Untreated Stage IV Melanoma Patients Exhibit Abnormal Monocyte Phenotypes and Decreased Functional Capacity

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Abstract
Monocytes may contribute to tumor progression in part by mediating tumor-induced immunosuppression. Alterations to the monocyte populations and functions in untreated patients with late-stage melanoma are not fully understood. To characterize these alterations, we compared the frequency, phenotype, and functional capacity of peripheral blood monocytes and other myeloid cells in untreated, newly diagnosed stage IV melanoma patients (n = 18) with those in healthy volunteers. Stage IV untreated melanoma patients exhibited a sizeable decrease in the percentage of monocytes (P < 0.0001) that included a drop in the percentage of the CD14⁺CD16⁻ classical monocyte pool (P = 0.006). Although there was not a significant difference in the CD14⁺HLA-DR<low/>⁻ monocyte population between the patients with melanoma and the healthy volunteers, the HLA-DR levels were considerably lower in the patients' CD14⁺CD16⁻ intermediate (P < 0.0001) and CD14⁺CD16⁺ nonclassical monocyte populations (P = 0.001). Decreased surface expression of CD86 (P = 0.0006) and TNFRII (P = 0.0001) and increased expression of tissue factor and PD-L1 (P = 0.003) were identified on monocytes from patients with melanoma. Furthermore, these monocytes had decreased ability to upregulate CD80 expression and cytokine production following stimulation with agonist of Toll-like receptor 3 (TLR3). Peripheral blood dendritic cell subsets were decreased in untreated stage IV melanoma patients. Our study demonstrates that untreated late-stage melanoma patients exhibit monocytopenia in addition to phenotypic and functional deficiencies that may negatively affect their immune function. These findings open new avenues into examining the role of monocyte populations in melanoma development.

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Introduction
Approximately 10% of the circulating leukocytes in humans consist of monocytes that can differentiate into both macrophages and dendritic cells (DC; ref. 1). Monocytes can be subdivided into three distinct populations based on the differential expression of CD14 and CD16 (2). Classical monocytes are short-lived cells that are CD14⁺CD16⁻, intermediate monocytes are CD14⁺CD16⁺, and nonclassical monocytes are CD14⁺CD16⁻ (1–5). In humans, approximately 80% of the circulating monocytes are of the highly phagocytic classical subset. The nonclassical monocytes are considered to be important in both proinflammatory and infectious disease states (6, 7). The intermediate monocytes are functionally distinct from the other two subsets, due to their anti-inflamatory properties such as the secretion of interleukin (IL)-10 in response to lipopolysaccharide stimulation (3).

Recent evidence has highlighted the importance of monocytes and other myeloid cells in tumor-mediated immunosuppression in patients with metastatic melanoma (8–10). In particular, the loss of HLA-DR expression on CD14⁺ monocytes has been identified as a potential mechanism whereby the melanoma tumors can cause systemic immunosuppression in the patients (8, 10, 11). However, comparisons between these studies are problematic due to the variations in the types of treatments and the stages of diseases in each cohort. We have reported an increase in CD14⁺HLA-DR<low/>⁻ monocytes (often referred to as monocyctic myeloid-derived suppressor cells) in patients with B-cell non-Hodgkin lymphoma, glioblastoma multiforme, and chronic lymphocytic leukemia in which these cells cause or contribute to the systemic immunosuppression and the aggressive disease (12–14).

The immunosuppressive function of other myeloid-derived suppressor cells (MDSC) has also been evaluated although their inhibitory capacity in humans appears to be less than that observed in the murine model (9). Studies of melanoma in humans and in the murine model have demonstrated important species-specific differences in immune responses with the mouse antigen-presenting cells unable to respond to VEGF stimulation (15). Studies involving global analysis of cell subsets, gene expression, and serum cytokine profiles in patients...
with stage I to IV melanoma have demonstrated that the repolarization of the immune system is partially due to the VEGF-orchestration of chronic inflammation leading to a subsequent Th-2 bias (15, 16). In addition, DCs have a role in the systemic immune dysregulation that is seen in patients with cancer (17–19). DCs are classically divided into myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC). mDCs and pDCs differ in the expression of Toll-like receptors (TLR), which leads to divergent cytokine production following TLR stimulation (20–22).

In this study, we evaluated the phenotype and functions of monocytes, DCs, and other myeloid cells from untreated stage IV melanoma patients. This cohort allows us to measure the tumor's influence on peripheral blood cells without the complicating effects of treatment. Our results demonstrate that untreated patients with significant tumor burdens have a dysregulated monocyte population. Although the levels of CD14⁺HLA-DR⁻ monocytes were not elevated in this cohort, there was a dramatic decrease of the HLA-DR levels on the intermediate and nonclassical monocytes. Melanoma patient monocytes showed decreased levels of inflammatory markers and increased expression of the inhibitory programmed death ligand 1 (PD-L1/B7-H1/CD274). In addition to a lower frequency in the circulating DCs, monocytes isolated from these patients also failed to properly respond to TLR3 stimulation. These findings demonstrate that substantial changes in the monocyte phenotypes and functions exist in patients with untreated malignant melanoma.

Materials and Methods

Patient population

Blood samples were collected in sodium (Na)-heparin Vacutainer 10 mL blood collection tubes (Becton Dickinson) from patients with newly diagnosed stage IV melanoma with no previous treatment and age-matched healthy volunteers. Samples were processed as soon as possible and all within 18 hours of collection. Unfractionated whole blood was used for immunophenotyping and cells from the remaining blood were isolated by density gradient centrifugation (Lymphoprep, MP Biomedicals LLC). Monocytes were isolated by incubating PBMCs with anti-CD3, anti-CD7, anti-CD16, anti-CD19, anti-CD56, anti-CD123, and glycoporphin A (Monocyte Isolation Kit II, Miltenyi Biotec) as per the manufacturer’s instructions. The magnetically purified monocytes were stimulated with polyinosinic-polycytidylic acid (poly I:C; Imgenex) for 6 hours. Proteins were measured using the Milliplex MAP cytokine/chemokine panel (Millipore) as per the manufacturer’s instructions. Luminex plate reader (Millipore) was used to detect cytokines and chemokines. Protein concentrations were determined using a standard curve generated using multiplex assay analysis software (Millipore).

Statistical analyses

Immunophenotype values from volunteers and patients were tested for statistical significance using the two-tailed nonparametric Mann-Whitney test for unpaired samples. Statistical analyses and graphs were performed using Prism, version 5.0 software (GraphPad Software).

Results

Clinical characteristics

Peripheral blood samples from age-matched healthy volunteers (n = 18) and untreated stage IV melanoma patients (malignant melanoma; n = 18) were collected for analysis. Patient and healthy volunteers’ demographics are shown in Table 1. The malignant melanoma cohort included 2 patients diagnosed with metastatic melanoma without known primaries; metastatic melanoma was noted in 1 patient during a routine colonoscopy and in the other patient during the biopsy of an enlarged inguinal lymph node. All the remaining patients in the study had prior biopsies demonstrating melanoma; all cases of ocular melanoma were excluded in this study. There were 2 patients with a history of additional carcinomas; one patient had breast cancer and was treated with chemotherapy and the

| Table 1. Malignant melanoma patient and healthy volunteer demographics |
|------------------------|-------------------|-------------------|
| Age, mean (range)      | 60.2 (45–80)      | 61.2 (48–74)      |
| Gender (%)             |                   |                   |
| Patients with malignant melanoma | Male | 11 (61%)         | Female | 7 (39%) |
| Healthy volunteer controls | Male | 9 (50%)          | Female | 9 (50%) |
| AJCC stage at time of sampling (%) | IV (100%) | |
| Metastatic disease with unknown primary at time of diagnosis (%) | 2 (11%) |
| Time to metastatic diseases mean (range) | 3.4 years (0–12.4) |

Abbreviation: AJCC, American Joint Committee on Cancer.

Peripheral blood immunophenotyping and antibodies

Peripheral blood samples were directly stained with antibodies and analyzed by flow cytometry as described previously (13). Antibodies used for this study are listed in Supplementary Table S1. Samples were run on a BD FACSCalibur flow cytometer (Becton Dickinson), calibrated the day of use, and the data files were analyzed with Flowjo, Cell Quest (BD), and/or Multiset (BD) software.

Monocyte isolation and cytokine analysis

Fresh peripheral blood monocytes (PBMC) were isolated using density gradient centrifugation (Lymphoprep, MP Biomedicals LLC). Monocytes were isolated by incubating PBMCs with anti-CD3, anti-CD7, anti-CD16, anti-CD19, anti-CD56, anti-CD123, and glycoporphin A (Monocyte Isolation Kit II, Miltenyi Biotec) as per the manufacturer’s instructions. The magnetically purified monocytes were stimulated with polyinosinic-polycytidylic acid (poly I:C; Imgenex) for 6 hours. Proteins were measured using the Milliplex MAP cytokine/chemokine panel (Millipore) as per the manufacturer’s instructions. Luminex plate reader (Millipore) was used to detect cytokines and chemokines. Protein concentrations were determined using a standard curve generated using multiplex assay analysis software (Millipore).
Monocytes in Melanoma Patients Have an Inhibitory Phenotype

Figure 1. Abnormal monocyte distribution and loss of HLA-DR in patients with stage IV melanoma. Peripheral blood was analyzed by flow cytometry. A, frequency of monocytes as measured by forward scatter and side scatter in the leukocyte population of patients with malignant melanoma (MMP) and healthy volunteer controls (HV). B, frequency of CD14+CD16+ classical, CD14+CD16− intermediate, CD14−CD16+ nonclassical monocytes subsets as a percentage of the monocytes pool in both healthy volunteers and malignant melanoma patients. C, frequency of CD14+HLA-DRint− % of monocytes as a percentage of CD14+ monocytes. D, HLA-DR expression as measured by mean fluorescence intensity (MFI) on the monocyte subsets. Box and whisker plots: horizontal line, median; box, 25th and 75th percentiles; whiskers, min and max; **, P < 0.01.

Abnormal monocyte distribution in untreated stage IV melanoma patients

We used a panel of antibodies that was developed and subsequently expanded for this study [Supplementary Table S1 and Gustafson et al. (23)]. Flow cytometric analysis of unfractionated peripheral blood has become a valuable method to assess the immune status of individuals and our gating strategy is outlined in the Supplementary Figures. We applied this method to assess the monocyte and DC populations in untreated patients with malignant melanoma. Analysis of the monocytes by forward/side scatter properties demonstrated that stage IV untreated melanoma patients had a significantly lower frequency of circulating monocytes (P < 0.0001; Fig. 1A), whereas no differences were observed in the granulocyte and lymphocyte populations compared with healthy volunteers (Supplementary Fig. S1). Within the monocyte population, we measured the distribution of classical (CD14+CD16−), intermediate (CD14+CD16−), and nonclassical monocytes (CD14−CD16+) and found a specific decline of the classical monocytes in patients with malignant melanoma (P = 0.006) with no detectable changes in the other compartments (Fig. 1B and Supplementary Fig. S2). We did not observe significant differences in the circulating levels of immunosuppressive CD14+HLA-DRint−/neg monocytes between patients with malignant melanoma and healthy volunteers (Fig. 1C, Fig. S3). However, when we examined the HLA-DR levels on each of the three subgroups of monocytes, we found lower HLA-DR expression on the intermediate (P < 0.0001) and nonclassical monocytes (P = 0.001), but no measureable differences were observed on the classical monocytes (Fig. 1D). These results reveal changes to the immunophenotypes of PBMCs in patients with malignant melanoma that have not been identified previously.

Monocytes in patients with malignant melanoma have an inhibitory phenotype

We hypothesized that there might be other markers on the patients’ monocytes that could provide insight into how these monocytes might cause immunosuppression (Supplementary Table S1). Compared with healthy volunteers, patients with malignant melanoma had lower frequencies of CD86-positive monocytes (P = 0.0006) but not CD80-positive monocytes (Fig. 2A). The TNF receptor 2 (TNFR2) plays an important role in enabling lymphocyte activation and proliferation (24). Expression of this immune marker was significantly lower in patients with malignant melanoma (P = 0.0001; Fig. 2B).

Increased expression of monocyte tissue factor (TF) has been reported in pancreatic cancer and hepatocellular cancer (25, 26). The expression of TF in the tumor microenvironment is thought to be important for angiogenesis in patients with solid tumors (26, 27). In patients with malignant melanoma, the expression of TF on monocytes was significantly increased (P = 0.003) in comparison to the healthy volunteers (Fig. 2B). Patients with malignant melanoma also had increased expression of the inhibitory molecule PD-L1 (P = 0.003) on their monocytes (Fig. 2D). We found a negative correlation between the expression of HLA-DR and PD-L1.
MDSCs are a heterogeneous population that play a role in immunosuppression and have been well characterized in the murine model. In our study, we did not observe differences in the frequency of MDSC populations, including Lineage⁻/CD33⁺/HLA-DR⁻/CD15⁺/CD14⁻ or IL-4Ra/CD124⁺ monocytes in patients with malignant melanoma and in healthy volunteers (data not shown). Taken together, these results suggest that monocytes from patients with malignant melanoma exhibit a profile of immunosuppressive characteristics leading to suboptimal antigen presentation/costimulation and the expression of receptors that inhibit T-cell proliferation and/or function.

Evaluation of DC subsets

Monocytes are precursor cells of the myeloid CD1c DCs. Given the significant differences noted in the classical monocytes of patients with malignant melanoma, we characterized the frequency of the circulating DC subsets. These patients with malignant melanoma had lower populations of circulating CD1c DC (P = 0.04), plasmacytoid CD303 DC (P < 0.0001), and CD141 DC (P = 0.014; Fig. 3). However, we noted no differences in the expression of CD83, CD86, or HLA-DR on CD1c or CD303 DCs from patients with malignant melanoma (Supplementary Figs. S4 and S5). These data suggest a frequency deficit of DCs but not necessarily defective activation of DCs in patients with malignant melanoma.

Decreased activation and cytokine production following poly (I:C) stimulation of monocytes

All normal monocytes express TLRs that enable them to initiate immune responses against invading pathogens. We evaluated the ability of monocytes from patients with malignant melanoma...
malignant melanoma to secrete cytokines following TLR3 stimulation. Both positively and negatively selected monocytes isolated from density gradient purified PBMCs were stimulated with poly (I:C). A minimal response to the stimulation was observed in the positively selected monocyte populations (data not shown). We also stimulated monocytes in fresh whole blood with poly (I:C) for 6 hours. As shown earlier, the circulating monocytes in patients with malignant melanoma expressed similar levels of the costimulatory molecule CD80 in comparison with healthy volunteers (Fig. 2A). However, malignant melanoma monocytes failed to properly induce CD80 expression upon poly (I:C) stimulation (Fig. 4). Results from these experiments suggest that monocytes from patients with malignant melanoma have a functional deficit in addition to phenotypic changes.

Using the negatively selected monocytes we measured the concentration of 42 cytokines following stimulation with poly (I:C) and increased production of IL-1α, IL-1β, IP-10, and TNF-α was observed in the samples from patients with malignant melanoma (Fig. 5A–D). However, the increase in cytokine production was not as robust as that observed for the healthy volunteers when we measured the concentration of IL-1α (P = 0.03, patient with malignant melanoma; P = 0.008, healthy volunteers) and TNF-α (P = 0.03, patients with malignant melanoma; P = 0.008, healthy volunteers). A similar increase in the expression of IL-1β was noted in the patients with
malignant melanoma ($P = 0.03$) and healthy volunteers ($P = 0.02$); for IP-10 a significant increase was noted only in the healthy volunteer population ($0.007$). These results demonstrate that following TLR3 stimulation with poly (IC), monocytes in patients with malignant melanoma exhibit impaired inflammatory cytokine production.

**Discussion**

We hypothesized that monocytes from untreated stage IV melanoma patients would show evidence of phenotypic and functional changes resulting from metastatic disease. To test this, we analyzed and compared the immunophenotypes and functional responses to TLR stimulation of monocytes from patients with malignant melanoma and from healthy volunteers. In our small cohort ($n = 18$), patients with malignant melanoma had not received any prior chemotherapy treatments that could have affected the frequency, phenotype, and/or function of the monocytes. As such, this cohort allows us to assess the differences in the frequency of monocyte subsets associated with malignant melanoma without the confounding factors of previous treatment and different stages of disease. The changes in monocyte immunophenotypes in patients with melanoma may provide insights into as yet unidentified mechanisms of how melanoma tumors cause immunosuppression. We found that in patients with untreated late-stage melanoma, monocytes are present in much lower frequencies when compared with healthy volunteers. Even within the smaller monocyte compartment the classical monocytes were not different between malignant melanoma patients and healthy volunteers. In our small cohort although 4 patients exhibited high levels of these immunosuppressive monocytes (>2 SDs above the healthy volunteers mean: 24.1%), however, when we examined the HLA-DR levels on each of the three monocytic subgroups, we found a significant decrease of HLA-DR surface expression on the intermediate and nonclassical monocytes. To our knowledge, this is the first observation of differential downregulation of HLA-DR on monocyte subgroups in patients with cancer. One potential reason that we did not see a difference in CD14$^+$ HLA-DR$^{low/-}$ monocytes between the patient and the healthy volunteers control groups is that the levels of HLA-DR on monocyte subsets were not different between malignant melanoma patients and healthy volunteers. As the classical monocytes are the largest monocyte subgroup, it may mask the HLA-DR drop when monocytes are analyzed as a whole. Therefore, we would suggest that future studies continue to analyze the three monocyte compartments separately. The role of each of the three monocyte subgroups remains unclear in melanoma pathology; however, studies have shown that these subsets are functionally different and do not have the same cytokine production profile.

To gain further insight into the differences in the frequency of monocyte subsets associated with malignant melanoma, we evaluated the expression of a variety of immune markers involved in the activation of monocytes and signaling to T cells. The expression of costimulatory molecules CD80 and CD86 are required for T-cell activation and they play an important role in modulating immune responses, including antitumor responses ($28$). Our study demonstrated no measurable changes in the expression of CD80 in patients with malignant melanoma in comparison with the decrease noted in CD86 expression. Both CD80 and CD86 are required for efficient activation of T cells and these results, along with the loss of HLA-DR, suggest that monocytes have an impaired ability to present antigen to T cells in patients with late-stage melanoma. Monocytes can also negatively affect T-cell proliferation and function by expressing inhibitory ligands like PD-L1. Clinical trials evaluating antibodies that block the PD-1/PD-L1 interaction in melanoma are ongoing and the initial results have been favorable ($29$). Not only was PD-L1 expression increased in malignant melanoma monocytes, the expressions of PD-L1 and HLA-DR were inversely correlated. The expression of TNFR2 was decreased in malignant melanoma monocytes suggesting that these monocytes may not be able to fully respond to TNFα signals.

We also examined other myeloid subsets in patients with malignant melanoma. A decrease in the frequency was noted in the circulating DC subsets yet there was little difference in the expression of costimulatory molecules CD83, CD86, or HLA-DR. These results suggest that the deficiency may be a result of the numbers of DCs and not the baseline functional status of DCs. Further mechanistic studies will be required to establish the relationship between the decline in monocytes and in circulating DCs in patients with malignant melanoma. We did not detect differences in Lineage$^+$ CD33$^+$ HLA-DR$^+$, CD15$^+$ CD14$^+$, or IL-4Rα/CD124$^+$ MDSCs. In agreement with other groups, we did not find differences in MDSCs and thus their role in melanoma development remains in question.

To evaluate the functional capacity of the monocytes, we stimulated the isolated monocytes with poly (IC) and using a multiplex ELISA cytokine concentrations were measured 12 hours poststimulation. Monocytes from patients with malignant melanoma were not able to fully recapitulate the induction of cytokines observed in healthy volunteers and unable to upregulate CD80 expression. Although we did not directly test the ability of malignant melanoma monocytes to respond to granulocyte macrophage-colony stimulating factor and IL-4, our previous work has shown that monocytes with reduced expression of HLA-DR and TNFR2 are unable to fully differentiate into mature DCs ($13, 14$). Taken together, these results suggest that monocytes from patients with malignant melanoma have a decreased functional capacity. Further studies will be necessary to test patient monocytes on T-cell mixed lymphocytes reactions as our study had insufficient material for such analyses.

Since these analyses were performed in untreated patients, the changes that we observed are likely a direct effect of the tumor. Other factors like age and gender are likely not contributing to the monocytes changes as we did not see age- or gender-related differences in our melanoma cohort. We hypothesize that monocyte interactions with melanoma cells and/or exposure to tumor-derived factors are the probable mechanisms for the monocyte defects observed in this cohort. The data presented here suggest that, in addition to the deficit
in the circulation, monocytes in patients with untreated malignant melanoma express inhibitory signals that negatively affect T cells, are unable to fully present antigens with an adequate costimulatory signal, and unable to respond to inflammatory signals like TNF-α or TLR agonists. These factors therefore may contribute to an environment of systemic immunosuppression in the patients with malignant melanoma through a mechanism that has yet to be defined. The patients with malignant melanoma in our study were placed into various clinical trials at our institution and consequently we were unable to correlate our current findings with survival studies. However, our data provide the impetus to begin to dissect the functions of the monocyte subgroups during tumor development in future clinical trials. In addition, these findings have considerable implications for the development of novel immunotherapeutic approaches for treating melanoma.

In conclusion, our results demonstrated significant changes in the monocyte populations in this relatively small cohort of untreated patients with late-stage melanoma. Future studies with larger cohorts will be required to further define the contributions of each of the monocyte subgroups in melanoma development and tumor-mediated immunosuppression. Results from our study indicate that the development of better treatment strategies must take into consideration the immunosuppressive characteristics of the monocytes in patients with melanoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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