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Role of Macrophage Migration Inhibitory Factor in the Regulatory T Cell Response of Tumor-Bearing Mice

Susanna Choi,* Hang-Rae Kim,† Lin Leng,‡ Insoo Kang,‡ William L. Jorgensen,§ Chul-Soo Cho,*,¶ Richard Bucala,‡ and Wan-Uk Kim*,¶

Macrophage migration inhibitory factor (MIF) is involved in tumorigenesis by facilitating tumor proliferation and evasion of apoptosis; however, its role in tumor immunity is unclear. In this study, we investigated the effect of MIF on the progression of the syngenic, CT26 colon carcinoma and the generation of tumor regulatory T cells (Tregs). The results showed that the tumor growth rate was significantly lower in MIF knockout ($MIF^{-/-}$) mice than in wild-type ($MIF^{+/+}$) mice. Flow cytometric analysis of both spleen and tumor cells revealed that $MIF^{-/-}$ mice had significantly lower levels of tumor-associated $CD4^+$Tregs than $MIF^{+/+}$ mice. The splenic cells of $MIF^{-/-}$ mice also showed a decrease in $CD8^+$Tregs, which was accompanied by an increase in CD8-induced tumor cytotoxicity. Interestingly, the inducible Treg response in spleen cells to anti-CD3/CD28 plus IL-2 plus TGF-β was greater in $MIF^{-/-}$ mice than in $MIF^{+/+}$ mice. Spleen cells of $MIF^{-/-}$ mice, stimulated with anti-CD3/CD28, produced lower levels of IL-2, but not TGF-β, than those of $MIF^{+/+}$ mice, which was recovered by the addition of recombinant MIF. Conversely, a neutralizing anti-MIF Ab blocked anti-CD3–induced IL-2 production by splenocytes of $MIF^{+/+}$ mice and suppressed the inducible Treg generation. Moreover, the administration of IL-2 into tumor-bearing $MIF^{-/-}$ mice restored the generation of Tregs and tumor growth. Taken together, our data suggest that MIF promotes tumor growth by increasing Treg generation through the modulation of IL-2 production. Thus, anti-MIF treatment might be useful in enhancing the adaptive immune response to colon cancers. ***The Journal of Immunology*****, 2012, 189: 3905–3913.**

Within the immune system, macrophage migration inhibitory factor (MIF) is considered a broad-spectrum proinflammatory cytokine. MIF was originally identified as a T cell-derived lymphokine (1). T cells activated by specific Ag, mitogens, or anti-CD3 Ab show increased expression of MIF mRNA and protein (2). Anti-MIF Abs inhibit T cell proliferation and Ab production from B cells (2). MIF also is known to be involved in angiogenesis, tumor growth, and metastasis (3). For example, administration of anti-MIF mAb into mice bearing the human melanoma tumor, G361, significantly decreases tumor growth and neovascularization (4). In EG7 tumor-bearing mice, treatment with anti-MIF Abs results in an accumulation of $CD4^+$ and $CD8^+$ T cells at the tumor sites (5). However, in tumor conditions, it is still unclear whether and how host-derived MIF affects T cell immunity, independent of the angiogenic and proliferative effect of MIF on tumor cells.

All solid tumors are embedded in a stromal microenvironment consisting of immune cells, such as macrophage and lymphocytes, as well as nonimmune cells. In particular, tumor-derived $CD4^+$ regulatory T cells ($CD4^+$Tregs) have been extensively studied in many different types of cancer (6, 7). Ag-specific $CD4^+$Tregs at tumor sites significantly suppress immune responses, leading to immune tolerance to tumor cells (8). Inducible $CD4^+$Tregs suppress immune responses through a cell contact or soluble factor-dependent mechanism once they are activated by exposure to a specific Ag (9). Although the origin of $CD4^+$Tregs remains largely unknown, they may be converted from $CD4^+CD25^-$ naive T cells and Ag-experienced effector cells in the suppressive cytokine milieu of tumor sites or they may arise by proliferation and expansion of naturally occurring $CD4^+CD25^+$ T cells after Ag stimulation (10). $CD4^+$Tregs in turn may promote local tumor growth and also contribute to the systemic progression of tumors into the peripheral blood or lymphoid organs (11). Thus, an increased Treg response may be a major obstacle for immunotherapy of cancer. It has become clear that a better understanding of the mechanisms and actions of Tregs in tumor immunity is critical in developing an effective tumor vaccine or immunotherapy (12). However, the function of MIF in the biology of Tregs is unknown.

In this study, we have investigated the role of MIF in tumor progression and the generation of tumor-associated Tregs. We demonstrated first that tumor growth rate was significantly lower in $MIF^{-/-}$ mice than in $MIF^{+/+}$ mice when syngeneic colon cancer (CT26) cells were injected. Flow cytometric analysis of both spleen and tumor cells revealed that $MIF^{-/-}$ mice had significant lower levels of tumor-associated Tregs than $MIF^{+/+}$ mice. The splenic cells of $MIF^{-/-}$ mice also showed a decrease in $CD8^+$ Tregs, which was accompanied by an increase in CD8-induced tumor cytotoxicity. In contrast, when splenic T cells were induced

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Abbreviations used in this article: GITR, glucocorticoid-induced TNF-related protein; LDH, lactate dehydrogenase; MIF, macrophage migration inhibitory factor; Treg, regulatory T cell.

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by anti-CD3/CD28 plus IL-2 plus TGF-β, the number of Tregs was significantly higher in $MIF^{-/-}$ than in $MIF^{+/+}$ mice, suggesting that these stimuli are associated with a reduction in Treg development in $MIF^{-/-}$ mice. As expected, $MIF^{-/-}$ mice showed significantly lower levels of IL-2 production by spleen cells stimulated with anti-CD3/CD28 than $MIF^{+/+}$ mice. Moreover, the administration of IL-2 into $MIF^{-/-}$ mice restored both the generation of Tregs and colon carcinoma growth. We conclude that MIF promotes tumor progression in mice by increasing intratumor Tregs through the regulation of IL-2 production.

Materials and Methods

Mice

Mice genetically deficient in *mif* ($MIF^{-/-}$ mice) were backcrossed onto the BALB/c background (generation N10) (13). Age- and sex-matched wild-type BALB/c mice ($MIF^{+/+}$) were used as a control. All mice were 8–12 wk of age, unless specified otherwise. The mice were maintained in specific pathogen-free conditions and were used according to guidelines of the Institutional Animal Care Committee.

Induction and determination of tumor growth in mice

To determine the effect of MIF on tumor growth, CT26 tumor cells (an undifferentiated colon cancer cell line) were injected into syngeneic $MIF^{-/-}$ and $MIF^{+/+}$ mice, as described previously (14). Briefly, CT26 cells were cultured in DMEM (Welgene, Daegu, South Korea) supplemented with 10% FBS (Wisent Bioproducts, St. Bruno, QC, Canada). The cultured cells were resuspended in PBS, and 1×10^6 cells (suspended in 0.1 ml PBS) then were injected s.c. into the upper flank of $MIF^{-/-}$ and $MIF^{+/+}$ mice. Tumor size was estimated every day by orthogonal linear measurements made with Vernier calipers according to the following formula: volume (mm^3) = ([width, mm]^2 × [length, mm])/2 (15).

Flow cytometry analysis

Single-cell suspensions were prepared from the tumor tissues and spleens of $MIF^{-/-}$ and $MIF^{+/+}$ mice after tumor inoculation. The cells of tumor tissues and spleen, obtained from $MIF^{-/-}$ and $MIF^{+/+}$ mice, were resuspended in staining buffer (HBSS; Welgene) containing 2% FBS and stained for 1 h with the following Abs conjugated with FITC, PE, or allophycocyanin: anti-CD4 Ab (eBioscience, San Diego, CA), anti-CD8 Ab (eBioscience), anti-CD122 Ab (eBioscience), anti-CD132 Ab (BD Pharmingen, San Diego, CA), anti-CTL-associated Ag-4 (CTLA4) Ab (eBioscience), anti-glucocorticoid–induced TNF-related protein (GITR) Ab (eBioscience), anti-Foxp3 Ab (eBioscience), anti-CD25 Ab (eBioscience), and anti-CD44 Ab (eBioscience). Isotype Ab (eBioscience) was used as a control. For the staining of Foxp3 and CTLA4, the cells were fixed and treated with permeabilization buffer (eBioscience). The three-color samples were acquired using a FACSCanto (BD Biosciences, San Jose, CA) equipped with Diva software. Data were analyzed with Flowjo (Tree Star, Ashland, OR) software. Representative dot plots for $CD4^+CD25^+Foxp3^+$ T cells are shown in Supplemental Fig. 1A.

In vitro culture of splenic cells

Spleens were isolated from $MIF^{-/-}$ and $MIF^{+/+}$ mice and prepared as single-cell suspensions. The splenic cells then were resuspended in RPMI 1640 (Welgene) supplemented with FBS (Wisent Bioproducts). To induce Tregs, splenic cells were plated at a concentration in 96-well plates, and stimulated with precoated anti-CD3 Ab and anti-CD28 Ab (BD Pharmingen) in the absence or presence of murine IL-2 (1 ng/ml; R&D Systems, Minneapolis, MN) plus TGF-β (3 ng/ml; PeproTech, Rocky Hill, NJ). Cells were cultured for 72 h, harvested, and used for flow cytometry analysis. In some experiments, recombinant MIF or anti-MIF Ab was added to spleen cells stimulated with anti-CD3 Ab plus anti-CD28 Ab to determine the effect of MIF on IL-2 production by splenic T cells. Mouse recombinant MIF was prepared as the native protein from an *Escherichia coli* expression system and purified free of endotoxin by C8 chromatography, as described previously (16). Anti-MIF Ab (NIHIII.D.9) and nonimmune IgG were isolated from mouse ascites by protein A IgG purification kit (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions. The MIF receptor antagonist, 3-(3-hydroxybenzyl)-5-methylbenzooxazol-2-1 (a.k.a. Debio1036), was synthesized, as described previously (17).

ELISA for cytokines

Cytokine concentration was determined in the culture supernatants of splenic cells and in the sera by ELISA. Spleen cells of tumor-bearing $MIF^{-/-}$ and $MIF^{+/+}$ mice were plated in flat-bottom 96-well plates, and then stimulated with plate-bound anti-CD3 (BD Pharmingen) and anti-CD28 Ab (BD Pharmingen) for 48 or 72 h. Levels of IL-2, IFN-γ, IL-10, and TGF-β in the culture supernatants were measured by ELISA, according to manufacturers' instructions (R&D Systems). Serum MIF levels were also determined by ELISA kit (Unique Product Superb Quality Client Favorite Nicest Service Life Science, Wuhan, China).

Immunohistochemical staining of tumor tissues

Immunohistochemical staining for CD3 was performed in tumor tissues to assess the infiltration of $CD3^+$ T cells. Tumor tissue was dissected, fixed in a formalin solution (Sigma-Aldrich, St. Louis, MO), and paraffin embedded. Nonspecific binding was blocked by treating the sections (5 μm) with 10% normal goat serum at 37°C for 1 h. The sections were incubated overnight at 4°C with rabbit anti-CD3 Ab (Abcam, Cambridge, MA) and then for 60 min at room temperature with biotinylated secondary Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Sections then were treated for 30 min at room temperature with 3,3′-diaminobenzidine to reveal the Ag. Counterstaining was performed using hematoxylin.

Detection of MIF mRNA

Total RNA was isolated from CT26 tumor cells and spleen cells of $MIF^{+/+}$ and $MIF^{-/-}$ mouse using the RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Reverse transcription was performed using oligo(dT) as the random primer for reverse transcription and specific MIF primers and GAPDH primers for PCR. Reverse transcription was carried out using MyCycler (Bio-Rad Laboratories, Hemel Hempstead, U.K.). The following primers were used to amplify a 174-bp fragment of MIF, 5′-ACGACATGAACGCTGCCAAC-3′ and 5′-ACCG-TGGTCTCTTATAAACC-3′; GAPDH, 5′-GCAGTGGCAAAGTGGAG-ATT-3′ and 5′-TAGTAGAGGCGGGGAAGACG-3′. The amplification profile for MIF was 28 cycles of denaturation at 94°C (30 s), annealing at 60°C (45 s), and extension at 60°C (1 min), followed by extension for 10 min at 72°C. Afterward, the PCR products were resolved by electrophoresis on a 1% agarose gel.

Cell-mediated cytotoxicity assay

The CT-26 target cells (5×10^4/well) were added to effector $CD8^+$ T cells, which were obtained from the spleens of $MIF^{+/+}$ or $MIF^{-/-}$ mice 30 d after the tumor inoculation, in 96-well flat-bottom plates at E:T cell at a ratio of 40:1. After coincubation of both cell types for 4 or 8 h at 37°C, cytotoxicity was assessed by measuring the concentrations of lactate dehydrogenase (LDH) released in the culture supernatants using the CytoTox 96 Assay kit (Promega, Madison, WI). The specific lysis is calculated by the equation of ([experimental release] − [spontaneous release]/[target maximum] − [target spontaneous release]). Spontaneous LDH release in the absence of effector cells was <10% of the maximal LDH release by a detergent. All experimental procedures and assays were performed in triplicate more than two times, which showed similar results.

rIL-2 treatment in vivo

To determine the role of IL-2 in tumor progression and Treg generation in $MIF^{-/-}$ mice, the mice were injected i.p. with IL-2 (2,500 or 12,500 IU/day/mouse) 14 times every other day after the tumor cell inoculation; carrier-free anti-mouse rIL-2 Ab was purchased from R&D Systems. For comparison, the tumor-bearing $MIF^{-/-}$ and $MIF^{+/+}$ mice without IL-2 injection were maintained under the same conditions. After 30 d, tumor tissue cells and spleen cells were isolated from the three groups of mice. The cells were cultured, harvested, and used for flow cytometry analysis for Tregs.

Statistical analysis

Data are expressed as the mean ± SD. Comparisons of the numerical data between groups were performed by paired or unpaired Mann–Whitney *U* test or ANOVA. The p values <0.05 were considered statistically significant.

Results

Tumor growth is suppressed in $MIF^{-/-}$ mice

Recent studies have demonstrated that MIF is critically involved in the processes of cell proliferation and tumor angiogenesis (3–5, 14). However, it remains unclear whether MIF of host origin or produced by tumors independently affects tumor progression. To address this question, we established an animal model of colon cancer by inoculating CT-26 mouse colon carcinoma cells (1×10^6)

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into syngeneic MIF$^{-/-}$ and MIF$^{+/+}$ mice. We first observed a sustained reduction in tumor growth in MIF$^{-/-}$ mice when compared with MIF$^{+/+}$ mice (Fig. 1A). In parallel, MIF$^{-/-}$ mice showed a smaller change in body weight than MIF$^{+/+}$ mice, as observed for 30 d after tumor inoculation (Fig. 1C). Moreover, the weight of tumors, which were excised 30 d after tumor injection, was significantly lower in MIF$^{-/-}$ than in MIF$^{+/+}$ mice (Fig. 1B), whereas spleen weights were higher in MIF$^{-/-}$ mice (Fig. 1D), indicating that host MIF is crucial to CT-26 tumor cell growth. Similarly, when 4T1 mouse mammary carcinoma cells (1×10^6) were injected into the mice by the same protocol, their growth also was significantly reduced in MIF$^{-/-}$ mice ($n = 9$ for each group; Supplemental Fig. 1B), suggesting that the effect of host MIF on tumor growth is not limited to the CT-26 colon cancer.

Tumor growth can be affected by several factors, one of which is regulation of growth-promoting cytokines (18, 19). For example, anti-MIF Ab treatment of EG.7 tumor-bearing mice increases T lymphocyte infiltration of tumors (5). To investigate whether the inhibition of tumor growth in MIF$^{-/-}$ mice is associated with T cell-mediated antitumor immunity, we analyzed the composition of T cells infiltrated in the tumor tissues of MIF$^{-/-}$ and MIF$^{+/+}$ mice. By immunohistochemical staining, the percentage of CD3$^+$ T cells was significantly higher in tumor tissues of MIF$^{-/-}$ mice than those of MIF$^{+/+}$ mice (Fig. 1E), indicating that MIF regulates T cell trafficking into tumor tissues (5).

It has been reported that CT-26 tumor cells intrinsically produce significant amounts of MIF (3), and that tumor-derived MIF may replenish the deficiency of host MIF in MIF-deficient mice. To address this issue, we determined MIF expression in the CT-26 tumor cells and the sera of MIF$^{-/-}$ mice. As reported previously (3), we found that CT26 cell line expressed MIF mRNA (Fig. 1F). MIF was also detectable in the sera of MIF$^{-/-}$ mice, but its levels were significantly lower in MIF$^{-/-}$ mice ($n = 14$) than in MIF$^{+/+}$ mice ($n = 14$) (mean [±SD] serum concentration: 0.28 ± 0.17 ng/ml for MIF$^{-/-}$ mice versus 0.49 ± 0.26 ng/ml for MIF$^{+/+}$ mice, $p < 0.05$; Fig. 1G). Given that host tissues of MIF$^{-/-}$ mice did not express MIF (Fig. 1F), the source of MIF in the sera of MIF$^{-/-}$ mice is the CT26 tumor cells.

Reduction of Tregs in tumor-bearing MIF$^{-/-}$ mice

Our next experiment investigated the potential role of Tregs in the differential growth of CT26 colon cancer cells in MIF$^{-/-}$ and MIF$^{+/+}$ mice. Under tumor microenvironments, Tregs can be differentiated, expanded, recruited, and activated via multiple mechanisms (12, 20). As a result, Tregs are commonly found in the tumor itself, the peripheral blood, or the lymphoid organs of the tumor-bearing host, and they in turn critically contribute to tumor growth and metastasis. To determine the role of MIF in the generation of Tregs, we first examined the levels of CD4$^+$CD25$^+$ Foxp3$^+$ T cell (CD4$^+$Tregs) in MIF$^{-/-}$ and MIF$^{+/+}$ mice. As seen in Fig. 2A–C, the percentage of CD4$^+$Tregs over total CD4$^+$ T cells was significantly lower in tumor tissue and the spleens of MIF$^{-/-}$ mice than in those of MIF$^{+/+}$ mice. Morover, the percentage of CD4$^+$Tregs over total CD4$^+$ T cells also was reduced in tumor tissue from MIF$^{-/-}$ mice implanted with 4T1 mouse mammary carcinoma cells (Supplemental Fig. 1C), which is in

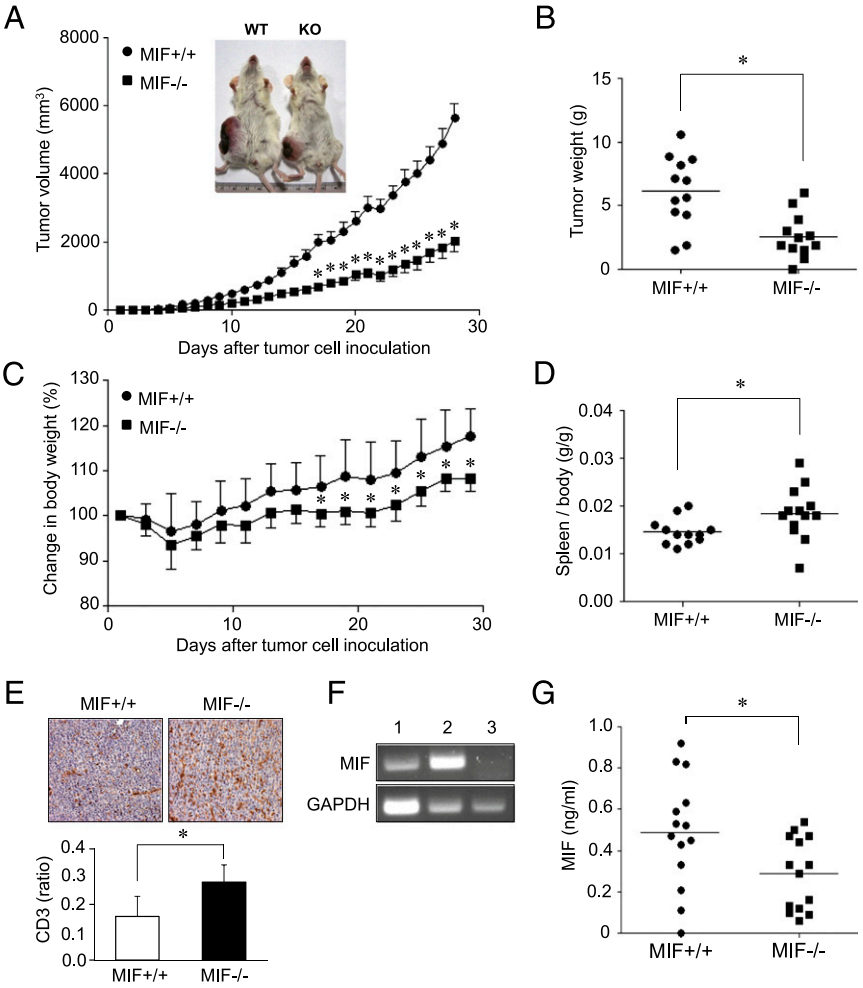

FIGURE 1. Reduced growth of the CT26 colon carcinoma in syngeneic MIF$^{-/-}$ mice. (**A** and **B**) Decrease in tumor size and weight in MIF$^{-/-}$ mice. CT26 colon cancer cells (1×10^6/mouse) were administered s.c. into the right flank of MIF$^{-/-}$ mice and wild-type (MIF$^{+/+}$) mice. The tumor volume was calculated using the formula of width^2 × length × 0.5 (length > width). Four weeks after the tumor inoculation, tumor mass was excised and its weight was measured. *$p < 0.001$. Representative photographs are shown inside the figure. (**C**) Changes in body weight of MIF$^{-/-}$ mice versus MIF$^{+/+}$ mice after the tumor injection. *$p < 0.05$. (**D**) Comparison of spleen weight between MIF$^{-/-}$ and MIF$^{+/+}$ mice, which was assessed at 4 wk after the tumor injection. *$p < 0.05$. (**E**) Immunohistochemical staining (original magnification ×100) of tumor mass of MIF$^{-/-}$ mice ($n = 6$) and MIF$^{+/+}$ mice ($n = 6$) using anti-CD3 Ab. The number of T cells was manually counted and presented as a ratio of T cells over non-T cells, including tumor cells. Data are the mean ± SD of CD3 ratios. (**F**) MIF mRNA expression in CT-26 tumor cells. Total RNA was isolated from CT26 colon cancer cells (*lane 1*), spleen cells of MIF$^{+/+}$ mouse (*lane 2*), and spleen cells of MIF$^{-/-}$ mouse (*lane 3*). The mRNA expressions in these cells were determined by RT-PCR analysis. Expression of GAPDH mRNA is presented as a control. (**G**) MIF concentrations in the sera of tumor-bearing MIF$^{+/+}$ mice ($n = 14$) and MIF$^{-/-}$ mice ($n = 14$). Four weeks after the tumor inoculation, the sera were collected from the two groups of mice, and circulating MIF levels were measured by ELISA. *$p < 0.05$. KO, Knockout; WT, wild type.

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parallel with the data on the decrease in 4T1 tumor growth in MIF$^{-/-}$ mice (Supplemental Fig. 1B). We next measured the expression of functional markers for Tregs, including GITR and CTLA4 by CD4$^+$Foxp3$^+$ T cells in MIF$^{-/-}$ mice and MIF$^{+/+}$ mice injected with CT-26 tumor cells. The result showed that GITR and CTLA-4 expression levels by CD4$^+$Foxp3$^+$ T cells in both spleen and tumor tissue were not different between the two groups (Fig. 2A, 2B), suggesting that their regulatory function may not be altered in MIF$^{-/-}$ mice.

Although infiltration of lymphocytes is almost always observed in both human and experimental animal cancers (21, 22), complete rejection of tumors is extremely rare. Interestingly, we identified three cases (15%) of complete rejection of 20 MIF$^{-/-}$ mice within 30 d after tumor inoculation, but never in wild-type mice ($n > 30$; $p < 0.05$). When we compared the composition of T cells in the spleen of three groups of mice, tumor-bearing, tumor-rejected, and tumor-naive mice, the percentages of splenic CD4$^+$ and CD8$^+$ T cells were increased to normal levels in tumor-rejected, MIF$^{-/-}$ mice and were comparable to those observed in the spleen of tumor naive mice (Fig. 2D–F). Tumor-rejected MIF$^{-/-}$ mice also had lower levels (%) of CD4$^+$Tregs within the splenic composition of CD4$^+$ T cells than tumor-bearing MIF$^{-/-}$ mice or MIF$^{+/+}$ mice (Fig. 2G). Moreover, tumor size, as determined 30 d after the tumor inoculation, correlated well with the percentages of CD4$^+$ Tregs when assessed in MIF$^{-/-}$ and MIF$^{+/+}$ mice ($r = 0.39$; Fig. 2H).

Taken together, MIF$^{-/-}$ mice harbored more CD4$^+$ and CD8$^+$ T cells, but fewer CD4$^+$Tregs in the spleen and/or tumor tissues than MIF$^{+/+}$ mice, which may account for diminished tumor growth in MIF$^{-/-}$ mice.

Increase in inducible CD4$^+$Tregs in tumor-bearing MIF$^{-/-}$ mice

T cells cultured with IL-2, TGF-β, and TCR-triggering stimuli have more inducible Tregs than T cells cultured with media or TCR-triggering stimuli alone (23, 24). We next compared the

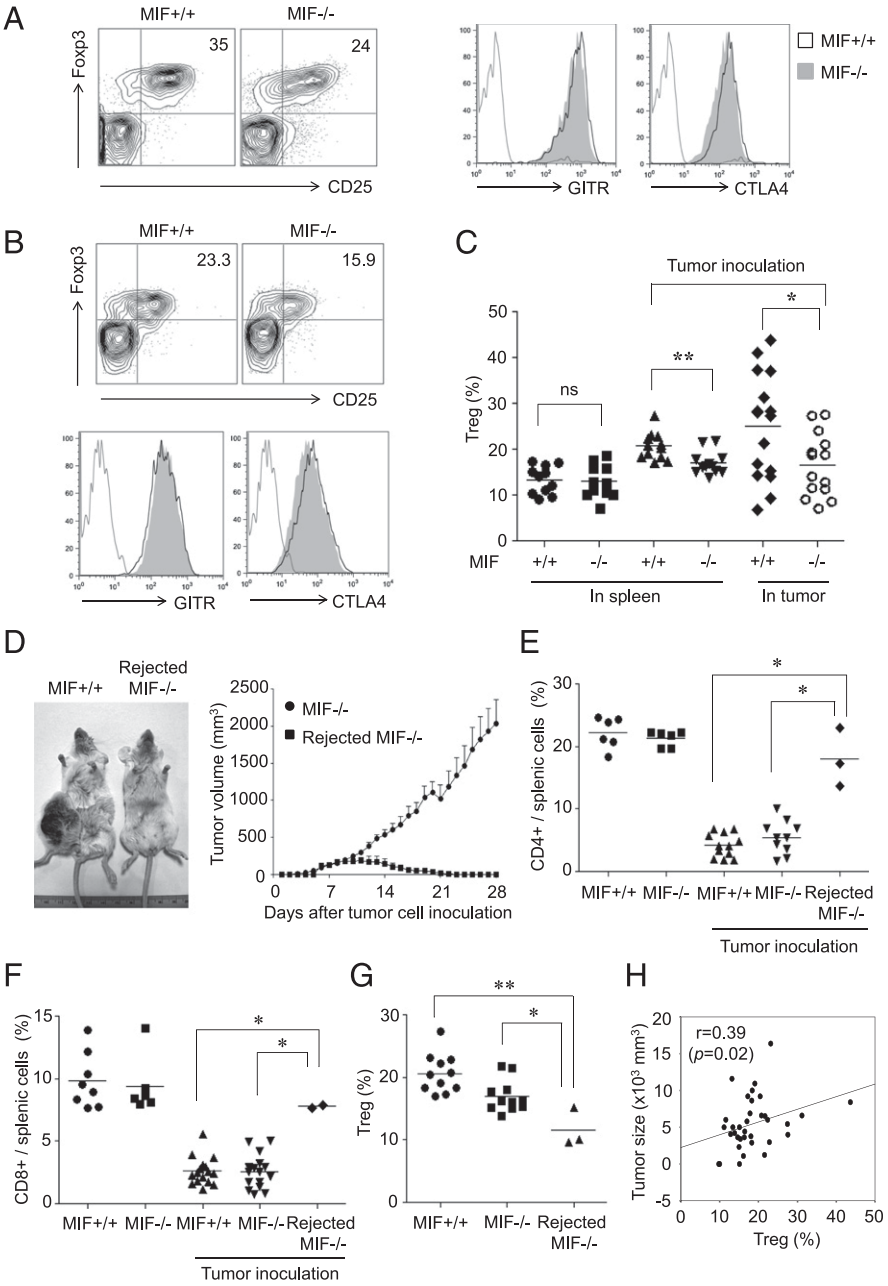

FIGURE 2. Decrease in CD4$^+$Tregs in MIF$^{-/-}$ mice. (**A**) Decrease in CD4$^+$Tregs in MIF$^{-/-}$ mouse as compared with MIF$^{+/+}$ mouse. Representative data of similar results in more than five mice are shown. Using flow cytometry analysis, the frequency of CD4$^+$ CD25$^+$Foxp3$^+$ T cells, CD4$^+$GITR$^+$ T cells, and CD4$^+$CTLA4$^+$ T cells was determined in single-cell suspensions of tumor mass of MIF$^{-/-}$ versus MIF$^{+/+}$ mouse. (**B**) Representative flow cytometry analysis of CD4$^+$Tregs in the spleen of MIF$^{-/-}$ versus MIF$^{+/+}$ mouse. (**C**) Comparison of the percentage of CD4$^+$ Tregs in the spleen and tumor tissue between tumor-bearing MIF$^{-/-}$ and MIF$^{+/+}$ mice. The frequency of Tregs was calculated as the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells in the CD4$^+$ T cell population. Mice without tumor inoculation were used as a control. $^{**}p < 0.01$. (**D**) Tumor rejection in MIF$^{-/-}$ mice. *Left panel*, Represents a photograph of tumor-bearing and tumor-rejected MIF$^{-/-}$ mouse taken 4 wk after tumor inoculation. *Right panel*, Shows changes in tumor volume in tumor-rejected versus tumor-bearing MIF$^{-/-}$ mice. Representative data are shown. (**E** and **F**) Recovery of CD4$^+$ and CD8$^+$ T cells in the spleen of tumor-rejected MIF$^{-/-}$ mice. The frequency of CD4$^+$ or CD8$^+$ T cells was determined by flow cytometry and calculated as the percentage of CD4$^+$ or CD8$^+$ cells in the splenocytes from mice. Tumor-naive MIF$^{-/-}$ and MIF$^{+/+}$ mice were used as a control. $^*p < 0.05$. (**G**) Decrease in CD4$^+$ Tregs in the spleen of tumor-rejected MIF$^{-/-}$ mice, as compared with that of tumor-bearing MIF$^{-/-}$ and MIF$^{+/+}$ mice. The frequency of Treg was calculated as the percentage of CD4$^+$CD25$^+$ Foxp3$^+$ cells in the CD4$^+$ T cell population. $^*p < 0.05$, $^{**}p < 0.01$. (**H**) Correlation of the frequency of CD4$^+$Tregs with tumor size determined at 4 wk after tumor injection, in all of the MIF$^{-/-}$ and MIF$^{+/+}$ mice tested.

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number of inducible Tregs in spleen cells activated by the aforementioned stimuli in MIF$^{-/-}$ mice and wild-type mice. As expected, when sorted CD4$^+$ T cells were stimulated by anti-CD3/CD28 Ab plus IL-2 plus TGF-β, the frequency of CD4$^+$Tregs was markedly increased. Interestingly, in contrast with basal Tregs, the number of inducible Tregs was significantly greater within the CD4$^+$ T cell population of MIF$^{-/-}$ than MIF$^{+/+}$ mice (Fig. 3A–C). The percentage of inducible CD4$^+$Tregs (%) among total splenic cells stimulated with anti-CD3/CD28 Ab plus IL-2 plus TGF-β showed similar results (data not shown). We postulated that insufficient generation of these stimuli under tumor conditions may be associated with decreased Tregs in MIF$^{-/-}$ mice. To address this question, we measured the levels of IL-2, IFN-γ, IL-10, and TGF-β production by spleen cells stimulated with anti-CD3 plus anti-CD28 Ab to simulate TCR triggering by tumor Ags (25). Spleen cells from tumor-bearing MIF$^{-/-}$ mice showed significantly lower levels of IL-2 production than spleen cells from tumor-bearing MIF$^{+/+}$ mice (Fig. 3D). A decrease in IL-2 production also was observed in the splenic cells of MIF-deficient mice without tumor inoculation (data not shown). Nevertheless, the production of IFN-γ, IL-10, and TGF-β by spleen cells was not different between the MIF$^{-/-}$ and MIF$^{+/+}$ groups (Fig. 3E, 3F). These data indicate that MIF may act as upstream of IL-2 production and IL-2–mediated Treg genentation, but it may not affect IL-10 and TGF-β production by splenocytes.

To address this isssue, we added anti–IL-2 Ab to splenic CD4$^+$ T cells stimulated with anti-CD3/CD28 Ab plus TGF-β (3 ng/ml). As reported previously (24), anti–IL-2 Ab strongly inhibited the inducible CD4$^+$Treg response (Fig. 4A). We next treated recombinant MIF to MIF-deficient splenocytes. The result showed that addition of MIF (500 ng/ml) restored IL-2 release by MIF$^{-/-}$ splenocytes stimulated with anti-CD3 Ab plus anti-CD28 Ab (Fig. 4B). Conversely, neutralizing anti-MIF Ab, but not isotype control Ab, blocked anti-CD3–induced IL-2 production by splenocytes of MIF$^{+/+}$ mice (Fig. 4C). Moreover, Debio1036, a benzoxazolone compound that specifically inhibits MIF binding and activation of its transmembrane receptor CD74 (17), also suppressed anti-CD3–induced IL-2 production by splenocytes of MIF$^{+/+}$ mice (Fig. 4D). In parallel, anti-MIF Ab treatment suppressed the CD4$^+$Treg generation in the splenic CD4$^+$ T cells of MIF$^{+/+}$ mice stimulated with anti-CD3/CD28 Ab plus TGF-β, which was completely recovered by exogenous IL-2 (5 ng/ml) (Fig. 4E). Taken together, these results suggest that defective IL-2 production is an important factor explaining reduced Treg generation in MIF$^{-/-}$ versus MIF$^{+/+}$ mice.

Increased cytotoxicity and decreased CD8$^+$Tregs in tumor-bearing MIF$^{-/-}$ mice

To be effective, tumor-specific cytotoxicity must not only be initiated, but also be vigorous and sustained so as to achieve successful tumor regression (26, 27). Although a decrease in CD4$^+$Tregs may be linked to the regression of tumor growth in MIF$^{-/-}$ mice, it also is possible that CD8$^+$ cells exhibit an enhanced, intrinsic cytotoxicity against tumor cells in the setting of MIF deficiency. To test the potential role of MIF in CD8$^+$ cell-mediated cytotoxicity, CT-26 target cells were cocultured with effector CD8$^+$ T cells obtained from the spleens of MIF$^{+/+}$ and MIF$^{-/-}$ mice, and cytotoxicity was assessed by a LDH release assay. As shown in Fig. 5A, CD8$^+$ effector T cells sorted from the spleens of MIF$^{-/-}$ mice showed a significant increase in the cytotoxicity response when compared with corresponding cells sorted from MIF$^{+/+}$ mice. We next investigated the presence of immunosuppressive

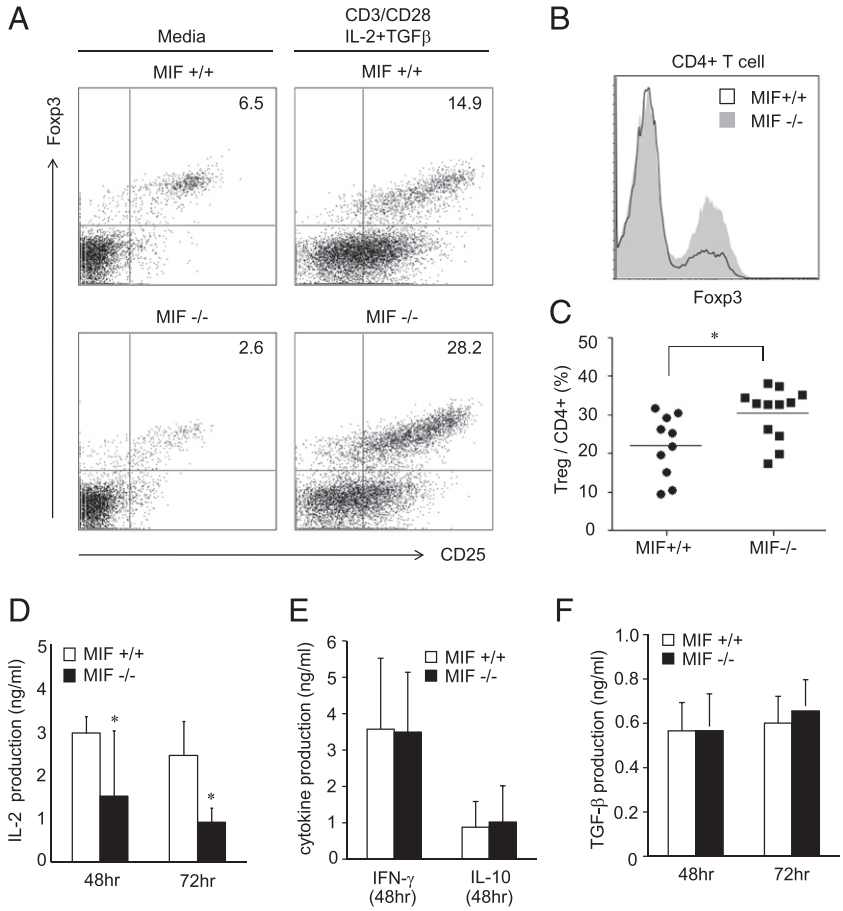

FIGURE 3. Inducible CD4$^+$Treg response and cytokine production in spleen cells of MIF$^{-/-}$ versus MIF$^{+/+}$ mice. (**A** and **B**) Representative data and quantification of inducible CD4$^+$Tregs. Sorted CD4$^+$ T cells of the spleen of tumor-naive mice were stimulated with or without anti-CD3/CD28 Ab plus IL-2 (1 ng/ml) plus TGF-β (3 ng/ml). After 72 h of stimulation, the frequency of CD4$^+$CD25$^+$ Foxp3$^+$ in CD4$^+$ cells was determined by flow cytometry. Overlay histogram for Foxp3 is also shown in (B). (**C**) Comparison of the percentage of inducible CD4$^+$Tregs between tumor-naive MIF$^{-/-}$ and MIF$^{+/+}$ mice. $*p < 0.05$. (**D–F**) Cytokine production by splenic T cells of tumor-bearing MIF$^{-/-}$ versus MIF$^{+/+}$ mice. The spleen cells (1×10^6) of the two groups of mice were stimulated with anti-CD3/CD28 Ab for 48 or 72 h. Levels of IL-2, IFN-γ, IL-10, and TGF-β in the culture supernatants were measured by ELISA. $*p < 0.05$.

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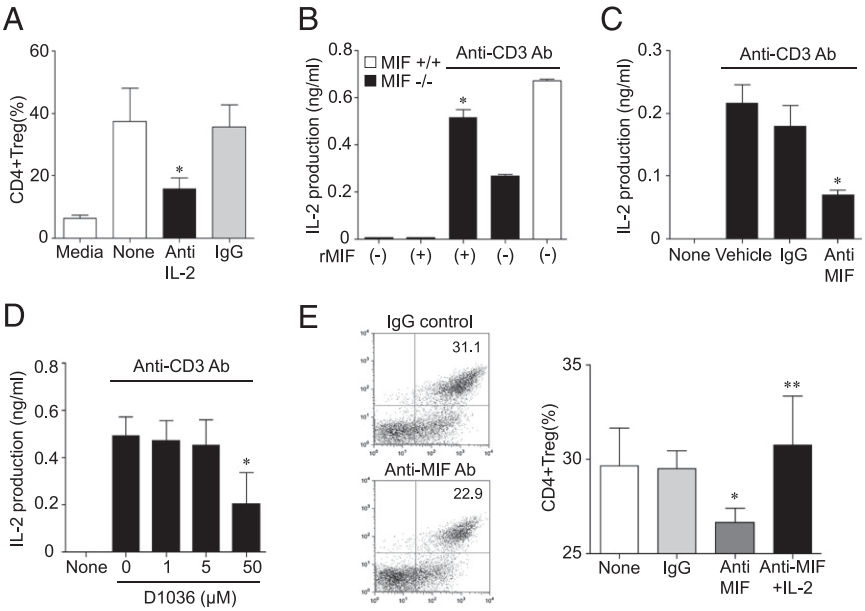

FIGURE 4. MIF controls IL-2–mediated Treg generation. (**A**) Suppression of inducible CD4$^+$Tregs by anti–IL-2 Ab. Splenic CD4$^+$ cells of MIF$^{+/+}$ mice were stimulated with anti-CD3/CD28 Ab (CD3/CD28) plus TGF-β (3 ng/ml) in the presence of anti–IL-2 Ab (0.1 μg/ml) or isotype control IgG. After 72 h of incubation, the frequency of CD4$^+$CD25$^+$Foxp3$^+$ T cells over CD4$^+$ cells was determined by flow cytometry analysis. Data show mean ± SD. *$p < 0.05$ versus isotype control Ab. (**B**) Restoration of IL-2 production by recombinant MIF. Splenic T cells (2×10^5) of MIF$^{-/-}$ mice were stimulated with anti-CD3/CD28 Ab in the presence of recombinant MIF (500 ng/ml) for 48 h. IL-2 levels in the culture supernatants were measured by ELISA. *$p < 0.05$ versus IL-2 production by splenic cells of MIF$^{+/+}$ mice treated with anti-CD3/CD28 Ab alone. (**C** and **D**) Inhibition of IL-2 production by neutralizing anti-MIF Ab (C) or MIF receptor antagonist (D). Splenic cells (2×10^5) of MIF$^{+/+}$ mice were stimulated with anti-CD3/CD28 Ab in the absence or presence of anti-MIF Ab (100 μg/ml) for 48 h or D1036 (a MIF receptor antagonist: 1, 5, and 50 μM) for 24 h. Isotype control IgG (100 μg/ml) was used as a control. IL-2 levels in the culture supernatants were measured by ELISA. *$p < 0.05$ versus isotype control Ab or anti-CD3/CD28 Ab alone. (**E**) Effect of anti-MIF Ab on the inducible CD4$^+$Treg production. Splenic CD4$^+$ cells of MIF$^{+/+}$ mice were cultured in RPMI 1640 supplemented with 10% FBS, and stimulated with anti-CD3/CD28 Ab (CD3/CD28) plus TGF-β (3 ng/ml) in the presence of anti-MIF Ab (50 μg/ml) or isotype control IgG (50 μg/ml). After 72 h of incubation, the frequency of CD4$^+$Tregs was determined by flow cytometry analysis. A representative is shown on the *left*. Bar graph on the *right* shows the mean ± SD of six independent experiments. *$p < 0.05$ versus isotype control Ab. **$p < 0.01$ versus anti-MIF Ab alone.

CD8$^+$ regulatory cells, CD8$^+$Tregs, which have also been identified as a T cell population with immune suppressive properties in vitro (28). We tested whether tumor-bearing MIF$^{-/-}$ mice also have fewer CD8$^+$Tregs within the total CD8$^+$ T cell effector population that is used in the cytotoxicity assay, as shown above for CD4$^+$ T cells. As illustrated in Fig. 5B, tumor-bearing MIF$^{-/-}$ mice indeed had a lower number of CD8$^+$Tregs in the spleen than MIF$^{+/+}$ mice. The number of CD8$^+$Tregs in the tumor mass also was lower in MIF$^{-/-}$ mice (Fig. 5C). Similar to CD4$^+$Tregs, the frequency of CD8$^+$Tregs of total splenic T cells stimulated with anti-CD3/CD28 Ab plus IL-2 plus TGF-β was significantly higher in MIF$^{-/-}$ than in MIF$^{+/+}$ mice (Fig. 5D, 5E). Taken together, these results suggest that MIF suppresses tumor-specific cytotoxicity by CD8$^+$ T cells to promote the generation of CD8$^+$Tregs.

IL-2 treatment restores tumor growth and Treg generation in MIF$^{-/-}$ mice

The dominant function of IL-2 appears to be in the maintenance of immune homeostasis and tolerance to self. IL-2 has been critically linked to the biology of Tregs (29, 30). Most Tregs constitutively express the high-affinity IL-2R, which is comprised of the IL-2Rα, IL-2Rβ, and IL-2Rγ protein (31). Based on the data on IL-2 production levels in MIF$^{-/-}$ mice, we investigated whether exogenous IL-2 restores the generation of Tregs and tumor growth. To this end, we injected rIL-2 i.p. into MIF$^{-/-}$ mice 14 times every other day for 30 d after the tumor cell inoculation. As shown in Fig. 6A–C, the rate of tumor growth was partially restored in MIF$^{-/-}$ mice by the administration of IL-2, and this effect was associated with an increase in the frequency of CD4$^+$Tregs in the spleen (Fig. 6C). In parallel, IL-2–injected MIF$^{-/-}$ mice had an increased level of GITR and CTLA-4 in tumor-infiltrated CD4$^+$ T cells when compared with vehicle-treated MIF$^{-/-}$ mice (data not shown). The frequency of CD8$^+$Tregs also was significantly higher in the spleens of MIF$^{-/-}$ mice treated with IL-2 than in those of control mice (Fig. 6D). Together, these results indicate that IL-2 promotes the generation of CD4$^+$ and CD8$^+$Tregs, which in turn may contribute to the restoration of tumor growth in MIF$^{-/-}$ mice.2

Discussion

Tumor-infiltrating immune cells are crucial to combating cancer, and their activity correlates with disease prognosis and survival (32, 33). The identification of Ags from various tumors that are recognized by T cells further supports the importance of antitumor immunity and sets the stage for the development of more effective and Ag-specific cancer immunotherapies (34). Various clinical trials indicate that immunotherapy with cancer Ag can induce Ag-specific immune responses in the majority of patients (34, 35), but such immune responses are too weak or transient to produce therapeutic benefit to cancer patients. One of the factors contributing to this therapeutic failure is the presence of Tregs (12). Tregs induce immune tolerance by suppressing host immune responses against self or non-self Ags, thus limiting antitumor immunity and promoting tumor growth (6–12).

MIF was discovered as a cytokine secreted by activated T cells (1). Bacher et al. (2) have reported that MIF plays an important regulatory role in the activation of T cells. Mitogen- or Ag-activated T cells express significant quantities of MIF mRNA and protein, and neutralization of MIF inhibits IL-2 production and T cell proliferation in vitro, decreasing the Th cell response to soluble Ag in vivo (2). It also has been shown that neutralizing

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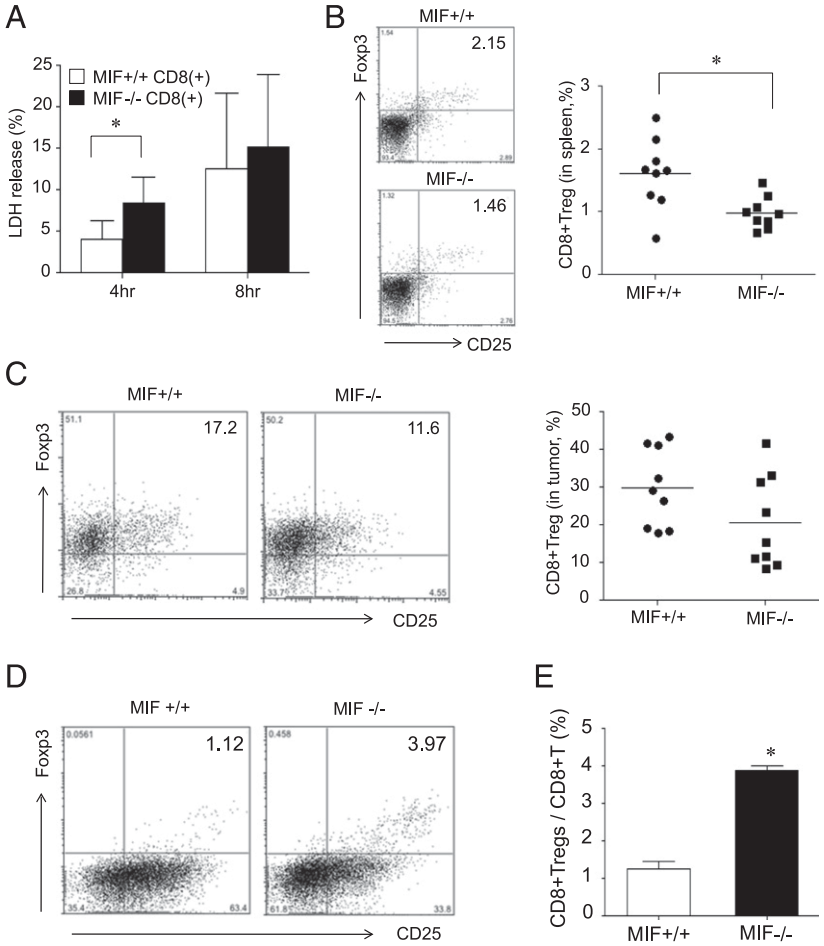

FIGURE 5. Changes in tumor-specific cytotoxicity and CD8$^+$Tregs in MIF$^{-/-}$ mice. (**A**) Increase in tumor cell death induced by CD8$^+$ T cells of MIF$^{-/-}$ mice. The CT26 carcinoma cells were s.c. injected in MIF$^{-/-}$ mice and MIF$^{+/+}$ mice, and CD8$^+$ T cells in the spleens of the two groups of mice then were isolated using anti-CD8 microbeads. The CD8$^+$ effector cells were cocultured with CT26 target cells with E:T ratios of 40:1 for 4 and 8 h. Cytotoxicity was measured by LDH release. *$p < 0.05$. (**B** and **C**) Decrease in CD8$^+$Tregs in tumor-bearing MIF$^{-/-}$ mice. Four weeks after the tumor injection, spleen cells and tumor tissues were harvested from MIF$^{-/-}$ mice and MIF$^{+/+}$ mice. The frequency of CD8$^+$CD25$^+$ Foxp3$^+$ T cells was assessed by flow cytometry and calculated as the percentage of CD8$^+$CD25$^+$Foxp3$^+$ cells in the CD8$^+$ T cell population. The representative data are shown in the *left panel*. *$p < 0.05$. (**D** and **E**) Inducible CD8$^+$ Tregs in tumor-naive MIF$^{-/-}$ mice ($n = 3$) and MIF$^{+/+}$ mice ($n = 3$). The splenic cells were stimulated by anti-CD3/CD28 Ab plus IL-2 plus TGF-β, as described in *Materials and Methods*. The number of CD8$^+$CD25$^+$Foxp3$^+$ T cells was determined by flow cytometry. *$p < 0.05$ versus MIF$^{+/+}$ mice. A representative is shown in the *left panel* in (D).

anti-MIF Ab inhibits T cell proliferation and IL-2 production in vitro and suppresses Ag-driven T cell activation and Ab production in vivo (36). However, the effect of MIF on tumor-associated Tregs remains undefined. In this study, we demonstrated first that the growth rate of the CT26 colon carcinoma was significantly lower in MIF$^{-/-}$ mice than in MIF$^{+/+}$ mice. MIF$^{-/-}$ mice showed a higher infiltration of T cells in tumor tissues, whereas MIF-deficient hosts had lower levels of tumor-associated Tregs in the spleen and tumor tissue than MIF$^{+/+}$ mice. The decrease in tumor growth and Treg infiltration into the tumor tissue was similarly noted in MIF$^{-/-}$ mice implanted with 4T1 breast cancer cells, suggesting that the effect of host MIF on tumor growth and Treg production is not limited to CT-26 colon cancer. Together, these results suggest that MIF plays a role in the generation of Tregs in tumor-bearing mice.

Given that MIF promotes angiogenesis (4, 14), it is noteworthy that T cell infiltration into tumor tissues was enhanced in MIF$^{-/-}$ mice, where angiogenesis is markedly repressed (37). Because Tregs inhibit the proliferation and activation of T cells by tumor Ags (38), a decrease in Tregs in MIF$^{-/-}$ mice may increase T cell activation and proliferation triggered by tumor Ags in the tumor tissues, leading to a reduction in tumor mass by a CTL response. It is also a possibility that MIF has a role in the regulation of antitumor T cell trafficking. The finding that anti-MIF Ab promotes the migration of T lymphocytes into EG.7 tumors and augments CD8$^+$ T cell-specific antitumor activity supports this notion (5).

A direct inhibitory function for MIF in Th1-dependent CTL responses also has been described by Abe et al. (5). These authors found that administration of a neutralizing anti-MIF mAb to tumor-bearing mice significantly increases the CTL response by splenic cells, but the exact mechanism of this effect remains unclear. By using a coculture system of CT26 cells and sorted CD8$^+$ T cells, we demonstrated that CD8$^+$ effector cells from MIF$^{-/-}$ mice show a significant increase in an anti-CT26 CTL response that was accompanied by a decrease in CD8$^+$Tregs. In these tumor-bearing mice, CD8$^+$Tregs expressed both CD25 and Foxp3 molecules, which are shared by CD4$^+$Tregs. CD8$^+$Tregs have been identified to mediate immunosuppression in cancer and other diseases (39, 40), although some groups have questioned the immunosuppressive properties of these cells (41). For example, CD8$^+$ Tregs suppress Ag-activated CD4$^+$ T cells in a TCR-specific manner restricted by the MHC class Ib molecule, Oa-1 (40, 42). In colorectal cancer, CD8$^+$CD25$^+$Foxp3$^+$Tregs are present in tumor-infiltrating lymphocytes and suppress antitumor T cell immunity (43). Our results, together with previous reports (40, 42, 43), suggest that MIF promotes the generation of CD8$^+$Tregs, which may in turn play a role in promoting tumor growth by decreasing CTL response.

Another possibility for the increased CD8 cytotoxicity in MIF$^{-/-}$ mice could be alteration in antigenicity. It has been reported that MIF mediates many of its inflammatory properties by interacting with the CD74-CD44 MIF receptor complex (44). We and others have demonstrated that the CT26 cell line expresses MIF (3) (Fig. 1F) as well as the MIF-binding receptor CD74 (45). We also found that these cells express the MIF signaling receptor component, CD44, as determined by flow cytometry analysis (data not shown). Therefore, tumor-derived MIF in MIF$^{-/-}$ mice may increase the antigenicity of the tumor in an autocrine manner to enhance a CD8$^+$ T cell, antitumor immune response. If this were the case, then CT26 cells may be rejected by tumor-infiltrating CD8 T cells in MIF$^{-/-}$ mice (as seen in Fig. 2D) via recognition of MHC class

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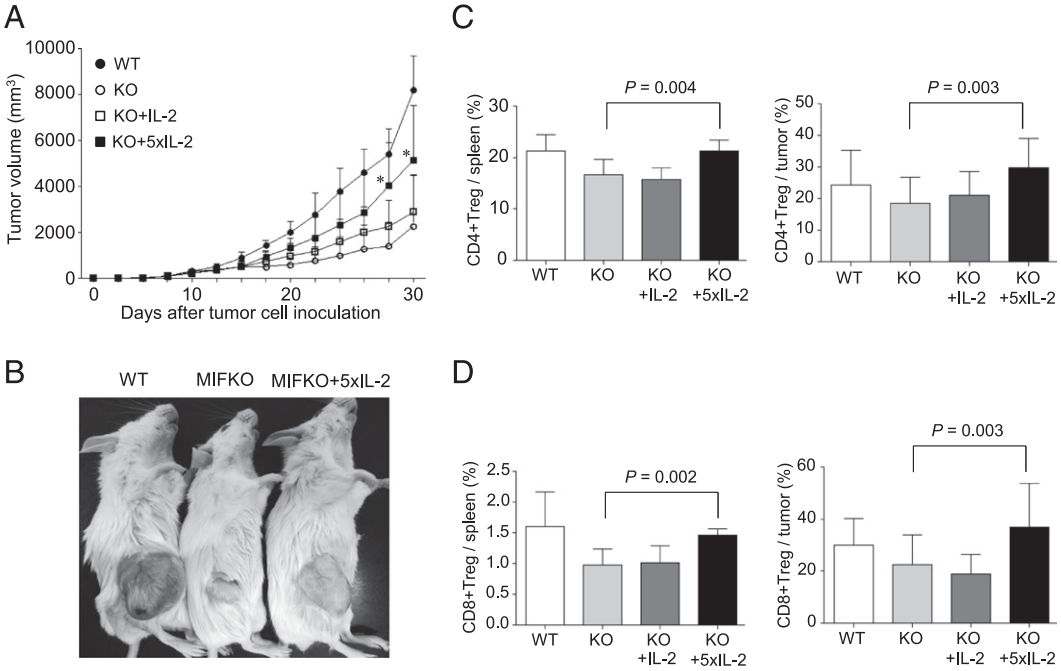

FIGURE 6. rIL-2 reverses tumor growth and Treg generation in MIF$^{-/-}$ mice. (**A**) Tumor-bearing MIF$^{-/-}$ mice were injected with 2,500 IU rIL-2 (IL-2, $n = 3$) or 12,500 IU rIL-2 (5 × IL-2, $n = 9$) every other day for 30 d after the tumor cell inoculation. MIF$^{+/+}$ ($n = 5$) and MIF$^{-/-}$ mice ($n = 9$) injected with vehicle alone were used as a control. Tumor growth rate was monitored every day. $*p < 0.05$. (**B**) Appearance of vehicle-treated MIF$^{+/+}$ mice, vehicle-treated MIF$^{-/-}$ mice, and IL-2–injected MIF$^{-/-}$ mice at 4 wk after tumor cell inoculation. (**C** and **D**) The percentage of CD4$^+$ and CD8$^+$Tregs in the spleens and tumor tissues of IL-2–treated MIF$^{-/-}$ mice ($n = 3$ for IL-2, $n = 9$ for 5 × IL-2), vehicle-treated MIF$^{-/-}$ ($n = 9$), and vehicle-treated MIF$^{+/+}$ mice ($n = 5$), determined by flow cytometry analysis.

I-associated MIF-derived peptides. In a similar way, it is also possible that the MIF$^{-/-}$ mice might not be completely syngeneic with the tumors.

What is the possible mechanism by which the generation of CD4$^+$ and CD8$^+$Tregs is suppressed in the CT26 colon carcinoma-bearing MIF$^{-/-}$ mice? Recent studies have linked chronic inflammation to cancer progression (46–49). Cancer is concomitantly associated with inflammation that may create a specific cytokine environment favoring the expansion of Tregs (50, 51). Suppressive cytokines, such as IL-10, TGF-β, and IL-2 that are secreted by tumor cells, tumor-infiltrating T cells, and macrophages, not only recruit Tregs to tumor sites, but also favor the conversion of nonsuppressive T cells into Tregs with suppressive function (50, 51). Our work underscores the importance of MIF, as an upstream of IL-2, in the generation of Tregs and in the suppression of tumor immunity. We demonstrated that IL-2 secretion by splenic cells stimulated with anti-CD3/CD28 Ab was significantly lower in MIF$^{-/-}$ mice, and that addition of recombinant MIF restored IL-2 release by MIF$^{-/-}$ splenocytes stimulated with anti-CD3 Ab. Conversely, neutralizing anti-MIF Ab or the small molecule, Debio1036, which inhibits the interaction between MIF and its receptor CD74, blocked anti-CD3–induced IL-2 production by splenocytes of MIF$^{+/+}$ mice and suppressed the inducible CD4$^+$Treg generation. Moreover, administration of rIL-2 into tumor-bearing MIF$^{-/-}$ mice restored the generation of Tregs and tumor growth. These observations indicate that MIF controls the generation of CD4$^+$ and CD8$^+$ Tregs through the induction of IL-2, suggesting that IL-2 plays a critical role in the activation and maintenance of tumor-associated Tregs in vivo, particularly in MIF-deficient conditions.

It is unexpected that inducible CD4$^+$ and CD8$^+$Treg responses by stimulation with anti-CD3/CD28 Ab plus IL-2 plus TGF-β were greater in MIF$^{-/-}$ mice than in MIF$^{+/+}$ mice. It has been reported that, in EG.7 tumor-bearing mice, anti-MIF Ab treatment significantly enhances expression of the common γ-chain (γ_c) (CD122) of IL-receptor complex (5), which is required for intracellular signaling of IL-2 (29, 30) and mature CD8$^+$ T cell survival. However, we did not find any difference in the expression of γ_c in the CD4$^+$ and CD8$^+$ T cells stimulated without or with anti-CD3/CD28 or with anti-CD3/CD28 Ab plus IL-2 plus TGF-β between the two groups of mice. Therefore, it seems likely that an increase in inducible Treg response in MIF$^{-/-}$ mice is not due to γ_c overexpression in the CT26 colon carcinoma model, and other mechanisms, such as a compensatory increase in IL-2 signaling efficiency, may be involved in this process.

With regard to cancer biology, a better understanding as to how Tregs are generated in the tumor microenvironment and how their suppressive function can be blocked is fundamentally important to improve the therapeutic potential of cancer vaccines. Our data suggest that MIF promotes tumor growth by increasing Treg generation and upregulating IL-2 production. These findings raise the possibility that MIF may be employed therapeutically to augment the suppressive function of Tregs in human disease, and that anti-MIF Ab might be useful to enhance the adaptive immune response against certain cancers.

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Disclosures

The authors have no financial conflicts of interest.

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