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Tumor-derived microRNAs induce myeloid suppressor cells and predict immunotherapy resistance in melanoma

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Abstract
The accrual of myeloid-derived suppressor cells (MDSCs) represents a major obstacle to effective immunotherapy in cancer patients, but the mechanisms underlying this process in the human setting remain elusive. Here, we describe a set of microRNAs (miR-146a, miR-155, miR-125b, miR-100, let-7e, miR-125a, miR-146b, miR-99b) that are associated with MDSCs and with resistance to treatment with immune checkpoint inhibitors in melanoma patients. The miRs were identified by transcriptional analyses as being responsible for the conversion of monocytes into MDSCs (CD14^{+}HLA-DR^{neg} cells) mediated by melanoma extracellular vesicles (EVs) and were shown to recreate MDSC features upon transfection. In melanoma patients, these miRs were increased in circulating CD14^{+} monocytes, plasma and tumor samples, where they correlated with the myeloid cell infiltrate. In plasma, their baseline level clustered with the clinical efficacy of CTLA-4 or PD-1 blockade. Hence, MDSC-related miRs represent an indicator of MDSC activity in cancer patients and a potential blood marker of a poor immunotherapy outcome.
Introduction

Immunotherapy with immune checkpoint inhibitors (ICIs) has achieved a remarkable improvement in overall survival (OS) in different tumor histologies, albeit the majority of patients display intrinsic resistance (1). The response to PD-1 blockade requires a pre-existing and flourishing immune response and involves determinants of adaptive immunity, such as a high number of tumor DNA mutations or neoantigens, the interspersed distribution of infiltrating effector T cells, a high level of the expression of PD-L1 or an IFN-related gene signature (2,3). In contrast, resistant tumors lack signs of immunogenicity and are definable as “immune-desert” based on the absence of local T cell reactivity (4). Resistant tumors are also endowed with the innate anti-PD-1 resistance signature (IPRES) (5), which involves specific genetic programs, including wound healing, epithelial-mesenchymal transition (EMT), extracellular matrix remodeling, TGF-β signaling, hypoxia, macrophage chemotaxis and angiogenesis. Most of these mirror the transcriptional profile of myeloid-derived suppressor cells (MDSCs), a population of immature myeloid cells pathologically associated with cancer and known for their potent inhibitory activity on antitumor T cell immunity (6). In addition, MDSCs directly coordinate cancer cell plasticity, triggering the EMT phenotype (7,8) and activating signaling pathways often associated with drug resistance, stroma remodeling and angiogenesis (9,10). MDSCs distribute systemically in cancer patients, accumulating in the peripheral blood, lymph nodes (LN) and distant organs, where they facilitate cancer dissemination by forming the so-called pre-metastatic niche (6). A high frequency of MDSCs in the peripheral blood, mainly comprising monocytic and polymorphonuclear subsets (9,11), is associated with poor prognosis (6,12) and represents a possible mechanism of resistance to immunotherapies (11,13-17).

MDSC generation is believed to stem from conditioning of the bone marrow (BM) by soluble tumor factors, including GM-CSF, M-CSF, G-CSF and other proinflammatory...
cytokines (6). A delivery of tumor factors could be facilitated by shuttling organelles (18). Extracellular vesicles (EVs), nanoparticles abundantly secreted by cancer cells, are involved in the intercellular transfer of proteins, lipids and genetic material, such as RNAs and microRNAs (miRs) (19-21). Because of their ability to recirculate in body fluids, EVs represent an ideal candidate in the process of BM conditioning that leads to MDSC generation (22,23). In the present work, we identified a panel of circulating miRs associated with monocytic MDSC (M-MDSC) activity in melanoma patients, representing a potential tool to assess the role of these cells in the resistance to ICIs.
Results

The monocyte-MDSC transition is mediated by melanoma EVs

We and others have previously defined cancer-associated human M-MDSCs as CD11b<sup>+</sup>CD33<sup>+</sup> myeloid cells expressing the hallmark CD14<sup>+</sup>HLA-DR<sup>neg</sup> phenotype and exerting immunosuppressive activity on T cells (9,11). In an attempt to mimic this phenotype in vitro, we opted to use tumor EVs to convert normal myeloid cells. EVs were purified by differential centrifugation of conditioned medium (CM) from melanoma cell lines and evaluated for their morphology and marker profiles (Supplemental Figure 1A). Here, we report that CD14<sup>+</sup> monocytes, when exposed for 24 h to melanoma EVs isolated from short-term (INT12, LM38) and long-term (Mel501, 624.38) cultured cell lines, show down-regulated HLA-DR expression, increase of IL-6 and CCL2 transcription and secretion together with suppressive properties on activated T cells (Figure 1A, B and Supplemental Figure 1B and C), a phenotype we have named EV-MDSCs. Monocyte conditioning strictly relies on the presence of vesicles, as EV-depleted melanoma CM loses the ability to down-regulate HLA-DR expression (Supplemental Figure 1D).

CD14<sup>+</sup>HLA-DR<sup>neg</sup> M-MDSCs accumulate in the peripheral blood of metastatic melanoma patients as compared to healthy donors (HD, Figure 1C) (11,24), and show spontaneous production of the immunosuppressive and protumor factors such as IL-6, CCL2 and TGFβ (10,11,25), detected ex vivo as RNA and proteins (Supplemental Figure 1E). Their suppressive potential is documented by the boost or decrease of T cell proliferation upon depletion or re-addition of CD14<sup>+</sup> cells from patients’ PBMCs (Supplemental Figure 1F). Interestingly, CD14<sup>+</sup> cells from patients retain the potential to acquire EV-MDSC features measured as HLA-DRA down-regulation in the presence of melanoma EVs (Figure 1D). Furthermore, we could ascertain the induction of functional EV-MDSCs in an autologous setting, i.e. with EVs purified from CM of a patient’s melanoma cell line, which we
incubated with the patient’s monocytes isolated from PBMCs; these EV-MDSCs were able to inhibit autologous T cell proliferation (Figure 1E).

Parallel experiments have been performed in a murine setting with EVs released by the Ret melanoma cell line established from skin melanomas developed by RET transgenic mice (ret-EVs) (26). Ret-EVs convert BM immature myeloid cells (IMCs) into MDSCs in vitro, boosting NO, iNOS and arginase-I expression and immunosuppressive activity on T cell proliferation (Supplemental Figure 2A-C). Upon i.p. administration to naïve C57BL/6 mice, fluorescently labeled EVs interact with Gr1+CD11b+ IMCs and, to a lesser extent, with F4/80+Gr1neg macrophages in the spleen, BM and LNs (Supplemental Figure 2D). Repeated administration of ret-EVs leads to a significant increase only in the frequency of Gr1+CD11b+ Ly6Chigh M-MDSCs, detectable in the BM (Supplemental Figure 2E).

Since tumor EVs can circulate in body fluids together with EVs originating from immune and other cells, we tested whether plasma EVs from advanced melanoma patients could also promote the EV-MDSC phenotype in vitro. EV screening of unprocessed plasma by nanoparticle tracking analysis (NTA) shows that patients’ samples contain EVs in increased number and mean size as compared to those from HDs. Notably, the size of EVs directly correlates with the frequency of circulating MDSCs in matched peripheral blood samples, suggesting a potential link to the disease (Figure 1F and supplemental Figure 3A). To obtain vesicles for functional studies, we applied a two-step centrifugation protocol aimed at enriching for larger (f1, obtained at 16,500 g) versus smaller EVs (f2, obtained with subsequent centrifugation of f1 supernatant at 118,000 g), as indicated by transmission electron microscopy (TEM) and bead-calibrated flow cytometry (Supplemental Figure 3B). Interestingly, f1 EVs from melanoma patients’ plasma down-regulate HLA-DRA at RNA level in HD CD14+ monocytes upon in vitro co-culture, suggesting the presence of EV-MDSC-converting vesicles potentially derived from tumor cells (Figure 1G). This effect was less frequent with f2 EVs from melanoma patients, which
in contrast induced an increase of HLA-DRA expression in HD monocytes. The up-regulation of HLA-DRA at the RNA level could be ascribed to monocyte activation possibly due to the presence of stimulating EVs in f2 plasma fraction likely originating from APCs, similarly to what we observed with f1 and f2 from plasma of HDs (Figure 1G). Reports have indeed shown that plasma exosomes can activate monocytes by inducing the up-regulation of HLA-DR and CD86 expression in healthy and pathological conditions (27,28). Similarly to normal monocytes, also monocytes from melanoma patients down-regulate their HLA-DRA expression upon co-culture with autologous plasma f1 and f2 EVs, accentuating the potential relevance of this phenomenon in vivo. Of note, EV-MDSCs generated with autologous plasma f1 and f2 EVs display an increased suppressive effect on T cell proliferation with respect to unconditioned ones (Figure 1H). The actual presence of melanoma EVs in patient’s plasma could be confirmed by protein analysis, showing that in contrast to the HD, both f1 and f2 EVs of the patient express gp100 melanoma marker with a tendency to accumulate in f1. This data may contribute to explaining the down-regulation of HLA-DRA in HD monocytes that we observed predominantly with f1 plasma EVs from melanoma patients as shown in Figure 1G. An increase in f1 could be also assessed for the EV markers Rab5B, CD63 and CD49b/VLA-2 integrin, while both fractions show similar levels of the LAMP-2 EV marker (Figure 1I).

**The transcriptional regulation of EV-MDSCs involves miR modulation**

We next investigated the molecular mechanisms leading to the in vitro induction of EV-MDSCs. Genome-wide transcriptional analysis revealed 662 genes, 514 up-regulated and 148 down-regulated (FC>\(|2|\), FDR<0.05), as differentially expressed in EV-MDSCs with respect to untreated monocytes (Supplemental Table 1). Gene Ontology (GO) enrichment analysis indicated that most of the up-regulated genes are involved in various immune cell functions, including inflammatory response, apoptosis signaling pathways, signal
transduction regulation and wound healing (Supplemental Table 2). Notably, the expression pattern of bona fide MDSC hallmark genes in the human setting is detectable in EV-MDSCs at the transcriptional level. We also identified key chemokines for protumor myeloid cell functions and hematopoietic stem cell maintenance, including CCL1, CCL22, CXCL5, CCL7, CSF1 and PPBP/CXCL7 among the top hits. Several transcriptional factors were up-regulated, including HIF1, mTOR, NOTCH1, NFKB2, RUNX1, MYC, ETS1, factors involved in STAT3 downstream signaling pathways (MYC, CCND1 and MCL1) and different SMAD genes, most of which have been reported to be involved in MDSC functions (29-31). Genes involved in increased survival and anti-apoptotic effects (e.g., BCL2 and CDKN2A), are enhanced, together with some molecular hallmarks of murine MDSCs, including S100A8 and MMP9 (Figure 2A). Notably, approximately 10% of genes differentially expressed in EV-MDSCs were associated with EMT, as revealed by analysis of the overlap in a list of EMT-related genes; this analysis included an EMT database (32), gene sets associated with EMT and/or wound healing in the IPRES signature (5) and genes common to mesenchymal and myeloid cells (33) (Figure 2B). The modulation of critical genes, including the down-regulation of HLA-DRA, HLA-DPA1, CD1D, and TLRs (TLR4, TLR5, TLR7) and the up-regulation of CD274/PD-L1, TNFRSF8/CD30, genes promoting angiogenesis and metastasis (PTGS2 and VEGFA) and genes controlling cell survival and proliferation (BCL2A1 and CDKN2A), have been confirmed by qPCR analysis (Figure 2C).

The CIBERSORT assessment of transcriptome data (34) shows a significant enrichment of the M0 fraction of nonpolarized monocytes (Supplemental Figure 4) rather than to M1 and M2 polarized macrophage fractions (35). In silico analysis of EV-MDSC-regulated genes also indicated a consistent overlap with an MDSC gene list described in colon tumor tissue datasets (36). To confirm the overlapping features of our EV-MDSCs with myeloid cells from cancer patients, we compared the transcriptomic profile of EV-MDSCs with those of
monocytes sorted from the blood of patients with different tumor types (37-39) and found a significant enrichment of the overexpressed genes (Figure 2D).

Monocyte skewing to EV-MDSCs also shows changes in miR expression. Via profiling, 98 miRs were identified as differentially expressed between monocytes and EV-MDSCs (FC |1.2|, FDR <0.05); 34 were differentially expressed with a fold change of ≥|2|, including miRs such as miR-146a, miR-125 and miR-155, described as being involved in the regulation of classical and alternative myeloid cell activation (Figure 3A and Supplemental Table 3) (40-42). Integrative analysis of miRs and gene expression profiling data revealed regulatory networks governing key myeloid cell functions, including the chemotaxis, adhesion, differentiation, and recruitment of phagocytes, macrophages and antigen-presenting cells (Supplemental Figure 5).

**miRs induce the EV-MDSC phenotype in CD14⁺ monocytes**

Since EVs are known miR carriers (43-45), we hypothesized that EV-mediated miR cargo is involved in the process of in vitro EV-MDSC conversion.

Profiling enabled us to identify 104 miRs detectable in both melanoma EVs and matched tumor cells (Supplemental Table 4). By crossing this set of miRs with those overexpressed in EV-MDSCs and associated with gene down-regulation, we selected the top up-regulated miRs - namely, miR-146a, miR-155, miR-125b, miR-100, let-7e, miR-125a, miR-146b, and miR-99b - as MDSC-associated miRs for subsequent studies (Figure 3A; Supplemental Table 5). The evaluation of these miRs in plasma EV fractions f1 and f2 of melanoma patients shows that they are all detectable, and that five out of eight miRs (miR-146a, miR-146b, let-7e, miR-99b and miR-125b) are enriched in the f1 EV fraction, while the others appear equally distributed (Figure 3B). Similarly, melanoma cell line-derived EVs show comparable levels of MDSC-miRs in f1 and f2, compatible with the release of larger and smaller EVs by melanoma cells in vitro. Of note, the miR content associates
with the induction of EV-MDSCs, as shown by HLA-DRA down-regulation in HD monocytes (Supplemental Figure 6). The contribution of MDSC-miRs in MDSC conversion was then explored with the help of corresponding mimics transfected into HD monocytes. Similar to EV-MDSCs, miR-transfected monocytes display down-regulation of HLA-DRA and the induction of IL6 and CCL2 expression (Figure 3C). In line with their MDSC nature, these cells also exert suppressive activity on T cells, as suggested by the impairment of proliferation, CD25 expression, and IFNγ and TNFα secretion in TCR-triggered autologous T cells (Figure 3D). Conversely, the transfection of melanoma patient-derived monocytes with miR inhibitors led to their functional recovery (Figure 3E), indicating that MDSC-miRs play a role in sustaining myeloid cell-related protumor effects. The potential link of MDSC-miR expression and the accumulation of MDSCs in melanoma patients’ peripheral blood is also supported by a significantly higher expression of six out of eight MDSC-miRs in the patients’ samples, which we could observe upon comparing the levels of MDSC-miRs in CD14+ cells sorted from PBMCs of HD with those of metastatic stage IIIC-IV melanoma patients at treatment baseline (i.e., at ≥ one month apart from any systemic therapy) (Figure 3F).

We next assessed the contribution of direct MDSC-miR transfer from a tumor to monocytes via EVs with the aid of EVs derived from melanoma cells silenced for miR-146a, miR-100, miR-125b and miR-155. miR-devoid EVs lose the ability to convert monocytes into EV-MDSCs in terms of HLA-DRA down-modulation, CCL2 and IL6 induction and MDSC-miR up-regulation (Figure 4A and B). Similarly, the contemporaneous inhibition of these MDSC-miRs by adding miR inhibitors to melanoma EV-exposed monocytes from HD abrogates the acquisition of the EV-MDSC phenotype as well as suppressive activity on T cell activation and cytokine production (Figure 4C-E). Of note, the addition of miR inhibitors to a patient’s monocytes conditioned with EVs deriving from
the autologous melanoma cell line also restores T cell activity and cytokine release (Figure 4F).

Taken together, these results show that MDSC-miRs display an increased expression in monocytes of patients, circulate within EVs in patients’ plasma and condition myeloid cells via EV-mediated transfer.

**MDSC-miRs are enriched in correlation with myeloid cell accrual**

The relevance of MDSC-miRs was next investigated ex vivo in human melanoma specimens. As expected, we detected high miR-146a, miR-155, miR-125b, miR-100, let-7e, miR-125a, miR-146b and miR-99b levels in subcutaneous, visceral and lymph node metastases from stage III-IV melanoma patients (n=58) by qPCR, confirming the expression of these miRs by this tumor type (Supplemental Figure 7). Interestingly, MDSC-miRs correlated with myeloid markers CD163, CD14, CD209, CD68, ITGAM (CD11b/Mac-1) and CD33 detected in the same samples but not with T cell markers CD3, CD4, CD8 or with tumor-related markers PMEL, TYR, MLANA, miR-21 and miR-211; the only exception were miR-155 and miR-100 which correlated with CD4 (Figure 5A and Supplemental Table S6). Multivariate statistical analysis showed a significant correlation between the MDSC-miR cluster and the expression of all myeloid markers (P=0.0062, r=0.5491). Additionally, the correlation of CD14 and CD163 expression with the miR-146a, miR-100, miR-125b and miR-155 clusters was also significant (P=0.0455, r=0.2088). The association of MDSC-miRs with infiltrating myeloid cells at the RNA level was confirmed by the correlation of miR-146a, miR-125b and miR-146b with the number of CD163+ cells in tumor sections (Figure 5B). Indeed, in situ hybridization revealed that miR-146a, miR-100, miR-125b and miR-155 expression was localized in both CD163+ and melanoma cells (Figure 5C). This data suggests that these miRs might be indicators of MDSC activity at the tumor site.
**MDSC-miRs are enriched in the plasma of melanoma patients and cluster with resistance to immunotherapy**

To assess MDSC-miRs in the circulation, we measured their expression levels in plasma obtained from melanoma patients with advanced disease (stage IIIC unresectable and stage IV) (n=20) and from a group of age- and gender-matched HD (n=20). The analysis showed increased levels of all MDSC-miRs in the patients, with statistically significant differences shown for miR-146a, let-7e, miR-125a, and miR-146b (Figure 6A). Notably, the expression levels of the studied miRs appeared more inter-correlated in patients than in HD (data not shown), further indicating an association with disease.

To test the potential for using MDSC-miRs as peripheral blood-based markers, we conducted a retrospective analysis in a set of plasma samples obtained from metastatic melanoma patients (n=87) receiving either ICIs (ipilimumab or nivolumab) or targeted therapy (TKIs; vemurafenib or the combination of dabrafenib and trametinib). Enrolled patients included 49 and 37 patients in the ICI and TKI groups, respectively (Supplemental Table 7). The expression levels of MDCS-miRs in the baseline samples were analyzed and correlated with overall survival (OS) and progression-free survival (PFS), both singly and jointly, in the latter case via a machine learning tool known as Adaptive Index Modeling (AIM). By estimating the joint contribution of the 5 miRs showing an association with disease outcomes in univariable assessments (let-7e, miR-125a, miR-99b, miR-146b and miR-125b), we could divide patients into 5 groups on the basis of an index score comprising the number of miRs showing increased expression, as detailed in Methods. Regarding OS, by pooling low scores (0-1; consisting of no or only one increased miR) versus high scores (>1; having 2 to 5 altered miRs), we observed that the low scores were associated with significantly better OS than were the high scores (Figure 6B). A comparable trend was observed when PFS was analyzed (Supplemental Figure 8). Most importantly, when these data were divided by the type of therapy, the difference in OS
between low and high scores was much more evident, with a P-value of 0.003, for ICI-treated patients; no difference was detected for TKI-treated patients. Comparable data were observed for PFS; however, the curves for ICI patients were less divergent than were those for OS due to the known minor impact of ICIs on PFS (2). These data clearly indicate that our MDSC-miRs may represent predictive markers of the response to or the benefit of ICIs in advanced melanoma patients.
Discussion

In the present work we discovered a set of miRs that associates with the phenotypic and functional features of M-MDSCs in melanoma patients. These miRs are detected in plasma as associated with EVs, in blood monocytes and in tumor biopsies, as correlated with the frequency of altered myeloid cells. Most importantly, higher circulating levels of these miRs cluster with shorter PFS and OS in patients receiving ipilimumab and nivolumub but not in those treated with BRAF/MEK inhibitors. These latter findings, if prospectively validated, pave the way for the development of what, to our knowledge, represents the first predictive peripheral blood biomarker of resistance to ICIs in cancer. The assessment of MDSC-miRs would make use of the concept of liquid biopsy (46), which has traditionally focused on genetic tumor features (47) but surely could be implemented with information from the systemic environment, including host immunity (48).

The evidence that factors associated with myeloid-related immune suppression might influence disease outcome in melanoma patients is not unexpected, as higher MDSC frequency is a constant in poor disease prognosis, even during ICI administration (14-17). The true predictive role of the MDSC-miRs here identified might instead reflect specific functional properties that MDSCs could exhibit once immune responses are unleashed by the ICIs (29). This hypothesis is in line with preclinical data showing that tumor resistance to PD-1 or CTLA4 blockade can be reverted by myeloid cell depletion (49, 50).

Major evidence, albeit primarily in murine models, indicates a central role of miR-mediated post-transcriptional regulation in MDSC differentiation and function (40, 51). Via in vitro studies on monocyte conditioning, we found that hallmarks of human MDSCs - i.e., down-regulation of HLA-DR; secretion of IL-6, TGFβ and CCL2; and an inhibition of T cell proliferation and function (9,10) - can be reconstituted by in vitro co-transfection of the eight MDSC-miRs; this finding held true even when the requirement was decreased to four
miRs (miR-146a, miR-100, miR-125b and miR-155) in some donors (data not shown). The chance of capturing such a complex regulatory scenario likely stems from the use of tumor EVs for MDSC generation. Indeed, EVs are known to carry distinct sets of miRs and to deliver them into target cells for the induction of specific functions, a key mechanism of intercellular cross-talk (52). In the context of cancer, transformed cells exploit EV-mediated miR transfer to mold host tissues by triggering angiogenesis, EMT, invasion and metastasis, immune escape and multidrug resistance (23, 43). Here, we provide experimental proof that melanoma EVs are embedded with a set of miRs ready to convert monocytes into bona-fide MDSCs upon direct transfer. As this process is mimicked by ret-EVs injected in vivo into C57BL/6 mice (Supplemental Figure 2E), it is reasonable to hypothesize that MDSC accumulation in melanoma patients might also involve in vivo myeloid conditioning by tumor EVs. The ability of these nanoparticles to distribute in body fluids (20) and reach the BM as well as other immunoregulatory sites could then account for the alteration of myelopoiesis, as depicted by our mouse studies as well as by previous preclinical data (18).

An in vitro tumor-mediated education of differentiated monocytes to MDSC-resembling cells was already reported by others (53). This process can be explained by the high plasticity of monocyte differentiation that cancer can arrest and shift toward immunoregulatory/suppressive mediators, as originally described for sepsis and cardiovascular diseases (54). This evidence supports the recently introduced hypothesis that cancer-associated myeloid dysfunctions could stem not only from progenitors conditioning in the BM but also from the peripheral reprogramming of circulating differentiated monocytes and neutrophils (55, 56).

Most of the miRs here identified are involved in myeloid cell differentiation and polarization by participating in pathways often associated with cancer-related immunosuppression (57-59). For instance, miR-146a, one of the most studied myeloid miRs (40), is reported to
serve as negative feedback in the TLR4-mediated activation of NFkB-related genes (60) and to promote M2 polarization in both humans and mice (42). miR-155, in addition to its key role in T cell responses (61), is the top up-regulated miR in GM-CSF- and IL6-induced MDSCs and is involved in STAT3 activation via SHIP-1 and PTEN targeting (62). This miR has also been shown to facilitate tumor growth by promoting MDSC accrual, survival and function through SOCS1 inhibition (63-66). Similarly, miR-125b and miR-125a have been implicated in monocyte differentiation toward immunosuppressive phenotypes (40,42) via a pathway involving Lin28A (67), a key factor in hematopoietic stem cell growth and survival (68). miR-125b is produced with miR-100 from a common primary polycistronic transcript; miR-99b, let-7e and miR-125a are produced via a similar mechanism (69), which explains their coordinated expression in tumor biopsies and in circulating monocytes from melanoma patients (Figure 5A and Figure 3F). The miR-99b/let-7e/miR-125a miR cluster was also shown to play a role in the mechanisms that stabilize STAT-3 activity in tolerogenic APC (70). Specific insights into the pathways targeted by MDSC-miRs derive from matching their predicted gene panels with the transcriptional profile of EV-MSDCs, an analysis that provides key information about the vital processes of cancer-related myeloid dysfunctions involving chemotaxis, adhesion and differentiation, with nodes including expected as well as novel patterns (Supplemental Figure 5).

Overall, our study sheds light on the poorly defined patterns of human MDSC activity, providing strong evidence about the role of defined miRs in driving cancer-related myeloid cell reprogramming. The involvement of MDSC activation and expansion in limiting the clinical efficacy of ICIs is also strengthened by our results, pointing to the need of drugs for the correction of myeloid dysfunctions (51), to overcome immunotherapy resistance in melanoma patients. Lastly, the evaluation of MDSC-miRs plasma levels holds promise as a potential biomarker to assess and monitor systemic immunosuppression in cancer patients, possibly predicting their chance of responding to ICIs.
Methods

Human studies. Peripheral blood samples from fasting patients and HD were processed within 2 h for plasma collection and PBMC isolation and storage (11). PBMCs from stage III-IV patients obtained before surgery or medical treatment were studied for MDSCs by immunophenotyping, and sorted CD14+ cells were assayed for cytokine, miR expression, MDSC conversion and suppressive function. Plasma from stage IIIC-IV patients at baseline prior to any medical treatment were studied for circulating EVs and miRs. Metastatic tumor specimens obtained from stage IIIC-IV melanoma patients were snap frozen and processed for RNA extraction and analyses.

Mouse studies. C57BL/6 mice were purchased from Charles River Laboratories. In vivo and ex vivo interaction studies investigating the effects of ret melanoma EVs on myeloid cells involved the i.p. injection of 200 μg EVs, labeled with Cell Proliferation Dye eFluor 670 (Thermo Fisher Scientific) for interaction, or unlabeled EVs injected 3 times every 48 h. Four days after the last injection, the animals were sacrificed to collect the BM and spleen cells for flow cytometry evaluation. CD11b+Gr1+ immature myeloid cells (IMCs) \((10^5)\) were cultured with 5 μg EVs overnight. NO production was measured by Griess reagent (Promega), and arginase-I and iNOS expression was measured by flow cytometry. Treated IMCs were washed twice by centrifugation to remove EVs and co-incubated with CFSE-labeled T cells stimulated with CD3/CD28 mAbs (T cells:IMCs=1:1).

Cell cultures. Melanoma cell lines were generated in our laboratory from human melanoma specimens (71). The ret mouse melanoma cell line was established from the skin tumors of ret transgenic mice (26). Mycoplasma contamination was tested periodically.
Isolation of extracellular vesicles (EVs). According to a standardized protocol optimized for large-scale EV production, the supernatant of $10^9$ human melanoma cells, cultured for 72 h in RPMI 1640 medium containing 10% EV-depleted FCS (obtained by centrifuging FCS 2 h at 118,000 g), 2 mM L-glutamine and 200 U/ml penicillin/streptomycin, was centrifuged at 300 g for 10 minutes, 0.22 μm vacuum filtered (Millipore) and ultracentrifuged (Sorvall WX Ultra 100, Thermo Fisher Scientific) at 100,000 g for 2 h at 4°C. Subsequently, collected EVs were suspended in PBS and washed by ultracentrifugation (1 h, 118,000 g, 4°C). After protein quantification (Bradford Protein Assay, Bio-Rad), aliquots were frozen at -80°C until further use. *Ret* murine melanoma EVs were purified from 48 h serum-free conditioned media of *ret* cells by sequential centrifugation (300 g for 10 min, 4,000 g for 20 min), 0.22 μm vacuum filtering and ultracentrifugation at 100,000 g for 4 h at 4°C. Further processing was analogous to the process for EVs from human melanoma cell lines. Plasma samples were obtained from EDTA-blood samples after centrifugation at 3000 g for 10 min, followed by plasma centrifugation at 3000 g for 10 min. For EV isolation, 500 μl plasma diluted 1:8 times with 0.1 μm-filtered PBS was centrifuged at 16,500 g for 10 min to obtain the fraction 1 EV pellet, and the supernatant was centrifuged at 118,000 g for 1.5 h to sediment the fraction 2 EVs. The same protocol was also applied to isolate f1 and f2 human melanoma EVs from CM.

Nanoparticle tracking analysis (NTA). The size distribution and concentration of EVs purified from conditioned media of melanoma cells (5 μg diluted 1:10,000) and of EVs in unprocessed plasma samples (5 μl diluted 1:10,000) or cell-depleted conditioned medium of melanoma cells (dilution 1:2) were determined using an LM10-HS NanoSight instrument and evaluated with NTA software (Malvern Instruments). Preparations were analyzed five times for 30 s.
Transmission electron microscopy (TEM). Freshly isolated plasma EV preparations were fixed with 4% paraformaldehyde and deposited on Formvar-carbon-coated nickel grids. Grids were doubly stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (CM 10 Philips; FEI).

Western blot analysis. Proteins obtained from purified EVs and cells were separated on 4% to 12% Bis-Tris precast gels (Invitrogen), transferred to nitrocellulose membranes (Amersham) and incubated with Abs. Reactive proteins were visualized using HRP-conjugated secondary antibodies (Amersham) and enhanced chemiluminescence (Amersham, SuperSignal). The list of used Abs is provided in Supplemental Table 8.

Flow cytometry. To perform immunophenotyping studies, human and mouse cells were incubated with specific fluorochrome-conjugated Abs or isotype controls, provided in Supplemental Table 8, washed and acquired. Flow cytometry of EVs from melanoma cells was performed using sulfate/aldehyde 5 μm latex beads (Thermo Fisher Scientific) (72). A SmartFlare detection probe for miR-146a was used according to the manufacturer’s instructions (Merck Millipore). Samples were evaluated using a FACSCalibur (BD Biosciences) and a Gallios or Cytoflex flow cytometer (Beckman Coulter) and FlowJo software (TreeStar Inc.). Morphology of plasma EV fractions was assessed by acquisition with the Gallios flow cytometer (Beckman Coulter) after calibration with 200, 500 and 1000 nm beads (Polysciences).

Monocyte studies. Monocytes (purity >95%) were purified from buffy coats of HD and from patients PBMC by sorting with anti-CD14+ beads (MACS, Miltenyi Biotech) according to the manufacturer’s instructions. For the generation of EV-MDSCs, CD14+ cells (0.3x10^6 in 300 μl) were co-cultured with melanoma EVs (50 μg) in complete medium supplemented with 10% EV-depleted FCS for 24 h. For the transfection of miR inhibitors or
miR mimics (Qiagen), 50 nM oligos in HiPerfect transfection reagent (Qiagen) were admixed with monocytes or melanoma cells for 4 h before the addition of FCS-containing medium. For miR inhibition in melanoma cells, EVs were isolated from conditioned medium 3 days after transfection with inhibitors as described above. For proliferation studies, human T cells were stained with CFSE (Thermo Fisher Scientific) prior to stimulation with anti-CD3 and anti-CD3/CD28 mAbs and assessed by flow cytometry after 3 days. Cytokines were assessed in cell-free supernatants from cultures of EV-MDSCs and from melanoma patients’ CD14+ cells purified from PBMCs using a Cytokine Bead Array (CBA, BD Biosciences), according to the manufacturer’s instructions.

**RNA extraction and quantitative real-time PCR (qPCR) analysis.** RNA was extracted from monocytes, EV-MDSCs, melanoma cells and from melanoma specimens with mirVana miRNA Isolation Kit (Ambion). qPCR analysis was performed according to the manufacturer’s instructions using Thermo Fisher Scientific and Exiqon reagents to evaluate gene and miR expression levels, respectively. The list of assays is provided in Supplemental Table 8. The endogenous controls used for normalization were GAPDH and ACTB for genes, and U6 snRNA and SNORD48 for miRs. qPCRs were carried out in triplicates and run on the ABI Prism 7900HT instrument, and analysis was performed using SDS software version 2.2.2. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method and expressed in arbitrary units, and fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. For plasma and EV samples, synthetic spike-in ath-miR-159a was added to monitor RNA isolation efficiency, and NucleoSpin miRNA Plasma kit (Macherey-Nagel) was used for RNA isolation. For plasma 250 µl were used. Before performing RNA extraction, EV samples were treated with RNAse A (10 µg/ml, Sigma-Aldrich) for 1 hour at 37°C. miR expression levels were evaluated using Thermo Fisher reagents; a pre-amplification step was included in this process. To evaluate MDSC-miRs in plasma of patients, RNA was
reverse transcribed and pre-amplified with Megaplex RT and PreAmp Primers Human Pool A, and tested in duplicate with TaqMan Array MicroRNA Cards (Thermo Fisher Scientific). To set up miRNA analysis by customized cards, the system conditions relative to spike-in use, retrotranscription and preamplification conditions was defined in titration experiments and ath-miR-159a to control RNA extraction and U6 snRNA were used for quality control. All samples were tested in the same batch of cards. The cards were run on QuantStudio 7 Flex, and analysis was performed with QuantStudio 6 and 7 Flex software.

**Gene expression and miR microarray profiling.** Gene expression profiles were generated using the HumanHT-12 v4 WGDASL BeadChip array (Illumina), and miR profiles were obtained using custom SurePrint Human miRNA Microarrays (Agilent). Raw gene expression data were log2-transformed and normalized with robust spline normalization using the “lumi” package (73). Probes with a detection P-value < 0.01 in at least one sample were kept, and for each gene, the probe with the highest detection rate was selected. Raw miR data were background-corrected with the “normexp” method and quantile-normalized using the “limma” package (74). Only probes with intensity higher than 10% of the 95th percentile of the negative controls in at least one sample were kept. The intensities of replicate probes were averaged, and the probes with the highest mean expression across samples were selected for each miR. Differential expression analysis was carried out with the “limma” package. P-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR). Genes and miRs with FDR < 0.05 and absolute fold change ≥ 1.2 were considered significant. The data were deposited in the Gene Expression Omnibus repository (GSE102011). Functional analyses were generated through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) and Gene Set Enrichment Analysis (GSEA) (75). In silico evaluation of subpopulations was assessed with CIBERSORT (34).
**IHC and ISH.** Melanoma sections were stained for CD163 after antigen retrieval performed by heating in a pressure cooker with citrate buffer at pH 6, 5 mM for 15 min and using the Dako REAL Detection System Alkaline Phosphatase/RED kit and red AP as a chromogen (Dako Agilent). Sections were scanned using the Aperio ScanScope XT systems (Aperio Technologies, Leica Microsystems), and signal quantifications were conducted by image analysis by MIAQuant as described previously (76). ISH analysis for miR visualization was performed by hybridization with double-DIG-LNA probes and positive and negative controls (Exiqon) as previously described (77). High-resolution Z stacks were acquired using Olympus BX63 equipped with DP89 camera and cellSens software (Olympus).

**Statistics.** Data were analyzed using GraphPad Prism 5 and 7 Software (GraphPad Software Inc.). Unless otherwise indicated, the results are presented as mean ± SD and in box and whiskers plots, the box boundaries represent the 25th and 75th percentiles, the middle line is the median value and the whiskers represent the minimum and maximum values. The results were analyzed using 2 tailed unpaired or paired Student's t-tests, Mann-Whitney U-tests, and Pearson and Spearman correlations, as specified. A P value less than 0.05 was considered significant. Multivariate analysis, simple correlations, partial correlations and canonical correlations of variables were calculated using SAS and R statistical software. Variables were also ranked in order to standardize values before calculating the abovementioned correlations. Plasma miRs were investigated as putative prognostic factors and their association with OS was first examined with univariable Cox proportional hazard models. Their joint association with OS was then investigated by means of a machine learning algorithm known as “adaptive index modeling”. This approach was used to obtain an index score based on the following steps: 1) forward selection of the miRs significantly associated with the clinical endpoints of interest, OS and
PFS, in a proportional-hazard Cox regression model; 2) definition of an optimal cutoff for each selected miR (based on Ct); 3) construction of the index score, as an enumeration of miRs above the cutoff value. The index score built on data included five miRs; hence we could split the patients cohort into two groups based on index score=0-1 (none or one miR with altered expression) or index score=2-5 (two or more miRs with altered expression). OS and PFS curves according to the levels of the selected score were finally estimated with the Kaplan-Meier method.

**Study approval.** The study protocol (code INT39/11 and INT40/11) for human studies was approved by the IRB of Fondazione IRCCS Istituto Nazionale dei Tumori and the Independent Ethics Committee. All participants, including melanoma patients and blood donors, gave their informed written consent. Animal care was in accordance with government and institutional guidelines and regulations.
Author contributions: VH, VV, MR, LR, VU and PA conceived the study and wrote the manuscript. VF, XH, AC, MD, ES, RS, EV, PF, ADL, BV, AG, AV, MC and VB performed the experiments and data analyses. EC developed image quantification software. LDG, FDB, RP and FA conceived the experimental design in a clinical setting. LL and LM performed the statistical analysis.

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69. UCSC Genome Browser on Human Dec. 2013 GRCh38/hg38 Assembly


Figure 1. Conversion of myeloid cells into MDSCs by melanoma EVs. (A) HD-CD14+ cells (Mono) incubated 24 h with melanoma EVs (Me EVs) down-regulate HLA-DR (left, representative plot and summary of n=7 HD), increase production of cyto-chemokines (middle), modulate HLA-DRA, IL6 and CCL2 gene transcription (right) and (B) suppress proliferation of activated CFSE-labeled T cells (percent proliferation is indicated). (C) CD14+HLA-DR<sup>reg</sup> cell frequency and HLA-DR expression on gated CD14<sup>+</sup> cells in PBMCs of melanoma patients (Pts, n=31) and HD (n=15) by flow cytometry. (D) HLA-DRA down-regulation of HD (n=5) and patients' (n=4) monocytes cultured with Me EVs. (E) Induction of EV-MDSCs in CD14<sup>+</sup> cells from a patient by autologous melanoma cell line EVs (left), suppressive activity on activated CD25<sup>+</sup> T cells (percent is indicated, right). (F) NTA evaluation of EV size in plasma samples of patients and HD (n=27/group) (above); correlation of EV mean size and frequency of CD14<sup>+</sup>HLA-DR<sup>reg</sup> in gated CD14<sup>+</sup> cells of melanoma patients (below). (G) EV-MDSC converting potential of f1 and f2 plasma EVs from patients and HD (n=5/group) shown as HLA-DRA down-regulation in monocytes (Mono) from two different HD; control: melanoma EVs. Data are presented as mean ± SEM. (H) Autologous (auto) plasma EVs f1 and f2 convert melanoma patient’s CD14<sup>+</sup> cells, as shown by modulation of HLA-DRA, IL6 and CCL2 transcripts (above). EV-MDSCs generated with autologous plasma EVs f1 and f2 of a melanoma patient inhibit T cell proliferation (percent is indicated, below). (I) Western blot of plasma EV fractions (f1, f2) of an HD and a patient. gMFI, geo mean fluorescence intensity; FC, fold change by using as a calibrator untreated monocytes; RE, relative expression. P<0.001 (A right, E), P<0.01 (A left) and P<0.05 (H above) by paired Student’s t-test. P<0.05 (D) by Mann-Whitney U-test. *P<0.05, ***P<0.001 by unpaired Student’s t-test (C, F). Data are representative of 2 (E,H,I) and 3 (A,B) experiments.
Figure 2. Transcriptional regulation of EV-MDSCs at the gene level. (A) Heatmaps of the genes regulated in EV-MDSCs (+EVs) compared to untreated monocytes (Mono, n=5 HD) clustered according to the most representative functional categories. (B) EMT-related genes selected by a list of 839 genes from melanoma IPRES, EMT and wound healing (5); from the literature-based EMT database (32); and from mesenchymal myeloid markers (33). (C) The fold change (FC) of expression levels of selected genes in EV-MDSCs compared to untreated monocytes measured by qPCR. P<0.01 by paired Student’s t-test. A representative HD of 5 tested in A is shown. (D) GSEA plots obtained with the top 100 up-regulated genes in EV-MDSCs showing a significant enrichment in datasets comparing the transcriptomic profiles of blood monocytes from colorectal, pancreatic and breast cancer patients to those of monocytes obtained from HD; GSE47756 colon cancer (37); GSE60601 pancreatic cancer (38), GSE65517 metastatic breast cancer (39).
Figure 3. miR regulation in EV-MDSCs. (A) Volcano plot of the miRs regulated in EV-MDSCs (n=5 HD) compared to untreated monocytes (Mono) based on microarray results, identification strategy of MDSC-miRs, and relative expression of selected miRs in EV-MDSCs compared to untreated monocytes assessed by qPCR in a representative HD. (B) Expression of MDSC-miRs in f1 and f2 plasma EVs of melanoma patients (n=16). Box and whiskers (Tukey). (C) Monocytes from HD transfected with MDSC-miR mimics (MmiRs) modulate HLA-DRA, IL6 and CCL2 gene expression compared to monocytes treated with scrambled control used as calibrator. (D) Immunosuppressive activity of mono+MmiRs on autologous activated CFSE-labeled T cells, as evaluated by CD25 expression and proliferation (percentage is indicated, left), and release of IFNγ and TNFα (right). (E) Loss of immunosuppressive activity of monocytes from a melanoma patient transfected with miR inhibitors (ImiRs) prior to co-incubation with autologous activated CFSE-labeled T cells, as evaluated by flow cytometry (left) and cytokine release (right). (F) Expression of MDSC-miRs in CD14+ cells isolated from PBMCs of melanoma patients (n=31) and HD (n=15). FC, fold change. AU, arbitrary units. P<0.05 by paired Student’s t-test (A,C) and by Mann-Whitney U-test (F). *P<0.05, **P<0.01 by paired Student’s t-test (B, D, E). Results are representative of 5 (C), 4 (D) and 3 (E) experiments.
Figure 4. Inhibition of MDSC-miRs rescues monocytes from the acquisition of a suppressive phenotype. (A) Effect of transfection with miR inhibitors (ImiRs) or scrambled control (Scr) on MDSC-miR expression in INT12 melanoma cells and the respective EVs (left). Reduced expression of miR-146a in silenced melanoma cells (Me) was confirmed by flow cytometry using an APC-fluorescent SmartFlare probe (right). (B) EVs derived from miR-silenced melanoma cells (+I-EVs) impaired the induction of the EV-MDSC phenotype compared to EVs derived from scrambled control cells (+S-EVs) (left); MDSC-miR expression was reduced in monocytes treated with I-EVs compared to those treated with S-EVs (right). (C) Effect of transfection with ImiRs or Scr of HD monocytes cultured in the presence of melanoma EVs, as evaluated by qPCR and flow cytometry, and (D) IL-6 and CCL2 release. (E) Loss of immunosuppressive activity of EV-MDSCs, generated from HD in the presence of ImiRs or Scr, on activated CFSE-labeled T cells, as evaluated by flow cytometry (left) and cytokine release (right), and (F) loss of immunosuppressive activity of EV-MDSCs generated from a patient with autologous melanoma-derived EVs. Percentage of CD25 and CFSE expression is indicated. FC, fold change, cells transfected with scrambled control were used as a calibrator (A, B, C). P<0.05 by paired Student's t-test (A, B, C), P<0.001 by 1-way ANOVA (D). *P<0.05, **P<0.01 by paired Student's t-test (E, F). Experiments were repeated twice and performed in triplicates (A-D, F). Data are representative of 2 HD tested (E).
Figure 5. MDSC-miRs are expressed in tumor specimens and monocytes of melanoma patients. (A) Correlation matrix of the expression levels of MDSC-miRs and CD163, CD14, CD209, ITGAM, CD33, CD68, CD3D, CD4, CD8A, PMEL, TYR and MLANA in metastatic melanoma specimens (n=58); PMEL, TYR, MLANA, miR-21 and miR-211 are used as unrelated controls. r values from univariate Spearman analysis in correlations with P < 0.05. (B) Immunostaining of CD163 infiltrate in melanoma (left) and correlation of the quantified CD163 signal with the expression levels of MDSC-miRs (right) as determined by qPCR (n=20). (C) ISH images showing the expression of miR-146a, miR-100, miR-125b and miR-155 in representative tumor sections. miR signals appear as brown dots localized both in CD163+ infiltrating cells and in melanoma cells. Bars 100 μm. Representative images are shown in (B) and (C).
Figure 6. MDSC-miRs are enriched in plasma from melanoma patients and associate to resistance to immunotherapy. (A) MDSC-miR detection in the plasma of patients (Pts) and HD (n=20/group). miR expression levels were normalized using ath-miR-159a as reference miR. RE, relative expression. *P<0.05 for miR-146a and P<0.01 for other miRs by Mann-Whitney U-test. (B) Overall survival (OS) of metastatic melanoma patients based on the expression levels of MDSC-miRs in plasma samples obtained at baseline of therapy, assessed by multivariable index score approach and AIM, in the global population (n=87; left panel) and in the subsets of patients receiving immunotherapy (ICIs, n=49) or targeted therapy (TKIs, n=37). One patient was excluded from the latter because receiving chemotherapy. Patients with low scores (0-1; showing no or only one increased miR) had a significantly better OS with respect to patients with high score (>1; having 2-5 increased miRs) only if receiving ICIs. Kaplan-Meier survival curves with Log-rank p-values are shown.