Control of Tumor-Associated Macrophage Alternative Activation by Macrophage Migration Inhibitory Factor

Kavitha Yaddanapudi, Kalyani Putty, Beatriz E. Rendon, Gwyneth J. Lamont, Jonathan D. Faughn, Abhay Satoskar, Amanda Lasnik, John W. Eaton and Robert A. Mitchell

*Published online 6 February 2013
http://www.jimmunol.org/content/early/2013/02/06/jimmunol.1201650*
Control of Tumor-Associated Macrophage Alternative Activation by Macrophage Migration Inhibitory Factor

Kavitha Yaddanapudi,* Kalyani Putty,* Beatriz E. Rendon,* Gwyneth J. Lamont,* Jonathan D. Faughn,* Abhay Satoskar,† Amanda Lasnik,‡ John W. Eaton,* and Robert A. Mitchell*

Tumor stromal alternatively activated macrophages are important determinants of antitumor T lymphocyte responses, intra-tumoral neovascularization, and metastatic dissemination. Our recent efforts to investigate the mechanism of macrophage migration inhibitory factor (MIF) in antagonizing antitumor immune responses reveal that macrophage-derived MIF participates in macrophage alternative activation in melanoma-bearing mice. Both peripheral and tumor-associated macrophages (TAMs) isolated from melanoma bearing MIF-deficient mice display elevated proinflammatory cytokine expression and reduced anti-inflammatory, immunosuppressive, and proangiogenic gene products compared with macrophages from tumor-bearing MIF wild-type mice. Moreover, TAMs and myeloid-derived suppressor cells from MIF-deficient mice exhibit reduced tumor immunosuppression and neo-angiogenic potential by TAMs, MIF deficiency confers protection against transplantable s.c. melanoma outgrowth and melanoma lung metastatic colonization. Finally, we report for the first time, to our knowledge, that our previously discovered MIF small molecule antagonist, 4-iodo-6-phenylpyrimidine, recapitulates MIF deficiency in vitro and in vivo, and attenuates tumor-polarized macrophage alternative activation, immunosuppression, neoangiogenesis, and melanoma tumor outgrowth. These studies describe an important functional contribution by MIF to TAM alternative activation and provide justification for immunotherapeutic targeting of MIF in melanoma patients.

The Journal of Immunology, 2013, 190: 000–000.

Patients diagnosed with early-stage (stage I) melanoma have a generally favorable prognosis, whereas the 5-y survival rate for those diagnosed with stage IV metastatic disease is only 5–10% (1). Because of the highly immunogenic nature of melanocytic tumors, immunotherapeutic targeting strategies have largely focused on this malignancy (2). Despite this fact and encouraging results from clinical trials with anti–CTLA-4 in patients with late-stage melanoma (3), overall survival percentages among advanced melanoma patients have not improved significantly (4). Contributing to the relative lack of clinical responses with current cancer immunotherapies is the exacerbation of both innate and adaptive immunosuppressive pathways by alternatively activated macrophages within the tumor microenvironment (5). Tumor-dependent polarization of peripheral and stromal macrophages promotes tumor progression by inducing regulatory T cell generation (6). Programmed death 1–dependent lymphocyte immunosuppression (7–9), and tumor-associated neoangiogenesis (10, 11). The identification of new immunotherapeutic targets that are ready “druggable” is critical to the elucidation of an individual or combinatorial immunotherapeutic strategy that will provide meaningful and durable clinical responses in late-stage cancer patients.

Despite its well-documented activities as a proinflammatory determinant of innate immune responses (12–14), macrophage migration inhibitory factor (MIF) has T lymphocyte immunosuppressive activities in malignant disease settings (15, 16). For example, systemic inhibition of MIF during murine tumor outgrowth significantly enhances antitumor CTL Th1 responses (15). Moreover, MIF silencing in neuroblastoma cells induces robust antitumor CTL responses in transplanted mice compared with MIF-expressing neuroblastoma cells (16). Finally, MIF overexpression in ovarian and melanocytic cancers antagonizes NK cell–mediated cancer cell cytolysis (17, 18). Combined, these findings suggest that MIF actively suppresses antitumor lymphocyte responses, and MIF inhibition breaks this immunosuppression, resulting in enhanced cancer cytolytic responses. What is less clear is how MIF promotes immunosuppressive adaptive immune response phenotypes when MIF is so tightly linked to proinflammatory innate immune responses (19).

We now report that in malignant disease settings, MIF is an important mediator of macrophage alternative activation. MIF deficiency or small-molecule antagonism reduces B16 melanoma tumor outgrowth and B16F10 metastatic melanoma lung colonization in a manner that coincides with decreased anti-inflammatory and increased proinflammatory effector expression in bone marrow–derived, peripheral, and tumor stromal macrophages. We also demonstrate that tumor-associated macrophage (TAM) proangiogenic effector expression and angiogenic potential are reduced in...
MIF-deficient and MIF$\textsuperscript{+}$ small-molecule inhibited macrophages. Combined, these findings provide important and novel evidence that MIF is a critical mediator of protumorigenic macrophage alternative activation and ensuing melanoma genesis.

Materials and Methods

Mice

Wild-type male C57BL/6 mice (MIF$\textsuperscript{+}$) were obtained from Harlan Laboratories (Dublin, VA). OT-1 transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were used at 6–8 wk of age. All mice were handled in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care international guidelines and with the approval of the Institutional Animal Care and Use Committee at the University of Louisville.

Tumor models

B16F0 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA). To establish s.c. tumors, we s.c. injected $1 \times 10^5$ B16 tumor cells into the left flanks of MIF$\textsuperscript{+}$ and MIF$\textsuperscript{-/-}$ C57BL/6 mice. The Lewis lung carcinoma (LLC) model used was established by injecting $1 \times 10^5$ LLC tumor cells s.c. into mice. Tumor growth was monitored three times a week by measuring with digital calipers. To establish the experimental metastasis model of melanoma, we injected $1 \times 10^5$ B16-F10-hu2 cells (Caliper Life Sciences, Mountain View, CA) into the tail vein of C57BL/6/MIF$\textsuperscript{+}$ and MIF$\textsuperscript{-/-}$ mice. For in vivo imaging, mice were anesthetized with 75 mg/kg ketamine and 7.5 mg/kg xylazine, and injected i.p. with the reporter substrate, D-luciferin, potassium salt (150 mg/kg) or vehicle control (corn oil) daily for the next 14 d. Tumor growth was monitored three times a week by measuring with digital calipers.

Isolation and treatment of peritoneal macrophages

Peritoneal exudate cells (PECs) were obtained by peritoneal lavage from naive and B16 tumor-bearing MIF$\textsuperscript{+}$ and MIF$\textsuperscript{-/-}$ C57BL/6 mice 18–21 d after tumor challenge. For isolation of CD11b$\textsuperscript{+}$ macrophages, tumor-bearing mice were injected i.p. 4 d before harvest with 2 ml thiglycollate broth (BD Biosciences, San Jose, CA). The CD11b$\textsuperscript{+}$ macrophages were enriched using the autoMACS ProSeparator (Miltenyi Biotec, Auburn, CA). Isolated cells were treated for 24 h with 4-IPP (50 μM) or DMSO (vehicle control) and LPS (0.2 μg/ml; Sigma-Aldrich). For anti-MIF Ab treatment, TAMs were treated for 16 h with either neutralizing anti-MIF Ab (100 μg; clone 3D9, a generous gift of Dr. Richard Bucala, Yale University; IgG1 isotype) or IgG1 isotype control Ab (BD Biosciences) and F4/80$\textsuperscript{+}$ cells were positively selected using the PhenomIAGER (Biospace Lab, Paris, France). Bioluminescence was quantified from the in vivo signals emitted from ventral views (days 18–25) using the Biospace software. The mean photon emission was calculated and expressed as photons/second/steradian (ph/s/σ).

Isolation and treatment of peritoneal macrophages

Freshly isolated TAMs or MDSCs (granulocytic or monocytic subpopulations) were added in triplicates to 96-well plates at the indicated cell numbers (10$^6$) of the population expressing the Ag of interest. Absolute number (10$^6$) of the population was determined using the comparative CT method ($\Delta$CT method). The $\Delta$CT was calculated as the difference between the normalized CT values ($\Delta$CT = CT of target gene − CT of endogenous control gene) of the treatment and the control samples: $\Delta$CT = CT treatment − CT control, $\Delta$CT was then converted to fold change by the following formula: fold change = 2 $^{-\Delta\Delta\text{CT}}$.

Quantitative PCR analysis

For RNA extraction, cells were lysed in buffer RLT (Qiagen, Valencia, CA), homogenized, and purified using RNeasy mini kit (Qiagen) following manufacturer’s instructions. Integrity of RNA was checked using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The RNA was reverse transcribed with MultiScribe reverse transcriptase and oligo(dT) primers (Applied Biosystems, Bedford, MA). Quantitative assessment of mRNA levels was done by real-time RT-PCR on an ABI 7500 FAST instrument with either TaqMan fast advanced master mix (Applied Biosystems) or SYBR green ROX quantitative PCR (qPCR) master mix (Qiagen), 0.2 μM forward and reverse primers (Invitrogen, Grand Island, NY). Relative expression profiles of mRNAs were then calculated using the comparative CT method ($\Delta$CT method). The $\Delta$CT was calculated as the difference between the normalized CT values ($\Delta$CT = CT of target gene − CT of endogenous control gene) of the treatment and the control samples: $\Delta$CT = CT treatment − CT control, $\Delta$CT was then converted to fold change by the following formula: fold change = 2 $^{-\Delta\Delta\text{CT}}$.

Isolation and treatment of s.c. and lung TAMs

s.c. and lung TAMs were isolated from B16 tumor-bearing MIF$\textsuperscript{+}$ and MIF$\textsuperscript{-/-}$ C57BL/6 mice 18–21 d after tumor challenge. s.c. and lung tumors were enzymatically digested, and F4/80$\textsuperscript{+}$ cells were positively selected using the autoMACS ProSeparator (Miltenyi Biotec). Purity of isolated cells was checked by flow cytometry. s.c. and lung F4/80$\textsuperscript{+}$ TAMs were washed and treated with 16 h with 4-IPP (50 μM) or DMSO (vehicle control) and LPS (0.2 μg/ml; Sigma-Aldrich). For anti-MIF Ab treatment, TAMs were treated for 16 h with either neutralizing anti-MIF Ab (100 μg; clone 3D9, a generous gift of Dr. Richard Bucala, Yale University; IgG1 isotype) or IgG1 isotype control Ab (BD Biosciences) and F4/80$\textsuperscript{+}$ cells were positively selected using the autoMACS ProSeparator (Miltenyi Biotec). Purity of cell populations was confirmed by flow cytometry and was >98%.

Phenotypic and quantitative analysis of splenocytes, peritoneal cells, and tumor-infiltrating leukocytes

Phenotypic and quantitative analysis of splenocytes, peritoneal cells, and tumor-infiltrating leukocytes

Single-cell suspensions from spleen, PECs, and tumors were obtained from naive or melanoma-bearing MIF$\textsuperscript{+}$ and MIF$\textsuperscript{-/-}$ C57BL/6 mice and stained with relevant Abs (F4/80, CD11b, CD206, MHC-II, CD11c, CD80, CD86, Ly6G, Ly6C, CD4, and CD8), and tumor-infiltrating leukocytes (TAMs) were added in triplicates to 96-well plates at the indicated cell numbers and stained with relevant Abs (F4/80, CD11b, CD206, MHC-II, CD11c, CD80, CD86, Ly6G, Ly6C, CD4, and CD8), and cell counts were acquired using a FACS (Becton Dickinson and Company, Franklin Lakes, NJ), and results were analyzed with FlowJo software (Tree Star, Ashland, OR). To calculate the absolute number of a splenocyte subset in the total pool of splenocytes, we multiplied the absolute number of total splenocytes (obtained via counting the cells on a hemocytometer) by the relative prevalence of that subset (%) obtained via flow cytometry analysis. The results were expressed as the absolute number (10$^6$) of the population expressing the Ag of interest. Absolute number of macrophages in PECs and tumor-infiltrating leukocytes were analyzed as described earlier.

Functional assays

Functional assays

Freshly isolated TAMs or MDSCs (granulocytic or monocytic subpopulations) were added in triplicates to 96-well plates at the indicated cell numbers; and, in some cases, pretreated with 4-IPP (50 μM) or DMSO (vehicle control) for 16 h. Splenocytes from OT-1 mice were then added at the appropriate dilution in triplicate to wells containing TAMs or MDSCs in presence of the OVA (200 μg/ml; Sigma-Aldrich) and cultured for an additional 72 h. Eighteen hours before harvesting, cocultures were pulsed with [3H]thymidine (1 μCi/well; MP Biologicals). [3H]thymidine uptake was quantified using a liquid scintillation counter, and relative cpm were used to determine percentage inhibition of proliferation.
free media, counted, plated on Matrigel transwell chambers (BD Biosciences), and cocultured in supernatants from MIF+/+, MIF−/−, MIF+/− control, and MIF+/− 4-IPP–treated TAMs for 24 h at 37°C with 5% CO2. The migrated cells were fixed with 4% formaldehyde and stained with crystal violet (0.1% in 20% ethanol). HUVEC migration was quantitated by manually counting the number of cells on the inserts under low power at ×40 magnification.

**HUVEC tube formation assay.** HUVECs were resuspended in conditioned media from MIF+/+, MIF−/−, MIF+/− control, and MIF+/− 4-IPP–treated cultures and dispensed into wells precoated with Matrigel and incubated for 24 h. Tubes were quantified by counting the number of connecting branches between discrete endothelial cells.

**ELISAs**
Cytokines were measured by ELISA in supernatants from PEC or TAM cultures. ELISA kits used were the murine IL-10, TNF-α, VEGF, MMP-9, and IL-12 kits obtained from R&D Systems (Minneapolis, MN).

**Western blotting**
B16 and B16F10 cells were cultured overnight in serum-free media, and 500 μl media was concentrated ~25 times using 10 kDa molecular mass Microcon concentrators (Millipore). Concentrated supernatants and lysates of adherent B16 and B16F10 cells were probed with an Ab that recognizes murine MIF (Torrey Pines Biolabs).

**Arginase activity and NO assays**
Arginase activity was quantified in cell lysates by measuring the production of urea using the QuantiChrom arginase Assay Kit (DARG-200; BioAssays Systems). Nitrite concentrations in culture supernatants were determined using Griess reagent (Sigma-Aldrich).

**Generation and treatment of bone marrow–derived macrophages**
Bone marrow cells were harvested from femur and tibias of 6- to 10-wk-old C57BL/6 mice. The cells were cultured in RPMI 1640 supplemented with 10% FBS (Life Technologies), recombinant mouse M-CSF (10 ng/ml; R&D Systems), and L929 conditioned medium (15%) in Nunclon surface cell culture plates. Nonadherent cells were collected after 24 h and were cultured for 7 d in the supplemented medium in Corning/Costar ultralow attachment polystyrene culture plates, changing the medium once on day 4. On day 7, live cells were purified by centrifugation over Ficoll/Lite-LM (Atlanta Biologicals). The resulting cell population was >98% CD11b+.

The cells were resuspended in RPMI 1640 with 10% FBS, dispensed into 24-well cell culture plates, and treated for 24 h with 4-IPP (50 μM) or DMSO (vehicle control) in the presence of LPS (0.2 μg/ml; Sigma-Aldrich).

**Statistical analysis**
GraphPad Prism 5.0 software (GraphPad Prism Software, La Jolla, CA) was used for all statistical analyses. Comparisons between groups were done by two-tailed Student t tests. For all tests, statistical significance was assumed where p < 0.05.

**Results**
**MIF deficiency or small-molecule antagonism reduces murine melanoma outgrowth and enhances peripheral macrophage proinflammatory responses**
Because MIF promotes evasion from antitumor CTL responses (15, 16), we set out to determine how or whether MIF regulates protumorigenic phenotypes and macrophage responses in tumor-bearing mice. B16 murine melanoma cells were injected s.c. into syngeneic C57BL/6 MIF+/+ and MIF−/− mice, and tumor outgrowth was followed by caliper measurements. As shown in Fig. 1A, melanomas from MIF-deficient mice grew out at a significantly slower rate than those developing in mice with functional MIF (Fig. 1A). Consequently, MIF-deficient mice showed increased survival when compared with MIF-wild type.
mice (Fig. 1B). We next determined the relative proinflammatory status of peritoneal macrophages from MIF−/− and MIF+/+ tumor-bearing mice using surrogate proinflammatory marker expression in PECs. As shown in Fig. 1C, TNF-α mRNA and protein levels, IL-12 mRNA/protein, COX-2 mRNA, and inducible NOS mRNA and corresponding NO levels were substantially higher in PECs derived from tumor-bearing, MIF-deficient mice than those from MIF wild-type mice, suggesting that MIF functionally promotes a reduced proinflammatory macrophage phenotype in tumor-bearing mice. In support of a functional role for host effector cell–derived MIF, as opposed to implanted tumor cell–derived MIF, in suppressing the inflammatory phenotype observed in tumor-bearing mice (Fig. 1C), PECs from B16 tumor-bearing MIF−/− mice treated ex vivo with the MIF small-molecule suicide antagonist, 4-IPP (20), expressed significantly greater levels of TNF-α mRNA and protein than control PECs (Fig. 1D).

We next tested whether systemic in vivo neutralization of MIF with 4-IPP recapitulates MIF deficiency in reducing established B16 melanoma progression. 4-IPP treatment resulted in a significant impairment of B16 outgrowth and progression, but with only a modest increase in survival rates compared with vehicle alone control mice (Fig. 2A, 2B). Importantly, PECs from 4-IPP–treated mice expressed proinflammatory effectors at significantly greater levels than those from control mice (Fig. 2C), effectively phenocopying MIF deficiency (Fig. 1C). To ascertain that 4-IPP treatment had no off-target effects, we treated MIF−/− mice with 4-IPP or vehicle control and monitored B16 melanoma tumor growth. No difference in tumor progression or percentage survival was observed between vehicle control and 4-IPP–treated MIF-deficient mice (Supplemental Fig. 1A, 1B). Furthermore, the proinflammatory cytokine responses of peripheral macrophages were similar in vehicle and 4-IPP–treated MIF-deficient mice, confirming the lack of any residual in vivo 4-IPP activity in the absence of its target: MIF (Supplemental Fig. 1C).

Prior studies have shown that tumor-bearing mice develop alternatively activated macrophages in distal sites such as the spleen and peritoneal cavity that ultimately serve to suppress peripheral inflammatory and immune responses (21, 22). Consistent with these reports, resident peritoneal cells from B16 melanoma-bearing mice display increased protumoral, M2-type marker expression (arginase-1 [ARG-1], IL-10, VEGF-A) and decreased M1-type marker expression (TNF-α and IL-12) compared with peritoneal cells from naive mice (data not shown), indicating that, in tumor-bearing hosts, peripheral macrophages are skewed toward an M2-like, tumor-promoting phenotype. Hypothesizing that the proinflammatory polarization profile observed in MIF-deficient/inhibited PECs is indicative of reduced peripheral alternative macrophage activation (23, 24), we determined the expression of several M2 markers, ARG-1 (25), IL-10 (25), and stabilin-1 (26), in relation to the classical (M1) macrophage activation marker, TNF-α, in 4-IPP–treated MIF+/+ peritoneal macrophages. As shown in Fig. 3, ARG-1 mRNA (Fig. 3A) and activity (Fig. 3B), IL-10 mRNA/protein (Figs. 3A, 3B), and stabilin-1 mRNA (Fig. 3A) expression were significantly reduced, and TNF-α mRNA/protein was increased (Fig. 3A, 3B), in 4-IPP–treated, CD11b-purified peritoneal macrophages isolated from melanoma-bearing MIF+/+ mice. Importantly, this same trend was observed in 4-IPP–treated, bone marrow–derived macrophages isolated from MIF+/+ tumor-bearing mice (data not shown).

Because alternatively activated stromal macrophages within malignant lesions are important determinants of local tumor immunosuppression (5), we next investigated the polarization profiles and functional activities of B16 melanoma TAMs from MIF wild-type and MIF-deficient mice. As shown in Fig. 4A, F4/80−MIF promotes alternative activation markers and reduces classical activation markers of peripheral macrophage polarization in tumor-bearing mice

![FIGURE 2](http://www.jimmunol.org/) Pharmacologic inhibition of MIF delays in vivo melanoma tumor outgrowth and enhances peripheral macrophage proinflammatory profiles. (A) C57BL/6 MIF−/− mice were injected with B16 cells (s.c.). Seven days after tumor inoculation, mice were treated i.p. with 4-IPP (80 mg/kg in corn oil) or vehicle control (corn oil) for 14 d and tumor volumes were plotted. Data represent the average tumor volumes of 10 mice/group ± SEM and are representative of two independent experiments. (B) Tumor growth was monitored daily in all animals until sacrifice because of tumors exceeding 5% of body weight. (C) Resident PECs from C57BL/6 mice bearing an s.c. melanoma tumor (n = 10) were pooled and activated in vitro with LPS and either DMSO (vehicle control) or 4-IPP (50 μM) for 24 h. mRNA, protein, and NO levels were analyzed from indicated cells. Data represent the average ± SEM of duplicate samples representative of three independent experiments. **p ≤ 0.005, ***p ≤ 0.0005.
cells isolated from B16 lesions from MIF\(^{-/-}\) mice had significantly reduced expression of the alternative activation marker, ARG-1, and increased classical activation marker, TNF-\(\alpha\) mRNA (Fig. 4A, top panel), and activity/protein (Fig. 4A, bottom panel). In contrast with peritoneal monocyte/macrophage populations (Fig. 1C), IL-12 expression was undetectable in F4/80\(^+\) TAMs from MIF\(^{+/+}\) wild-type mice consistent with prior studies (27–29), and MIF deficiency/inhibition was unable to reverse this effect in TAMs (data not shown). Consistent with a reduced alternative activation immunosuppressive phenotype, MIF-deficient TAMs were less active in suppressing Ag (OVA)-induced splenocyte activation/proliferation (30) than TAMs from MIF wild-type mice (Fig. 4B).

TAMs from MIF\(^{+/+}\) mice were isolated, treated ex vivo with 4-IPP, followed by expression and phenotypic profiling to further evaluate the relative importance of macrophage-derived MIF in promoting the polarization profile and phenotype of alternatively activated tumor stromal macrophages. As shown in Fig. 4C, 4-IPP effectively reduced ARG-1 expression and increased TNF-\(\alpha\) similar to the trend observed with peritoneal macrophages (Fig. 3) and MIF-deficient TAMs (Fig. 4A). Importantly, 4-IPP pretreatment of TAMs reduced the immunosuppressive phenotype of alternatively activated TAMs (Fig. 4D) similar to that observed with MIF deficiency (Fig. 4B) consistent with the reduced anti-inflammatory, and enhanced proinflammatory, expression profile (Fig. 4C). For further validation of a functional role for MIF in

FIGURE 3. Alternative activation of peritoneal macrophages from tumor-bearing mice is altered by in vitro 4-IPP treatment. (A and B) CD11b\(^+\) peritoneal macrophages from melanoma-bearing C57BL/6 MIF\(^{+/+}\) mice (\(n = 10\)) were treated in vitro with LPS and either DMSO (vehicle control) or 4-IPP (50 \(\mu\)M) for 24 h. (A) mRNA and (B) arginase activity and protein expression were analyzed from indicated cells. Data represent the average \(\pm\) SEM of duplicate samples representative of two independent experiments. *\(p \leq 0.05\), **\(p \leq 0.005\).

FIGURE 4. TAM alternative activation profile and phenotype is attenuated by MIF deficiency and 4-IPP treatment. (A and C) F4/80\(^+\) TAMs from MIF\(^{+/+}\) and MIF\(^{-/-}\) C57BL/6 mice (\(n = 10\)) bearing an s.c. melanoma tumor were pooled and activated in vitro with (A) LPS alone or (C) LPS in the presence of either DMSO (vehicle control) or 4-IPP (50 \(\mu\)M) for 24 h. Cell lysates were analyzed for mRNA, protein expression, and arginase activity. (B and D) F4/80\(^+\) TAMs from tumor-bearing MIF\(^{+/+}\) and MIF\(^{-/-}\) C57BL/6 mice (\(n = 10\)) were untreated or pretreated for 16 h with 4-IPP (50 \(\mu\)M) or DMSO (vehicle control). Splenocytes from OT-1 mice were added in triplicate to wells containing TAMs in the presence of OVA (200 \(\mu\)g/ml) and cultured for 72 h. Eighteen hours before harvesting, cocultures were pulsed with \(^{[3]}\)Hthymidine. Data represent the average \(\pm\) SEM of triplicate samples (A, C) or average \(\pm\) SD of triplicate samples (B, D) representative of three independent experiments. *\(p \leq 0.05\), **\(p \leq 0.005\), ***\(p \leq 0.0005\).
promoting macrophage alternative activation in tumor-bearing mice, qPCR analyses of PECs and TAMs revealed that the mRNA expression of several additional well-characterized M2 alternative activation markers (Retnla/FIZZ1, Mrc-1, and Chi3l3/ Ym1) was reduced in MIF-deficient PECs (Supplemental Fig. 2A) and TAMs (Supplemental Fig. 2B). Importantly, ex vivo treatment of MIF+/+ PECs and TAMs similarly resulted in significantly decreased FIZZ-1, Mrc-1, and YM1 expression (data not shown). PECs from MIF-deficient mice also displayed significantly increased expression of the M1 classical activation marker IRF5 (Supplemental Fig. 2A), whereas IRF5 expression was unchanged in TAMs from MIF+/+ mice (Supplemental Fig. 2B). Flow cytometric analyses of CD45^+ F4/80^+ MIF+/+ and MIF^−/− TAMs also revealed that the expression of cell-surface-associated M2 markers, CD206 and CD23, were reduced, whereas M1 cell-surface-associated markers, MHC-II, CD11c, CD80, and CD86, were increased in MIF^−/− TAMs compared with MIF+/+ TAMs (Supplemental Fig. 2C). Finally, it is important to note that the total numbers of CD11b^+ F4/80^+ macrophages within splenocytes, peritoneal cells, and CD45^+ tumor-infiltrating leukocytes were not significantly different between MIF+/+ and MIF^−/− B16 tumor-bearing mice (data not shown). Combined, these studies strongly indicate that macrophage-derived MIF is an important determinant of peripheral and intratumoral macrophage alternative activation in B16 melanoma-bearing mice.

**MIF promotes melanoma metastases and TAM polarization in colonized organs**

Alternatively activated macrophages promote metastatic dissemination and colonization (31, 32). Luciferase-expressing B16F10 metastatic melanoma cells were injected i.v. into syngeneic MIF+/+ or MIF^−/− mice and progression followed by whole-body luminescence to investigate whether stromal MIF contributes to melanoma pulmonary metastases and colonization. Imaging on day 21 post i.v. injection of B16F10 cells (Fig. 5A, 5B) or days 18, 21, and 23 (Fig. 5B) revealed significantly reduced lung tumor burden in MIF^−/− mice compared with MIF+/+ mice. Lungs from MIF-deficient mice exhibited reduced metastatic tumor burden both visually (Fig. 5C) and as a function of total lung mass (Fig. 5D). F4/80^+ cells were purified from metastatic tumor-bearing lungs from MIF+/+ and MIF^−/− mice and evaluated for M2 and M1 marker expression by qPCR to determine whether macrophage polarization was altered in the lungs of MIF-deficient versus wild-type mice. As shown in Fig. 6A, the immunosuppressive effectors, ARG-1 and IL-10 mRNA and activity/protein, were significantly reduced, whereas TNF-α mRNA and protein were elevated in lung TAMs from MIF-deficient mice. Notably, MIF is expressed and secreted at very high levels in both B16 and B16F10 murine melanoma cells (Fig. 6B), suggesting that tumor cell–derived MIF does not independently dictate intratumoral macrophage polarization, at least in initial melanoma outgrowth, and that macrophage-derived MIF dominates the macrophage phenotype. This is consistent with our observations that 4-IPP, added ex vivo to polarized MIF^+/+ TAMs, similarly reverts TAMs from an M2 expression profile toward an M1 profile (Fig. 4C, Supplemental Fig. 3A).

Because MIF-deficient TAMs from s.c. melanoma lesions were found to be less immunosuppressive than those from MIF wild-type mice (Fig. 4B), we next tested whether MIF-deficient lung TAM immunosuppression was similarly dysfunctional. As shown in Fig. 6C, TAM-mediated inhibition of Ag-induced splenocyte proliferation was reduced in MIF-deficient lung TAMs when compared with lung TAMs from MIF wild-type mice. In addition, and consistent with the defective macrophage alternative activation profile observed with ex vivo inhibition of macrophage-derived MIF (Supplemental Fig. 3A), pretreatment of MIF+/+ lung TAMs with 4-IPP reduced the immunosuppressive potential of these cells (Supplemental Fig. 3B).

**MIF-dependent TAM polarization promotes macrophage angiogenic potential**

Alternative activation of TAMs represents a critically important component of neo-angiogenic potential in developing neoplasms (33, 34). Because MIF has previously been shown to promote the expression of proangiogenic growth factors from macrophages in vitro settings (35), we hypothesized that macrophage-derived MIF contributes to not only the anti-inflammatory and immunosuppressive phenotype of TAMs, but may also promote proangiogenic TAM potential within malignant lesions. F4/80^+ TAMs from the lungs of MIF+/+ and MIF^−/− mice were examined for relative expression of proangiogenic effectors. As shown in Fig. 7A, MIF-deficient lung TAMs expressed reduced mRNA and protein levels of both VEGF and MMP-9, two critically important mediators of TAM angiogenic potential (11, 36), compared with MIF wild-type lung TAMs. To determine whether MIF-deficient lung TAMs were functionally defective in promoting tumor vascularization, we cocultured supernatants from MIF+/+ and MIF^−/− lung TAMs with HUVECs, and endothelial cell migration and

**FIGURE 5.** MIF deficiency decreases lung metastasis in an experimental metastasis model of melanoma. B16-F10-luc2 cells were injected into the tail vein of MIF+/+ and MIF^−/− mice (n = 10). (A) Ventral images taken on day 21 are shown for representative mice (n = 5). (B) Bioluminescence was quantified from the in vivo signals emitted from ventral views. Data shown are representative of three independent experiments (n = 5). (C) Mice were sacrificed on day 25, and lungs were perfused, harvested, and photographed. (D) Lung tumor burden in tumor-bearing MIF+/+ and MIF^−/− mice. Data represent the average weights of four sets of lungs/group ± SEM and are representative of two independent experiments. *p ≤ 0.05.
tube formation were assessed. Supernatants from MIF+/+ lung TAMs were significantly more active in inducing HUVEC migration (Fig. 7B) and tube formation (Fig. 7C) in vitro than lung TAMs from MIF-deficient mice. Similarly, decreased VEGF and MMP-9 expression, as well as HUVEC tube-forming potential, was reduced by ex vivo treatment of MIF wild-type lung TAMs with 4-IPP (Supplemental Fig. 4A, 4B). Finally, 4-IPP ex vivo treatment of MIF+/+ TAMs from B16 s.c. lesions (Supplemental Fig. 4C), PECs from B16 tumor-bearing MIF +/+ mice (Supplemental Fig. 4D), and bone marrow–derived macrophages from B16 MIF+/+ mice (Supplemental Fig. 4E) resulted in a similar reduction of VEGF and MMP-9 expression. Combined, these studies indicate that TAM-derived MIF is a critical determinant of melanoma TAM polarization, immunosuppression, and angiogenic potential.

MIF deficiency or small-molecule inhibition reduces splenic MDSC immune suppression in tumor-bearing mice

MDSCs promote tumor progression through both immune-dependent (37) and -independent (38) mechanisms. When MDSCs populate the tumor microenvironment, they become F4/80+ (39) and, therefore, represent an important subpopulation of the immunosuppressive TAM population in malignant lesions (Figs. 4, 6, 7). To determine whether MIF participates in measurable MDSC-specific immunosuppression, we determined relative MDSC immunosuppressive activities in the spleens of tumor-bearing MIF+/+ and MIF+/− mice.

Initial attempts to isolate sufficient numbers of splenic MDSCs from B16 tumor-bearing mice were unsuccessful. To circumvent this problem, we inoculated MIF+/+ and MIF−/− mice with the well-characterized peripheral MDSC-eliciting cell line, LLC (40, 41). Pooled spleens were used to purify both GR-1hi Ly-6G+ granulocytic and GR-1 dim Ly-6G− monocytic MDSCs from either MIF+/+ or MIF+/− LLC tumor-bearing mice and were evaluated for their relative suppression of OVA-induced lymphocyte proliferation (40). GR-1hi Ly-6G+ and GR-1 dim Ly-6G− monocytic MDSCs from either MIF+/+ or MIF−/− LLC tumor-bearing mice were evaluated for their relative suppression of OVA-induced lymphocyte proliferation (40). GR-1hi Ly-6G+ and GR-1 dim Ly-6G− monocytic MDSCs isolated from LLC-tumor-bearing, MIF-deficient mice were significantly less immunosuppressive than MIF wild-type MDSCs (Fig. 8A, 8B). Importantly, MIF deficiency caused no significant changes in the raw numbers of spleen-derived CD11b+GR1hi or CD11b+GR1dim MDSCs, or in the total MDSCs when compared with MDSCs numbers obtained in MIF+/+ mice (data not shown). Finally, this MIF-deficient MDSC phenotype was fully recapitulated by 4-IPP pretreatment of MIF+/+ granulocytic and monocytic MDSCs isolated from LLC tumor-bearing, MIF-deficient mice (Fig. 8C, 8D), again suggesting that effector cell–derived MIF is necessary for the observed alleviation of MDSC immunosuppressive activity.

Discussion

Our findings suggest that monocyte/macrophage-derived MIF contributes to TAM polarization and functionally promotes an immunosuppressive, proangiogenic, and alternatively activated (M2-like) phenotype in tumor-bearing mice. MIF induces prion-
Inflammatory cytokine responses in several disease pathologies including arthritis (42), acute respiratory distress syndrome (43), atherogenesis (44), glomerulonephritis (45), and type I diabetes (46). However, our results clearly indicate that, in malignant disease settings, MIF is a central participant in macrophage M2-like, anti-inflammatory cytokine responses. As an autocrine-acting cytokine, MIF exerts signal initiating events via interaction with its cognate cell surface receptor, CD74 (47). Proinflammatory signaling in macrophages by MIF requires CD74 and its first identified coreceptor, CD44 (48, 49). However, recent studies show that CD74 also forms MIF-sensitive coreceptor complexes with several chemokine receptors including CXCR2, CXCR4, and CXCR7 (50–52). Given this differential coreceptor usage by soluble MIF, it is tempting to speculate that MIF signals to proinflammatory, classically activated pathways or anti-inflammatory, alternatively activated pathways in a manner that is dictated by which CD74 coreceptors are preferentially expressed on a given cell type. That being said, in vitro neutralization of MIF with an mAb was unable to recapitulate MIF deficiency or 4-IPP antagonism in decreasing M2 and increasing M1 marker expression profiles in PECs from tumor-bearing mice (data not shown). Because 4-IPP interferes with MIF secretion (53), it is conceivable that there is a distinct and stringent requirement for local MIF secretion and/or receptor dynamics in macrophage populations that is absent in other cell types where autocrine-acting MIF is readily inhibited by neutralizing Abs (54, 55).

In vivo reprogramming of macrophage polarization by loss or inhibition of host MIF coincides with a significant reduction in s.c. and metastatic melanoma tumor growth and progression (Figs. 1, 2, 5). Our observed defects in MIF-deficient TAM and MDSC immunosuppressive activities (Figs. 4, 6, 8), coupled with prior studies showing that MIF neutralization in tumor-bearing hosts induces CTL activities (15), is suggestive of an important role for MIF in tumor-induced innate and adaptive immune tolerance and...
immunosuppression. One caveat to these studies is that tumor size differences could, in theory, influence relative TAM polarization states and phenotypes in an MIF-independent manner. However, our results indicate that when equal-sized tumors across different comparison groups are analyzed, we still find significant re-orientation of TAM polarization/immunosuppressive function in MIF-deficient and 4-IPP-treated mice, suggesting that TAM re-polarization associated with MIF deficiency/inhibition is not attributable to differences in tumor size.

It is interesting to note that, like other M2-elicited cytokines/growth factors, MIF promotes functional tumor-promoting phenotypes in malignant cells (56–59). These autocrine tumor-promoting activities of secreted MIF on malignant disease processes coincide with paracrine effects on stromal macrophages. For example, tumor cell-derived MIF regulates CXCL8 (IL-8) and VEGF expression in human monocytes (35, 60), consistent with our findings (Fig. 7). As such, soluble MIF elicited from TAMs within a tumor’s stromal compartment likely promotes not only M2 TAM polarization in an autocrine manner, but also amplifies paracrine signaling on tumor cells. In this scenario, tumor-derived MIF would also be expected to promote M2 TAM polarization. Interestingly, our studies in MIF-deficient mice suggest that the bulk of MIF’s M2 promoting activity in early melanoma outgrowth/progression (Figs. 1, 5) derives primarily from stromal, host-derived MIF as B16 and B16F10 melanoma cell lines express copious amounts of MIF (Fig. 6B). In further support of this scenario, 4-IPP has negligible antitumor efficacy in immuno-deficient SCID mouse models of established tumorigenesis (data not shown), suggesting that 4-IPP inhibition of established melanoma outgrowth (Fig. 2A) is independent of melanoma-derived MIF inhibition and is dependent on inhibition of host immune effector cell–derived MIF (Fig. 1C, 2B).

During the preparation of this article, a study was published by Wang and colleagues (61) describing a contributing role for MIF in teratoma progression through its ability to promote macrophage-dependent angiogenesis. Our study validates and extends these findings by demonstrating an important role for MIF in murine melanoma TAM alternative activation. We further demonstrate that, in addition to regulating TAM angiogenic potential, MIF contributes to TAM and MDSC immunosuppressive phenotypes in tumor-bearing mice. An important component of Wang and colleagues’ (61) study was the use of MIF−/−embryonic stem cells to demonstrate the importance of stromal cell–derived MIF to teratoma outgrowth. In contrast, our efforts to use lentiviral short hairpin RNA to deplete melanoma MIF in B16 and B16F10 cell lines were unsuccessful (data not shown), but the findings by Wang and colleagues (61) support our conclusion that, at least during initial melanoma outgrowth, stromal macrophage MIF contributes dominantly to macrophage polarization. In further support for this conclusion, a study by Dranoff’s group (62) provides clinically relevant evidence that MIF promotes tumor-promoting TAM angiogenic properties in late-stage malignant melanoma patients. MIF antibodies were identified in chemotherapy-responding melanoma patients, and these neutralizing MIF Abs functionally attenuated Tie-2 and MMP-9 expression in human TAMs, leading to disrupted tumoral vasculature and lymphocyte/granulocyte infiltrates (62).

Simpson et al. (63) recently reported that in an aggressive 4T-1 model of metastatic breast cancer, tumor-derived MIF promotes tumor growth and pulmonary metastasis by influencing the differentiation of inflammatory cells within the tumor microenvironment. Our studies additionally indicate that the MIF-dependent effects on alternative activation observed with PECs and TAMs are not restricted to melanoma tumors. Analysis of PECs and TAMs from LLC-bearing mice reveals that MIF deficiency and 4-IPP inhibition similarly skew TAM from an M2-like expression pattern/phenotypic profile toward an M1-like expression pattern/phenotype (data not shown). Clearly, more studies are needed to fully demonstrate the precise contribution of aberrant macrophage polarization to defective melanoma outgrowth and lung metastases in MIF-deficient mice but, combined with other recent studies (61–63), there is now considerable evidence to suggest that MIF is an important determinant of TAM protumorigenic activities.

In conclusion, these studies describe a functional role for MIF in contributing to the alternative activation of macrophages in tumor-bearing hosts. Perhaps more importantly, our discovery that the loss or inhibition of MIF effectively reprograms TAM polarization coinciding with attenuated melanoma progression and pulmonary metastases suggests that immunotherapeutic targeting of MIF may one day provide significant clinical benefits for melanoma patients.

Acknowledgments

We thank Drs. Jill Sutcliffe, Robert Stout, and Jun Yan for helpful discussion and insight on TAM alternative activation. We also thank Huaiyu Zheng for assistance with mouse tumor imaging and imaging analyses.

Disclosures

R.A.M. is an inventor on patents pertaining to 4-IPP as a novel anticancer therapeutic agent targeting MIF. The other authors have no financial conflicts of interest.

References


53. Bernhagen, J., R. Krohn, H. Lue, J. L. Gregory, A. Zernecke, R. R. Koenen, M. Dewor, I. Georgiev, A. Schob, L. Leng, et al. 2007. MIF is a noncognate ligand of CXCR4 chemokine receptors in inflammatory and athereogenic cell re-


55. Tarnowski, M., K. Grywula, R. Liu, J. Tarnowska, J. Drulaka, J. Ratajczak, R. A. Mitchell, M. Z. Ratajczak, and M. Kucia. 2010. Macrophage migration inhibitor factor is secreted by rhabdomyosarcoma cells, modulates tumor me-


57. Mitchell, R. A. C. Metz, T. Peng, and R. Bucala. 1999. Sustained mitogen- activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitor factor (MIF). Regulatory role in cell pro-

58. Ren, Y., H. M. Chan, Z. Li, Y. Tian, Y. Li, and B. Li. 2010. Macrophage migration inhibitor factor contributes to angiogenesis by up-regulating IL-8 and correlates with poor prognosis of patients with primary nasopharyngeal carci-

