MIF Is Necessary for Late-Stage Melanoma Patient MDSC Immune Suppression and Differentiation

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Abstract

Highly aggressive cancers "entrain" innate and adaptive immune cells to suppress antitumor lymphocyte activity. Circulating myeloid-derived suppressor cells (MDSC) constitute the bulk of monocytic immunosuppressive activity in late-stage melanoma patients. Previous studies revealed that monocyte-derived macrophage migration inhibitory factor (MIF) is necessary for the immunosuppressive function of tumor-associated macrophages and MDSCs in mouse models of melanoma. In the current study, we sought to determine whether MIF contributes to human melanoma MDSC induction and T-cell immunosuppression using melanoma patient-derived MDSCs and an ex vivo coculture model of human melanoma-induced MDSC. We now report that circulating MDSCs isolated from late-stage melanoma patients are reliant upon MIF for suppression of antigen-independent T-cell activation and that MIF is necessary for maximal reactive oxygen species generation in these cells. Moreover, inhibition of MIF results in a functional reversion from immunosuppressive MDSC to an immunostimulatory dendritic cell (DC)–like phenotype that is at least partly due to reductions in MDSC prostaglandin E2 (PGE2). These findings indicate that monocyte-derived MIF is centrally involved in human monocytic MDSC induction/immunosuppressive function and that therapeutic targeting of MIF may provide a novel means of inducing antitumor DC responses in late-stage melanoma patients.

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Introduction

Stage IV melanoma is a highly aggressive and resistance-prone malignancy that carries a 5-year survival rate of approximately 15%. Melanoma cells are unusually immunogenic and, consequently, are adept at inducing host innate and adaptive immunosuppressive mechanisms that, collectively, serve to attenuate antitumor lymphocyte responses (1). Adaptive cell types and effectors involved in melanoma-associated immune suppression include regulatory T lymphocytes (Treg), cytotoxic T lymphocyte antigen-4 (CTLA-4), and programmed cell death 1 (PD-1)—of which the latter two are currently being evaluated as therapeutic targets in late-stage melanoma patients (2).

It is becoming increasingly evident that tumor-entrained innate immune effectors—e.g., tumor-associated macrophages (TAM), tumor-associated neutrophils (TAN), tolerogenic dendritic cells (DC), and MDSCs—also provide highly significant degrees of immune escape to aggressive malignancies (3–7). In patients with advanced melanoma, circulating monocyte-like MDSCs provide the bulk of monocyte-associated immune suppression (8), negatively affect patient survival, and inversely correlate with the presence of functional antigen-specific T cells (9).

Previous studies from our laboratory established a novel functional role for monocyte-derived macrophage migration inhibitory factor (MIF) in dictating alternative activation phenotypes in mouse melanoma TAMs; loss or inhibition of MIF reduces melanoma TAM- and MDSC-mediated immune suppression (10). In a related study using the 4T1 mouse model of breast cancer, Simpson and colleagues showed that tumor-derived MIF promotes MDSC accumulation and immunosuppressive activity (11). Reconstitution of wild-type MIF cDNA into 4T1 MIF shRNA knockdown cells, but not an enzymatically inactive MIF mutant (proline-2 to serine-2, P2S) cDNA, was capable of reconstituting tumor-derived, MIF-dependent MDSC induction. This result was in line with our finding that small-molecule inhibitors of MIF’s enzymatic activity fully phenocopy MIF deficiency in their ability to dictate the immunosuppressive activities of monocytes/macrophages in tumor-bearing hosts (10).

Studies by the Dranoff laboratory have identified MIF as a target of naturally developing auto-antibodies in late-stage melanoma patients who had successfully responded to a trial immunotherapy consisting of autologous GM-CSF secreting tumor cell vaccines followed by CTLA-4 blockade (ipilimumab; ref. 12). MIF auto-antibodies disrupted MIF-dependent effects on human monocytes/macrophages, suggesting that the beneficial effects of these MIF-targeting auto-antibodies in advanced melanoma patients are due to inhibition of MIF-dependent innate immune stromal cell phenotypes. Although this finding suggests a
clinically relevant role for MIF in human melanoma disease progression/survival, no studies have directly investigated the functional and/or mechanistic contributions of MIF to innate immune cell–mediated immunosuppression in melanoma patients. Using our well-characterized, small-molecule MIF enzymatic antagonist (4-ido-6-phenylpyrimidine, 4-IPP; refs. 10, 13–15), we investigated MIF contributions to human melanoma MDSC induction, phenotype, differentiation status, and mechanistic effectors. We show that human MDSCs derived from late-stage melanoma patients and those induced in vitro by tumor cells rely on MIF to suppress T-cell activation. MIF reliance corresponds with reactive oxygen species (ROS) and cyclooxygenase-2 (COX-2)/PGE2 production elicited by MDSCs. Unexpectedly, when MDSC-derived MIF is inhibited during short-term ex vivo culture of MDSCs, their differentiation is redirected toward a more DC-like phenotype. These MIF-inhibited monocytic MDSCs induce antigen-specific T-cell stimulatory function in these cells. Together, our results support a crucial protumorigenic contribution by MIF to the immune suppression and differentiation of circulating melanoma MDSCs and provide justification for therapeutic targeting of MIF in patients with advanced melanoma.

Materials and Methods

Patient samples and cell lines
Peripheral blood was collected from 27 patients with metastatic melanoma stage III to IV, and from 12 healthy donors. Melanoma patients included in this study were not undergoing therapy when their samples were collected, and they all had progressive disease. Patient samples were collected after informed consent was obtained by staff of the JG Brown Cancer Center Biorepository and covered under University of Louisville IRB protocol number 08-0388. The melanoma cell line A375 (ATCC CRL-1619) was purchased from ATCC and maintained in DMEM containing 10% (v/v) FBS. We do not culture this cell line longer than 6 to 8 weeks, and all of our stocks come from thawed vials that were frozen at passage 2 after they were received from ATCC. The A375 cell line was authenticated by the ATCC cell bank using short tandem repeat profiling.

Mice
Wild-type male C57BL/6 mice (MIF+/+) were obtained from Harlan Laboratories. OT-I and OT-II transgenic mice were obtained from The Jackson Laboratory. All mice were handled with the approval of the Institutional Animal Care and Use Committee at the University of Louisville (Louisville, KY).

MDSC isolation and 4-IPP treatment
Monocytic CD14+ MDSCs from melanoma patients were isolated from peripheral blood mononuclear cells (PBMC) using anti-CD14 magnetic microbeads and the autoMACS Pro Separator (Miltenyi Biotec), per manufacturer’s instructions. One million MDSCs were plated in complete Iscove’s modified Dulbecco’s medium (IMDM; supplemented with 10% human AB serum; Sigma-Aldrich; 2 mmol/L L-glutamine, and penicillin/streptomycin) per well in a 6-well plate (BD Falcon) and treated with 4-IPP (50 μmol/L) or DMSO (vehicle control) for 24 hours. For functional experiments, autologous T cells were isolated from PBMCs using the Pan T-cell Isolation Kit (Miltenyi Biotec).

In vitro generation of human MDSCs
CD14+ cells (1 x 10^9) isolated from PBMCs obtained from healthy donors were cocultured with 5 x 10^5 A375 tumor cells in complete IMDM per well in a 6-well plate (16). Tumor/monocyte cocultures were treated twice with 4-IPP (100 μmol/L on day 0 and 50 μmol/L on day 2) or 0.1% DMSO (vehicle control). A375 cocultured monocytes (both untreated and 4-IPP treated) and control monocytes cultured without tumor cells were harvested by gently scraping after 64 to 68 hours of culture and CD11b+ cells were purified. Details for cell isolation techniques used are provided in the Supplemental Methods section.

Mouse bone marrow–derived MDSCs
Tibias and femurs from MIF+/+ and MIF−/− C57BL/6 mice were removed using sterile techniques, and bone marrow was flushed. To obtain bone marrow–derived MDSCs, bone marrow cells were cultured for 4 days with GM-CSF (40 ng/mL), and IL6 (40 ng/mL) cytokines, as previously described (17). MIF+/+ and MIF−/− bone marrow cultures were treated with 0.1% DMSO (vehicle control) or with 4-IPP (50 μmol/L) during the last 48 hours of the culture period. For functional assays, CD11b+ GR1+ bone marrow MDSCs were isolated from bone marrow cultures using CD11b and GR1 microbeads followed by magnetic separation (Miltenyi).

Antibodies and flow cytometry
Untreated or 4-IPP–treated melanoma patient MDSCs and tumor cell line–induced MDSCs (A375-MDSC) were stained with anti-human antibodies according to the manufacturer’s recommendations. Details on flow cytometry staining and antibody panels are provided in the Supplemental Methods section and in Supplementary Table S1.

Functional studies
To evaluate the suppressive functions of melanoma patient–derived MDSCs and A375-MDSCs, autologous T cells were labeled with 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) and seeded at 100,000 cells per well in a 96-well U-bottom plate. For patient samples, freshly purified CD14+ cells or CD14+ cells that were pretreated with or without 4-IPP for 24 hours were added to T cells at ratios of 2:1, 1:1, or 1:2. T cells were activated by the addition of anti-CD3/CD28 mAb-coated beads (Invitrogen) per well for 4 days. T-cell activation was measured by flow cytometry, and IFNγ concentrations in the supernatants were determined by ELISA. Controls included nonactivated T cells or T cells activated with beads alone. For A375-MDSCs, CD11b+ monocytes, or CD11b+ HLA-DR+ cells purified from tumor cocultures with or without 4-IPP treatment for 64 hours were added to T cells at ratios of 1:2 or 1:4, and T-cell activation was measured as above.

DC phenotype and function
Melanoma patient MDSCs and A375-MDSCs were either untreated or treated with 4-IPP (50 μmol/L), PGE2 (10 μmol/L), or with 4-IPP plus PGE2 for 72 hours and were analyzed for the expression of human DC markers by flow cytometry. For mouse DC phenotype studies, bone marrow MDSCs from MIF+/+ and MIF−/− mice were cultured for 48 hours and analyzed for mouse DC marker expression by flow cytometry. DC function in A375-MDSCs was analyzed using Tetanus Toxoid (TT) antigen presentation assays. A375-MDSCs were either untreated or treated with...
4-IPP (50 μmol/L) or 4-IPP (50 μmol/L) plus PGE2 (10 μmol/L) for 72 hours. MDSCs were added to autologous, CFSE-labeled T cells at ratios of 1:5 (20,000 MDSCs) or 1:10 (10,000 MDSCs). T cells were activated with 1.0 μg/mL of T-cell mitogen for 48-hour culture period. CFSE dilution was measured by flow cytometry. DC function in mouse bone marrow MDSCs was determined using the OT-II TCR transgenic mice and ovalbumin (OVA) antigen presentation. MIF+/− and MIF−/− bone marrow MDSCs harvested after 48-hour culture period were added to CFSE-labeled CD4+ T cells purified from OT-II splenocytes at 1:5 and 1:10 ratio in the presence of 200 μg/mL of OVA (Sigma-Aldrich) per well for 5 days. CD4+ T-cell activation was estimated by flow cytometry.

Quantitative PCR analysis

Total RNA and real-time analysis was performed as previously described (10). Taqman probes (Applied Biosystems) for genes Hs00153133_m1 (FAM), MIF (Hs00225534_m1; FAM), COX-2 (Hs00153133_m1; FAM), and NOX4 (Hs00418356_m1; FAM) were used according to the manufacturer's instructions.

Microarray analysis

Total RNA from cultured monocytes, vehicle-treated, and 4-IPP–treated A375-MDSCs were isolated, and microarray analysis was performed according to the manufacturer's instructions. Details of the instrumental set-up and analysis are described in the Supplemental Methods section. The microarray datasets discussed in the current study have been deposited in NCBI's Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number GSE73333.

ROS detection

The oxidation-sensitive dye DCF-DA was used to measure ROS production in untreated or 4-IPP–treated melanoma patient MDSCs or A375-MDSCs. Details of ROS estimation by flow cytometry are provided in the Supplemental Methods section.

Western blotting

Lysates of cultured monocytes and A375-MDSCs were probed with antibodies that recognize human MIF and human GAPDH (Santa Cruz Biotechnology, Inc.).

ELISAs

Cytokines were measured by ELISA in supernatants from T-cell: MDSC cocultures and from MDSC cultures. ELISA kits used were the human IFNγ and PGE2 kits obtained from R&D Systems.

Statistical analysis

GraphPad Prism 5.0 software (GraphPad Prism Software, Inc.) was used for all statistical analyses. Two-group comparisons between control and test samples (groups compared are indicated in the respective figures) were done by two-tailed Student t tests. Multiple data comparisons were derived by one-way ANOVA followed by the Tukey post hoc test. For all tests, statistical significance was assumed where P < 0.05.

Results

Circulating CD14+HLA−DR−/low MDSCs with potent immunoregulatory activities have been identified in the peripheral blood of patients with ovarian cancer (18), hepatocellular cancer (19), and late-stage melanoma (20–22). In an effort to extend our previous findings that monocyte cell–derived MIF provides functional contributions to MDSC immunosuppressive activity (10), we first analyzed the frequency and phenotype of circulating monocyte MDSCs in stage III/IV metastatic melanoma patients using multicolor FACS analysis. Representative dot plots for one of the melanoma patients and one of the normal donors are included in the study to illustrate the gating strategy used (Fig. 1A). The percentage of circulating lineage−/HLA−DR−/low monocyte MDSCs is significantly elevated in melanoma patients' freshly isolated peripheral blood compared with that in normal donors (Fig. 1A and B; refs. 21, 22).

Consistent with prior studies (21, 22), in our study purified CD14+ melanoma monocytes exhibited potent inhibitory activity against autologous T-cell activation (Fig. 2A and B) and IFNγ production (Fig. 2C) induced by anti-CD3/anti-CD28, compared with cultured CD14+ monocytes from normal donors (Fig. 2D–F). This finding is also consistent with prior studies (8) demonstrating that both HLA−DR+ and HLA−DR−/low CD14+ circulating myeloid cell populations represent highly immunosuppressive MDSCs. We next determined whether inhibition of melanoma MDSC MIF with our small-molecule MIF enzymatic antagonist, 4-IPP (10, 13–15), affected MDSC immunosuppressive activity. Treatment of melanoma patient–derived CD14+ monocytes with 4-IPP for 24 hours significantly reduced their T-cell inhibitory activity (Fig. 2G and H). No toxicity or loss of viability was observed in MDSCs treated with either 4-IPP or vehicle (Supplementary Fig. S1A), although there was a slight decrease in MDSC suppressive activity compared with freshly isolated MDSCs (compare Fig. 2A with Fig. 2G). This loss of T-cell suppressive activity in short-term cytokine-free cultures of MDSCs is likely a result of ex vivo culture in the absence of tumor-derived MDSC polarizing factors.

Because we were interested in pursuing validation and mechanism-based studies—both of which necessitate greater numbers of cells than would be practical using patient-derived peripheral blood samples—we established an in vitro model of melanoma cell line–induced MDSCs that faithfully recapitulates patient-derived CD14+HLA−DR−/low monocyte MDSC phenotype and function (16). This model utilizes a coculture system consisting of A375 human melanoma cells and normal donor CD14+ monocytes cocultured for approximately 68 hours (16). We characterized the phenotype of the monocyte MDSC-like cells induced during the A375-monocyte coculture with multicolor flow cytometry. The percentage of CD14+CD11b+CD33+HLA−DR−/low cells was substantially increased in A375-monocyte cocultures in comparison with that in monocytes cultured in the absence of melanoma cells (Supplementary Fig. S2). Furthermore, CD11b+ cells purified from the A375-monocyte cocultures exhibited a significant reduction in HLA−DR and increases in CD14, CD33, PD-L1, and DC-SIGN markers (Supplementary Fig. S3A and S3B)—an expression signature that closely corresponds to monocyte MDSCs isolated from late-stage melanoma patients (16, 21, 23). Although we refer to the CD11b+ cells isolated from the cocultures of A375 cells and monocyte as ‘A375-MDSCs,’ these cells represent a heterogeneous population of cells, similar to the MDSCs isolated from melanoma patients (8). MIF mRNA and protein expression was increased in A375-MDSCs compared with monocytes cultured without tumor cells for the same period of time (Supplementary Fig. S3C and S3D).
To determine whether MIF inhibition during MDSC induction influences the acquisition of MDSC phenotype/function, 4-IPP was added at the beginning of the A375:monocyte coculture. Changes in cell-surface marker expression of A375-MDSCs and relative T-cell suppressive activity were assessed 68 hours later. MIF inhibition during the MDSC induction phase by melanoma cells resulted in reductions of CD14, CD33, and PD-L1 and an increase in CD-SIGN expression, whereas HLA-DR or CD11c expression was not significantly altered (Supplementary Figs. S3A and S3B).

With respect to functional immunosuppressive activities, A375-MDSCs were potent suppressors of autologous T-cell activation and IFNγ production compared with fresh monocytes and tumor cell–free cultured monocytes, whereas A375-MDSCs from 4-IPP–treated cocultures (A375-MDSCs + 4-IPP) possessed little to no suppressive activity on T-cell proliferation/IFNγ production (Fig. 3). The induction of suppressive function in monocytes relied upon direct cell contact with the tumor cells: Monocytes cultured in the presence of A375 tumor cell–conditioned media did not suppress autologous T-cell activation (Supplementary Fig. S4A). This finding is consistent with previous observations using the same A375:monocyte coculture model system to induce MDSCs (16). To evaluate whether the diminished suppressive activity observed with MDSCs from 4-IPP–treated A375:monocyte cocultures was simply due to a reduced number of MDSCs present in the 4-IPP–treated cultures, we isolated CD11b+ HLA-DR− MDSCs from both untreated and 4-IPP–treated A375:monocyte cocultures and compared their respective immunosuppressive functions. CD11b+ HLA-DR− MDSCs from 4-IPP–treated cocultures were significantly less suppressive than CD11b+ HLA-DR− MDSCs from untreated cocultures (Supplementary Fig. S4B). Neither melanoma nor monocyte cell viability was significantly affected by the presence of 4-IPP during coculture (Supplementary Fig. S1B), but the possibility that 4-IPP may...
be influencing the expression/secretion of tumor-derived MDSC-polarizing factors, including active, tumor cell–derived MIF, was not ruled out (11).

To determine whether MIF was necessary for the suppressive function of established A375-MDSCs, isolated A375-MDSCs from A375:monocyte cocultures were treated with 4-IPP for 24 hours. This treatment partially attenuated established A375-MDSC inhibition of T-cell proliferation (Supplementary Fig. S5A and S5B), suggesting that MDSC-derived MIF was necessary for maximal MDSC immunosuppressive functions. The effects of MDSC inhibition of T-cell proliferation (Supplementary Fig. S6A and S6B), suggesting that MDSC-derived MIF was necessary (Supplementary Fig. S6A and S6B). To rule out the possibility that the lower suppressive activity observed with MIF-deficient bone marrow MDSCs was not simply due to fewer MDSCs present in the differentiated MIF-deficient bone marrow MDSCs, we isolated CD11b+GR1+ MDSCs from both MIF wild-type and MIF-deficient bone marrow cultures and compared their immunosuppressive functions. CD11b+GR1+ MDSCs from wild-type bone marrow MDSCs exhibited potent inhibitory activity on antigen-specific T-cell proliferation, compared with MIF-deficient CD11b+GR1+ MDSCs (Supplementary Fig. S7A). When 4-IPP was added during differentiation of wild-type CD11b+GR1+ bone marrow MDSCs, they were significantly less immunosuppressive compared than vehicle-treated CD11b+GR1+ bone marrow MDSCs (Supplementary Fig. S7B)—effectively phenocopying 4-IPP–treated human MDSCs and MIF-deficient bone marrow MDSCs (Supplementary Fig. S7A). To confirm that 4-IPP treatment had no off-target effects, MIF-deficient bone marrow MDSCs were treated with 4-IPP or vehicle control. No difference in immunosuppressive activity was observed between vehicle control and 4-IPP–treated MIF-deficient bone marrow MDSCs (Supplementary Fig. 7A), confirming the lack of any residual in vitro 4-IPP activity in the absence of its target, MIF.

Figure 2.
Melanoma MDSCs suppress autologous T-cell activation in an MIF-dependent manner. A–C, melanoma patient-derived CD14+ MDSCs were cultured with CFSE-labeled autologous T cells and anti-CD3/anti-CD28 beads for 4 days and T-cell activation was determined. Representative histograms (A) and bar graphs showing the percentage of proliferated T cells (B) and IFNγ production (C). D–F, healthy donor CD14+ monocytes were cultured with CFSE-labeled autologous T cells and anti-CD3/anti-CD28 beads for 4 days and T-cell activation was determined. Representative histograms (D) and bar graphs showing the percentage of proliferated T cells (E) and IFNγ production (F). G and H, melanoma MDSCs were pretreated with or without 50 μM/L 4-IPP for 24 hours and then added to CFSE-labeled autologous T cells and anti-CD3/anti-CD28 beads for 4 days. Representative histograms (G) and bar graphs (H) showing the percentage of proliferated T cells. Data, average ± SEM of three independent experiments. **, P < 0.005; ***, P < 0.0005.
To identify potential mechanistic effectors and/or pathways associated with melanoma monocytic MDSCs, we performed mRNA microarray analyses on cultured monocytes and on A375-MDSCs obtained from either untreated or 4-IPP–treated A375:monocyte cocultures. Expression profiles from A375-MDSCs were markedly different from those for cultured monocytes (Supplementary Fig. S8A).

When MIF was inhibited during A375-MDSC induction, a large subset of gene products reverted back to levels observed in monocytes cultured in the absence of tumor cells (Supplementary Fig. S8B). Inflammatory cytokines, chemokines/chemokine receptors, matrix metalloproteases, angiogenic growth factors and arachidonic acid/prostaglandin-generating enzymes were all differentially expressed in A375-MDSCs and restored to "normal" expression by MIF inhibition (Supplementary Fig. S8C).

One gene product of particular interest is that of the NADPH oxidase 4 enzyme (NOX4; Supplementary Fig. 8C). Because NADPH oxidases convert molecular oxygen into superoxide anion upon activation by protein kinase 3 (25), so we next evaluated the relative ability of phorbol myristic acid (PMA) to induce dichlorofluorescein (DCF)-detectable ROS in A375-MDSCs. DCF fluorescence was more strongly induced by PMA in A375-MDSCs than in A375-MDSCs obtained from 4-IPP–treated cocultures (Figs. 4B and C).

In accordance with published results (21), CD14⁺ monocytes from freshly isolated PBMC from melanoma patients had more ROS than healthy donor CD14⁺ monocytes (Supplementary Fig. S1C), and treatment of isolated melanoma MDSCs with 4-IPP for 24 hours significantly reduced DCF-detectable ROS in these cells (Fig. 4D and E).

Murine MDSCs, when cultured in the presence of appropriate growth factors, can differentiate into DCs (26, 27). Human melanoma MDSCs are characterized by higher levels of the DC markers, CD80, CD83, and DC-SIGN compared with normal human monocytes (21). Short-term, cytokine-free culture of human melanoma MDSCs moderately increases the expression of DC markers—including and especially HLA-DR—but without loss of CD14 expression. The retention of CD14 expression on these cells is indicative of a lack of lineage-specific differentiation.
(21). In an effort to determine whether MIF inhibition in melanoma MDSCs maintains NADPH oxidase 4 (NOX4) expression and ROS levels in melanoma MDSCs. A, quantitative PCR analysis of NOX4 mRNA in healthy donor monocytes or presence of A375 cells. A375:monocyte cocultures were either untreated (A375-MDSCs) or treated with 4-IPP (A375-MDSCs+4-IPP: 100 μmol/L, day 0 and 50 μmol/L, day 2). Data, average ± SEM of triplicate samples. B and C, representative histogram (B) and bar graph (C) of mean fluorescent intensities (MFI) of DCF-detectable ROS in untreated and PMA-treated A375-MDSCs and A375-MDSC+4-IPP. D and E, DCF-detectable ROS levels in CD14+ late-stage melanoma patient-derived MDSCs (n = 3) that were pretreated with either DMSO or 4-IPP (50 μmol/L) for 24 hours. Representative histogram (D) and bar graph (E) showing expression of DCF-detectable ROS in melanoma MDSCs and 4-IPP-treated MDSCs. Data, average ± SEM of three independent experiments. **, P ≤ 0.005; ***, P ≤ 0.0005.

To determine whether these phenotypic marker changes correspond to an increase in antigen-specific T-cell functional responses, established A375-MDSCs were cultured with or without 4-IPP for 72 hours (Fig. 5A). Treatment with 4-IPP resulted in significant increases in the percentage of cells (Fig. 5B, top) and relative expression (MFI, Fig. 5B, bottom) of DC markers CD80, CD83, CD86, CD40, and perhaps more importantly, significant reductions in CD14 and PD-L1 on HLA-DR+ MDSCs.

We next sought to recapitulate these findings using MDSCs derived from the A375:monocyte coculture model. We tested two independent models (please see the diagram, Supplementary Fig. S9) for different timing of MIF inhibition by 4-IPP: (i) culturing A375 with monocyte MDSCs in the presence of 4-IPP during the induction phase, followed by culturing purified A375-MDSCs for an additional 72 hours with no other treatments (4-IPP during MDSC induction = A375-MDSC + 4-IPP; Supplementary Fig. S9B), and (ii) addition of 4-IPP after MDSC induction during the 72-hour differentiation phase (4-IPP after MDSC induction = A375-MDSCs treated with 4-IPP; Supplementary Fig. S9C).

Changes in DC marker upregulation were similar, whether 4-IPP was added during MDSC induction (A375-MDSC + 4-IPP) or after MDSC induction (A375-MDSCs treated with 4-IPP) compared with control, untreated A375-MDSCs (Fig. 6A). Specifically, markers associated with DCs—CD80, CD83, CD40, CD1A, CD86, and CD11c—showed a trend toward increased expression in both 4-IPP treatment conditions of HLA-DR+ A375-MDSCs, although not all conditions resulted in statistically significant increases (Fig. 6A).

To determine whether these phenotypic marker changes correspond to an increase in antigen-specific T-cell functional responses, established A375-MDSCs were cultured with or without 4-IPP for 72 hours (Fig. 6B and C). In contrast, 4-IPP-treated A375-MDSCs induced an approximately 4-fold increase in tetanus toxoid–specific T-cell proliferation, indicating that MIF inhibition promotes established MDSC differentiation that results in antigen-specific T-cell responses.

We next validated these findings in the murine MIF-deficient model, using bone marrow MDSCs. Like human melanoma MDSCs treated with 4-IPP, murine MIF-deficient bone marrow MDSCs cultured for 48 hours in cytokine-free media express elevated CD80, CD83, CD86, and MHC II-IA-IE compared with wild-type bone marrow MDSCs (Supplementary Fig. S10A and S10B). These MIF-deficient bone marrow MDSCs could induce ovalbumin-specific CD4+ T-cell proliferation to a significantly greater extent than bone marrow MDSCs from MIF wild-type mice (Supplementary Fig. S10C and S10D). Together, these findings indicate that loss or inhibition of MIF promotes MDSC differentiation toward a more DC-like cell phenotype that results in noticeably improved T cell–mediated antigen-specific immune responses.

Prostaglandin E2 (PGE2) is a critical determinant of MDSC immunosuppressive activity and, perhaps more importantly,
can redirect the differentiation of human DC toward functionally stable MDSCs (16, 28, 29). PGE₂ is generated from a prostaglandin synthase 2 (PTGS2, aka COX-2)–dependent conversion of arachidonic acid (released as a product of phospholipase A₂ catalysis) to PGH₂, which is then converted to PGE₂ by prostaglandin E synthase (PTGES). Because the expression of cytosolic PLA₂ (PLA2G4), COX-2 (PTGS2), and PTGES are all increased in A375-MDSCs in an MIF-dependent manner (Supplementary Fig. S8C), we next sought to determine whether reductions in PGE₂ in 4-IPP–treated melanoma MDSCs were mechanistically linked to MDSC differentiation toward DC-like cells.

As COX-2 is generally considered to be the rate-limiting step associated with PGE₂ production and release, we first determined whether COX-2 expression is elevated in MDSCs from patients with late-stage melanoma. The average mRNA expression of COX-2 in peripheral blood CD14⁺ cells isolated from advanced melanoma patients (n = 5) was approximately 10-fold greater than that of CD14⁺ cells isolated from normal donors (n = 5; Fig. 7A). Inhibition of MIF with 4-IPP in both patient-derived MDSCs (Fig. 7B) and A375-MDSCs (Fig. 7C) significantly reduced COX-2 mRNA expression, consistent with several studies that demonstrated a central regulatory role for MIF in dictating COX-2 expression (30–32). The reduced COX-2 expression in melanoma patient–derived MDSCs treated with 4-IPP established that both A375-MDSCs and A375:monocyte cocultures correlated with significant reductions in PGE₂ concentrations (Fig. 7D–F).

Next, we asked whether reconstituting PGE₂ to 4-IPP–treated MDSC cultures was sufficient to reverse the effects of MIF inhibition on MDSC → DC-like differentiation (Fig. 7G and H). PGE₂ added to 4-IPP–treated melanoma MDSCs efficiently reduced the 4-IPP–mediated increases in both the proportion of HLADR⁺ MDSCs (% positive cells; Fig. 7G) and the expression (MFI; Fig. 7H) of CD80, CD83, and CD40 markers. It also increased the 4-IPP–dependent reductions in percentages and expression of CD14 on HLADR⁺ MDSCs (Fig. 7G and H). Consistent with the observed reversion in immunophenotype, PGE₂ reconstitution of 4-IPP–treated MDSCs effectively inhibited their ability to induce tetanus toxoid–specific T-cell proliferation (Supplementary Fig. S11). Taken together, these data suggest that MIF is an important and previously unrecognized determinant of human melanoma mononuclear MDSC induction and immunosuppressive function. Perhaps more importantly, inhibition of MIF in

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**Figure 5.** MIF inhibition induces a DC phenotype in melanoma patient–derived MDSCs. A and B, CD14⁻ melanoma patient–derived MDSCs (n = 3) were cultured ex vivo for 72 hours in the absence or presence of 4-IPP (50 μmol/L) and analyzed for the expression of DC markers by flow cytometry. Representative dot plots (A) and bar graphs showing percentages and mean fluorescent intensities (MFI; B) of DC marker expression on HLADR⁺ MDSC and 4-IPP-treated MDSCs. Data, average ± SEM of three independent experiments. *P ≤ 0.05; **P ≤ 0.005.
established melanoma MDSCs induces the differentiation of immunosuppressive MDSCs into cells with DC-like phenotype and function. These findings provide compelling justification and rationale for therapeutic targeting of MIF in immunosuppressive human malignancies.

Discussion

Our data describe the important functional contribution made by MIF to human monocytic MDSCs. We show that MIF is necessary for CD14<sup>+</sup>HLADR<sup>low</sup> MDSC induction, immunosuppression, and in vitro differentiation. Using CD14<sup>+</sup> MDSCs derived from advanced-stage melanoma patients, we show that the small-molecule MIF antagonist 4-IPP strongly reduced MDSC-mediated suppression of T-cell activation and IFNγ production. MIF inhibition in short-term, cytokine-free MDSC cultures led to enhanced human DC differentiation strategies could be highly effective in the treatment of late-stage cancer patients (35).

In cancer patients, defective DCs have been implicated in promoting tumor growth and adversely affecting antitumor efficacy of vaccines. Inhibition of VEGF signaling with VEGF-Traps treatment improves DC differentiation/maturation in cancer patients; these effects, however, are insufficient to improve antigen-specific immune responses (35). This lack of immune response is linked to the increased presence of MDSCs in the peripheral blood of the treated patients. Our findings that a safe, bioavailable, and highly efficacious in vivo pharmacologic MIF inhibitor is sufficient to induce MDSC differentiation into functionally immunostimulatory DCs suggest that a multifaceted approach combining anti-MIF therapeutics with established DC maturation strategies could be highly effective in the treatment of late-stage cancer patients (35).

Although several studies describe important functional contributions by MIF to murine innate tumor stromal cell phenotypes, none of these previous studies—including our own (10)—have identified mechanistic effectors and signaling pathways of MIF-dependent functions (11, 36). In an attempt to
identify downstream MIF effectors that could be responsible for MIF-dependent phenotypic and/or functional contributions to human MDSCs, we did a microarray analysis of normal monocytes, A375-MDSCs, and A375-MDSCs + 4-IPP cells. Although several candidate effector mRNAs were identified that are potentially regulated by MIF, we initially chose to focus on ROS- and PGE2-regulatory gene products for the following reasons: (i) ROS are a necessary component of MDSC-dependent immune suppression (21, 24, 26); (ii) COX-2 inhibitors attenuate human monocyte MDSC immunosuppression (16); (iii) COX-2-dependent PGE2 generation maintains MDSC phenotype and function while inhibiting DC development (28, 29); and (iv) MIF is well documented to regulate ROS-regulatory and PGE2-regulatory mechanisms in a variety of cell types (31, 37–39). Our current findings indicate that MIF is an important determinant of several PGE2-regulatory enzymes’ expression—most notably, COX-2. MIF inhibitor treatment reduces MDSC PGE2 levels and exoge- ously reconstituted PGE2 maintains DC marker expression while reducing DC-like phenotype in these cells. These findings suggest that MIF maintains MDSC suppressive phenotype, at least in part, via PGE2 production.

It is less clear how MIF mechanistically dictates MDSC immunosuppressive activity. Our results clearly indicate an important functional role for MIF in maintaining NOX-4 expression and DCF-detectable ROS in MDSCs. At the same time, MIF is centrally important to COX-2 expression and maintaining PGE2 levels that are necessary for MDSC immunosuppressive function (16). It is likely that both of these effector mechanisms (ROS and PGE2 maintenance)—and potentially others—are involved in MIF-dependent MDSC suppression of T-cell activation, but what is less clear is how MIF regulates such a broad array of immunosuppressive and differentiation-regulating gene products. Although we are currently evaluating the signaling requirements for the MIF receptor CD74 (39), in MIF-dependent MDSCs.
phenotypes, we cannot rule out the possibility that MIF’s influences on these cells may be receptor independent. This is based on the fact that enzymatically inactive, CD74-binding competent, N-terminal proline MIF mutants are entirely unable to reconstitute MIF-dependent MDSC induction/function (11, 40). This, coupled with the fact that the MIF enzymatic inhibitor 4-IPP reportedly has little to no MIF:CD74 antagonist activity (41) but very effectively phenocopies MIF deficiency in monococytes/macrophages (current study and ref. 10), is highly suggestive of a CD74-independent signaling function. If this is so, in fact, the case, alternative mechanisms for MIF-dependent modulation of MDSC functionality include alternative outside-in signaling via non-cognate MIF receptors (42, 43) or an intracellular mechanism of action via known (44), or presently unknown, pathways. It will be important to identify the precise mechanism of action going forward as it will not only provide important information regarding a seemingly crucial central node for MIF-dependent regulation of innate immune function in both mice and humans, but also because it could point to a previously unknown function for the highly druggable enzymatic active site of MIF.

Although MIF has been shown to regulate MDSC induction and suppressive activity in murine models of cancer (10, 11), these results illuminate the functional contribution by MIF to human MDSCs. Our findings introduce a role for MIF in regulating human melanoma MDSC differentiation. Our data demonstrating that 4-IPP effectively reduces in vitro MDSC immunosuppression while increasing DC-like antigen-specific T-cell responses suggest that in vivo MIF therapeutic targeting may simultaneously reduce cancer-induced MDSC-mediated T-cell inactivation while enhancing antitumor antigen-specific T-cell responses in metastatic melanoma patients.

It is not yet known whether current therapies that target adaptive immune tumor suppressive checkpoints, such as anti-CTLA-4 (ipilimumab) and anti–PD-1 (pembrolizumab or nivolumab), could act in synergy with 4-IPP targeting of MIF. Some evidence supports the former possibility: A trial immunotherapy consisting of ipilimumab (in combination with irradiated, GM-CSF-expressing, autologous tumor cells) provokes a humoral response resulting in the elicitation of clinically relevant anti-MIF autoantibodies (12). Given that the targeting of CTLA-4 with ipilimumab is efficacious in patients with metastatic melanoma (45, 46), it is possible that combinatorial targeting of adaptive immunosuppressive mechanisms (anti–CTLA-4) and innate immunosuppressive mechanisms (anti-MIF) may provide synergistic clinical responses in patients with advanced-stage melanoma.

Disclosure of Potential Conflicts of Interest

R.A. Mitchell is an inventor on patents pertaining to 4-IPP as an anticancer therapeutic agent targeting MIF.

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MIF Is Necessary for Late-Stage Melanoma Patient MDSC Immune Suppression and Differentiation

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