, such as DCs, monocytes, macrophages, and natural killer (NK) cells, as well as with adaptive immune cells, such as T and B cells, or related subpopulations [22]. Moreover, longevity of neutrophils increases several-fold during inflammation [46] and there is growing evidences of the key role of neutrophils in tumour transformation, including orchestration of the immune response. In fact into cancer microenvironment, neutrophils have been linked with immunosuppression, angiogenesis, tumor development and metastasis [47-49].

The neutrophils found in the tumor are often referred to as tumor-associated neutrophils (TAN). Recent data have suggested that tumours manipulate neutrophils, sometimes early in their differentiation process, to create diverse phenotypic and functional polarization states able to alter tumour behaviour [50]. Neutrophil polarization states have been divided into N1 or N2 [51]. In fact, TAN can have an anti-tumorigenic (N1) or a pro-tumorigenic (N2) phenotype (fig.4). It has been shown that blockade of TGFβ polarize the protumoral, immunosuppressive N2 to the antitumor, immunostimulatory N1 neutrophils [51]. The most TAN within the tumour microenvironment appear to have an N2 phenotype and thus contribute to tumor growth and immunosuppression producing large amounts of arginase 1, which inactivates T cell activation [52]. Moreover, neutrophils also exert their immunosuppressive function through production of ROS that at high concentrations, induce apoptosis in T cells [53]. Compared with healthy subjects, neutrophils isolated from patients with hepatocellular carcinoma release more CCL2 (C-C Motif Chemokine Ligand 2) that inhibits the production of IFNγ [54]. In addition, N2 TAN recruit immunosuppressive regulatory T cells (Treg) into tumors through secretion of CCL17 (C-C Motif Chemokine Ligand 17) [55].

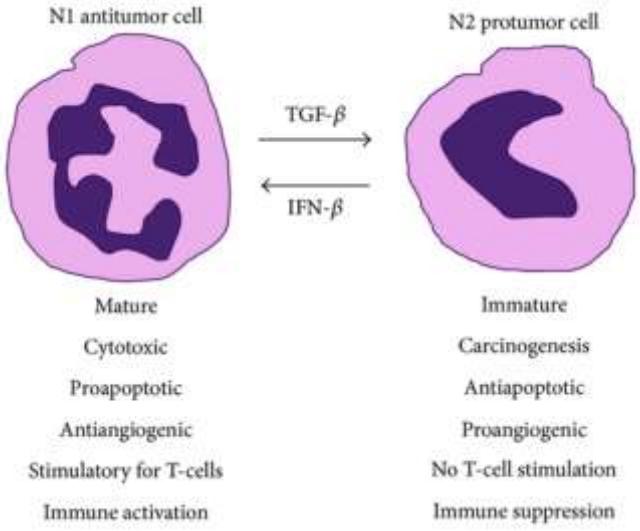

Figure 4. Neutrophil polarization. Neutrophils may be divided into N1 antitumor and N2 protumor cells. TGFβ is a potent driver of the transition from N1 to N2 phenotype whereas IFN-β is a potent driver of the transition in the opposite direction.

Since we have no definitive markers yet, we do not know whether the N2 neutrophils within the tumors are actually granulocytic like MDSC or whether they are mature neutrophils converted to an N2 phenotype by the tumor microenvironment [51]. Therefore, the restrictive term MDSC may be re-evaluated because self-limiting. For this reason we consider MDSC as neutrophils with immunosuppressive capabilities (N2 neutrophils).

3. *Mesenchymal stem cells (MSC) and Cancer associated fibroblasts (CAF)*

MSC is a subset of non hematopoietic stem cells existed in bone marrow (BM) and originating from the mesodermal germ layer [56]. They have the ability to differentiate into multiple lineages such as chondrocytes, osteocytes, adipocytes, myocytes, and astrocytes; so MSC could be considered as a potential source of stem cells for cellular and genetic therapy [57].

MSC are identified by the absence of the CD34 and CD45 hematopoietic cell markers and expression of CD29, CD90 and CD105 [58]. MSCs express the major histocompatibility complex (MHC) class I but do not express MHC class II, B7-1, B7-2, CD40 and CD40L molecules [59].

MSC have been shown to have both stimulating and inhibiting effects on tumor progression. They, through a complex crosstalk with neighboring cells/factors, can inhibit many effector

functions of immune cells, thereby promoting an immunosuppressive state in the tumor microenvironment that allows tumor cells and their associated stroma to overcome the immune surveillance (fig.5) [60].

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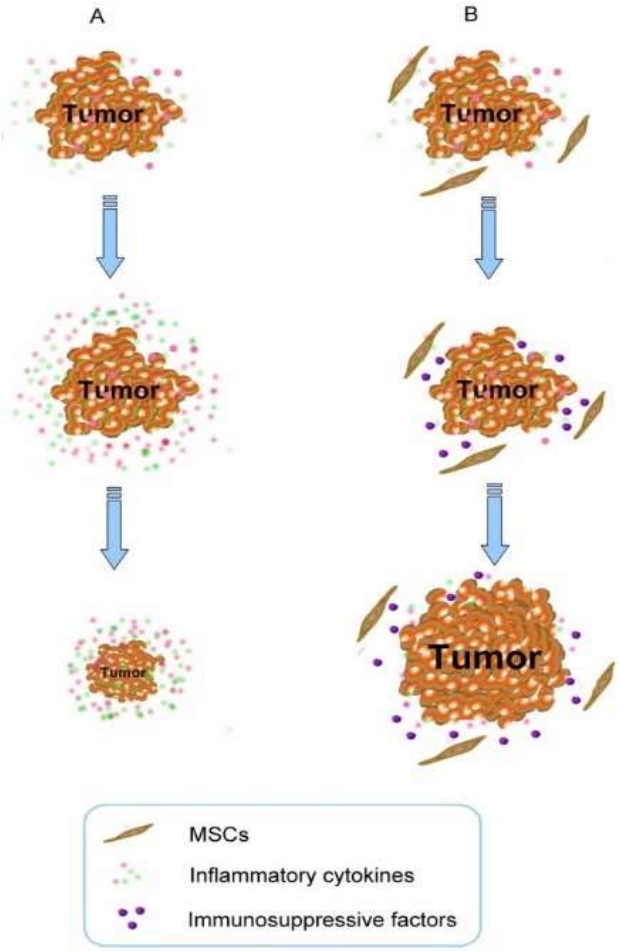

Figure 5. MSC have a tropism for tumors. MSC in tumor inflammatory microenvironment may be elicited of immunosuppressive function, which will help tumor to escape from the immunity surveillance.

MSC can home and engraft to cancer, including breast [61-63], lung [64, 65], pancreatic [66], colon [67], ovarian [68] and prostate carcinomas [69, 70], melanoma [71], glioma [72], Kaposi's sarcoma [73] and osteosarcoma [74]. Their chemotactic responses resemble those of immune cells. MSC express chemokine receptors, growth hormone receptors, adhesion molecules and Toll-like receptors (TLR) [68]. Recently, it has been demonstrated a connection between the stimulation of specific TLR and MSC activation status. They are type-I transmenbrane glycoproteins that recognize ‘‘danger’’ signals leading to profound

cellular and systemic responses that mobilize innate and adaptive host immune cells [75]. MSC express several TLR and their ability to migrate, invade and secrete immune modulating factors was drastically affected by specific TLR-agonist engagement [76]. In the last few years, in a clear parallel with macrophage polarization, it has been demonstrated that MSC can polarize into two different types with distinct phenotypes defined MSC1 and MSC2 [77]. In particular, TLR4 stimulation polarizes MSC toward a pro-inflammatory MSC1 phenotype, while stimulation of TLR3 results in the polarization toward an immunosuppressive MSC2 phenotype. Accordingly, the in vitro co-culture of MSC1 with several cancer cell lines inhibited tumor growth, whereas co-cultures with MSC2 had opposite effects. Moreover, in immunecompetent models the treatment of tumors with MSC1 resulted in reduction of tumor growth and metastasis while an MSC2-treatment led to tumor growth [77].

It has been shown that engrafted MSC can develop into α-smooth muscle actin (SMA)-expressing myofibroblasts, usually recognized as cancer associated fibroblasts (CAF), in the tumor microenvironment [78]. In particular, BM-MSC co-cultured in vitro with cancer cells can be activated and may have a CAF-like phenotype. CAF have the capacity to promote tumor growth and metastasis, either via direct interaction with tumor epithelial cells or via the recruitment of inflammatory cells [79]. MSC and CAF show more similarities than differences including the expression of cell surface markers (HLA-DR, CD29, CD90, CD44, CD73, CD106 and CD117), expression of cytoskeleton proteins like vimentin, αSMA and nestin, and trilineage differentiation potential (to adipocytes, chondrocytes and osteoblasts) (tab.1) [80].

Table 1. Synopsis of MSCs and TAFs similarities and differences

	MSCs	TAFs
Pluripotency (trilineage differentiation potential)	Yes	Yes
Cell surface molecules		
CD14	–	–
CD29	+	+
CD31	–	–
CD34	–	–
CD44	+	+
CD45	–	–
CD73	+	+
CD90	+	+
CD106	+	+
CD117	+	+
HLA-DR	–	–
CXCR4	–	–
VEGF-R1 (Flt-1)	–	–
VEGF-R2 (Kdr)	–	–
TGF-β RII	–	–
TGF-β RII	–	–
Cytoskeleton and extracellular matrix proteins		
Vimentin	Yes	Yes
α-SMA	Yes	Yes
Nestin	Yes	Yes
Cytokeratin	No	No
E-cadherin	No	No
Ultrastructural details		
Cytoplasmic elongations	No	Yes
Lamellar content lysosomes	No	Yes
Intermediate filaments	Yes	No
Cytokines, chemokines and growth factors secretion		
IL-4	Low	High
IL-10	Low	High
IL-13	Low	High
TGF-β_1	Low	High
TNF-α	Low	High
VEGF	Low	High

+: expression; –: lack of expression.

CAF and MSC exhibit major differences in their ultrastructural features. On the contrary of MSC, CAF have nuclei with lobulated morphology, few mitochondria, highly developed endoplasmic reticulum with dilated cisternae and lamellar content lysosomes [80]. The function of the peculiar lysosomal structures might be involved in the capture and sequestration of tumor derived antigens, thus becoming unavailable to the anticancer immune cells. In fact, CAF have potent immunosuppressive ability like MSC, with also pro-tumoral effects. Therefore, since the differences between CAF and MSC are only functional (i.e.

cytokines production, proliferation rate), CAF could be MSC bearing an activated status that better 'serve' the cancer cell.

Immune escape in haematological diseases

Hematological malignancies are cancers that affect blood, bone marrow and lymphonodes, thus maintaining a slight contact with immune system cells. Multipotent, hematopoietic stem/progenitor cells (HSC/HPC), which are the dominant hematopoietic population in the BM, possess both self-renewal and differentiation abilities; their growth and maintenance is dependent on cytokine and niche factors. BM microenvironment involved in regulation and control of maintenance of HSCs is called 'niche' [81]. It is a dynamic system with bidirectional signals that ensure the regulation of normal HSCs numbers [82] and maintenance of the quiescent long-term HSC pool [83].

Overall, leukemias are malignant disorders of hematologic cells that result in the overproduction of undifferentiated and immature leukocytes that function abnormally within the BM, the circulation and at extramedullary sites. HSC/HPC are the source of leukemic cells and immune evasion mechanisms play a central role supporting tumor microenvironment transformation [84].

As in a variety of solid cancers, MDSC have been shown to play a central role in anti-tumor immune response in hematological malignances including acute and chronic myeloid leukemia (AML and CML), chronic lymphocytic leukemia (CLL), multiple myeloma and lymphoma [85-90]. Moreover, our group demonstrated that neutrophils isolated from myeloma and CML patients are immunosuppressive cells indicating a common altered pathway of myeloid maturation [91].

Proliferation, survival and drug-resistance of leukemic cells are largely dependent on their interplay with the bone marrow (BM) microenvironment, in which mesenchymal stromal cells (MSC) are important components. Indeed, MSC favor or impede LSC expansion representing a possible target for treatment of leukemias [92]. Since BM is a store of undifferentiated MSC, tumor cells may affect MSC activity in the tumor niche favouring a deep cross-talk between LSC and MSC [93].

Into the tumor milieu, MSC also play an important role for their immunological regulation ability that can interfere with the immune recognition of tumor cells creating an "immune protection site" in the cell microenvironemnt. Indeed, MSC can interfere with the recognition of tumor cells by immune system producing and releasing immunoregulatory factors as TGFβ, prostaglandin E2 (PGE2), tumor necrosis factor α (TNFα), indolamine 2,3-dioxygenase (IDO), hemeoxygenase (HO), NOS2, ARG1-2, IL10 [94-96]. MSC express

programmed death ligand 1 (PD-L1) that after its engagement with PD-1 expressed on T lymphocytes lead to the inhibition of T cell activation and proliferation with an inefficient immune response [97].

1.1 Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a myeloproliferative disease that originates from a haemopoietic stem cell (HSC) as a result of the t(9;22) leading to the Philadelphia chromosome and expression of the oncogenic tyrosine kinase BCR/ABL [98, 99]. The oncoprotein is central to the pathogenesis of CML and is the target of tyrosine kinase inhibitors (TKI) used for CML treatment. These drugs have drastically changed the treatment outcome of CML patients. More than 85% of CML treated patients achieve a complete cytogenetic response and approximately 40% of these patients achieve a complete molecular remission (CMR) [100]. Imatinib was the first TKI approved and has been considered the standard of care. Although the therapy with Imatinib is considered a major advance in oncology, a significant group of patients still develops drug resistance. Second generation TKI, Dasatinib and Nilotinib, are highly effective in those who fail imatinib as well as in newly diagnosed patients [101]. RQ-PCR–based molecular monitoring of BCR-ABL transcripts is the most sensitive tool for assessing disease burden in patients with CML. The European LeukemiaNet and the National Comprehensive Cancer Network recommend RQ-PCR–based molecular monitoring every 3 months until a deep response (i.e., major molecular response [MMR] or CCyR) occurs and every 3–6 months thereafter [102]. Achieving MMR is extremely important in the course of CML in order to avoid relapse.

In CML patients, immune system is dysfunctional. CD4+ T cells which are central components of effective immune response against cancer cells, are anergic against leukemic cells [103, 104] showing the downregulation of the TCR-ζ chain [105]. Furthermore, the number of NK cells is decreased and their function is impaired [106]. T-reg are significantly increased in CML patients with intermediate or high-risk Sokal scores compared to the low risk patients [107]. In addition, CML myeloid cells (including also CD34+ stem cells) over-expressed programmed death receptor ligand 1 (PD-L1) that binding its receptor PD-1 expressed on T lymphocytes lead to inactivation of T cells [108]. Recently, we observed higher levels of G-MDSC and M-MDSC in patients at diagnosis which decreased to normal levels after treatment with Imatinib [91]. Their percentage did not correlate neither with age, nor with leukocytosis or Sokal risk. In addition, no correlation was observed between MDSC and the response to Imatinib. Both subpopulations expressed BCR/ABL confirming that they are part of the tumor clone. Expression of arginase 1 and its circulating levels in the serum

resulted higher in patients at diagnosis in respect to healthy controls and decreased during TKI treatment. Furthermore, the percentage of G-MDSC correlated with arginase 1 protein levels in the serum. We demonstrated also an immunosuppressive activity of CML neutrophils, suggesting a strong potential immune escape mechanism created by CML cells, which control the anti-tumor reactive T cells [91].

Although the therapy with TKI have drastically changed the treatment outcome of CML patients, the therapy is hampered by chronic mild toxicities that may have, especially in a long time frame, a significant impact on patient quality of life [109]. In recent years, several studies have been conducted to evaluate the safety of imatinib discontinuation in patients who have reached durable CMR [110, 111]. Campiotti et al reported that CML molecular relapse occurs mainly during the first 6 months after imatinib discontinuation. Fifty-five percent and 80% of cumulative molecular relapses occurred in the first 3 months and in first 6 months, respectively [109]. However, late molecular relapses, up to 22 months post discontinuation, were also observed. Molecular relapse is presumably due to the reactivation of dormant CML LSC that are resistant to TKI-induced leukemic cell ablation. Unfortunatly, there is a lack of specific prognostic factors and a follow-up strategy which could determine the restarting of the leukemic growth in imatinib-discontinuing patients [112]. There is increasing evidence suggesting that NK-cells are important in controlling the leukemic cells: increased NK-cell counts seem to correlate with the successful imatinib discontinuation [113]. It could be of interest the monitoring of MDSC in patients who have discontinued imatinib treatment in order to see if their increase could correlate with the restarting of the leukemic growth.

1.2 Multiple Myeloma

Multiple Myeloma (MM) is a malignant plasma cell disorder characterized by the accumulation of neoplastic plasma cells (PC) in the bone marrow (BM) and the presence of monoclonal immunoglobulin in the blood and/or urine. MM is the second most common hematological malignancy and constitutes 1% of all cancers and 13% of all hematological cancers. It is a multistep progressing disease starting with an asymptomatic monoclonal gammopathy of undetermined significance (MGUS) through smoldering Multiple Myeloma (SMM), up to the most aggressive, symptomatic MM and plasma cell leukemia. Clinical features of this disease include anemia, bone resorption, renal failure, frequent occurrence of infections and hypercalcemia [114]. Osteolytic lesions are caused by rapid bone turnover, which occurs as a result of increased osteoclastic resorption that is not accompanied by a comparable increase in bone formation [115].

The development of MM is due not only to uncontrolled proliferation of PC but also BM microenvironment play a crucial role in disease progression to symptomatic myeloma. Here PC are hosted in niches that maintain their long survival and exert a protective effect on drug-induced apoptosis [116, 117]. Immune cells and BM mesenchymal stromal cells (MSC) are important components of this microenvironment.

Within the microenvironment, the host immune system has a pivotal role for the PC growth, proliferation, survival, migration and resistance to drugs and is responsible for some clinical manifestations of MM. In fact, Dysfunction of immune system is an important feature of MM patients and leads to infections and increased tumor growth [118-120]. Dendritic cells (DC) express lower levels of human leukocyte antigen (HLA) molecules and a reduced capacity in stimulating T cells [121]. Furthermore, MM cells are able to inhibit DC function through the secretion of IL-6, IL-10, and TGF-β [122, 123]. Immune responses are also impaired by immunosuppressive cell subpopulations including regulatory T cells, tumor-associated macrophages (TAM) and MDSC [124]. Different authors demonstrated an increase of MDSC in both peripheral blood and BM of MM patients [125, 126]. In addition to their immunosuppressive activity, MDSC show the potential to differentiate in functional osteoclasts thus contributing to osteolytic lesions which are a recognized hallmarks of MM [127, 128].

As MDSC, also mature MM neutrophils (N) have immune suppressive ability supporting the hypothesis of a functional alteration of the whole myeloid lineage (Romano et al, in submission). MM-N overexpressed ARG1 inhibiting T cells activaction and the addition of an ARG1-specific inhibitor partially reversed this inhibition.

AIM

SECTION-1/AIM1

MONOCYTIC MYELOID DERIVED SUPPRESSOR CELLS (M-MDSC) AS PROGNOSTIC FACTOR IN CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH DASATINIB

Recently, the prognostic role of MDSC accumulation has been documented for some hematological malignancies such as Hodgkin lymphoma, multiple myeloma, and acute leukemia patients, where they correlates with disease progression and persistence of minimal residual disease [15, 129]. This first study focused on defining the change of MDSC frequency in CML patients during therapy with imatinib (IM), nilotinib (NIL) or dasatinib (DAS). We also evaluated the ability of serum from CML patients and exosomes released from leukemic cells to generate CD14+HLADR- cells from healthy donor derived monocytes.

SECTION-2/AIM2

MESENCHYMAL STROMAL CELLS (MSC) AS KEY PLAYERS IN THE TUMOR MICROENVIRONMENT TRANSFORMATION

It is well known that MSC have a role in promotion of tumor growth, survival and drug-resistance. Recent reports indicated that granulocyte-like MDSC are increased in CML and MM patients and also mature neutrophils show immunosuppressive ability [34, 91]. Generally speaking, we name these myeloid immunosuppressive cells as neutrophils with a “N2” phenotype. To examine the role of MSC in promoting N2 polarization, we use a specific experimental model in vitro, co-culturing MSC with peripheral blood mononucleated cells (PBMC) from healthy individuals, in order to generate MSC-educated neutrophils and elucidate the different role of tumor-associated versus healthy MSC in promoting immune evasion.

MATERIAL AND METHODS

SECTION-1 (S-1)

(S-1) *Patients and sample collection*

This study has been approved by the local ethical committee (Azienda ospedaliero Universitaria Policlinico-Vittorio Emanuele, #34/2013/VE). After written informed consent, samples were collected from CML patients and age-matched healthy donors (HD) at Division of Hematology, AOU Policlinico – OVE, University of Catania. This study enrolled 59 CML patients and for 42 of them samples were collected at diagnosis too. Twenty patients were treated with IM (14 of whom evaluated also at diagnosis), 20 with NIL (15 evaluated at diagnosis) and 19 with DAS (13 evaluated at diagnosis). Among NIL treated patients, 6 were in second-line, while for DAS 5 patients were in second-line and 1 in third-line of treatment (all 6 patients changed TKI because of IM resistance). During treatment, all patients were followed with a monthly CBC count, molecular evaluation of the BCR/ABL transcript every 3 months and cytogenetic evaluation every 6 months, according to ELN guidelines. Clinical data of CML patients at diagnosis included in this study are shown in Table 2.

Patients	Gender	Age	BCR/ABL transcript levels	HGB (g/dL)	WBC (10^3/µL)	PLT (10^3/µL)	LDH (mg/dL)
1	M	67	81,37	11,2	72,2	355	2087
2	M	77	105,84	14,2	54,6	391	1426
3	F	73	59,77	11,1	30,7	651	-
4	M	56	66,29	12	164	526	2418
5	F	69	58,002	12.1	34	607	-
6	M	84	45,1	14	50,3	285	873
7	M	51	41,59	12,5	38,4	368	-
8	F	56	20,41	12,6	95	463	668
9	M	62	79,85	11,3	331	96	-
10	M	59	39,74	12,4	152,24	527	-
11	M	59	25,14	15,3	22,8	20	-
12	F	48	81,88	8,1	288	458	1043
13	F	71	48,16	9,2	70,5	338	2230
14	M	70	349,51	13,5	71,2	370	715
15	F	66	126,73	12,9	68,4	294	888
16	M	38	65,61	13,6	28,4	232	-
17	M	54	74,22	12,7	58	201	-
18	M	21	126,51	14,2	144	107	1247
19	M	53	142,78	16,6	34	311	288
20	M	61	33,66	14,4	51,6	399	983
21	M	48	40,33	9,9	256	350	1074
22	F	64	28,21	10,6	128,4	531	1147
23	F	57	122,97	11.4	156,6	273	1124
24	M	36	56,24	10,8	55	208	1352

25	M	52	14,89	10,2	46	418	1820
26	F	58	150,04	10,7	122,5	361	1683
27	F	65	191,66	15,3	87	252	688
28	F	72	71,98	13	111	168	345
29	F	78	48,26	11,7	77	651	-
30	M	37	153,59	12,8	91,6	344	1635
31	M	60	47	11	70	368	873
32	M	53	23,8	13	44	343	1820
33	F	62	31,5	11	22	98	-
34	F	73	68,8	14	315	543	723
35	F	67	58,7	12,6	120	521	-
36	M	58	23,00	12,4	23	44	-
37	M	47	78,2	11,9	98	98	1043
38	M	47	63	13,3	195	345	668
39	M	55	21	12.1	54	430	-
40	F	63	120	10,7	70	370	1683
41	M	43	44,6	12	122	333	2230
42	M	69	328	9	71	370	715

Table 2 part I

Patients	**Liver (cm)**	**Spleen (cm)**	**blast count**	**Sokal score**	**HASFORD score**	**M-MDSC (%)**	**Gr-MDSC (%)**
1	0	0	0	low	int	5,3	86,9
2	0	0	0	intermediate	low	31	85
3	0	0	0	low	-	0,7	72,7
4	2	0	0	high	intermediate	20,02	82
5	0	0	0	intermediate	low	10,2	81,4
6	0	0	0	intermediate	intermediate	23	79
7	0	0	0	low	low	26,9	87
8	0	-	0	intermediate	-	29,8	79,4
9	6	4	5	high	intermediate	37,7	85,4
10	2	3,5	0	intermediate	-	50	88,7
11	0	0	0	low	intermediate	18,9	87
12	2	14	3	high	intermediate	41	86
13	-	-	-	intermediate	intermediate	46,8	89
14	0	0	1	intermediate	intermediate	81,2	79
15	2	3	0	intermediate	intermediate	12,4	82
16	0	0	0	low	low	28,5	83,4
17	-	-	-	low	low	14,7	82
18	0	6	1	low	low	81,6	83,7
19	0	3	0	low	intermediate	63	83
20	0	0	1	low	low	25	81
21	7	14	1	intermediate	low	41	90
22	0	2	1	intermediate	intermediate	1	58,3
23	2	1	1	low	intermediate	61	88
24	7	8	1	low	low	42,4	87
25	0	2	1	low	intermediate	25	78
26	0	3	1	intermediate	intermediate	50	75
27	0	0	0	low	low	91,2	50
28	4	2	2	intermediate	intermediate	14,4	75

29	0	1	2	high	intermediate	5,4	64
30	0	0	0	low	low	2,8	82,5
31	0	0	0	intermediate	intermediate	28	88
32	0	2	1	low	intermediate	25	79
33	0	0	0	low	low	25,8	72
34	6	4	5	high	intermediate	55	75
35	2	2	0	intermediate	-	50	83
36	0	0	0	low	intermediate	16	87
37	2	0	3	high	intermediate	46	86
38	0	2	0	intermediate	-	44	86
39	0	0	0	intermediate	low	11	78
40	2	3	1	intermediate	intermediate	50	81
41	-	-	-	intermediate	intermediate	37	77
42	0	0	1	intermediate	intermediate	83,2	84

Table 2 part II

Table 2. Clinical disease characteristics of CML patients. The frequency and the fuctional characteristics of MDSC analyzed in the PB from CML patients at diagnosis. HD were age matched. (F, female; M, male; HGB, hemoglobin; WBC, white blood cells; PLT, platelets; LDH, lactate dehydrogenase. BCR/ABL transcipt levels are calculated as BCR-ABL/ABL).

(S-1) *Flow cytometry analysis of MDSC phenotype*

The amount of MDSC was evaluated in peripheral blood (PB). Analysis of MDSC was performed by multicolor FACS analysis using the following antibody (Beckman Coulter): CD14 PC5 (clone RMO52), HLADR ECD (clone IMMU-357), CD11b FITC (clone bear-1), CD33 PE (clone D3HL60, 251) and their respective isotype controls. Briefly, $1x10^6$ cells were stained with 10 µl of each of the above listed Abs and incubated for 20 minutes in the dark at room temperature. After lysing red cells with ammonium chloride, cells were analyzed by flow cytometer (Cytomics FC 500, Beckman Coulter) and analysis was performed using CXP Analysis software. Using sequential gating strategy (supplementary figure 1), G-MDSC cells were identified as cells $CD11b^+CD33^+CD14^-HLADR^-$, while the M-MDSC as $CD14^+HLADR^-$. The results were expressed as percentage.

(S-1) *Functional characterization of MDSC*

To evaluate the suppressive ability, G-MDSC and M-MDSC from CML patients and HD were first isolated using magnetic separation (CD66b-positive selection for G-MDSC and CD14-positive/HLA-DR-negative for M-MDSC, Miltenyibiotec) and then the purity and viability were tested by flow cytometry; viability was more than 90%. MDSC were co-cultured for three days with autologous Carboxyfluorescein succinimidyl ester (CFSE)-labeled T lymphocytes at ratio 1:4 [91]. For cell labeling, $5x10^5$ lymphocytes were incubated

at 37°C for 20 min in 1 ml PBS containing 1 µM CFSE (BD Pharmingen). T cells were stimulated with 5 mg/mL phytohemagglutinin (PHA) and incubated for 72 hours prior to flow cytometry. Controls included a positive T cell proliferation control (T cells plus PHA) and a negative one (T cells only). After three days, T cell proliferation was measured by CFSE dilution and analyzed using flow cytometry.

(S-1) *Western Blot Analysis*

Western Blot Analysis was performed according to the manufacturer‘s recommendations. The antibodies directed against the human Tsg101 and CD63 were obtained from Santa Cruz Biothecnology. The blots were scanned, and determinated using Scion Image software (New York, NY).

(S-1) *Soluble factors and exosomes for the generation of M-MDSC*

Purification of monocytes from PB of 4 HD was performed by a positive selection of these cells using a magnetic separation kit (EasySep, STEMCELL Technologies, Vancouver, BC, Canada). Cell purity was determined by flow cytometry and was >90%. Monocytes were cultured with RPMI-1640 medium with 1% penicillin-streptomycin supplemented with 20% FBS or HD (n=4) or CML sera (n=6). After 72 h of incubation, cells were stained with M-MDSC Abs for flow cytometry analysis.

HD monocyets were also cultured in the presence of exosomes (30 µg protein/10^6 monocytes) isolated from 5 CML serum patients at diagnosis.

(S-1) *Isolation of serum exosomes*

Serum exosomes were isolated and purified by differential ultracentrifugation according to a standard protocol for isolation of exosomes from viscous bodily fluids [130]. Serum was derived from heparinised blood, diluted 1:2 with PBS (phosphate -buffered saline) and centrifuged for 30 min at 2,000 x g at 4 C°. The supernatant was transferred to ultracentrifuge tubes and centrifuged using a 13.1 JS rotor (Beckman Instruments, Inc., Fullerton, CA) for 30 min at 12,000 x g, 4 C°. Supernatant was carefully transferred into fresh ultracentrifuge tubes and centrifuged using a SW28 rotor (Beckman Instruments, Inc., Fullerton, CA) at 110,000 x g for 2 h at 4°C. The resulting pellet , resuspended in 1 ml of PBS, was diluted with PBS, filtered through a 0.22-µm filter (Millex GP filter unit, Millipore, Billerica, MA) into fresh ultracentrifuge tubes and centrifuged in a SW28 rotor at 110,000 x g for 70 min at 4°C. Then the tube containing the pellet was resuspended in 1 ml of PBS, filled with PBS and centrifuged at 110,000 x g for 70 min at 4°C. The crude exosomes were resuspended in

50-100 µl of PBS for their characterization by scanning transmission and immunoelectron microscopy.

(S-1) *Scanning Electron Microscopy (SEM)*

Exosomes (20 µl) were fixed in 80 µl of 2% glutaraldehyde-0.1 M phosphate buffer and fixed overnight at 4°C. A drop of suspension was layered on a sterile cover glass coated with 0,1% poly-L-Lysine, postfixed in 1% osmium tetroxide (Merck, Darmstadt, Germany) in the same buffer for 1h at 4°C and washed in phosphate buffer. After dehydrating in a graded ethanol and critical point drying, the samples were sputtered with a 5nm gold layer using an Emscope SM 300 (Emscope Laboratories, Ashford, UK) and then observed. A Hitachi S-4000 (Hitachi High-Technologies America, Inc., Schaumburg, IL) field emission scanning electron microscope was used for the observations.

(S-1) *Transmission Electron Microscopy (TEM)*

Exosomes (20 µl) were fixed in 80 µl of 3% formaldehyde-0.1% glutaraldehyde overnight at 4°C. 5 µl of the above suspension was layered on a formvar copper coated nickel grids (Electron Microscopy Sciences, Fort Washington, PA) and allow to dry for 20 min to absorb exosomes. The grids, washed in PBS, were negatively stained with 4% uranyl acetate for 5 min and viewed using a Hitachi H-7000 transmission electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany). For immunogold labeling the grids were rinsed for 2x2 min with PBS and transferred in a TBS (Tris-buffered saline pH 7,4) solution containing 1% BSA (bovine serum albumin) (TBS/BSA) for 10 min at room temperature. Then the grids were incubated in blocking solution 5% BSA for 1,30 h at room temperature, rinsed with PBS, and incubated in a humid chamber overnight at 4°C with a mouse monoclonal antibody CD81 (Santa Cruz Biotechnology, Heidelberg, Germany) in a dilution 1:50 with TBS/BSA. After wash for 3x3 min with TBS/BSA, the grids were stained with a 10 nm gold-labeled secondary antibody Anti-mouse IgG (Sigma Aldrich) in a dilution 1:5 with TBS/BSA at 37°C for 1h in the dark. The grids were rinsed 2x2 with TBS/BSA, 2x2 with water and fixed with 1,5% glutaraldehyde in PBS for 10 min at room temperature. After rinsed again with water the grids were post-stained with 4% uranyl acetate for 5 min and allow to air drying. Observations were carried out using the transmission electron microscope. Negative controls were prepared in the absence of primary antibody but with secondary antibody-conjugate.

SECTION-2 (S-2)

(S-2) *MSC harvest, culture and characterization*

After written informed consent, BM samples were collected from patients with diagnosis of CML (n=10), of MGUS (n=10), smoldering (n=6) or active MM at first diagnosis (n=11) or relapse (n=5), and age-matched HD (n=10). Also MM-MSC from 3 refractory patients were collected. Clinical data of MM and MGUS patients included in this study are shown in Table 3-4.

BM mononuclear cells were obtained after density gradient centrifugation on Ficoll and cultured in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% L-glutamine. After 3 days in culture, non-adherent cells were removed, whereas MSC were selected by their adherence to the plastic-ware. The cultures were maintained at 7°C and 5% CO_2. MSC were expanded until the third or fourth passage and then trypsinized to be used for experiments. Selected MSC from both patients and HD at the third passage were also tested for MSC specific surface antigen expression (supplentary figure 2). Therefore, cells were labeled using combinations of monoclonal antibodies: anti-CD34-ECD (clone 581), anti-CD90-FITC (clone F15.42.1.5), anti-CD105-PE (clone 1G2) and anti-CD45-PC5 (clone J.33). The appropriate isotopic control was also included. Labeled MSC were acquired using a Beckman Coulter FC-500 flow cytometer.

Moreover, MSC osteogenic and adipogenic ability differentiation was confirmed in two CML-, MGUS-, MM- and HD-MSC. In brief, for osteoblastic and adipocytic differentiations, 80% confluent MSCs were grown in medium supplemented with 10mM b-glycerol phosphate (Sigma-Aldrich, St Louis, MO, USA), 50 mg/ml ascorbic acid and 10nM dexamethasone for osteoblasts, or with 10 μg/ml insulin, 0.5 mM dexamethasone, 0.5 mM isobutylmethylxanthine and 0.1 mM indomethacin for adipocytes [131, 132]. Osteocytic and adipocytic differentiation of MSC was evaluated using alkaline phosphatase and Oil-Red-O respectively (data not showed).

Table 3: Baseline clinical characteristics of patients included in the study.

	MGUS (n=10)	Newly-diagnosed MM (n=16)	Relapsed MM (n=5)	Smouldering MM (n=7)
Median age (range)	67 (49-70)	65 (45-68)	67 (38-75)	64 (49-77)
Males/Females	7/3	10/6	3/2	5/2
Isotype, n				
IgG	0	10	2	4
IgA	10	4	2	2
Light-chain only	0	2	1	1
Cytogenetics, n				
Normal	8	8	1	4
del 13	1	1	2	2
del 17	0	5	0	1
t(4;14)	0	1	2	0
not performed/failed	1	1	0	0
Haemoglobin, g/dl (range)	12.8 (12-14.5)	10.6 (6.5-13.8)	9.8 (6.6-12.8)	12.3 (12-13.9)
Platelets 1000/uL (range)	219 (180-315)	221 (90-384)	123 (43-225)	206 (113-315)
Bone marrow plasmocytosis >50%, n (%)	0	5	3	2
C-reactive protein median, mg/l (range)	0.1 (0.01-4)	4.4 (0.001-8.5)	5.3 (0.05-9.6)	0.7 (0.01-5)
LDH median, mm/h	195 (132-213)	209 (109-708)	240 (125-368)	198 (134-250)
ESR median, mm/h	17 (0-26)	72 (6-134)	84 (10-138)	16 (0-32)
STAGE ISS, n				
1	N.A.	4	0	7
2	N.A.	8	4	0
3	N.A.	4	1	0

ESR: Erythrocyte Sedimentation Rate; ISS: International Staging System.

Table 4: Summary of refractory MM patients.

sex	age	Type	Number of treatment lines
F	72	IgA-λ	3
M	72	IgG-κ	2
F	64	IgG-κ	1

Type: paraproteins IgG or IgA, κ-light chains κ or λ-light chains λ

(S-2) *Induction and evaluation of neutrophils "N2" polarization*

Human peripheral blood mononucleated cells (PBMC) were isolated from healthy volunteer donors after density gradient centrifugation on Ficoll. PBMC were cultured alone or co-cultured with MSC derived from healthy subjects, patients with CML or MM (1:100 ratio) [133]. MSC were seeded to achieve confluence by 7 days. After one week, PBMC were collected and neutrophils were isolated using anti-CD66b magnetic microbeads (MiltenyiBiotec). Their

purity and viability were confirmed by cytofluorimetric analysis (supplentary figure 3). The immunosuppressive capacity of educated neutrophils (ed-N) was analyzed by evaluating T cell anergy when co-cultured with autologous CFSE-labeled T cells stimulated with 5 mg/mL phytohemagglutinin (PHA). T cells were isolated by magnetic cell separation using human CD3 microbeads (Miltenyi Biotec). For T lymphocytes labeling, $5x10^5$ lymphocytes were incubated at 37°C for 20 min in 1 ml PBS containing 1 μM CFSE. Controls included a positive T cell proliferation control (T cells plus PHA) and a negative one (T cells only). After three days T cell proliferation was analyzed by flow cytometry.

Bortezomib (BTZ, 5 nM), lenalidomide (LEN, 10 μM) and pomalidomide (POMA, 1 μM) were added during co-culture of PBMNC with MM-MSC (from patients at diagnosis or relapsed or refractory) to investigate the effects of proteosome ihibitor (BTZ) and immunomodulating drugs (LENA and POMA) on neutrophils polarization.

To evaluate their pro-angiogenic effect in vitro, educated neutrophils were co-cultured with Human Brain Microvascular Endothelial Cells (HBMEC) (1:2).

To investigate the ability of PC to activate healthy MSC in cells with the same immunological alteration of MM-MSC, commercially available stromal cell lines HS-5 cells and HD-MSC were incubated with human MM cell lines (U266, MM1S) for 24 h (1:10) before to performe co-culture with PBMNC.

(S-2) *Real-time RT-PCR for gene expression of MSC and educated neutrophils*

For gene expression studies, MSC were trypsinized from culture flasks both at Time 0 (cells at confluence incubated with standard medium only) and after 48 hours from start of co-culture experiments. In co-culture experiments, MSC and educated neutrophils were purified using respectively anti-CD271 and anti-CD66b magnetic microbeads (MiltenyiBiotec). After RNA extraction and reverse transcription, we evaluated expression of the following mRNA: ARG1, NOS2, PTGS2, TNFα, TGFβ, IL6, IL10, IL8, IL32 and IL1β. Their expression was assessed by TaqMan Gene Expression (Life Technologies) and quantified using a fluorescence-based real-time detection method by 7900HT Fast Start (Life Technologies). For each sample, the relative expression level of each studied mRNA was normalized using GAPDH as invariant controls.

(S-2) *Western Blot* analysis

Western Blot analysis was performed using antibodies directed against the human MyD88, TLR4 and β-actin. The blots were scanned and determinated using Scion Image software.

(S-2) *Immunofluorescence*

Cells were grown directly on coverslips before immunofluorescence. After washing with phosphate-buffered saline (PBS), cells were fixed in in 4% paraformaldehyde for 20 minutes at room temperature. After fixation, cells were three times washed in PBS for 5 minutes. Subsequently, the cells were incubated with primary antibody against IRF3 (anti-rabbit) and NFKB (anti-mouse) at dilution 1:100, overnight at 4°C. Next day, cells were three times washed in PBS for 5 minutes and incubated with secondary antibodies: TRITC (anti-rabbit) at dilution 1:200, and FITC (anti-mouse) at dilution 1:200 for 1h at room temperature. The slides were mounted with medium containing DAPI (4′,6-diamidino-2-phenylindole) to visualize nuclei. The fluorescent images were obtained using a Zeiss Axio Imager Z1 Microscope with Apotome 2 system and was performed by Image J Software.

(S-2) *Zebrafish as in vivo model*

1. *Zebrafish husbandry*

Adult (5–8 month-old) wild-type AB zebrafish (Danio rerio) were maintained under conditions according to the Organisation for Economic Cooperation and Development guidelines. Fish were kept in a flow-through system with a light/dark cycle of 14 h/10 h and were fed with SDS 400 food twice daily.

2. Xenotransplantation procedure

Zebrafish were anesthetized with 0.02% tricaine (Sigma-Aldrich) and injected with suspensions of MM cell lines (U266 or MM1S) mixed with HD- or MM-MSC in a 1:1 ratio [134, 135] in PBS ($5x10^4/5x10^4$) using a borosilicate glass capillary and a MICROINJECTOR system.

Prior to implantation, MM cells were labeled for coimplantation with MSC with DiIC18(5)-DS (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine-5,5'-Disulfonic Acid) (ThermoFisher) at a final concentration of 1 mmol/L for 5 minutes at 37°C in a 5% CO_2 atmosphere and 15 minutes at 4°C.

We evaluated the tumor xenografts by tomography 6 days post-injection, measuring the tumor volume and intensity of fluorescence with ImageJ software [136].

3. *Flow cytometry analysis*

MM cell xenograft was determined by flow cytometry. Wild-type adult zebrafish were anaesthetized with 0.02% tricaine and the kidney/marrow and other extracted organs were dissected and placed in PBS as described. Single cell suspensions were generated by passing through a nylon mesh and stained with propidium iodide (Sigma) was to exclude dead cells. Staining with the human monoclonal antobodies against CD138 was performed. To evaluate

MSC xenograft, human CD90 antibody was also used. All These human mAb did not show cross-reactivity with Zebrafish cells.

4. *Real time PCR*

By using Trizol reagent, total RNA was extracted from kidney/marrow and total of organs. After reverse transcription, we evaluated expression of the following mRNA: TBX21, GATA3, INFγ, IL4, IL13. Their expression was assessed by TaqMan Gene Expression (Life Technologies) and quantified using a fluorescence-based real-time detection method by 7900HT Fast Start (Life Technologies). Expression was normalized to the expression of the housekeeping gene, GAPDH.

Statistical analysis

The data are expressed as mean ± SD. Statistical analysis was carried out by paired Student's t-test, ANOVA test or Mann-Whitney test. For correlation analysis, the Pearson's correlation was assessed. A p value <0.05 was considered to indicate a statistically significant difference between experimental and control groups.

RESULTS

1. MONOCYTIC MYELOID DERIVED SUPPRESSOR CELLS (M-MDSC) AS PROGNOSTIC FACTOR IN CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH DASATINIB (SECTION 1)

1.1 MDSC are increased in CML patients

G-MDSC and M-MDSC percentages in CML patients at diagnosis were greater than HD subjects (respectively 84±9% vs 56.2±5.4% and 32±20% vs 5.9±4%, $p<0.0001$) (Figure 6A). Moreover, the frequency of M-MDSC significantly correlated with BCR/ABL transcript levels ($r=0.64$, $p<0.0001$) (Figure 6B). The percentages of G-MDSC and M-MDSC did not correlate neither with age, nor with leukocytosis or Sokal risk. To validate whether these increased myeloid subpopulations were MDSC cells, their immunosuppressive activity was investigated. For this purpose, we isolated by magnetic separation CD14-negative (representative of M-MDSC) and CD66b-positive (representative of G-MDSC) cells from both CML patients at diagnosis and healthy controls and incubated them with autologous CFSE-labeled T cells.

On the contrary of immature myeloid cells with G-MDSC or M-MDSC phenotype isolated from healthy donors, both G-MDSC and M-MDSC from CML patients were able to inhibit T cells proliferation in comparison to positive control (from 30±4.8% to 18.7±3.8% for G-MDSC, $p=0.0086$ and to 16.7±2.5% for M-MDSC, $p=0.0009$) (Figure 6C).

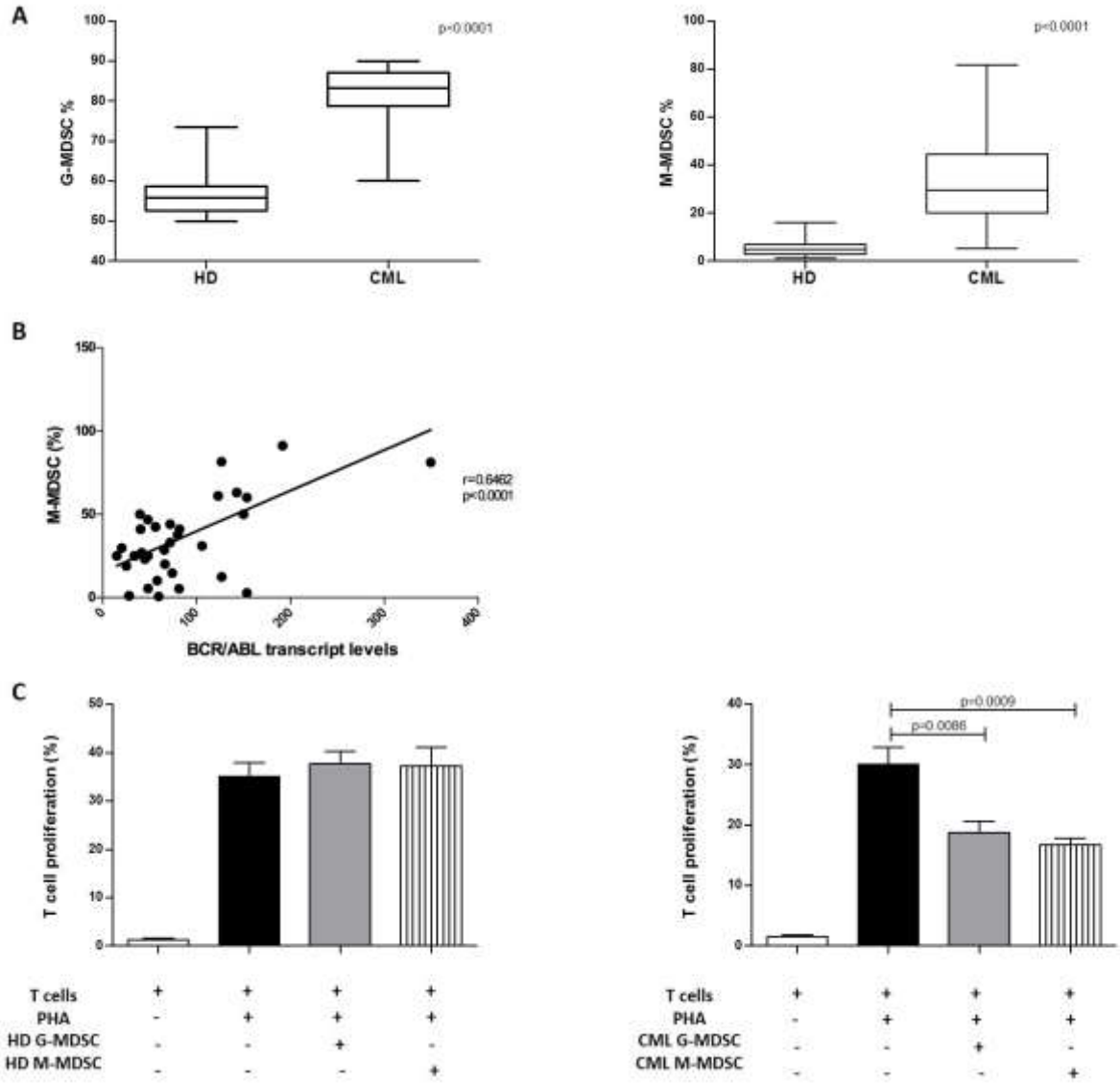

Figure 6. Increased frequency of MDSC in untreated CML patients. A. The percentages of circulating G-MDSC and M-MDSC were quantified in the peripheral blood. Flow cytometry analysis was performed with gates set on either $CD11b^{+}CD33^{+}CD14^{-}HLADR^{-}$ (G-MDSC) or $CD14^{+}HLADR^{-}$ (M-MDSC) cell populations. The bars represent the standard error of the mean. **B.** Correlation analyses of the peripheral M-MDSC count with BCR/ABL transcript levels calculated using the Pearson's correlation analysis **C.** Granulocytic and monocytic MDSC mediated T cell suppression in autologous co-cultures. MDSC was previously tested for cell viability by using cytofluorimetric analysis. Mean frequency of $CD3^{+}CFSE^{dim}$ cells±SD from four independent experiments in duplicate is shown. *HD: healthy controls.*

1.2 M-MDSC percentage correlates with MMR in dasatinb treated patients

CML patients were followed during therapy with IM, NIL or DAS. All TKI decreased the levels of G-MDSC at 3-6 months (from 82.5±9.6% to 55±17.3% after IM, to 60.9±9% after NIL and to 48.7±13% after DAS, $p<0.0001$) and 9-12 months (64±8% after IM, 61±6.3% after NIL and 32±15% after DAS, $p<0.0001$) of treatment (Figure 7A). The percentage of M-MDSC significantly decreased after DAS therapy only (from 33.6±19% to 6.8±12.6% at 6 months, $p=0.014$ and to 12±11.8% at 12 months, $p=0.004$). In fact, M-MDSC reduction was also observed but did not reach statistical significance after IM (22.2±24.5% and 20.8±18.6% respectively at 6 and 12 months) and after NIL treatment (21±19.9% and 19±17% at 6 and 12 months)with a great variability among patients.

MDSC accumulation correlates with disease progression and minimal residual disease in myeloma and leukemia patients [15, 129]. Therefore, we analyzed the correlation of MDSC with clinical response to TKI. On the contrary of IM and NIL treated patients (data not shown), we found a correlation between the MMR values and the number of persistent M-MDSC at 12 months. Indeed, a significant difference was observed comparing the percentage of M-MDSC in the MMR group (n=8) versus no MMR (n=11) ($p=0.0025$) (Figure 7B). Figure 7C shows the frequency of M-MDSC for patients evaluated both at diagnosis and after 12 months of treatment with dasatinib.

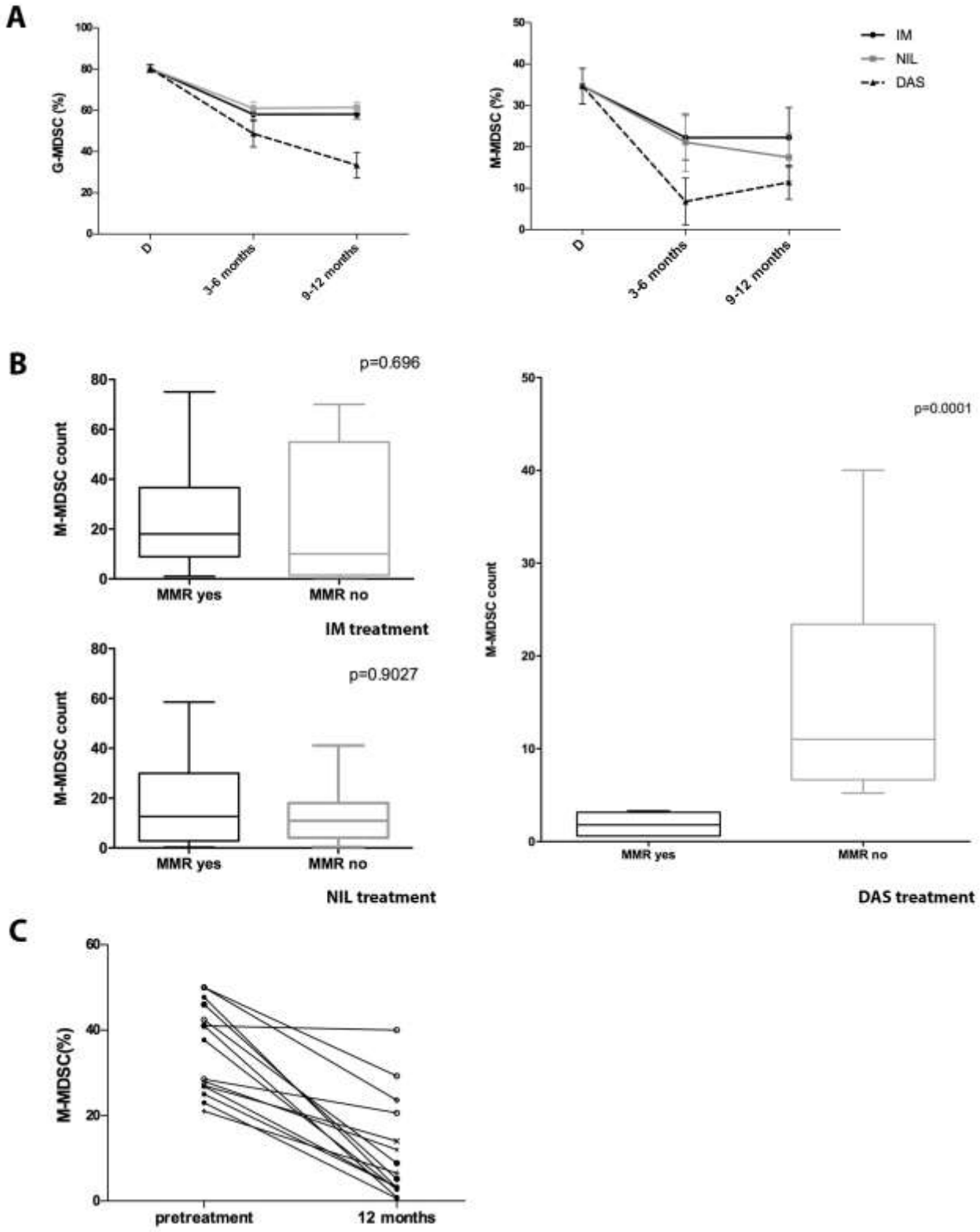

Figure 7. MDSC after TKI therapy. A. Changes in circulating G-MDSC and M-MDSC in CML patients treated with IM, NIL or DAS. The bars represent the standard error of the mean. G-MDSC at 3-6 and 9-12 months after IM, NIL and DAS: $p<0.0001$. M-MDSC after 3-6 months of DAS therapy: $p<0.05$; after 9-12

months of DAS treatment: p<0.01. **B.** The percentage of M-MDSC was compared between the MMR and no MMR groups. The bars represent the standard error of the mean. Statistical difference was calculated using Mann-Whitney test. **C.** M-MDSC count for 15 patients at diagnosis and after 12 months of therapy with DAS. Lines with empty circle represent patients no in MMR at 12 months. *MMR, major molecular response; BCR-ABL is ≤0.1%.*

1.3 CML cells are able to induce M-MDSC by secreting soluble factors

To evaluate whether leukemic cells were able to expand MDSC, we cultured monocytes isolated from healthy controls with sera from healthy subjects or CML patients at diagnosis. Monocytes displayed phenotypic conversion into $CD14^{+}HLADR^{-}$ only in conditions with CML sera where the percentage of M-MDSC increased by 29±13%, p=0.0006 (Figure 8A). No changes were observed by incubating monocytes with serum from healthy subjects. On the contrary, G-MDSC percentage did not change by addition of neither CML or healthy donor (HD) serum (data not showed). In line with their MDSC-like phenotype, CML serum-educated monocytes showed suppressive ability after incubation with autologous T lymphocytes (Figure 8B).

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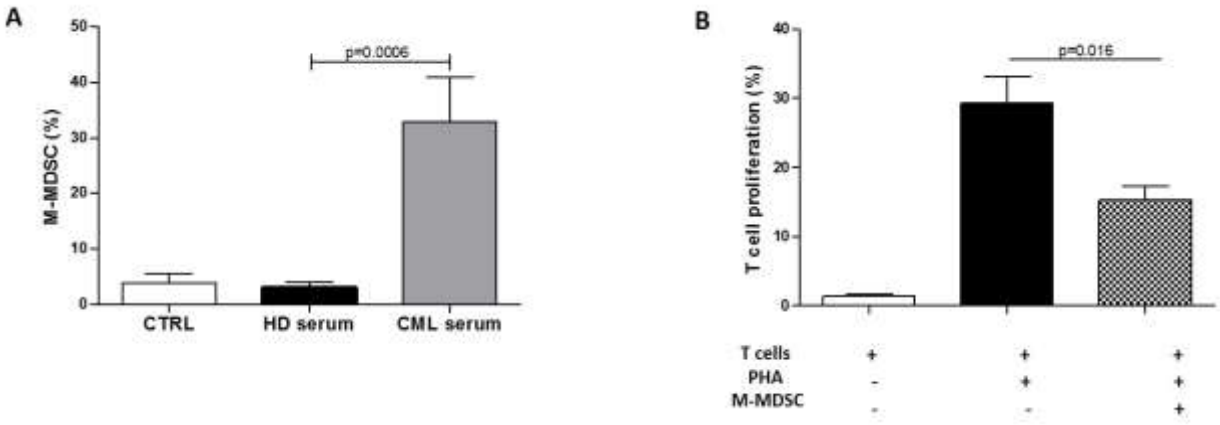

Figure 8. CML serum induces M-MDSC with T cell suppressive ability. A. Monocytes displayed phenotypic conversion into CD14+HLADR- after incubation with CML serum for 3 days. Results represent the means of four independent experiment; error bars denote SD. **B.** Suppressive activity of CML serum-educated M-MDSC

(CML s-ed M-MDSC) was evaluated in co-culture experiments with CFSE-labeled autologous T lymphocytes. Mean frequency of $CD3^{+}CFSE^{dim}$ cells±SD from four independent experiments in duplicate is shown.

1.4 CML-derived exosomes promote M-MDSC expansion

A number of studies have recently described tumor released exosomes as new key players in modulating the tumor microenvironment, promoting angiogenesis, tumor development and inhibition of immune cells [137, 138]. Exosomes isolated from CML serum satisfied the three major criteria as exosomes: the size of 50-100 nm in diameter (Figure 9A; a1-2), a density of 1.13 to 1.21 g/dl in a sucrose gradient and expressed CD80 (Figure 9A; a3), Tsg101 and CD63 proteins (Figure 9B). Incubating healthy monocytes with CML exosomes, we observed higher percentage of M-MDSC (from 9.4±2.7% in untreated condition to 17.4±5.5% in CML exosomes treated monocytes; p=0.006) (Figure 9C). We also demonstrated the immunosuppressive activity of CML Ex-educated M-MDSC in vitro (Figure 9D).

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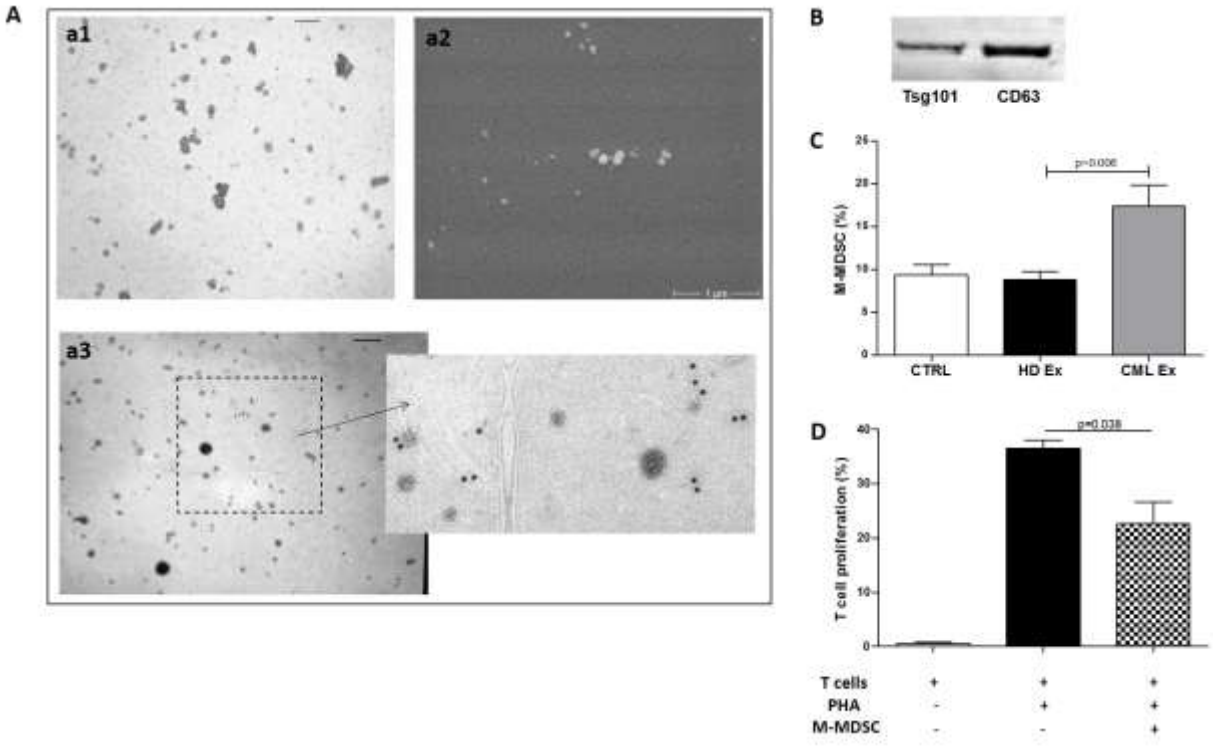

Figure 9. CML exosomes promote the generation of M-MDSC. A. a1: Representative TEM image of CML serum exosomes (Ex). The exosomes show a characteristic "deflated football –shaped" of 60-100 nm in size (Bar= 120 nm). a2: A SEM image of CML exosomes at high magnification (x 30.000). a3: The exosomes are positive for exosomal marker CD81 (Bar=120 nm). Right panel: boxed area shown at higher magnification. **B.**

Western blot analysis of protein extracted from exosomes. **C.** An increase of the percentage of CD14+/HLADR- cells was observed in vitro after incubation of HD monocytes with CML exosomes ($p<0.05$). Results represent the means of four independent experiment; error bars denote SD. **D.** Suppressive activity of CML exosomes-educated M-MDSC (CML Ex-ed M-MDSC) was evaluated in co-culture experiments with CFSE-labeled autologous T lymphocytes. Mean frequency of $CD3^{+}CFSE^{dim}$ cells±SD from four independent experiments in duplicate is shown.

2. Mesenchymal Stromal Cells (MSC) As Key Players in the Tumor Microenvironment Transformation (SECTION 2)

Since neutrophils from CML and MM patients are immunosuppressive and the levels of G-MDSC are increased, we investigated the role of MSC in the polarization of neutrophils toward a "N2" phenotype.

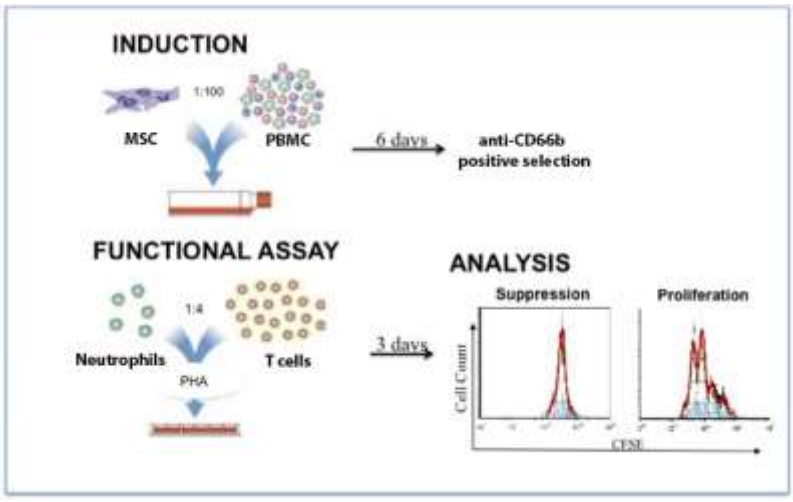

Figure 10. Model for education of neutrophils in vitro. After one week, PBMC were collected and neutrophils were isolated using anti-CD66b magnetic microbeads. Their immunosuppressive capacity was analyzed by evaluating T cell anergy when co-cultured with autologous CFSE-labeled T cells stimulated with PHA.

2.1 CML-MSC activate immature myeloid cells (IMC) in immunosuppressive neutrophils

2.1.1 N2 polarization is driven by disfunctional MSC

Following the model reported in figure 10, we cultured PBMC of healthy subjects in medium alone or with HD- or CML-MSC. After one week, both HD- and CML-MSC accumulated similar amount of neutrophils (Figure 11A). After magnetic cell separation, we analyzed if educated neutrophils (ed-N) were converted in immunosuppressive "N2", co-culturing them with autologous CFSE+ T cells. We found that only CML-MSCed-N inhibited T cell

proliferation (32±12% vs 63±5.9% observed in the condition with neutrophils isolated from PBMC cultured in medium alone) (p=0.003). On the contrary, HD-MSCed-N did not show any suppressive effect (Figure 11B).

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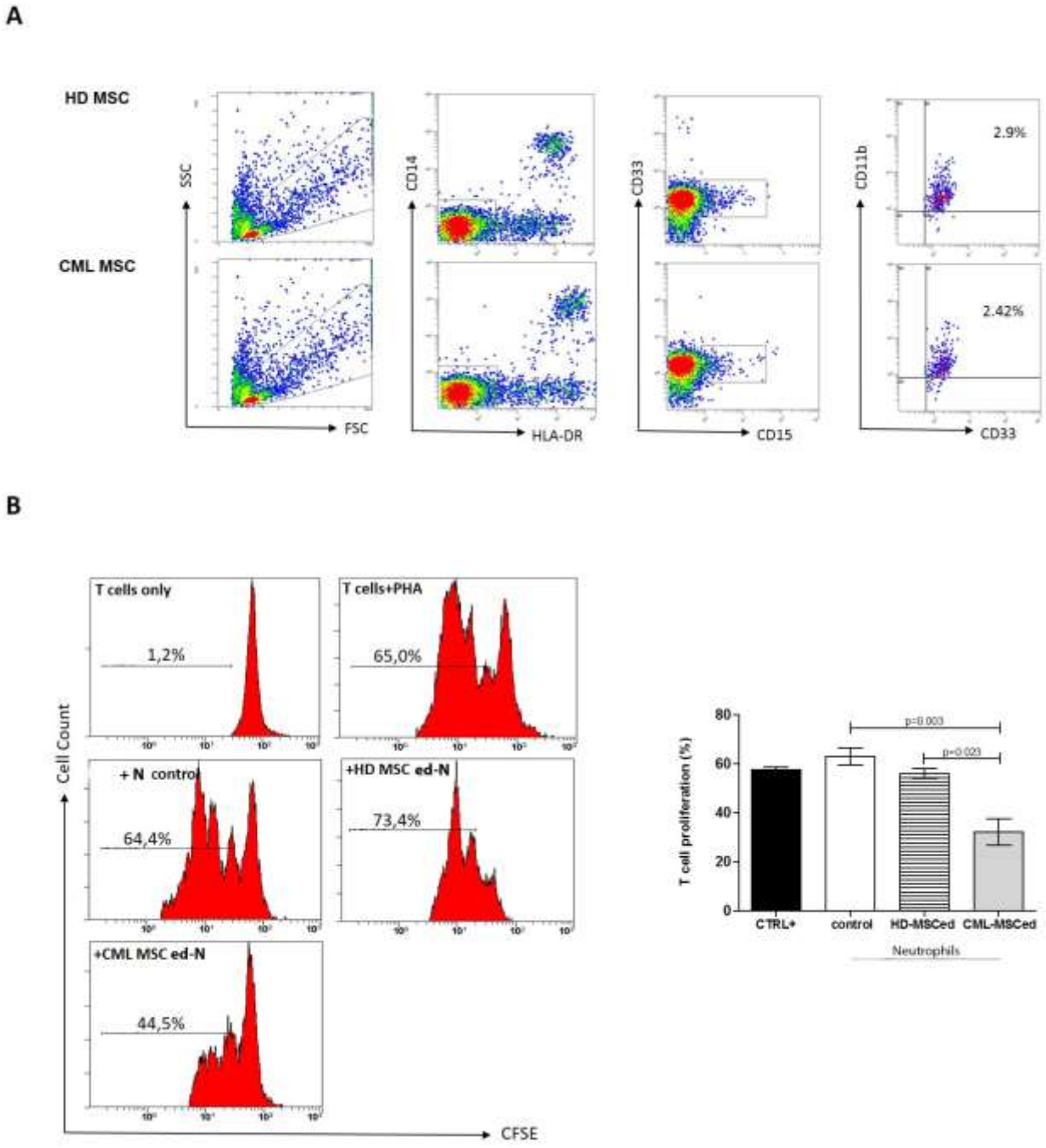

Figure 11. CML-MSC-educated neutrophils are immunosuppressive. A. HD- and CML-MSC generate similar amount of neutrophil-like cells. The figure shows a representative data from one experiment. Flow cytometry analysis was performed with gates set on CD11b+CD33+CD15+CD14-HLADR- cell population. **B.** MSCed-N were analyzed for their immunosuppressive activity against autologous T cells. Representative flow

cytometry dot-plots show the gating strategy for each experimental condition. The data represent mean±SD of all analyzed co-cultures in triplicate.

2.1.2 CML-MSC up-regulate immunomodulatory factors

It is well known that polarization of neutrophils in "N2" can be induced by multiple factors present in the tumor microenvironment. Immunomodulatory factors, including TNFα, TGFβ, IL6, IL10, IL1β, ARG1, NOS2 and COX2 are important to reprogram immature myeloid cells to become immunosuppressive neutrophils [23]. Therefore, we first analyzed their expression by MSC at Time 0. Despite a great variability among patients, we found a significant up-regulation of IL6 (5±2.8, p=0.04), COX2 (19±4.4, p=0.04) and TGFβ (6±3, p=0.01) by CML-MSC compared to HD- ones (Figure 12A). Expression of TNFα gene was down-regulated (0.55±1, p=0.027). After 48 h of co-culture with PBMC, CML MSC showed up-regulation of IL6 (54.3±23, p=0.003), TGFβ (4.8±3, p=0.04) and IL10 (5.6±2.8, p=0.03) expression (Figure 12B), suggesting that multiple mechanisms are involved in neutrophils polarization by CML-MSC.

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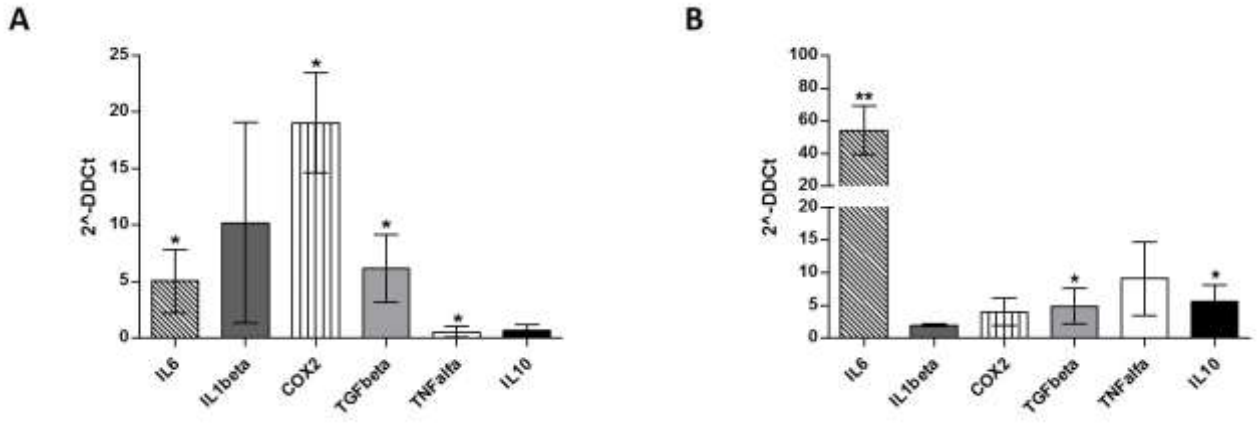

Figure 12. Expression of immunomodulatory factors by CML-MSC. Compared to HD-MSC, CML-MSC up-regulated IL6, COX2 and TGFβ at Time 0 (**A**) and overexpressed IL6, TGFβ and IL10 after 48 h of co-culture with PBMC (**B**). Calculated value of 2^-ΔΔCt in HD-MSC was 1.

2.1.3 Gene expression of immunomodulatory factors in CML-MSC educated-neutrophils

To test whether the changes of gene expression in CML-MSC during co-culture also occurred in CML-MSCed-N, we examined the expression of the same genes in neutrophils isolated after co-culture. Compared to neutrophils educated in co-culture with HD-MSC, CML-MSCed-N showed higher levels of ARG1 (23.5±11.9, p=0.02), IL6 (33.8±13.9, p=0.004), IL1β (47.3±25.2, p=0.001), COX2 (20.7±10.9, p=0.002) and TNFα (20.8±19.3, p=0.006) (Figure 13).

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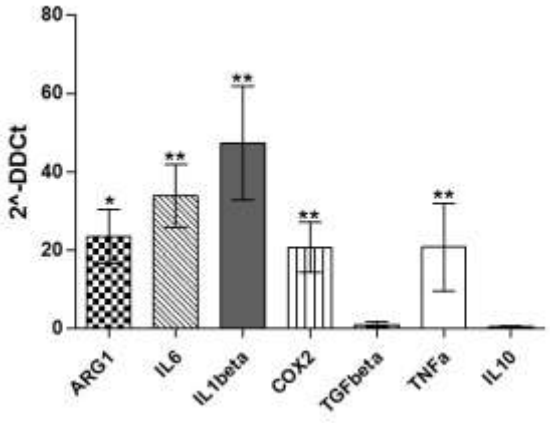

Figure 13. Expression of immunomodulatory factors by MSC educated-neutrophils. Compared to HD-, CML-MSCed-N expressed higher levels of ARG1, IL6, IL1β, COX2 and TNFα in respect with HD-MSCed-N. Calculated value of 2^- ΔΔCt in HD-MSCedG-MDSC was 1.

2.2 Mesenchymal Stromal Cells (MSC) as key players in promoting immunune escape and tumor microenvironment transformation from MGUS to Myeloma

2.2.1 MM-MSC polarized neutrophils versus a "N2" phenotype

Following the model reported in figure 10 and used to analyze CML-MSC, PBMC from healthy donors were co-cultured with healthy controls (HC)-, MGUS- or MM-MSC for one week. After magnetic cell separation, we found that only SMM-MSCed-N and MM-MSCed-N were able to suppress T cell proliferation (Figure 14A). No effects were observed after incubation of T lymphocytes with MGUS-MSCed-N or HC-MSCed-N or N control (isolated from PBMC cultured in medium alone). As MM-MSC from patients at diagnosis, also MM-MSC from subject with refractory MM induced neutrophils to become immunosuppressive.

Adding Bortezomib, Lenalidomide or Pomalidomide during co-culture with MM-MSC, isolated neutrophils did not lose immunosuppressive ability (Figure 14C).

Before incubation with T cells, the expression of our set of immune modulatory factors was investigated in MM-, SMM- and MGUS-MSCed-N using HC-MSCed-N as control. On the contrary of MGUS-MSCed-N, SMM- and MM-MSCed-N significantly up-regulated ARG1, NOS2 and TNFα (figure 14B). Up-regulation of ARG1 and NOS2 is the main mechanisms of MDSC-induced immune-suppression [34], while TNFα has been shown to arrest differentiation of immature myeloid cells and increase neutrophils suppressive activity [139].

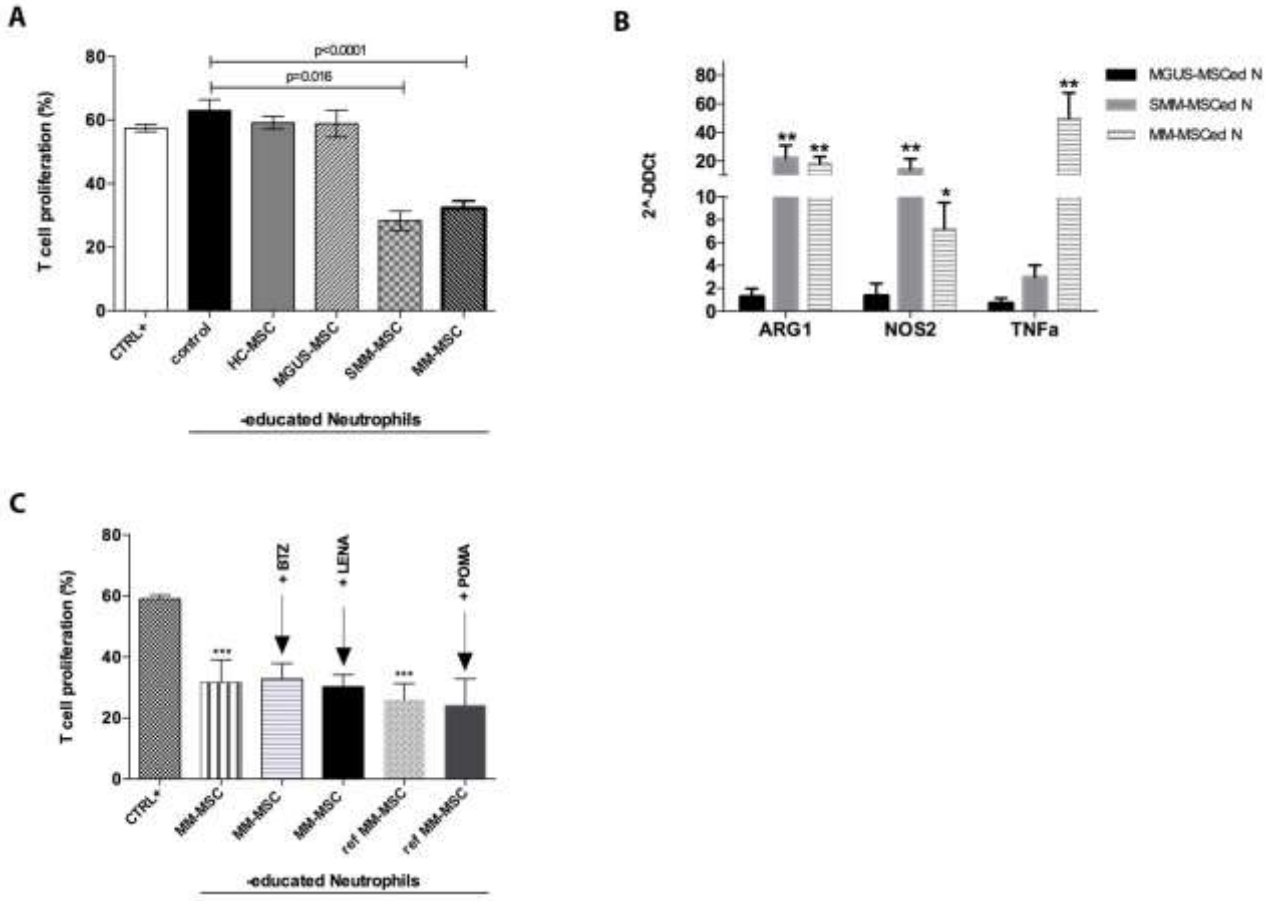

Figure 14. SMM- and MM MSC have the same immunological functional alterations. A. Only SMM- and MM-MSCed-N exhibited suppressive effects compared to N control (isolated from PBMC cultured without MSC). CTRL+: T lymphocytes incubated only with PHA (positive control). **B.** Expresssion of immunomodulatory factors by neutrophils educated with MGUS-, SMM- or MM-MSC in respect with HC-MSC ed-N. Calculated value of 2^-ΔΔCt in control (HC-MSC educated-neutrophils) was 1. *p<0.05; **p<0.001. **C.**

Adding BTZ (5 nM), LENA (10 uM) or POMA (1 uM) during co-culture with MM-MSC, isolated neutrophils did not lose immunosuppressive ability.

2.2.2 Molecular regulators of MM-MSC-mediated neutrophils activation

In many cancers, it has been demonstrated that tumor-associated microenvironment produces a large amount of immune-modulating factors involved in reprogramming immature myeloid cells to become immunosuppressive neutrophils and to attract them at the tumor sites. These immunomodulatory factors include PTGS2, TGFβ, NOS2, IL10, TNFα, IL1β, and IL6. Therefore, we analyzed their expression by MM-MSC in respect with HD-MSC at Time 0. A great variability of expression was observed among the patients, but no up-regulation of the genes above described was observed (Figure 15A). On the contrary after 48h from the start of co-culture with PBMC, MM-MSC showed higher expression of PTGS2 (5.8±5, p=0.018), TGFβ (27.8±34, p=0.03), NOS2 (20±25.8, p=0.04) and IL6 (20.7±22, p=0.02) expression (Figure 15B), suggesting that MM-MSC are functionally different from HD-MSC and are able to produce higher amount of immunomodulatory factors that could be involved in neutrophils activation versus a "N2" phenotype.

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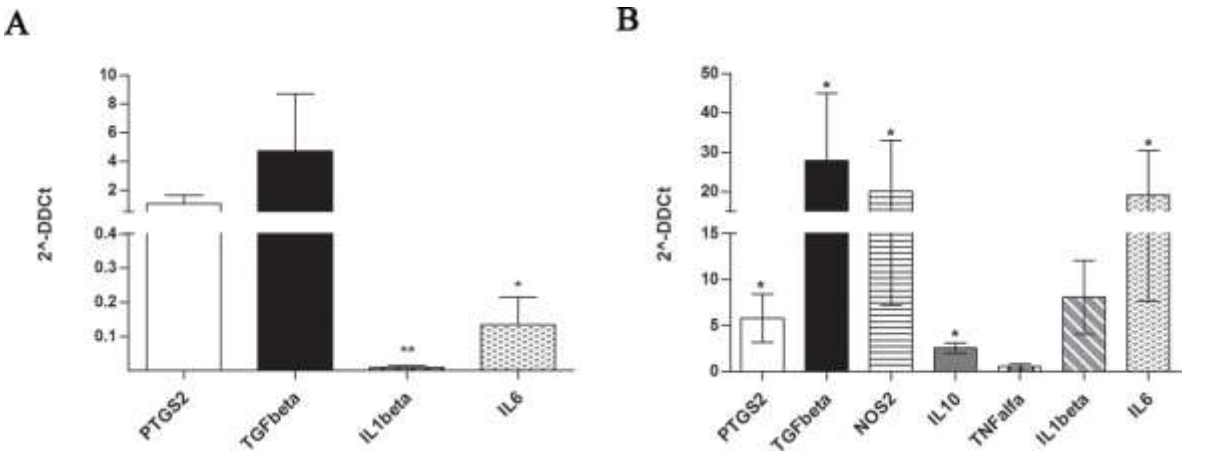

Figure 15: Expression of immune-modulatory factors by MM-MSC at Time 0 (A) and after 48 h of co-culture with PBMC (B). Only after incubation with PBMC, MM-MSC showed statistically signicant up-regulation of PTGS2, TGFβ, NOS2 and IL6 expression (p<0.05) compared with HD-MSC (calculated value of 2^-ΔΔCt in HD-MSC was 1).

2.2.3 MM-MSC educated neutrophils promote angiogenesis in vitro

A clinically relevant aspect of multiple myeloma BM microenvironment is neovascularization, a constant hallmark of disease progression. In addition to soluble factors directly secreted by the tumor cells, myeloma plasma cells also induce angiogenesis indirectly via recruitment and activation of stromal inflammatory cells such as macrophages and mast cells which secrete their own angiogenic factors [140]. In addition to suppress immune response, neutrophils with pro-tumor phenotype could promote tumor development by promoting angiogenesis. Therefore, we next investigated in vitro the pro-angiogenic effect of MM-MSCed-N and observed the increase of both tube length and meshes number compared to N control (isolated from PBMC cultured in medium alone) ($p<0.05$) (Figure 16). Also neutrophils isolated from co-culture with MSC obtained from refractory MM patients showed pro-angiogenic effects. Adding BTZ, LENA or POMA during co-culture with MM-MSC, isolated neutrophils lose their pro-angiogenic capacity.

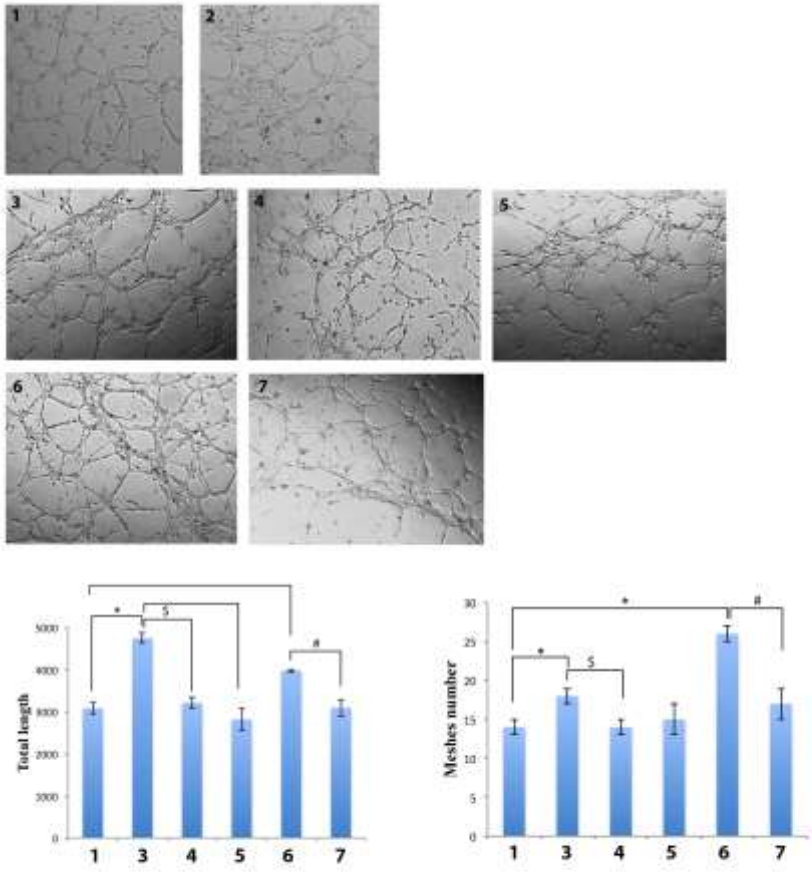

Figure 16. MM-MSCed-N have pro-angiogenic activity in vitro. IHBMEC were plated on Matrigel in the absence (1, control) or presence of VEGF-A (2, positive control), of MM-MSCed-N (3), MM-MSCed-N isolated from co-culture with BTZ (4) or LENA (5), refractory MM-MSCed-N (6) or refractory MM-MSCed-N isolated

from co-culture with POMA (7). After 5 hours, refreactory MM- and MM-MSCed-N induced tube formation. The pro-angiogenic effect was significantly reduced by the proteasome inhibitor and the immunomodulatory drugs. *p<0.05; **p<0.001; $ p<0.05; # p<0.05.

2.2.4 TLR4 signaling activates healthy MSC in stromal cells with the same functional alteration of MM-MSC

Since it has been demonstrated a connection between the stimulation of specific Toll-like receptors (TLR) and MSC activation status, including two distinct phenotypes defined MSC1 (TLR4-dependent) or MSC2 (TLR3-dependent), we hypothesized that MM-MSC could be stromal cells activated to better "serve" the cancer cells. Therefore, to investigate whether MSC polarization (MSC1/MSC2) may explain the immune alteration observed in MM-MSC, we pre-treated HC-MSC with LPS (lipopolysaccharide) or poly(I:C) as agonists, respectively, for TLR4 and TLR3. After 24h, HC-MSC were then cultured with PBMC from healthy donor. Only educated neutrophils isolated from co-cultures with HC-MSC pre-treated with LPS showed in vitro N2 phenotype with suppressive effects on T cell proliferation ($p<0.001$) (Fig.17A). No effects were observed after TLR3 stimulation. Therefore, we next investigated the ability of these ed-N isolated from co-culture with HC-MSC pre-tretaed with LPS to induce angiogenesis in vitro. As shown in figure 17B, only these neutrophils were able to increase the meshes number ($p<0.05$) and the total lenght ($p<0.05$) after incubation with IHBMEC. These data confirmed that LPS "activate" MSC inducing their commitment towards an inflammatory phenotype associated with polarization of neutrophils toward a N2 phenotype.

Moreover, western blotting analysis showed the up-regulation of myeloid differentiation 88 (MyD88), an important contributing protein in the TLR4 signaling cascade, in MM-MSC in respect with HC-MSC ($p<0.0001$; fig.17C). These data indicated that TLR4 signaling may play a role in tumor microenvironment transformation promoted by MSC.

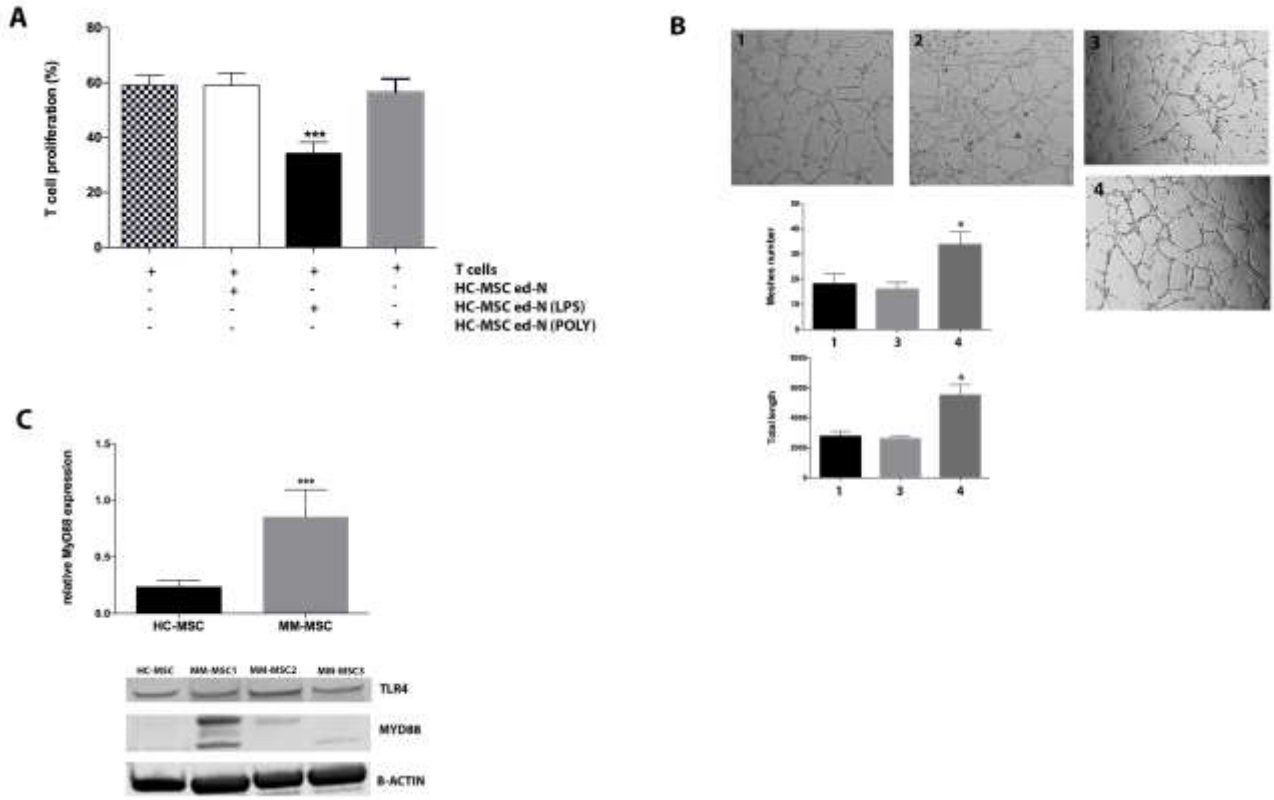

Figure17. LPS activate MSC toward an inflammatory phenotype associated with polarization of neutrophils versus a N2 phenotype. (A) Neutrophils isolated from co-culture with HC-MSC pre-treated with LPS are able to inhibit T cell proliferation. **(B)** After the pre-treatment with LPS, HC-MSC educated-neutrophils showed pro-angiogenic capacity in vitro. 1: IHBMEC control; 2: IHBMEC in presence of VEGF-A (positive control); 3: HC-MSCed-N; 4: plus ed-N isolated from co-culture of PBMC with HC-MSC pre-treated with LPS. **(C)** MyD88 expression was increased in MM-MSC in respect with HC-MSC. For analysis of western blot the optical density of the bands was measured using Scion Image software. All showed results represent the means of four independent experiments; error bars denote SD. *p<0.05; ***p<0.001.

2.2.5 MSC "activation" is induced by MM cells

To examine if plasma cells play a role in MSC polarization, before performing co-cultures with PBMC, we pre-treated HC-MSC or HS-5 cell line with MM cells (U266, MM1S). PC pre-treatment drives healthy MSC to activate neutrophils in immunosuppressive (Figure 18A) and pro-angiogenic cells just like SMM- and MM-MSC (Figure 18C). Moreover, exposure to PC induced up-regulation of pro-inflammatory factors as IL8, IL32 and PTGS2 (Figure 18B). We also observed an over-expression of CCL2 that regulates the recruitment of G-MDSC and enhances their immunosuppressive ability [141].

To confirm the role of TLR4 in MSC "activation", this pathway was investigated after exposure of HC-MSC to MM cell lines. Mitogen-activated Kinase (MAPK) cascade was not

activated and NF-kB did not translocate into the nucleus. Surplisingly, we found the activation of the transcription factor IRF3, indicating the involvement of the TLR4-MyD88-independent pathway (Figure 18D).

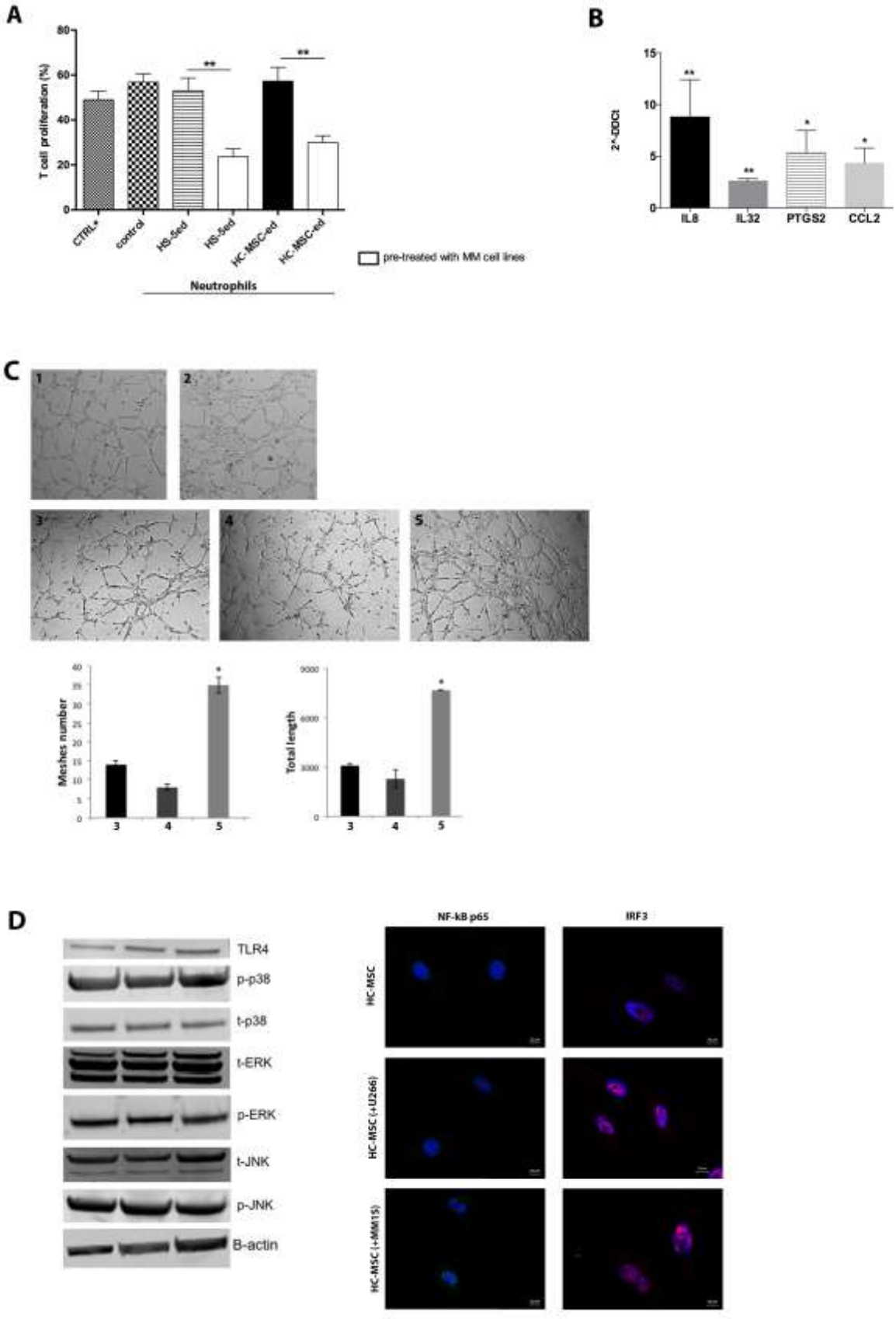

Figure18. PC activate MSC towards an inflammatory phenotype. (A) Neutrophils isolated from co-culture with HS-5 or HC-MSC pre-treated with MM cells were able to inhibit T cell proliferation. **(B)** Compared to HC-MSC not pre-treated, HC-MSC "educated" by MM cells up-regulated IL8, IL32, PTGS2 and CCL2. (**C**) After the pre-treatment with MM cells, HC-MSC-educated neutrophils showed pro-angiogenic capacity in vitro. 1: IHBMEC control; 2: IHBMEC in presence of VEGF-A (positive control); 3: plus N control (isolated from PBMC cultured without MSC); 4: plus HC-MSCed-N; 5: plus HC-MSCed-N isolated from co-culture with HC-MSC pre-treated with MM cells. (**D**) Western blotting analysis of MAPK cascade. Detection of NF-kB and IRF3 nuclear translocation was performed by incubation respectively with anti-mouse and anti-rabbit monoclonal antibodies followed by secondary antibodies conjugated to FITC (green) or TRITC (red). Counterstaining of cells was performed by using the nuclear dye, DAPI (blue). The photographs result from sequential analysis of the same microscopic field, followed by merging of different images with specific staining. All showed results represent the means of four independent experiments; error bars denote SD.*$p<0.05$; **$p<0.001$.

2.2.6 MM-MSC favour tumor engraftment and immune escape in vivo

We next explored in vivo the pro-tumor role of MM-MSC with respect to HC-MSC. After 7 days from implanting of mixtures of fluorescently labeled MM cells and HC- or MM-MSC into immunocompetent zebrafish, animals co-injected with U266 cells and MM-MSC showed higher human tumor cell engraftment calculated as tumor volume and fluorescence intensity compared with those injected with PC and HC-MSC ($p<0.05$) (Figure 19A). Cytofluorimetric analysis confirmed higher localization of human CD138+ and human CD90+ cells in organs (kidney marrow, spleen and liver) of zebrafish injected with PC and MM-MSC (respectively 36,4%±14,6 vs 4,9%±3,1 and 8,3%±0,35 vs 0,4%±0,2; $p=0.0039$ and $p=0.0007$; figure 19B). To examine changes in the Th1/Th2 balance in zebrafish after injection, we evaluated mRNA levels of master regulator transcription factors for Th1/Th2 lineage development: T-box transcription factor 21 (tbx21) and gata3. Tbx21 is a Th1 cell transcription factor important for Th1 lineage commitment and gata3 is a well-known regulator of Th2 cell differentiation. In addition, we evaluated the expression of IL-4 (IL4b) and IL-13 (Th2-type cytokines) and IFN-γ (ifnγ1-2) (a Th1-type cytokine) genes [142, 143]. Compared to animals co-injected with PC and HC-MSC, gata3, IL-4 and IL-13 were significantly up-regulated in zebrafish injected with PC plus MM-MSC (figure 19C), indicating that MSC from MM patients enhance mechanisms that circumvent the immune response.

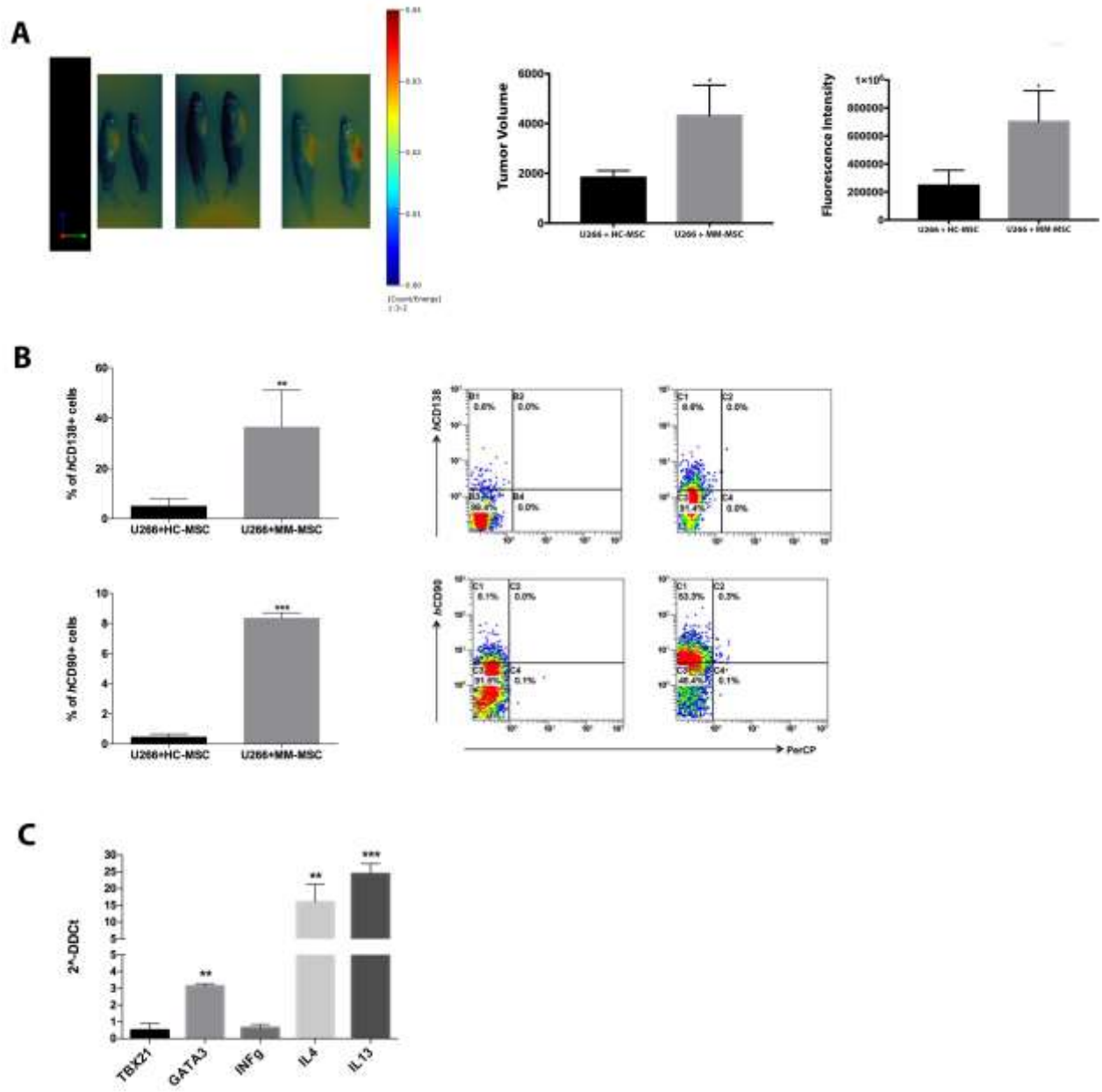

Figure18. MM-MSC enhance PC engraftment and immune escape mechanisms in immunocompetent Zebrafish. 3 animals were engrafted for every combination of PC with MM-MSC (n=6) or HC-MSC (n=4). **(A)** Evaluation of tumor xenografts by tomography. Prior to implantation, MM cells were labeled for co-implantation with MSC with DiIC18(5)-DS. The tumor volume and the intensity of fluorescence were measured using ImageJ software. **(B)** Detection of human U266 cells and human MSC in adult zebrafish using flow cytometry. **(C)** Analysis of changes in the Th1/Th2 balance in zebrafish using real time PCR. Results for animals co-injected with PC and MM-MSC are shown; calculated value of 2^-ΔΔCt in zebrafish injected with U266 and HC-MSC was 1. All analysis were performed 6 days after cellular mixture inhiection. *p<0.05;** p<0.001;***p<0.001.

DISCUSSION

The BM microenvironment consists of a cellular compartment, the extracellular matrix and soluble factors such as cytokines, chemokines and growth factors [144]. The cellular compartment is made up of ematopoietic cells including immune cells and non hematopoietic cells such as fibroblasts, endothelial cells, osteoblasts, osteoclasts and adipocytes. Complex interactions within the BM microenvironemnt between immune, non-immune and tumor cells influence the development and the progression of the hematological cancer, such as CML and MM. The theory of cancer immunoediting postulates that effector mechanisms of the immune system exert a dual role, eliminating cancer cells and promoting cancer progression through the selection of those cells whose genetic alterations allow tumor to progress in immuno-competent host [145, 146].

The ability of MDSC to support tumor growth and metastases can be divided into four functions: (a) protection of tumor cells from immune-mediated killing, (b) remodeling of the tumor microenvironment, (c) establishment of a premetastatic niche, and (d) interaction with tumor cells to induce "stemness" and facilitate the epithelial-to-mesenchymal transition (EMT) [147]. A large number of studies have demonstrated improvement of immune responses and antitumor immunity following depletion of GMDSC with a Ly6G-specific antibody in murine models [148-150].

We and other groups have previously demonstrated that there is a significant accumulation of MDSC in CML and MM patients which exert immunosuppression by releasing Arg1 [91]. As in other hematological malignancies, the prognostic role of MDSC accumulation has been documented in MM where they correlates with tumor progression and outcome of therapy [151]. For CML disease, a recent study demonstrated that both imatinib and dasatinib treatment efficiently decreased the amount of G-MDSC in CML patients, but it did not find a correlation between major molecular response (MMR) value and MDSC count [152]. Also our data (section 1) showed no correlation between G-MDSC number and MMR value both in IM, DAS and NIL treated patients. Nevertheless, a significant correlation was found between the number of persistent M-MDSC and MMR value in dasatinib treated patients. MDSC are supposed to provide a favorable microenvironment in which leukemia cells can evade host immunosurveillance and proliferate. Therefore, increased levels of M-MDSC in the follow-up of CML patients treated with dasatinib may indicate the delayed immune control and higher levels of residual leukemia cells. Studies by Sun et colleagues reported a similar observation in adult acute myeloid leukemia (AML) [153]. The authors found that the number of MDSC (identified as $CD33^{high}CD11b^{+}$ $HLADR^{low}$ cells) was significantly higher in the high minimal residual disease (MRD) group than that in the middle and low MRD groups. Moreover,

Gustafson et al. found that chronic lymphocytic leukemia (CLL) patients with higher levels of M-MDSC had a shorter time to disease progression compared to patients with lower levels [154]. These data suggest that M-MDSC could be predictive of poorer prognosis in different hematological malignancies. On the contrary of dasatinib therapy, the reduction of M-MDSC subset was not significant during imatinib and nilotinib treatment. This difference may be due in part to down-regulation of Src and NF-KB signal cascades as demonstrated for the inhibition of Treg [155] and MDSC in head and neck cancer [156]. Moreover, some studies reported that after dasatinib treatment, an immunostimulation can be observed with increased numbers of $CD8^+$ T cells, NK cells and decreased numbers of Treg [157, 158]. These effects seem to be dasatinib-specific because they were not observed with imatinib, nilotinib or bosutinib. Therefore, immunostimulatory ability of dasatinib may be in line with our data. CML patients can stop imatinib treatment without suffering disease relapse after achieving a complete molecular response (CMR) [111], suggesting that the immunesurveillance, whereby the immune cells inhibit tumor cell growth, plays a central role in restraining CML cells even in the absence of TKI treatment. The presence of relatively abundant NK cells and cytotoxic T lymphocytes (CTL) specific for CML antigens such as BCR-ABL1 or proteinase-3 are good candidates for predictive markers of safe TKI discontinuation [113, 159, 160]. It remains to be elucidated if MDSC might be candidate predictive markers of relapse risk following TKI discontinuation and their evaluation before and after discontinuation of imatinib involving a large patient cohort might be important. Moreover, this first study suggest the possible development in CML patients of a circuit primed by tumor cells that, through the release of soluble factors and exosomes, are able to expand M-MDSC, creating an immunotolerant environment that results in T cell anergy and facilitates tumor growth (Fig.19).

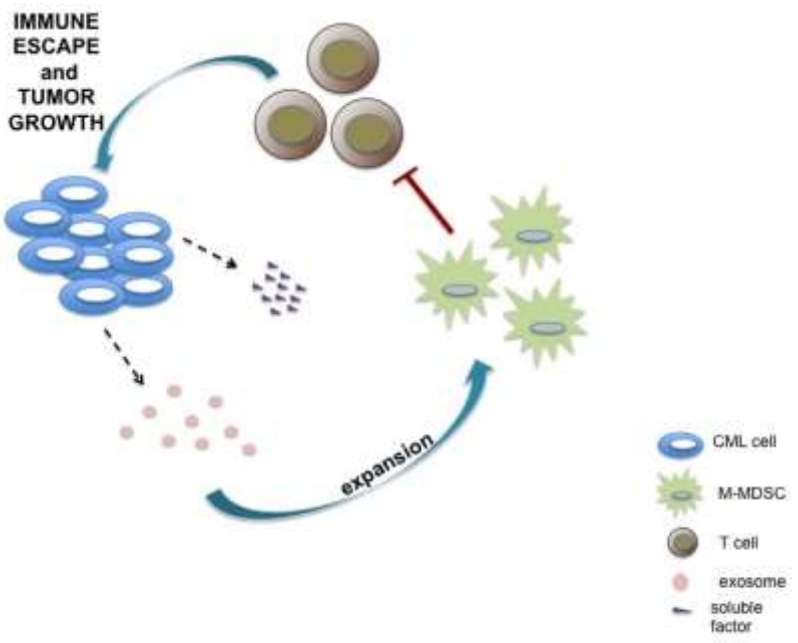

Figure 19. CML cells through the release of soluble factors and exosomes are able to expand M-MDSC, which in turn create an immunotolerant environment facilitating tumor growth.

(SECTION 2)

Although neutrophils are traditionally considered in the context of their antibacterial functions, it is becoming increasingly clear that tumor-associated neutrophils (TAN) play a major role in cancer biology. TAN can have an antitumorigenic (N1) phenotype or pro-tumorigenic (N2) phenotype capable of supporting tumor growth and suppressing the antitumor immune response. In untreated tumors, TAN develop a pro-tumorigenic phenotype termed 'N2 TAN' in analogy to the M2 macrophage phenotype. Depletion of these 'pro-tumorigenic' N2 neutrophils, therefore, inhibits tumor growth [47, 161] and reduces the level of immunosuppression in the tumor microenvironment [51, 162].

It has been demonstrated that both in CML and MM patients mature neutrophils are immunosuppressive cells as G-MDSC [91, 163], indicating a pro-tumoral role of both immature and mature neutrophils in cancer. Therefore, we named them N2 neutrophils. Studies have demonstrated specific examples of tumor-mediated signals (such as TGFβ) that induce N2 phenotype. We focused our attention on the role of MSC in tumor microenvironment transformation. They are an important component of BM niche and play an important role whitin an inflammatory milieu for their immunosuppressive ability which may

display negative effects in certain circumstances, promoting tumor evasion of immune surveillance [164, 165].

Even though their contribution in promoting tumor growth, survival and drug-resistance has been widely studied, the role of MSC in N2 phenotype activation and in promoting tumor immune escape within the microenvironemnt remains unexplored. Our present experiments demonstrated that MSC contribute to transform both the CML and MM microenvironment into an immune suppressive one by orchestrating neutrophils. Indeed, only CML- and MM- and SMM-MSC activate neutrophils into immunosuppressive N2. No suppressive effect was ever observed incubating T lymphocytes with HD- or MGUS-MSCed-N, demonstrating that tumor-associated MSC are functionally different from HD-MSC. Sa ́nchez et colleagues showed that immunosuppressive properties of MSC evolve along neoplastic transformation [166]. Using a murine model, the authors demonstrated that both normal and in vitro transformed MSC accumulated similar percentage of G-MDSC, but murine MDSC (IL4Rαhigh/GR1low) differentiated in presence of transformed MSC, exhibited an enhanced inhibitory effect on T cell proliferation. In human, it is still an open question to define a different role of tumor versus healthy MSC. Our data contribute to elucidate the different role of CML- and MM-MSC versus healthy MSC, confirming the alteration of their immune modulatory ability in CML and MM patients. Since this difference has been found for isolated CML- and MM-MSC after *in vitro* expansion, these stromal cells have a constitutive functional alteration in immune regulation.

Exploring the immunomodulatory factors expressed by CML-MSC at Time 0, we found a significant up-regulation of COX2, TGFβ, and IL6 compared to HD-MSC. These results reveal an acquired impairment by CML-MSC in their immunomodulatory functions. Moreover, during co-culture with PBMC, CML-MSC significantly up-regulated TGFβ, IL6, and IL10 expression, that reprogram immature myeloid cells to become immunosuppressive neutrophils [91, 167, 168]. Next, we examined the expression of immunomodulatory genes in MSC educated-neutrophils before incubation with T lymphocytes. Compared to HD-, CML-MSC ed-N up-regulated expression of ARG1, TNFα, IL1β, COX2 and IL6, providing thus evidence that CML-MSC transform myeloid cells in immunosuppressive neutrophils. Indeed, up-regulation of ARG1 is one of the main mechanisms of immunosuppression [34] and is highly expressed by both MDSC and polymorphonuclear leukocytes in CML patients [168]. TNFα has been shown to arrest differentiation of immature myeloid cells and increase MDSC suppressive activity [26, 139]. Also up-regulation of COX2 has been reported as mechanism of MDSC-mediated immunosuppression [26]. In addition, more recently, IL-6 has been found to stimulate NF-κB- mediated IDO upregulation in MDSC [169].

Similar results have been observed for MM-MSC. Exploring expression of immunomodulatory factors, we found a statistically signicant upregulation of PTGS2, TGFβ, NOS2, IL10 and IL6 expression in MM-MSC, suggesting that multiple mechanisms are involved in the activation of N2 phenotype. Since gene expression changes were not found at t0, the expression of the immune modulatory factors is influenced by interaction with PBMC *in vitro*.

Collectively, all these data show how CML-MSC and MM-MSC directly orchestrate neutrophils by driving activation of a N2 phenotype. Whether the alteration of the immunoregulatory abilities of MSC reveals an acquired capacity by MSC themselves, as consequence of a neoplastic transformation, or derived by interaction with tumor cells is a relevant question for clinical oncology. Zhanget and colleagues demonstrated the contribution of leukemia-induced alterations in the BM microenvironment that suppress normal HSC and provide a selective advantage to LSC [170]. Although the TKI reduce normal HSC inhibition by leukemic cells and facilitate their regrowth, it does not completely reverse leukemia-associated changes in the microenvironment [170]. Therefore, it is important to determine the mechanisms underlying these persistent changes and how leukemia-related alterations affect LSC response to TKI. Activating a N2 phenotype, CML-MSC are relevant in regulating T lymphocytes-mediated leukemia surveillance, becoming a potential target to act on leukemia microenvironment. When compared with HD-MSC, MM-MSC differ in cytokine production, show a decreased proliferative ability, a premature senescence and reduced ability to inhibit T cell proliferation [171, 172]. MM-MSC exhibit a distinctive gene expression profile compared to HD-MSC [173], suggesting that these differences could be attributed to the presence of genomic alterations in MM counterpart [174].

Patients affected by MM show increased neovascularization of the bone marrow stroma[175]. The grade of this neovascularization seems to increase during the evolution from MGUS to MM [176]. Our data show that MM-MSC are able to activate neutrophils in pro-angiogenic cells. Therefore they contribute both directly [135] and indirectly to the “angiogenic switch” that characterizes the transition from MGUS to MM. Moreover, since SMM-MSC have the same immunological functional alterations observed for MM-MSC, the activaction of an immunosuppressive microenvironemnt directed by MSC may contribute to the transition from MGUS to MM.

Toll-like receptors (TLR) are type I integral membrane glycoproteins with a crucial role in early host defence against invading pathogens [177, 178]. They are members of alarger superfamily that includes the interleukin-1 receptors (IL-1R) with a conserved cytoplasmic domain, that is known as the Toll/IL-1R (TIR) domain. Stimulation of TLR triggers the

association of the adaptor molecule MyD88 (myeloid differentiation primary-response protein 88), which in turn recruits IL-1R-associated kinases (IRAKs) and TRAF6 (tumour-necrosis-factor- receptor-associated factor 6)[179]. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1 (transforming growth factor activated kinase), TAB1 (TAK1-binding protein 1) and TAB2. After activation TAK1, in turn, phosphorylates both MAP (mitogen-activated protein) kinases and the IKK complex (inhibitor of nuclear factorB (IkB)-kinase complex), leading to its ubiquitynation and subsequent degradation and allowing nuclear translocation of NF-KB [179]. However, MyD88-deficient cells have revealed the existence of two different TLR4 signalling: MyD88-dependent and independent pathways, both of which mediate signalling in responce to LPS [180]. The MyD88-independent pathway activates IRF3 (interferon (IFN)-regulatory factor) and involves the late phase of NF-KB activation, both of which lead to the production of IFN-β and the expression of IFN-inducible genes [179]. MSC express several TLR and their ability to migrate, invade and secrete immune modulating factors was drastically affected by specific TLR-agonist engagement [76]. In particular, TLR4 stimulation polarizes MSC toward a pro-inflammatory MSC1 phenotype, while stimulation of TLR3 results in the polarization toward an immunosuppressive MSC2 phenotype [77]. Using specific agonists for TLR4 or TLR3, we observed that healthy MSC acquired the same immunological alteration of SMM- and MM-MSC after a pre-treatment with LPS. Indeed, stimulation of TLR4 in healthy MSC induced N2 neutrophil polarization with immusuppressive and pro-angiogenic capacity. Moreover, wester blotting analysis confirmed the up-regulation of MyD88 in MM-MSC compared to HC-MSC.

To examine if PC play a role in MSC polarization, before performing co-cultures with PBMC, we pre-treated HC-MSC or HS-5 cell line with MM cells. This pre-exposure to PC induced similar effects observed after pre-treatment with LPS. Therefore, we investigated if PC activate TLR4 pathway in healthy MSC. We did not observe nor activation of MAP kinases and NFKB translocation into the nucleus of MSC. Surplisingly, co-culture with PC induced IRF3 nuclear translocation, indicating the involvement of the TLR4-MyD88-independent pathway in MSC commitment.

Next, we explored the effects of the "activated" status of MM-MSC investigating their pro-tumor role in vivo. To further evaluate whether MM-MSC are stromal cells with a pro-tumoral behaviour, we used adult zebrafish as immune competent in vivo model. Implanting a mixtures of fluorescently labeled MM cells and HC- or MM-MSC, animals co-injected with U266 cells and MM-MSC showed higher human tumor cell engraftment. These data were also confirmed evaluating the percentage of *h*CD138 by flow cytometry. As in mice and

humans, also zebrafish has a genetically defined Th1/Th2 bias [143]. Therefore, we analyzed the expression of the master regulator transcription factors for Th1/Th2 and Th1- and Th2-type cytokines to better assess in vivo the involvement of the immune escape mechanisms promoted by co-injection of PC with MM-MSC. Our data revealed that MM-MSC and PC mixture promoted a Th2 responce, indicating that MSC from MM patients enhance mechanisms that circumvent the immune response.

In conclusion, our results highlight an important interplay between tumor cells, MSC and immune cells (Fig. 20). Indeed, tumor cells are able to promote MDSC proliferation and immune dysfunction in MSC with their consequent commitment toward an "activated" status to better 'serve' the cancer cells. Tumor microenvironment transformation from MGUS to MM is associated with progressive activation of MSC and TLR4 signaling may play a pivotal role inducing MSC commitment towards an inflammatory phenotype.

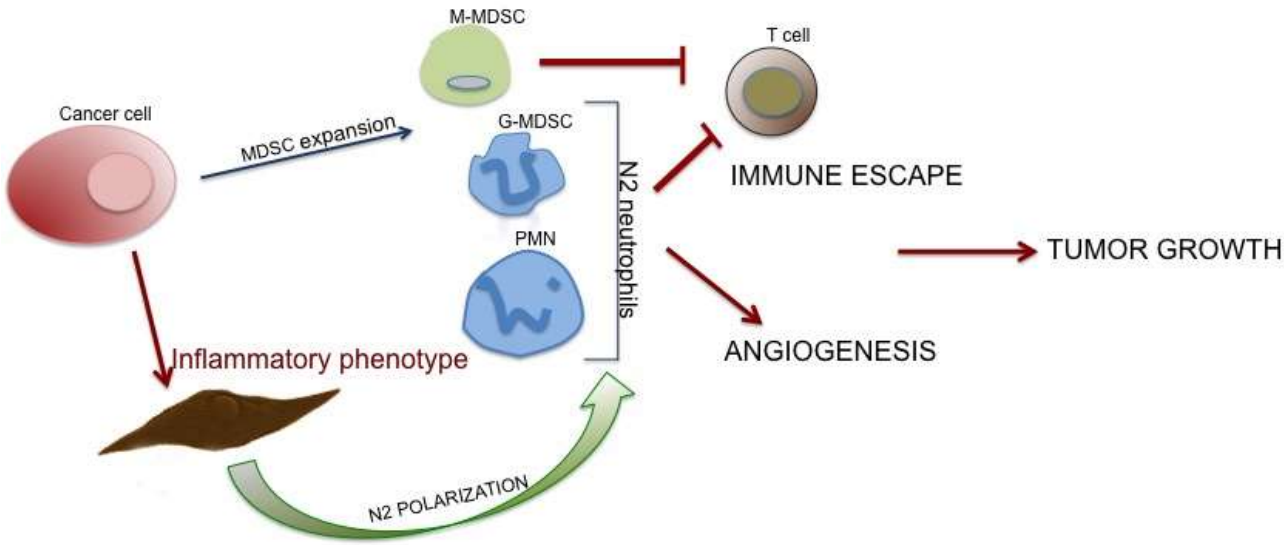

Figure 20. Tumor microenvironment transformation.

INFγ: interferon gamma
TNFα: tumor necrosis factor α
IL1: interleukin 1
IL6: interleukin 6
IL8: interleukin 8
IL10: interleukin 10
IL32: interleukin 32
IL1β: interleukin 1β
CSF1: colony stimulating factor 1
APC: antigen presenting cells
A2aR: adenosine A2a receptor
B7RP1: B7-related protein
BTLA: B and T lymphocyte attenuator
GAL9: galectin 9
HVEM: herpes virus entry mediator
ICOS: inducible T cell co-stimulator
KIR: killer cell immunoglobulin-like receptor
LAG3: lymphocyte activation gene 3
PD-1: programmed cell death protein 1
PDL-1: PD1 ligand
TGFβ: transforming growth factor-β
TIM3: T cell membrane protein 3
Arg1: arginase 1
IDO: indoleamine 2,3-dioxygenase
NK: natural killer
Treg: regulatory T cell
MDSC: myeloid derived suppressor cells
M-MDSC: monocytic-like MDSC
G-MDSC: granulocytic-like MDSC
PMN: polymorphonuclear neutrophils
LDN: low-density neutrophils
NDN: normal-density neutrophils
NOS2: nitric oxide synthase 2
ROS: reactive oxygen species
WBC: white blood cells
DC: dendritic cell
N1: anti-tumorigenic neutrophils
N2: pro-tumorigenic neutrophils
TAN: tumor-associated neutrophils
CCL2: C-C Motif Chemokine Ligand 2
CCL17: C-C Motif Chemokine Ligand 17
MSC: mesenchymal stromal cells
CAF: cancer associated fibroblasts
MHC: major histocompatibility complex
TLR: Toll-like receptors
αSMA: α-smooth muscle actin
HSC/HPC: hematopoietic stem/progenitor cells
BM: bone marrow
AML: acute myeloid leukemia
CML: chronic myeloid leukemia
CLL: chronic lymphocytic leukemia
PGE2: prostaglandin 2

HSC: haematopoietic stem cell
TKI: tyrosine kinase inhibitor
CMR: complete molecular response
TCR: T cell receptor
MM: multiple myeloma
MGUS: monoclonal gammopathy of undeterminated significance
SMM: smoldering multiple myeloma
HLA: human leukocyte antigen
TAM: tumor-associated macrophages
IM: imatinb
NIL: nilotinib
DAS: dasatinib
PBMC: peripheral blood mononucleated cells
HD: healthy donor
HGB: hemoglobin
PLT: platelet
LDH: lactate dehydrogenase
CFSE: carboxyfluorescein succinimidyl ester
PHA: phytohemagglutinin
SEM: Scanning Electron Microscopy
TEM: Transmission Electron Microscopy
Ed-N: educated neutrophils
MSCed-N: MSC educated neutrophils
BTZ: bortezomib
LEN: lenalidomide
POMA: pomalidomide
HBMEC: human brain microvascular endothelial cells
MyD88: myeloid differentiation 88
IRF3: interferon regulatory factor 3
NFKB: nuclear factor kappa-light-chain-enhancer of activated B cells
DAPI: 4′,6-diamidino-2-phenylindole
DiIC18(5)-DS: 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine-5,5'-Disulfonic Acid
zIL4: zebrafish interleukin 4
zIL13: interleukin 13
zTBX21: zebrafish T-box transcription factor
zGATA3: zebrafish GATA binding protein 3
GAPDH: Glyceraldeyde-3-Phosphate Dehydrogenase
COX2/PTGS2: cyclooxygenase-2/ Prostaglandin-Endoperoxide Synthase 2
LPS: lipopolysaccharide
Poly(I:C): Polyinosinic-polycytidylic acid
VEGFA: vascular endothelial growth factor A
Th1: T helper 1
Th2: T helper 2
***h*CD138**: human CD138
***h*CD90**: human CD90
MRD: minimal residual disease
CTL: cytotoxic T lymphocytes
IRAK: IL-1R-associated kinases
IL-1R: interleukin-1 receptor
TIR: Toll/IL-1R
TRAF6: tumour-necrosis-factor- receptor-associated factor 6

TAK1: transforming growth factor activated kinase
TAB1: TAK1-binding protein 1

SUPPLENTARY FIGURES

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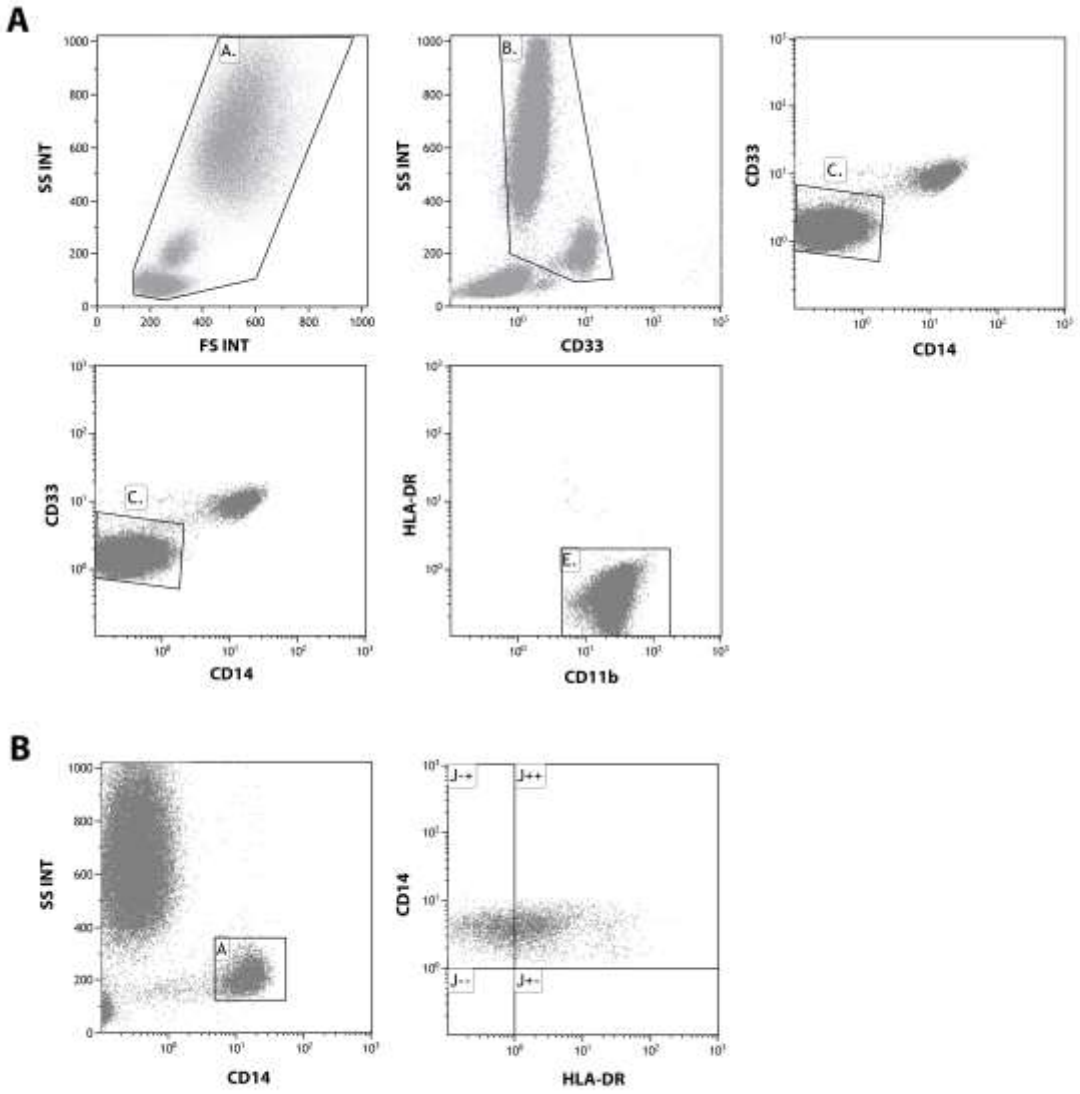

S-Figure 1. Flow cytometry plots with gating strategies for the identification of MDSC cells. The figure shows a representative cytoflorimetric analysis with gates set on CD11b $^{+}$CD33^{+}CD14^{-}HLADR^{-} (G-MDSC) (A) and CD14^{+}HLADR^{-} (M-MDSC) (B) cell populations.

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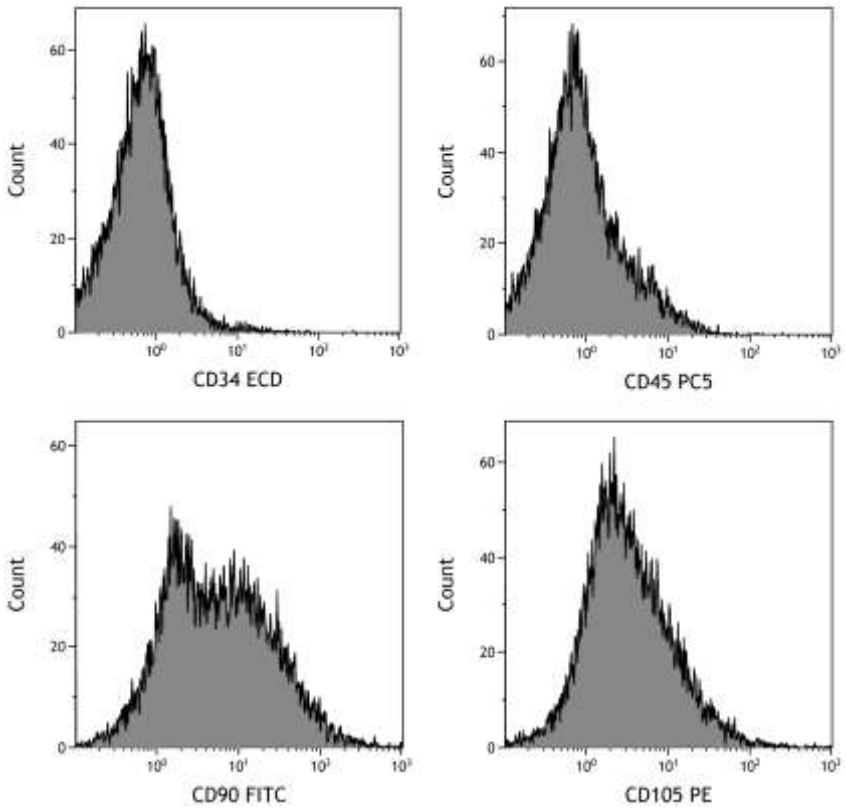

S-Figure 2: Evaluation of MSC specific surface antigen expression. Representative data from one MM-MSC sample. Flow cytometry analysis shows that MSC are positive for CD90 and CD105 and negative for CD34 and CD45.

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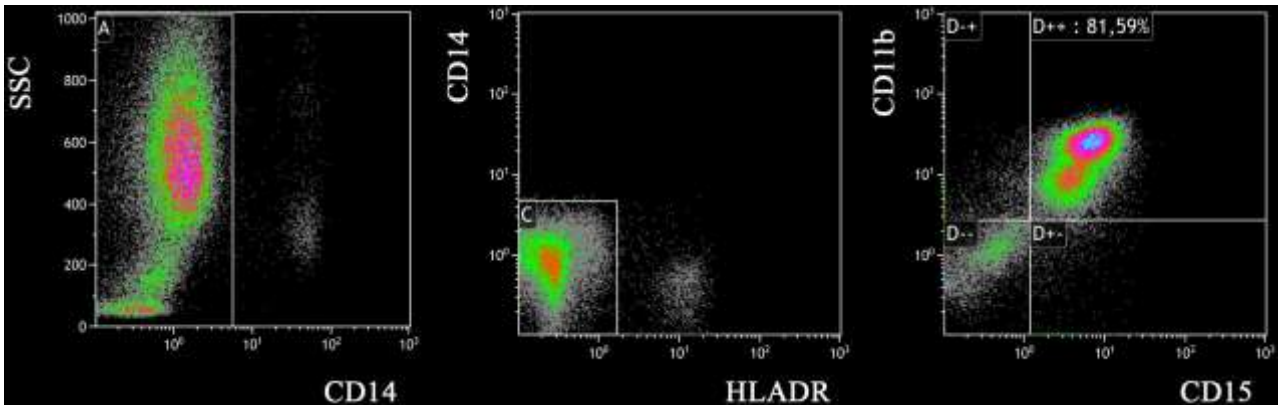

S-Figure 3: Purity of educated neutrophils after magnetic cell separation. After separation, the cells were incubated with fuorescently labeled anti-CD11b, anti-CD15, anti-CD14 and anti-HLADR antibodies, and the purity of the cells was analyzed by flow cytometry. The figure reports the representative flow cytometry dot plots showing the purity of educated neutrophils.

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