The Journal of Immunology
RESEARCH ARTICLE | JANUARY 15 2001

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J Immunol (2001) 166 (2): 747–753.
https://doi.org/10.4049/jimmunol.166.2.747

Regulation of the CTL Response by Macrophage Migration Inhibitory Factor

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Macrophage migration inhibitory factor (MIF) has been shown to be a pivotal cytokine that mediates host inflammatory and immune responses. Recently, immunoneutralization of MIF has been found to inhibit tumor growth in mice; however, the contributing mechanisms underlying this effect have not been well defined. We investigated whether MIF plays a regulatory role in the expression of CTL activity. In a mouse model of the CTL response using the OVA-transfected tumor cell line EL4 (EG.7), we found that cultures of splenocytes obtained from EG.7-primed mice secrete high levels of MIF following Ag stimulation in vitro. Notably, parallel splenocyte cultures treated with neutralizing anti-MIF mAb showed a significant increase in the CTL response directed against EG.7 cells compared with control mAb-treated cultures. This effect was accompanied by elevated expression of IFN-γ. Histological examination of the EG.7 tumors from anti-MIF-treated animals showed a prominent increase in both $CD4^+$ and $CD8^+$ T cells as well as apoptotic tumor cells, consistent with the observed augmentation of CTL activity in vivo by anti-MIF. This increased CTL activity was associated with enhanced expression of the common γ_c-chain of the IL-2R that mediates $CD8^+$ T cell survival. Finally, $CD8^+$ T lymphocytes obtained from the spleens of anti-MIF-treated EG.7 tumor-bearing mice, when transferred into recipient tumor-bearing mice, showed increased accumulation in the tumor tissue. These data provide the first evidence of an important role for MIF in the regulation and trafficking of anti-tumor T lymphocytes in vivo. ***The Journal of Immunology,*** **2001, 166: 747–753.**

The biological functions of the protein mediator known as macrophage migration inhibitory factor (MIF)[2] have only recently come under close scrutiny (reviewed in Ref. 1). Although MIF was first described nearly 4 decades ago as a soluble activity produced by activated T lymphocytes (2, 3), interest in MIF was rekindled when the mouse homologue of this protein was identified to be secreted from the anterior pituitary gland (4). Soon thereafter macrophages that had been previously considered to be a target of MIF action were found to be a significant source of MIF upon activation by microbial toxins or the cytokines TNF-α and IFN-γ (5). In vivo studies also established that MIF plays a critical role in the host response to endotoxin. Administration of recombinant MIF (rMIF) together with LPS exacerbates LPS lethality, while neutralizing anti-MIF Abs protect mice against lethal endotoxemia (4), exotoxemia (6), and peritonitis (7). Studies of MIF function also have established this protein to be required for the expression of IL-2 during the T cell activation response and for Ab production by B cells (8).

Two recent reports have identified an unanticipated role for MIF in tumor growth (9, 10). We observed that the administration of an anti-MIF mAb to mice significantly reduced the growth and vascularization of the syngeneic, s.c. implanted B cell lymphoma, 38C13 (9). Evidence was obtained that this anti-tumor effect was due in part to a requirement for MIF in endothelial cell proliferation and the tumor angiogenesis response (9). Similarly, anti-MIF mAb treatment of mice bearing the human melanoma tumor, G361, significantly decreased tumor growth and neovascularization (10). The previously described roles for MIF in macrophage and T cell activation responses suggested that MIF might exert significant pro-tumor effects by regulation of anti-tumor T lymphocyte responses. In the present study we examined the activity of MIF to modulate CTL responses. We report that neutralization of MIF can promote CTL activity, inhibit tumor growth, and increase T lymphocyte homing to sites of tumor invasion in vivo.

Materials and Methods

Experimental animals and cell lines

C57BL/6 (H-2^b) mice (female, 8–12 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal procedures were conducted according to guidelines of the North Shore University Hospital Institutional Animal Care and Use Committee under an approved protocol. EG.7 cells (produced by transfection of EL4 with a cDNA encoding OVA (11)), EL4 cells (MHC class II-negative, H-2^b murine thymoma), and YAC-1 cells were obtained from American Type Culture Collection (Manassas, VA).

Cytokines and Abs

Recombinant murine MIF (rMIF) was prepared as previously described (12, 13) (<1 pg endotoxin/μg protein). Neutralizing anti-MIF mAb (clone XIV.15.5, IgG1 isotype) was prepared as previously described (9, 14). An isotype control Ab (IgG1) was purified under similar conditions using the hybridoma, 5D4-11, which secretes Ab specific for type 3 dengue virus (American Type Culture Collection). FITC-rat anti-mouse CD3 Ab, PE-rat anti-mouse CD4, PerCP-rat anti-mouse CD8 Ab, PE-rat anti-mouse CD25 Ab, PE- rat anti-mouse CD28 Ab, FITC-rat anti-mouse CD44 Ab, PE-rat anti-mouse CD25 (IL-2Rα), PE-rat anti-CD122 (IL-2Rβ), PE- rat anti-mouse CD132 (shared γ-chain), and PE-rat anti-mouse H-$2K^b$ were purchased from PharMingen (San Diego, CA).

Generation of Ag-specific CTL

The generation of OVA-specific CTL has been described previously (11). In brief, spleen cells were obtained from mice primed 1–2 wk earlier by i.p. injection of 5×10^6 EG.7 cells. Isolated spleen cells (3×10^6) were incubated with irradiated EG.7 cells (20,000 rad; 10^6 cells) for 5 days (in the presence or the absence of cytokines or Abs; see below). Effector cells

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Received for publication August 2, 2000. Accepted for publication October 11, 2000.

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[2] Abbreviations used in this paper: MIF, macrophage migration inhibitory factor; γ_c, shared common γ-chain; TIL, tumor-infiltrating lymphocyte; FasL, Fas ligand.

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used in the in vitro CTL assay (see below) were collected from these cultures and recognized the $OVA_{257–264}$ (SIINFKEL) peptide in the context of H-$2K^b$ (15). To study the effect of MIF neutralization in vivo, EG.7-primed mice received an injection of anti-MIF mAb or control IgG (0.5 mg i.p.) on the day of tumor cell implantation and then daily for 1 wk. Spleen cells from anti-MIF- or control IgG-treated mice then were isolated and assessed for CTL activity in vitro as described below.

Cell-mediated cytotoxicity assay

EG.7 target cells (5×10^5/well) were added to serial dilutions of effector spleen cells (prepared as described above) in 96-well round-bottom plates at E:T cell ratios of 1:1 to 30:1 together with various concentrations of anti-MIF mAb, control IgG, or purified rMIF. After 4 h at 37°C, cytotoxicity was quantified by measurement of the cytosolic enzyme, lactate dehydrogenase (LDH) in the culture supernatant ($n = 3$) using the CytoTox 96 Assay (Promega, Madison, WI). Specific lysis for each E:T cell ratio is expressed as: specific lysis = [(experimental release) − (spontaneous release)/(target maximum − target spontaneous release)]. Spontaneous LDH release in the absence of CTL was <10% of the maximal cellular release by detergent lysis. All experimental procedures and assays were performed two or more times, with similar results.

NK assay

NK-sensitive YAC-1 cells were used as targets, and NK assays were performed as previously described (16).

Flow cytometric analysis

Single-cell suspensions free of erythrocytes were prepared from the spleens of experimental mice as indicated and analyzed by flow cytometry. All fluorescently labeled Abs were purchased from PharMingen and used according to the manufacturer's recommendation. Cells (10^6/aliquot) were resuspended in PBS containing 3% BSA and 0.1% sodium azide (FACS buffer) and were incubated with fluorescently labeled Abs for 30 min (4°C) followed by two washes in FACS buffer. Fluorescence data were acquired on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and were analyzed using CellQuest software (Becton Dickinson). This experiment was repeated once with similar results.

Analysis of cytokine production

Cytokine production was measured by analysis of culture supernatants by sandwich ELISA using murine IFN-γ, TNF-α, IL-2, and IL-12 kits purchased from R&D Systems (Minneapolis, MN). The ELISA for murine MIF was performed as previously described (14). Inclusion of neutralizing anti-MIF mAb in the culture complexes with biologically active MIF and renders the MIF inactive but still detectable by later ELISA.

Tumor growth in vivo

Experiments to determine the effect of anti-MIF mAb on EG.7 tumor growth were performed in C57BL/6 mice following methods described previously (9). Cultured EG.7 cells were washed and resuspended in PBS, and 5×10^6 cells (suspended in 0.1 ml of PBS) were injected s.c. into the upper flank of mice ($n = 5$/group). Mice received an i.p. injection of 0.3 ml of PBS, IgG1 isotype control Ab (0.5 mg), or purified anti-MIF mAb (0.5 mg) 1 h later and then every 24 h for 7 days. Tumor size was estimated on day 7 from orthogonal linear measurements made with Vernier calipers according to the formula: weight (mg) = [(width, mm)^2 × (length, mm)]/2 (17). This experiment was repeated twice with similar results.

Histologic studies

Tumors from control IgG- and anti-MIF-treated mice were excised at 7 days. Frozen tumor sections were stained using PE-CD4 (L3T4) and FITC-CD8 (Ly-2) mAbs (PharMingen). The $CD8^+$ and $CD4^+$ T cells were counted under a fluorescence microscope and expressed as the percent increase in the mean number of stained cells per tumor section compared with sections from the control IgG-treated mice. Ten fields per section were counted using a ×10 objective ($n = 5$ mice/group). Control sections incubated with a fluorescent-conjugated isotype control Ab showed no immunoreactivity.

In situ apoptosis detection

Cells undergoing apoptosis were detected using TUNEL according to the manufacturer's recommended procedure (R&D Systems). For statistical analysis, apoptotic cells were counted by light microscopy (×100) and expressed as the mean number (±SD) of apoptotic cells per tumor section. Five random fields per section (one section per mouse, five mice per group) were analyzed, and Student's t test was used to determine significance ($p < 0.05$).

In vivo lymphoid cell migration assay

Nontumor-bearing mice or mice bearing EG.7 tumors of similar size (~7 days after tumor cell injection), as described previously by Zou et al. (18), treated with daily injections of anti-MIF (0.5 mg/mouse i.p.) or control IgG, were used as the source of cells for this assay. Unfractionated spleen cells or purified splenic $CD8^+$ T cells (1×10^6 cells/ml) were obtained and labeled with PKH-26, a membrane-inserting red fluorescent dye (Sigma, St. Louis, MO). In vivo lymphoid migration assays were performed as previously described ($n = 5$ mice/group) (19). Briefly, labeled cells were injected i.v. into tumor-bearing recipient mice. Tumor masses were removed 24 h later, and cryostat sections were prepared. Sections were stained with FITC-anti-CD4 or FITC-anti-CD8 to determine T cell type. The presence of PKH-26 fluorescent donor cells was quantified by microscopy and expressed as the mean number of labeled donor cells per field of sectioned tumor tissue. For each section (one per mouse), 10 fields were enumerated using a ×10 objective. These experiments were repeated twice with similar results.

Adoptive immunotherapy

C57BL/6 mice were injected with 5×10^6 EG.7 cells s.c. and then treated with anti-MIF mAb or control IgG (0.5 mg/day i.p.) daily for 7 days ($n = 5$/group). One day after the last injection, spleen cells were isolated, and $CD8^+$ splenic T cells were purified using $CD8^+$ enrichment columns (R&D Systems). Unfractionated splenocytes or $CD8^+$ T cells (5×10^6 cells/mouse) were then transferred i.v. into recipient mice that had been injected with 5×10^6 EG.7 cells i.p. 1 day earlier. Tumor weights were determined on days 1–13 as described above. This experiment was repeated once with similar results.

Results

Anti-MIF enhances CTL activity in vitro

Previous studies established that MIF protein and mRNA is expressed as part of the macrophage and T lymphocyte activation response (5, 8, 20). To evaluate the potential role for MIF in the host response to tumor invasion, we first examined whether rMIF or a neutralizing anti-MIF mAb influenced Ag-specific, cytotoxic T cell responses in vitro. We isolated splenocytes from mice primed by the implantation of EG.7 cells and stimulated these spleen cell cultures for 5 days with irradiated EG.7 cells in the presence of rMIF, neutralizing anti-MIF mAb, or isotype control IgG1. As shown in Fig. 1*B*, the addition of anti-MIF mAb at 50 μg/ml significantly up-regulated the in vitro CTL response, whereas the addition of exogenous rMIF (Fig. 1*A*) or control IgG (Fig. 1*C*) did not affect CTL activity. Control studies showed that anti-MIF mAb treatment of splenocytes or EG.7 cells alone did not influence their survival or growth characteristics, and that in vitro pretreatment with anti-MIF mAb did not independently cause the development of cytotoxicity in unconditioned splenocyte cultures (data not shown).

We also studied a potential role for MIF in the effector phase of the CTL response by adding anti-MIF mAb or rMIF during the final 4-h assay period of splenocyte culture with EG.7 target cells. There was no effect of these agents on the in vitro cytotoxic activity during this assay period (data not shown). By contrast, we observed that anti-MIF mAb was most active in augmenting the CTL response in vitro when present within the first 2 days of the 5-day coculture period (Fig. 1*D*).

These data indicate that the immunoneutralization of MIF during the early phase of cytotoxic T cell activation in vitro potentiates later CTL activity. Not unexpectedly, therefore, we found that in vitro stimulation of splenocyte effector cells with irradiated EG.7 target cells produced a significant increase in the amount of MIF detectable in culture supernatant compared with splenocytes

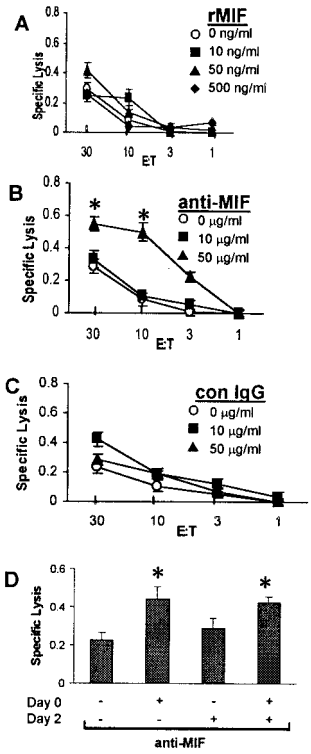

FIGURE 1. Anti-MIF mAb, but not rMIF or control IgG, enhance CTL activity in vitro. C57BL/6 mice primed with EG.7 cells 7 days earlier were the source of spleen cells (see *Materials and Methods*). Spleen cell cultures stimulated with irradiated EG.7 cells for 5 days in the presence of rMIF (*A*), anti-MIF (*B*), or control IgG1 (*C*). Fresh EG.7 target cells were added to spleen cells at various E:T cell cell ratios, and after a 4-h incubation at 37°C cytotoxicity measured by lactate dehydrogenase (LDH) release. *D*, The effect of anti-MIF mAb on in vitro CTL activity upon Ab addition at the onset of splenocyte-irradiated EG.7 cocultures (E:T cell ratio, 20:1; day 0) vs addition on day 2. $*$, $p < 0.05$ vs no addition, by Student's *t* test.

obtained from tumor-bearing mice cultured in the absence of irradiated EG.7 cells (Fig. 2*A*). Nevertheless, we observed no significant effect on CTL activity following the addition of bioactive rMIF to parallel splenocyte cultures, suggesting that there may already exist a maximum cellular response to MIF that is endogenously produced in these cultures (>30 ng/ml; Fig. 1*A*).

We next examined whether the immunoneutralization of MIF affected the production of cytokines known to play an important role in the expression of T cell cytotoxicity in vitro. We measured levels of IFN-γ, TNF-α, IL-2, and IL-12 present in the culture supernatants by specific ELISA. We found that among these, only IFN-γ showed a significant increase in concentration during the 2-day coculture period when anti-MIF mAb is most active in enhancing CTL activity compared with the control mAb-treated cultures (Fig. 2*B*). By contrast, incubation of splenocyte cultures from EG.7 tumor-bearing mice in the presence of anti-MIF mAb and irradiated EG.7 cells did not significantly alter IL-2, IL-12, or TNF-α protein expression compared with control IgG-treated cultures (data not shown). EG.7 cells cultured alone revealed no detectable levels of MIF, IFN-γ, IL-2, TNF-α, or IL-12 (data not shown). By flow cytometric analysis, neither rMIF nor anti-MIF treatment of cocultures influenced the percentage of cells displaying the cell surface markers $CD3^+$, $CD4^+$, $CD8^+$, $CD28^+$, and $CD44^{high}$ (data not shown).

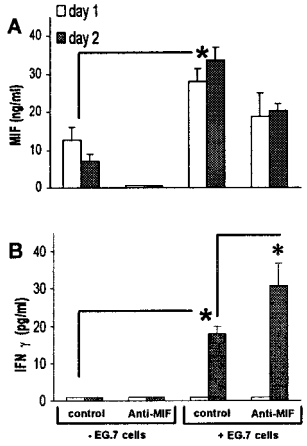

FIGURE 2. Secretion of MIF and IFN-γ is enhanced when primed spleen cells are cultured with irradiated EG.7 cells. Spleen cells were isolated from EG.7-primed mice and stimulated for 1 or 2 days with or without irradiated EG.7 cells together with an isotype control Ab (control) or anti-MIF mAb (50 μg/ml). Culture supernatants were analyzed by specific ELISA for MIF (*A*) and IFN-γ (*B*) as described in *Materials and Methods*. TNF-α and IL-12 values were below the limit of detection. $*$, $p < 0.05$, control plus EG.7 vs control without EG.7, by Student's *t* test.

Anti-MIF mAb treatment in vivo enhances CTL activity

We next compared the CTL response of splenocytes harvested from mice treated with anti-MIF mAb vs an isotype control IgG1 during the period of EG.7 tumor priming in vivo. These experiments showed that the administration of anti-MIF mAb daily for 1 wk after priming with EG.7 cells (on day 0) significantly enhanced the generation of CTL activity at E:T cell ratios of 30 and 10 (Fig. 3*A*). Inclusion of control IgG did not lead to enhanced CTL activity in this experimental system compared with either PBS alone or no addition.

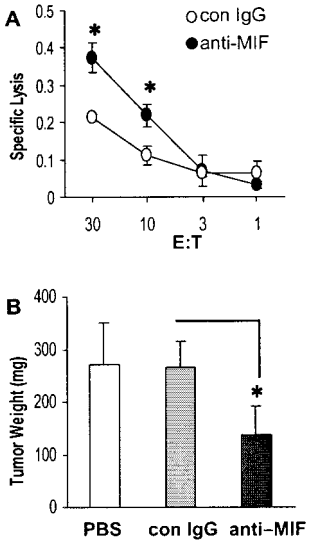

FIGURE 3. Anti-MIF treatment of EG.7 tumor-bearing mice increases CTL activity and inhibits tumor growth. C57BL/6 mice ($n = 5$/group) were injected with EG.7 cells and then treated with PBS, control IgG, or anti-MIF mAb (0.5 mg) daily. On day 7 the spleens were harvested, and isolated spleen cells were cocultured with irradiated EG.7 cells for 5 days, at which time cell lysis was measured in a 4-h CTL in vitro assay by LDH release (*A*). Tumor size was determined on day 7 (*B*). $*$, $p < 0.05$, anti-MIF treated vs control IgG treated, by Student's *t* test.

Recent studies have established a significant anti-tumor effect of anti-MIF mAb in mice bearing the 38C13 B cell lymphoma (9) and the G361 melanoma (10). In accordance with these data and the observed 2-fold enhancement of CTL activity by anti-MIF described above, we found that administration of anti-MIF mAb to mice bearing an EG.7 lymphoma tumor for 1 wk also resulted in a significant 2-fold reduction in tumor size compared with control IgG- or PBS-treated mice (Fig. 3*B*). In addition, we detected ~3-fold more tumor-infiltrating $CD8^+$ and $CD4^+$ cells following anti-MIF mAb treatment (Fig. 4).

CTLs kill tumor cell targets by inducing apoptosis (21). Consistent with the observed enhancement of host CTL activity by anti-MIF treatment, we found a significant increase (4- to 5-fold) in the number of apoptotic cells within the tumor masses obtained from the anti-MIF-treated mice (Fig. 5*A*) compared with tumors obtained from control IgG-treated mice (Fig. 5*B*). This difference in apoptosis was quantified by analyzing the average number of apoptotic cells per high power field in tumor sections from anti-MIF-treated mice (194 ± 63 cells/×100 field) vs the number from control IgG-treated mice (43 ± 22 cells/×100 field), and it was statistically significant ($p < 0.01$).

It was previously reported that rMIF inhibits NK cell activity in vitro (22). Accordingly, the inhibitory effect of anti-MIF mAb on tumor growth in vivo might be the result of enhanced NK cell activity. While we observed an increase in the NK activity of whole spleen cell preparations from EG.7-bearing mice compared with control, nontumor-bearing mice, we did not observe any changes in this activity in mice treated with anti-MIF Abs (data not shown).

Prior studies showed that MIF expression during Ag-driven $CD4^+$ T cell activation in vivo plays an important role in the immune response (8). We therefore examined whether the enhanced cytolytic activity observed with anti-MIF mAb treatment was associated with increased Ag-induced proliferation of $CD8^+$ T cells. In accordance with Bacher et al. (8), we found no augmentation in T cell proliferation in the presence of anti-MIF mAb treatment in vivo (data not shown). We also examined the effect of anti-MIF on IL-2R expression. The IL-2R is multimeric, consisting of the variably expressed α-chain (CD25) that regulates IL-2 affinity as well as two signaling subunits, the β (CD122) and the γ_c (CD132) chains (reviewed in Ref. 23). The γ_c subunit (also known as the common γ-chain) is a shared subunit of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors. Recruitment of the γ_c is required for intracellular signaling (24, 25), and its expression has been shown to be critical for mature $CD8^+$ T cell survival in vivo (26). Therefore, we examined the effect of anti-MIF treatment on γ_c expression. We observed that anti-MIF mAb treatment of tumor-bearing mice significantly enhanced expression of the γ_c-chain, but not that of the α or β subunits of the IL-2R on $CD8^+$ T cells (Fig. 6), compared with tumor-bearing animals treated with control IgG.

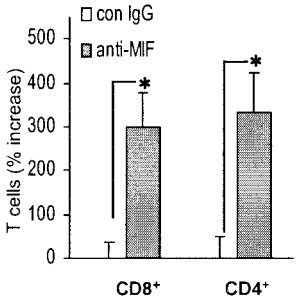

FIGURE 4. Anti-MIF treatment of EG.7 tumor-bearing mice increases T lymphocyte infiltration of tumors. Mice ($n = 5$/group) were treated daily for 7 days with control IgG or anti-MIF mAb. Then, EG.7 tumors were excised, and tumor sections were stained with PE-anti-mouse CD4 (L3T4) or FITC-anti-mouse CD8 (Ly-2) mAbs. $CD8^+$ and $CD4^+$ T cells were enumerated by fluorescence microscopy and expressed as the average percent increase (±SD) in immunoreactive infiltrating cells in the tumors of anti-MIF-treated animals compared with control IgG-treated animals. Sections incubated with a fluorescent-isotype control Ab showed no immunoreactivity. *, $p < 0.05$, anti-MIF vs control IgG treated, by Student's *t* test.

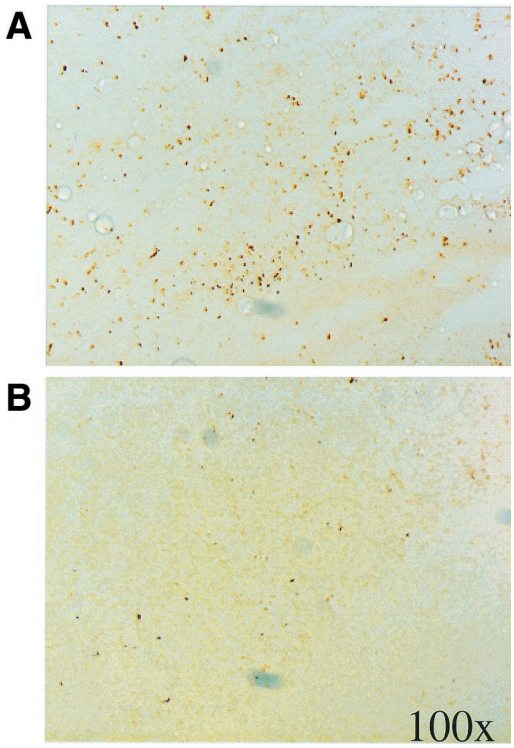

FIGURE 5. Anti-MIF treatment promotes EG.7 tumor cell apoptosis. Apoptotic cells were detected in situ by labeling DNA strand breaks by the TUNEL method. Numerous apoptotic (dark brown) EG.7 cells are visible in the tumor tissue obtained from mice treated with anti-MIF mAb (*A*). By contrast, fewer apoptotic bodies are observed in tumors obtained from mice treated with control IgG (*B*). Sections (×100) shown are representative of 10 tumor sections ($n = 5$ animals/group).

Anti-MIF promotes the migration of T lymphocytes into tumor tissue and augments $CD8^+$ T cell specific anti-tumor activity

To further test that the in vivo anti-tumor effect of anti-MIF mAb was attributable to specific effects on T cells, we next assessed the effects of anti-MIF treatment on trafficking of T lymphocytes into tumors. Control or EG.7 tumor-bearing mice were treated with either anti-MIF or control IgG for 7 days, and unfractionated spleen cells or purified splenic $CD8^+$ T cells were collected for labeling with PKH-26. Labeled unfractionated splenocytes or purified $CD8^+$ cells were transferred into EG.7 tumor-bearing recipients. The entry of PKH-26-labeled donor cells into tumors of recipient mice over 24 h was quantified by fluorescent microscopy of cryostat sections obtained from excised tumor tissue (Figs. 7, *A* and *B*, respectively). These experiments showed that spleen cells or purified $CD8^+$ T cells obtained from the anti-MIF mAb-treated, tumor-bearing mice entered tumor tissue in greater numbers (≥2-fold increase) than comparable cells obtained from the control mAb-treated, tumor-bearing mice.

Finally, we examined the effect of adoptively transferred $CD8^+$ cells (obtained from anti-MIF mAb-treated animals) on tumor growth in vivo. Five million unfractionated splenocytes or purified

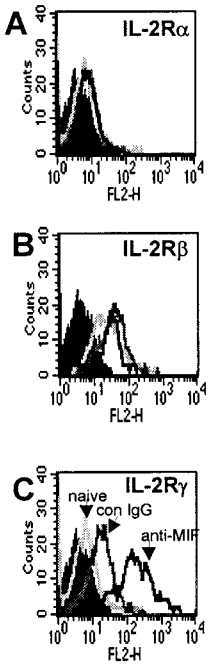

FIGURE 6. IL-2Rγ_c expression is up-regulated by treatment with anti-MIF in vivo. Spleen cells were collected from naive or EG.7 tumor-bearing mice ($n = 3$ mice/group) treated daily for 7 days with anti-MIF or control IgG. Spleens were pooled from individual groups and stained for CD8 and IL-2Rα (*A*), β (*B*), or γ_c (*C*) surface markers after gating on the $CD8^+$ T cell population. The shaded histogram represents the cells stained with isotype control Ab.

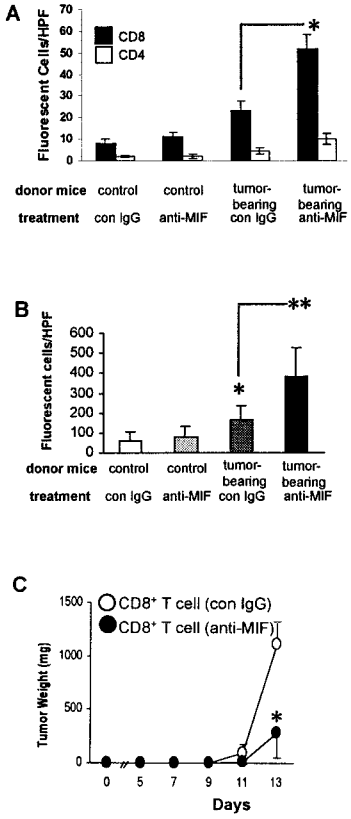

FIGURE 7. Treatment of donor tumor-bearing mice with anti-MIF increases the migration of transferred T lymphocytes into EG.7 tumors of recipient tumor-bearing mice and promotes $CD8^+$ T cell anti-tumor activity in recipient mice. Unfractionated spleen cells (*A*) or purified $CD8^+$ splenic T cells (*B*) from normal (control) or tumor-bearing mice were isolated 8 days after anti-MIF or control IgG treatment (0.5 mg for 7 days) and labeled with the fluorescent dye, PKH-26. Labeled cells then were transferred i.v. into tumor-bearing recipient mice ($n = 5$/group). One day later, the tumors were excised, cryostat sections were prepared, and the number of fluorescent cells per high power field (HPF) was enumerated (mean ± SD). *, $p < 0.05$; **, $p < 0.01$ (vs control Ab-treated mice, by Student's *t* test). *C*, C57BL/6 mice were injected with 5×10^6 EG.7 cells (i.p.) and then treated with anti-MIF mAb or control IgG (0.5 mg/day) for 7 days. Purified splenic $CD8^+$ T cells were transferred (5×10^6 cells/mouse i.v.). into recipient mice that had been inoculated s.c. with 5×10^6 EG.7 cells 24 h previously ($n = 5$/group). Tumor weights (average ± SD) were measured. *, $p < 0.05$ vs control Ab-treated mice, by Student's *t* test.

$CD8^+$ splenic T cells (Fig. 7*C*) from anti-MIF mAb or control IgG-treated, EG.7 tumor-bearing donor mice were transferred to mice that had been injected s.c. with EG.7 tumor cells 24 h previously. Tumor growth in vivo then was monitored for 2 wk. As shown in Fig. 7*C*, adoptive transfer of $CD8^+$ T cells obtained from anti-MIF-treated, tumor-bearing mice to untreated tumor-bearing mice showed a significant inhibitory effect on subsequent tumor outgrowth in recipient mice. In contrast, no significant difference in tumor weight was observed following the transfer of unfractionated splenocytes (5×10^6 cells; containing both $CD4^+$ and $CD8^+$ T cells and B cells) obtained from anti-MIF-treated vs control IgG tumor-bearing mice (data not shown). These data suggest the importance of a critical number of $CD8^+$ T cells obtained from anti-MIF-treated tumor-bearing animals to mediate a significant inhibition of tumor growth in adoptive transfer experiments.

Discussion

Emerging data from both experimental and human clinical studies indicate that tumor-associated Ags are sufficient to elicit an anti-tumor CTL response that can produce significant tumor regression (27, 28). Long term melanoma remissions have been achieved in a few cases by employing cell-based immunotherapeutic strategies aimed at enhancing CTL cytotoxicity by peptide immunization (29). However, despite the presence of tumor-specific Ags presented in the context of MHC class I, a robust tumor killing immune response is seldom detected in vivo. The generation of tumor-specific CTLs requires appropriate processing of tumor Ags, display of tumor Ags by MHC class I molecules, T lymphocytes expressing TCRs of appropriate specificity to recognize tumor Ags, and initial Ag presentation to the immune system in an immunologic context. This CTL response must not only be initiated, but must also be vigorous and sustained so as to achieve successful tumor regression.

The activity of several cytokines to enhance various aspects of the CTL response has been appreciated for some time. The early expression of IL-2, for example, is a critical factor in the proliferation and development of lytic potential by CTLs (30). Furthermore, IFN-γ (30), IL-1 and IL-6 (31), IL-2 together with IL-6 (32), IL-7 (33), IL-10 (34), and IL-12 (35–37) have all been identified to play a role in the activation, proliferation, and/or differentiation of CTLs. These mediators promote CTL activity by enhancing Ag presentation, $CD4^+$ Th cell function, and macrophage cell adhesion or by increasing the expression of critical costimulatory molecules. Anti-tumor effects mediated by the administration of recombinant cytokines, including IL-1 (38), IL-2 (39), IL-12 (40–42), IFN-α (43, 44), IFN-γ (45), and TNF-α (46), have been shown in tumor bearing-mice.

By contrast, only a few cytokines, including IL-4 (47, 48), and TGF-β (49), have been shown to suppress CTL differentiation or lytic activity. IL-4 inhibits the secretion of IFN-γ from $CD8^+$ T cells (50, 51) and appears to limit the activation and differentiation of $CD8^+$ T cells with high cytolytic potential (52). Furthermore,

CTL priming in the absence of IL-4 gives rise to a more potent response following challenge. The mechanisms by which these few cytokines inhibit CTL cytolytic activity are not well defined. In this study we have shown that MIF expression is up-regulated during the CTL response, and inhibition of MIF using specific mAbs promotes CTL activity in vitro and in vivo. Results from in vitro CTL studies revealed that immunoneutralization of MIF during the in vitro priming phase increased IFN-γ production in CTL cultures. Recognizing that MIF secretion is enhanced by the activation of Th2 cells, but not Th1 cells (8), it is possible that Ag stimulation induces MIF expression, which, in turn, inhibits CTL activation in vivo by suppressing the production of Th1 cytokines, including IFN-γ.

Previous studies have shown that MIF plays an essential role in the activation response to various mitogens or soluble Ag, an effect that is mediated by $CD4^+$ Th cells. Mitogen- or Ag-activated T cells express significant quantities of MIF mRNA and protein, and immunoneutralization of MIF inhibits IL-2 production and T cell proliferation in vitro and decreases the Th cell response to soluble Ag in vivo (8). In the present study we have shown that MIF expression is up-regulated in response to tumor Ag stimulation and that neutralization of MIF does not affect IL-2 secretion or Ag-induced proliferation of $CD8^+$ T cells. However, anti-MIF treatment significantly increased the expression of the IL-2R γ_c subunit that is required for intracellular signaling (25) and is important for $CD8^+$ T cell survival (26). Thus, the enhancement of T cell cytotoxicity by MIF neutralization cannot be attributed to an appreciable increase in the proliferation of $CD8^+$ T cells, but, rather, may be due to enhanced survival of a population of cytolytic $CD8^+$ T cells. Following the initiation of cytolytic activity by $CD8^+$ T cells, this cytolytic activity must be sustained to promote successful tumor regression. Accordingly, inhibition of MIF may act to prolong the CTL life span such that significant CTL antitumor activity becomes manifest both in vitro and in vivo.

Anti-MIF mAb treatment of EG.7 tumor-bearing mice significantly inhibited tumor growth in the context of enhanced CTL activity. Moreover, $CD8^+$ T cells transferred from anti-MIF treated tumor-bearing mice inhibited tumor growth in recipient mice. Given the observed increase in the number of apoptotic tumor cells found within the corpus of the tumor, we conclude that enhanced or sustained CTL cytotoxicity directly contributed to the suppression of tumor growth in anti-MIF-treated mice.

Recent reports have shown that tumor cells produce more MIF than nontransformed cells (10, 53, 54). Tumor cells can escape death by CTLs via the loss of the tumor Ag recognized by the CTLs or by the down-regulation of MHC expression that renders the tumor cell resistant to CTL-mediated lysis even when it expresses the appropriate tumor Ag (55). Although EG.7 cells constitutively secrete MIF ($\sim$10 ng/ml by 10^6 cells), neither rMIF nor anti-MIF influenced MHC class I expression by EG.7 cells (data not shown). Our data suggest that an additional mechanism for tumor evasion of the host immune response may occur by tumor cell secretion of MIF leading to a decrease in $CD8^+$ T cell survival.

Several studies have shown the expression of Fas ligand (FasL) by some tumor cells, and this raises the intriguing possibility that cancers might be sites of immune privilege. For example, apoptosis of tumor-infiltrating lymphocytes has been demonstrated in situ in FasL-expressing melanomas (56) and hepatocellular carcinomas (57). However, more recent in vitro and in vivo data have challenged the original hypothesis. These studies have revealed that some tumors lack FasL expression (58, 59) and that transfection of some tumor cells with FasL cDNA did not promote evasion of the immune system by tumor cells, but, rather, induced tumor regression (59, 60). Further studies have shown that FasL expression promotes rapid graft rejection (61, 62) and inflammation (63). In this study we did not examine the expression of FasL within the tumor. However, because anti-MIF inhibits the progression of several experimental models of inflammatory disease (reviewed in Ref. 1), suppresses tumor growth (9, 10), and prevents graft rejection (P. Heeger, unpublished observations), it would be interesting to examine the effect of MIF/anti-MIF on FasL expression in these systems.

We have also identified an important role for MIF in T cell trafficking. An increase in the accumulation of both $CD4^+$ and $CD8^+$ T cells within the tumors of anti-MIF-treated mice was observed. Tumor destruction by tumor-infiltrating lymphocytes (TILs) is known to involve both $CD4^+$ and $CD8^+$ T cells. Treatment of breast tumors in rats with IL-2 and TILs promotes tumor regression by the induction of apoptosis in the tumor cells (64), and a brisk accumulation of TILs in human melanoma is associated with a more favorable outcome for the patient (65). The observation that anti-MIF increases the migration of $CD4^+$ and $CD8^+$ T cells into the tumor mass provides an additional means by which anti-MIF may affect anti-tumor T cell function and may involve mechanisms such as altered chemokine or chemokine receptor expression.

In addition to modulating CTL activity, MIF appears to play a role in other aspects of tumor formation. Two independent laboratories have shown that MIF neutralization significantly inhibits tumor angiogenesis (9, 10), and Hudson and coworkers recently revealed that the addition of rMIF to fibroblasts inhibits p53 functions (both proliferation and apoptosis) by suppressing its transcriptional activity (66). Although a variety of host immune effector cells participate in the killing of tumor cells, tumor Ag-specific CTLs are highly effective in mediating tumor cell killing even at low Ag density expressed on the target cells (67). Accordingly, the therapeutic enhancement of $CD8^+$ CTLs by MIF immunoneutralization may provide an attractive basis for cell-based anti-tumor immunotherapies.

Acknowledgments

We thank K. Manogue for critically reading this manuscript.

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