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Macrophage Migration Inhibitory Factor Contributes to the Immune Escape of Ovarian Cancer by Down-Regulating NKG2D

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The proinflammatory cytokine macrophage migration inhibitory factor (MIF) stimulates tumor cell proliferation, migration, and metastasis; promotes tumor angiogenesis; suppresses p53-mediated apoptosis; and inhibits antitumor immunity by largely unknown mechanisms. We here describe an overexpression of MIF in ovarian cancer that correlates with malignancy and the presence of ascites. Functionally, we find that MIF may contribute to the immune escape of ovarian carcinoma by transcriptionally down-regulating NKG2D in vitro and in vivo which impairs NK cell cytotoxicity toward tumor cells. Together with the additional tumorigenic properties of MIF, this finding provides a rationale for novel small-molecule inhibitors of MIF to be used for the treatment of MIF-secreting cancers. The Journal of Immunology, 2008, 180: 7338–7348.

Epithelial ovarian carcinoma (OvCA) is the most common cause of death from gynecological malignancy. Even with extended surgery and chemotherapy, 5-year survival rates do not exceed 20–40% (1). Only noninvasive OvCAs of low malignant potential, here designated as borderline tumors, bear a more favorable prognosis (2). Because long-term survival strongly correlates with favorable immunological parameters (3), immunomodulatory therapies would be of major clinical interest. On the one hand, they should seek to relieve tumor-induced immunosuppression (4, 5); on the other hand, they need to consider inflammation, which not only is central to tumorigenesis (6) but also contributes to the formation of ascites (7, 8), thus correlating with a poor prognosis (9).

Macrophage migration inhibitory factor (MIF), a cytokine that promotes both processes (3), has initially been described as a mediator responsible for lethal shock syndrome after exposure to bacterial endotoxins (10). MIF has also been found to be critical for wound healing (11). A polymorphism in the MIF promoter could lead to high levels of MIF that antagonize glucocorticoid functions and contribute to autoimmune diseases like rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus and atopic dermatitis (12, 13). In addition, MIF can recruit inflammatory cells and promote atherosclerosis via noncognate binding to chemokine receptors CXCR2 and CXCR4 (14). In normal ovarian tissue, MIF is secreted by granulosa cells and is found in follicular fluids (15). In tumor biology, MIF has been proposed as a biomarker for prostate cancer (16). Further, MIF plays a critical role for angiogenesis, stimulates tumor cell migration, suppresses p53 activity (17, 18), and activates cyclin D1 and E2F transcription factors (Ref. 19; summarized in Ref. 20). The MIF-CD74 ligand-receptor complex (21) activates downstream nonreceptor tyrosine kinases via CD44 (22), a coreceptor that has been proposed as a tumor stem cell marker for breast cancer (23), leukemia (24), and other malignancies. The importance of MIF for tumorigenesis is strikingly demonstrated by the resistance of MIF-deficient fibroblasts toward malignant transformation induced by c-myc, H-ras, or dominant negative p53 (25). MIF also impairs antitumor immunity by inhibiting CTL and NK cell responses (26, 27), an effect proposed to be caused by MIF-induced T cell activation followed by activation-induced cell death (28), a mechanism that might reconcile immune inhibitory with proinflammatory potential. However, activation of NK cells by MIF has not been observed, which suggests that the MIF-mediated inhibition of NK cells is effected via a different mechanism (26).

The capacity of NK cells to potentially clear malignant cells depends on the recognition of stress- or transformation-induced molecules, most notably ligands for the activating NK cell receptor NK group 2D (NKG2D; 4). These ligands (MICA/B, ULBP1–4) are induced by DNA damage (29) and are found on virus-infected and tumor cells (30–32). In human cells, ligation of NKG2D leads to the activation of the adaptor protein DAP10. DAP10 signaling initiates a perforin-mediated cytolytic response (30, 32) that can lead to NK cell-mediated tumor clearance without prior activation.
Materials and Methods

Patient characteristics

All tumor tissues and ascites were obtained in the Department of Obstetrics and Gynecology of the University Hospital (Würzburg, Germany). Patients were between 25 and 82 years of age (median age, 59.5 years). Solid tumor tissues and ascites-derived cells were assessed by at least two experienced pathologists. Of the 38 epithelial ovarian malignancies included, 21 were classified as tumors of the serous-papillary subtype, 11 as mucinous, and 6 as endometrioid. Noninvasive OvCa of low malignant potential, so-called ovarian borderline tumors, bear a more favorable prognosis and were therefore regarded separately (n = 19). The experiments were approved by the local ethics committee, and all patients gave informed written consent.

Cell culture

Primary tumor cells were derived from ascitic fluid from ovarian cancer patients. Cells were centrifuged, washed with PBS, and transferred to MCDB105-M199 medium (PAA) supplemented with 10% heat-inactivated FCS (Biochrom), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 0.02% sodium pyruvate (all from PAA). Nonadherent cells were removed by washing after 72 h. The immortalized cell lines SKOV-3 (derived from a 64-year-old Caucasian woman diagnosed with ovarian adenocarcinoma, American Type Culture Collection (ATCC) CRL-1573) and OAW-42 (derived from ascites of a 12-year-old Caucasian diagnosed with ovarian teratocarcinoma, ATCC HTB-77), PA-1 (derived from ascites of a 12-year-old Caucasian diagnosed with ovarian teratocarcinoma, ATCC CRL-1572) and OAW-42 (derived from ascites of a patient diagnosed with ovarian cystadenocarcinoma, European Cell Culture Collection 85073102) were cultured in RPMI 1640 with 10% FCS.

Generation of cell culture and tissue supernatants (SN)

For the generation of SNs, 10^5 cells/well were seeded in a 6-well plate and adhered overnight before the cell culture medium was removed and replaced by 2 ml of fully supplemented RPMI 1640. SNs were collected at 72 h after the medium exchange. Tissue SNs were obtained by placing 2 mg of freshly resected tumor for 72 h into 2 ml of fully supplemented RPMI 1640.

Generation of MIF small interfering RNA and pcDNA3-MIF transfectants

The pSUPER plasmid was obtained from R. Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (40). A puromycin cassette was inserted into the Ndel site. The MIF-specific oligonucleotide sequences GATC CCCCCCCTATTTTTTGACAA (nucleotides 385-403) were obtained from Sigma Genosys and cloned into the BglII and SalI sites of pSUPER. The MIF-specific parts of the sequences are bold and underlined. For the generation of stable small interfering MIF transfectants, pSUPERpuro control or small interfering MIF plasmids were introduced using Transfectin transfection reagent (Bio-Rad). The cells were selected in medium containing 2 μg/ml puromycin (Carl Roth).

Real-time PCR

Total RNA was prepared using the RNeasy system (Qiagen) and transcribed with the RevertAid First-Strand cDNA Synthesis kit (Fermentas). For real-time PCR, cDNA amplification was monitored using SybrGreen chemistry on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The conditions for these PCR reactions were: 40 cycles at 95°C for 15 s, 60°C for 1 min, using the following specific primers: 18S up, 5′-CGGCTACCACTACCAAGGAA-3′ (nucleotides 450–469); 18S down, 5′-GCCCGGAGACGGGCTCTACA-3′ (nucleotides 272–289); MIF up, 5′-GCCCCGGACAGGGCTCTACA-3′ (nucleotides 349–369); MIF down, 5′-CCCTAGGCAAGTGTTTGTTTCA-3′ (nucleotides 349–369).
ELISA in ascitic fluid from OvCA patients (n = 18) and nonmalignant ascites (n = 5; p < 0.01). Effusion of MIF from OvCA tissues was further assessed by placing 2 mg of freshly resected the ΔΔCt method for relative quantification. Briefly, threshold cycles (Ct) for 18S rRNA (reference MIF) were determined in duplicates, and the relative change (ΔΔCt) in copy numbers was calculated according to the formulae 

$$
\Delta \Delta C_{t} = \Delta C_{t}^{\text{MIF}} - \Delta C_{t}^{\text{18S}}
$$

Dissociation curves and PCR products were analyzed to confirm the presence of a single specific PCR product at the expected size.

**Immunohistochemical staining**

All tissue specimens were from the tumor bank of the University of Würzburg School of Medicine (Würzburg, Germany) where they had been evaluated by at least two pathologists in routine diagnostics. Paraffin-embedded tissue samples of ovarian epithelial carcinomas, borderline tumors, and normal ovaries were cut at 2 μm, placed on slides (Superfrost; Langenbrink), deparaffinized with xylene, and rehydrated in a descending alcohol sequence. Anti-MIF Ab mAb289 (42) or irrelevant control IgG (Immunotools) and/or BMO2 IgG1, anti-MICB, AUMO1 IgG1 anti-ULBP1, BUMO1 IgG1 anti-ULBP2, and CUMO3 IgG1 anti-ULBP3 as described previously (38).

**Flow cytometric analysis of NKG2DL expression levels**

PBMC were obtained from healthy volunteers by density gradient centrifugation (Biocoll; Biochrom). Monocytes were depleted by adherence. PBMC were cultured on irradiated (30 Gy) RPMI 8866 feeder cells to obtain polyclonal NK cell populations (41). Where indicated, 10 μg/ml blocking anti-MIF Ab mAb289 (42) or irrelevant control IgG (Immunotools) and/or 500 μl of cell culture SN from primary ascites-derived OvCA cells or from OvCA cells in 2 ml of medium. Secreted MIF levels were determined by ELISA from the SN at 48 h. D, MIF mRNA levels were compared between ascites-free (n = 7) and ascites-forming (n = 8) OvCA.
MIF-short hairpin RNA or control transfected SK-OV-3 ovarian cancer cells were added on day 8. After 48 h, NK cells were harvested and used in different killing assays. Cytotoxicity against primary ascites-derived OvCA cells was assessed in 4-h 51Cr release assays in the absence or presence of mAb used at 10 μg/ml. NK cells were pretreated with normal human Igs to prevent Ab-dependent cellular cytotoxicity before they were incubated for 4 h with 1 × 105 51Cr-labeled target cells per well at various E:T ratios. Spontaneous 51Cr release was determined by incubating the target cells with medium alone. Maximum release was determined by adding Nonidet P-40 (2%). The percentage of 51Cr release was calculated as 100 × [(experimental release – spontaneous release)/(maximum release – spontaneous release)].

For assays involving SK-OV-3 OvCA cells, NK cells were labeled with PKH-26/Vibrant Dil solution (Cambridge), and lytic activity against CFSE-stained (Brilliant Green) target cells (100,000 target cells/well) was assessed in modified 4-h FATAL assays (43), using various E:T ratios. Cells were detached, and target cell lysis was determined by flow cytometric analysis of 50,000 target cells in a FACScan flow cytometer (BD Biosciences). PKH-26-negative target cells were selected by gating, and the percentage of CFSELabeled cells within this population was determined. Spontaneous leakage of CFSE was determined by incubating the target cells with medium alone.

For lysis experiments with purified NK cells, SK-OV-3 cells were stably transfected with a firefly luciferase plasmid, kindly provided by Dr. Michael Jensen (City of Hope National Medical Center and Beckman Research Institute, Duarte, CA) (44). NK cells were isolated from peripheral blood using a magnetic NK cell isolation kit (Miltenyi Biotech) and stimulated for 48 h with IL-2 (100 IU/ml) in the absence or presence of 5 ng/ml recombinant human (r) MIF (Lot 38220000; R&D Systems) or SN of primary ovarian cancer cells as described above for recovery experiments. Target cells were seeded into 96-well plates (105/well), and NK cells were added on day 8. After 48 h, NK cells were harvested and used in modified assays against SK-OV-3 targets. Target cell lysis was determined by flow cytometric analysis of different time points using an Orion II luminometer (Berthold). The ATP-dependent conversion of luciferin occurs only in viable luciferase-transfected cells and is thus directly proportional to cell viability (44).

**Regulation of NKG2D expression by patient or control serum, ascites, OvCA SNs, or rhMIF and TGF-β**

PBL were obtained from healthy volunteers or OvCA patients and incubated for 48 h with patient or control serum or ascites or with SN generated from primary ascites-derived OvCA cells or from SK-OV-3 control and MIF-knockdown cells. Likewise, rhMIF or TGF-β1 (Peprotech) were applied for 24 or 48 h. rhMIF was prepared at Yale University by a standardized protocol (45) and found to contain 32 EU/mg recombinant protein. A representative of three experiments is shown (p < 0.05 by ANOVA). SNs were generated from 10⁵ primary ascites-derived OvCA cells cultured for another 48 h before being analyzed as above.

**Statistics**

Experiments were performed at least three times with similar results. Analysis of significance was performed using the two-tailed Student t test or, where indicated, ANOVA (Excel, Microsoft). For the assessment of in vivo
FIGURE 4. OvCA-derived MIF induces a down-regulation of NKG2D. A, PBL were incubated with control medium or with SNs of SK-OV-3 pSUPERpuro control (1.6 ng/ml MIF) or SK-OV-3 pSUPERpuroMIF (0.4 ng/ml MIF) cells. At 48 h, the cells were stained with anti-NKG2D Ab or the respective isotype control. Abs directed against CD3, CD4, CD8, and CD56 were used to discriminate the various immune cell subsets. Cells were analyzed in a FACScan and gated for CD8<sup>+</sup> T (CD3<sup>+</sup> CD8<sup>+</sup>) and NK (CD3<sup>+</sup> CD56<sup>+</sup>) cells; the NKG2D expression was determined for each population. Expression levels are indicated as SFI values (n = 5). B, PBL were incubated with SNs of SK-OV-3 WT cells (1.3 ng/ml MIF) in the presence or absence of an isotype control or a blocking anti-MIF Ab (10 μg/ml) or the TGF-β receptor I kinase inhibitor SD-208 (1 μM). NKG2D expression on the various immune cell subsets was determined as in B. A representative of three experiments is shown. C, NKG2D expression was analyzed as in A, using freshly isolated CD8<sup>+</sup> T cells and NK cells from OvCA patients and healthy donors. Mean values and SDs are shown for four OvCA patients and five healthy individuals. Differences between the groups were assessed by a two-sided, unpaired t test (*, p < 0.05; **, p < 0.01). D, PBLs were isolated from OvCA patients...
expression levels, the scores for the relative staining intensities were compared between the various tumor entities using the Kruskal-Wallis test (*, p < 0.05; **, p < 0.01). For flow cytometry data, SDs are indicated as calculated from the raw data by Summit software (DakoCytomation).

**Results**

**MIF is strongly overexpressed in OvCA**

To investigate the in vivo expression of MIF in OvCA, paraffin sections from OvCAs (n = 11, 7 serous, 3 mucinous, 1 endometrioid), borderline tumors (n = 10), or healthy control tissue (n = 11) were stained with the MIF-specific mAb 289 (Fig. 1A). The percentage of MIF-positive tumor cells and the staining intensities were quantified on a scale ranging from 0 to 5 with 0 indicating the absence of staining and 5 being the maximum as outlined in *Materials and Methods*. Average scores were 0.3 ± 0.3 (range, 0–1) for normal ovaries, 1.2 ± 0.8 (range, 0–3) for borderline tumors (p < 0.01), and 4.6 ± 0.4 (range, 3–5) for OvCA (p < 0.001). No significant differences were found between the various OvCA subtypes.

In addition, we performed double stainings using anti-MIF mAb289 in combination with the tumor markers EpCAM (CD326) or cytokeratin 8 and with the macrophage marker CD68. Stainings of the tumor stroma showed that moderate MIF expression colocalizes with CD68 or with endothelial vessel structures. However, MIF levels were much higher in EpCAM (CD326) or cytokeratin 8-positive cells in the tumor center (Fig. 1B).

To quantify MIF mRNA expression relative to normal ovarian tissue (n = 3), solid tissues from borderline tumors (n = 4) and OvCA (n = 17, 9 serous-papillary, 5 mucinous, 3 endometrioid) as well as ascites-derived EpCAM-positive primary OvCA cells (n = 10, 5 serous-papillary, 3 mucinous, 2 endometrioid) were analyzed by quantitative real-time PCR revealing a highly significant overexpression of MIF mRNA in tissue from borderline tumors (7,000-fold on average; range, 4,000–10,000) and further increased MIF levels in solid OvCA tissue (25,000-fold on average; range, 3,300–70,000; Fig. 2A). MIF mRNA expression did not differ between solid tumor tissue and purified ascites-derived primary OvCA cells (18,000-fold overexpression on average; range, 70–49,000). cDNA samples prepared from splenocytes of MIF−/− mice served as negative controls which confirmed that the low MIF levels detected in normal ovarian tissue were still >8000-fold above background.

Immunoblotting of protein lysates from normal ovarian tissue (n = 6), borderline tumors (n = 3), OvCA tissue (n = 8, 5 serous-papillary, 2 mucinous, 1 endometrioid), and ascites-derived primary OvCA cells (n = 10, 5 serous-papillary, 3 mucinous, 2 endometrioid) confirmed increased MIF protein levels in OvCA compared with borderline tumors and normal ovarian tissues (Fig. 2B).

**High MIF levels correlate with the presence of ascites**

To obtain data on the presence of secreted soluble MIF, we measured MIF protein levels in ascitic fluids from 13 patients with OvCA and 5 patients with nonmalignant ascites by ELISA (Fig. 2C). Although MIF levels in malignant OvCA ascites ranged from 0.34 to 11.30 ng/ml (median, 2.70 ng/ml), ascites from patients with liver cirrhosis or cardiac ascites contained only 0.30–0.63 ng/ml (median, 0.50 ng/ml; p < 0.05). To further assess the secretion of MIF by OvCA cells, we placed 2 mg of freshly resected OvCA tissue in 2 ml of medium and measured the resulting MIF concentrations after 72 h of incubation (Fig. 2C). The high amounts of MIF secreted by OvCA tissue (median, 4.5 ng/ml; range, 4.3–4.9 ng/ml; n = 5) may explain the increase in MIF serum levels already reported for OvCA patients (47).

Having found that MIF levels are high in malignant ascites, we wondered whether the formation of ascites might correlate with MIF expression levels in the primary tumor (Fig. 2D). A two-sided, unpaired t test revealed that MIF expression levels were significantly higher in patients who had developed ascites (n = 8) at the time of tumor surgery than in ascites-free patients (n = 7; p < 0.01).

**MIF inhibits NK cell-mediated killing by decreasing NKG2D expression**

To elucidate a functional immunological role for MIF, we investigated whether a blockade of MIF would affect the NK cell-mediated killing of OvCA cells. To this aim, polyclonal NK cell cultures were generated and used as effector cells against 51Cr-labeled primary ascites-derived OvCA cells. Inclusion of a blocking anti-MIF Ab resulted in a moderate but significant increase in target cell lysis (Fig. 3A). In an alternative experimental setup, polyclonal NK cells were treated for 48 h with MIF-containing SN from primary ascites-derived OvCA cells before being used as effector cells against the same primary OvCA targets (Fig. 3B). A comparison with the previous experiment showed that target cell lysis is >50% inhibited by the tumor cell SN. However, this inhibition is greatly relieved when a blocking anti-MIF Ab is present during the preincubation period. To confirm these data with an established permanent cell line, we stably down-regulated MIF in SK-OV-3 cells. Next, we preincubated polyclonal human NK cell cultures with SNs of SK-OV-3 pSUPERpuro control (1.5 ng/ml MIF) or MIF-depleted SK-OV-3 pSUPERpuroMIF (0.3 ng/ml MIF). In line with the results obtained with primary cells, we also found the subsequent lytic activity of NK cells against SK-OV-3 WT cells to be significantly lower when they had been pretreated with MIF-containing SN than when they had been exposed to SN of pSUPERpuroMIF cells (Fig. 3C).

Accordingly, we hypothesized that MIF might down-regulate a receptor involved in the recognition of tumor cells by NK cells. One obvious candidate molecule in this context was NKG2D (30, 31), an activating receptor on NK and a costimulatory receptor on CD8+ T cells. In fact, NKG2D levels on CD8+ T cells and NK cells were determined by flow cytometry right after isolation and after 48 h in vitro culture. A representative experiment is shown. E, PBLs were isolated from OvCA patients (n = 3) and healthy donors (n = 5) and incubated for 48 h in vitro using either normal culture medium (RPMI 1640 with FCS) or cell culture medium with 50% ascites and 10-μg/ml amounts of a blocking anti-MIF Ab or an isotype control. NKG2D expression on CD8+ T cells and NK cells was determined by flow cytometry. To elucidate a functional immunological role for MIF, we investigated whether a blockade of MIF would affect the NK cell-mediated killing of OvCA cells. To this aim, polyclonal NK cell cultures were generated and used as effector cells against 51Cr-labeled primary ascites-derived OvCA cells. Inclusion of a blocking anti-MIF Ab resulted in a moderate but significant increase in target cell lysis (Fig. 3A). In an alternative experimental setup, polyclonal NK cells were treated for 48 h with MIF-containing SN from primary ascites-derived OvCA cells before being used as effector cells against the same primary OvCA targets (Fig. 3B). A comparison with the previous experiment showed that target cell lysis is >50% inhibited by the tumor cell SN. However, this inhibition is greatly relieved when a blocking anti-MIF Ab is present during the preincubation period. To confirm these data with an established permanent cell line, we stably down-regulated MIF in SK-OV-3 cells. Next, we preincubated polyclonal human NK cell cultures with SNs of SK-OV-3 pSUPERpuro control (1.5 ng/ml MIF) or MIF-depleted SK-OV-3 pSUPERpuroMIF (0.3 ng/ml MIF). In line with the results obtained with primary cells, we also found the subsequent lytic activity of NK cells against SK-OV-3 WT cells to be significantly lower when they had been pretreated with MIF-containing SN than when they had been exposed to SN of pSUPERpuroMIF cells (Fig. 3C).

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Because TGF-β exerts similar effects on the NKG2D system (38), we assessed possible interrelations between TGF-β and MIF. TGF-β levels were unaltered in MIF-depleted cells, as assessed by the TGF-β-specific CCL-64 bioassay (Ref. 46 and data not shown). Both a blocking anti-MIF Ab (mAb289) or the TGF-β
A

NKG2D expression

CD8

NK

B

NKG2D expression [SFI]

CD8

NK

no inhibitor SD-208 Mab289

C

NKG2D expression [SFI]

CD8

NK

48 h 48 h+48 h 48 h 48 h+48 h

D

Relative NKG2D mRNA expression

MIF (ng/ml)

TGF-β1 (ng/ml)

E

Lysis [%]

Effector:target ratio

untreated 4 ng/ml MIF

untreated + 48 h recovery

F

NK cells:

untreated + control IgG

untreated + anti-NKG2D

MIF-treated + control IgG

MIF-treated + anti-NKG2D
cells and NK cells. Surface levels of NKp30 were also reduced by 10% FCS before they were also subjected to FACS analysis. A

In human ovarian cancer patients, both MIF and TGF-β may affect NKGD2 expression in vivo. Consequently, we found NKGD2 surface expression levels to be greatly reduced in these patients (Fig. 4C). To obtain more information on the role of MIF in this context, we incubated PBLs from ovarian cancer patients (n = 3) or healthy donors (n = 5) with OvCa ascites (n = 5) or serum (n = 3) in the absence or presence of the blocking anti-MIF or an isotype control Ab. Serum from healthy donors (n = 4) and RPMI 1640 with FCS were also included as controls. These experiments showed that ovarian cancer patients display drastically reduced NKGD2 expression levels on freshly isolated PBL and on ascites-derived tumor-infiltrating lymphocytes (TIL) (Fig. 4, C–E).

However, NKGD2 expression recovers upon brief (48 h) in vitro culture in the absence of tumor-derived factors (Fig. 4, D and E). In contrast, NKGD2 expression on immune cells from healthy donors is decreased by ascites or serum from tumor patients (Fig. 4, D and E). Moreover, when PBL from either donor are cultured in the presence of ascites or serum, addition of a neutralizing anti-MIF Ab partly rescues NKGD2 expression. This effect is more pronounced with serum from tumor patients than from healthy controls (Fig. 4E). This confirms that MIF contained in serum or ascites from OvCa patients is partly responsible for the observed down-regulation of NKGD2, at least under ex vivo conditions. Another part of the effect may be due to TGF-β which was also detected in SNs from primary OvCa cells (median concentration for TGF-β1, 0.5 ng/ml; range, 0.08–1.25 ng/ml; n = 7).

Importantly, the effect of MIF-containing SN or ascites on NKGD2 expression was mimicked by rhMIF, which induced a concentration-dependent down-regulation of NKGD2 on CD8+ T cells and NK cells. Surface levels of NKp30 were also reduced by treatment with TGF-β1 (37), but not by MIF (Fig. 5A, lower right). Neither did MIF (4 ng/ml, 48 h) affect the expression of NKGD2A, NKGD2C, NKp44, NKp46, or NKp80 (data not shown).

To further dissociate MIF-dependent from TGF-β-mediated effects, we incubated PBLs with rhMIF or TGF-β1 (4 ng/ml each) for 48 h in the presence or absence of 1 μM SD-208 or the blocking anti-MIF Ab mAb289 used at 10 μg/ml. For each treatment, only the specific antagonist prevented the reduction of NKGD2 surface levels on CD8+ T and NK cells (Fig. 5B). The increased NKGD2 surface expression on SD-208-treated cells can be explained by SD-208 blocking auto and paracrine effects of lymphocyte-derived TGF-β.

Another difference between MIF and TGF-β became apparent when we investigated the recovery of NKGD2 expression on cytokine-treated cells: The effect of MIF was reversed 48 h after the cells had been switched to RPMI 1640 with 10% FCS + 100 IU/ml IL-2. In contrast, the effect of TGF-β largely persisted even 48 h after the medium exchange (Fig. 5C). This suggests that MIF and TGF-β may exert their effects on NKGD2 through different mechanisms. In fact, PBLs that had been treated with MIF showed a greatly reduced number of NKGD2 transcripts at 24 h (Fig. 5D), whereas NKGD2 mRNA was only slightly down-regulated by TGF-β1 (p = 0.17). Accordingly, MIF seems to have a strong transcriptional effect on NKGD2, whereas TGF-β appears to modulate NKGD2 expression levels largely by posttranscriptional mechanisms.

**Impaired cytotoxic capacity of MIF-treated NK cells is due to an MIF-dependent reduction in NKGD2 expression**

To confirm the functional significance of NKGD2 levels at the surface of NK cells, we first characterized the expression of NKGD2 ligands in SK-OV-3 cells. SFI values were 1.4 ± 0.2 for MICA, 1.1 ± 0.04 for MICB, 1.1 ± 0.1 for ULBP1, 1.9 ± 0.3 for ULBP2 and 1.7 ± 0.1 for ULBP3. Autocrine regulations of NKGD2 ligands (38) were not observed between control-transfected and pSUPERPuroMIF-transfected SK-OV-3 cells (data not shown). Having confirmed that ligands for NKGD2 are expressed by OvCa cells, we preincubated magnetically sorted NK cells for 48 h with 100 IU/ml IL-2 (to activate them) or with 100 IU/ml IL-2 + 4 ng/ml rhMIF or with 500 μl of tumor cell SN in the absence or presence of 10 μg/ml anti-MIF Ab or isotype control. When one-half of the cells were now used as effectors in a 4-h biophotonic lysis assay against SK-OV-3-fLuc cells, the MIF-treated cells showed clearly reduced lytic capacity (p < 0.05; Fig. 5E, top). However, the remaining cells were cultured for a further 48 h in medium low-dose IL-2 (10 IU/ml), but no MIF. Then, a subsequent lysis assay confirmed that the recovery of NKGD2 surface expression coincided with an almost complete restoration of the killing capacity of MIF-treated NK cells (p > 0.05; Fig. 5E, bottom). Likewise, when NK cells had been treated for 48 h with tumor cell SN (in the absence or presence of MIF Ab), MIF-dependent differences in target cell lysis almost disappeared after 48 h of recovery (data not shown). However, SN from primary

**FIGURE 5.** rhMIF inhibits NK cell activity toward OvCa targets through a transcriptional down-regulation of NKGD2. A, PBLs were incubated for 48 h with different concentrations of rhMIF or TGF-β1 (4 ng/ml) before NKGD2 and NKp30 expression levels were quantified as in Fig. 4A. Shown are histograms for NKGD2 in MIF-treated CD8+ T cells and NK cells and a summary of the findings on NKGD2 and NKp30 regulation (n = 5 for NKGD2, n = 3 for NKp30). B, PBL were preincubated with SD-208 (1 μM), blocking anti-MIF mAb 289 (10 μg/ml), or control medium for 1 h before they were treated with 4 ng/ml rhMIF or TGF-β1 for 48 h. SD-208 or the Ab were present during the whole course of the experiment. NKGD2 expression levels were quantified as in A (n = 3; a representative experiment is shown). C, PBLs were treated as indicated with rhMIF or TGF-β1. After 48 h, about one-half of the cells were used to determine NKGD2 expression as in Fig. 4A. The remaining cells were washed and cultured for a further 48 h in RPMI 1640 with 10% FCS before they were also subjected to FACS analysis. D, PBL were incubated for 24 h with different concentrations of rhMIF or TGF-β1 before total cellular RNA was extracted. The RNA was reverse-transcribed by standard techniques and analyzed for NKGD2 expression levels using a SybrGreen-based Taqman assay (n = 3). E, Magnetically sorted NK cells were activated for 48 h with 100 IU/ml IL-2 in the absence or presence of rhMIF (4 ng/ml), before they were incubated for 30 min with a blocking anti-NKGD2 Ab or an isotype control Ab (both used at 10 μg/ml). MIF-treated and untreated NK cells with and without blockade of NKGD2 were then used as effector cells in a 4-h biophotonic lysis assay directed against luc-transfected SK-OV-3 target cells. A representative of three experiments performed in triplicates is shown.
ascites-derived OvCA cells also induced MIF-independent long-term effects, such as reduced viability of NK cells and diminished killing capacity (data not shown).

Although these data show that the down-regulation of NKG2D expression correlates with the impaired lytic activity of MIF-treated NK cells, we also sought to prove a functional relationship between reduced NKG2D levels and killing capacity. Toward this aim, we repeated the 4-h killing assay with MIF-treated NK cells in the absence or presence of a blocking anti-NKG2D Ab (10 μg/ml) or the respective isotype control (Fig. 5F). Again, MIF-treated polyclonal NK cells displayed reduced lytic capacity when compared with untreated NK cells (p < 0.05). The addition of anti-NKG2D inhibited target cell lysis by both untreated (p < 0.01) and MIF-treated NK cells (p < 0.05), which still displayed residual NKG2D expression. Importantly, pretreatment with MIF showed no additional effect when NKG2D was blocked (Fig. 5F), indicating that MIF does not affect the NKG2D-independent lytic activity of the NK effector cells. These data therefore lead to the conclusion that the MIF effect on NKG2D expression is directly responsible for the MIF-induced inhibition of lytic activity.

Discussion

MIF was the first cytokine to be discovered (10), but its diverse role in immunity has only recently been elucidated (12). MIF-knockout mice display no obvious phenotype (48) apart from p53-dependent growth alterations (18). Nevertheless, gene array analysis of MIF-knockdown neuroblastoma cells revealed no less than 166 different genes regulated in an autocrine manner by MIF (49). Its most important physiological functions may be the sustainment of inflammatory reactions (50) and the promotion of tissue repair (11). A role for MIF in autoimmunity has been confirmed because MIF-knockout mice are resistant toward experimentally induced arthritis and autoimmune encephalomyelitis (51, 52). A critical role for MIF in tumor biology is strongly supported by the finding of MIF overexpression may be speculated whether proinflammatory effects exerted by MIF contribute to the formation of malignant ascites. Alternatively, lysophosphatic acid contained in malignant ascites may superinduce MIF levels.

To address a functional immunological role for MIF in OvCA, we generated a SK-OV-3 MIF-knockdown subline that secreted ~80% less MIF than control cells. Viability, proliferation, and morphological appearance of the MIF-depleted cells were unaltered (data not shown). SN from MIF-knockdown cells was considerably less suppressive for NK cytotoxicity than SN derived from control transfectants (Fig. 3C). Likewise, inclusion of a blocking anti-MIF Ab enhanced the NK cell-mediated lysis of primary ascites-derived OvCA cells (Fig. 3A). Moreover, the inhibitory effect of SN from primary ascites-derived OvCA cells could also be relieved by the blockade of MIF (Fig. 3B). Nevertheless, the lytic activity of polyclonal NK cells was still inhibited by OvCA SN even when a blocking anti-MIF Ab was included.

Because NK cell activation depends on the balance between activating and inhibitory signals transmitted by various NK receptors, we hypothesized that MIF might induce the down-regulation of an activating receptor. Flow cytometry and quantitative real-time PCR revealed that MIF decreases NKG2D expression in NK and CD8⁺ T cells (Figs. 4, A and B and 5, A–C). This effect was not restricted to rMIF or MIF contained in the SNs from SK-OV-3 cells. Serum or ascites from OvCA patients also decreased the NKG2D surface expression on CD8⁺ T and NK cells from healthy donors in a MIF-dependent manner. In contrast, serum from control persons had a much weaker effect which corresponds to the lower MIF levels described in the sera of healthy individuals (Fig. 4, D and E). This correlates with reduced NKG2D levels on tumor-infiltrating lymphocytes or PBLs from OvCA patients (Fig. 4, C–E). The reduced NKG2D expression levels on immune cells from tumor patients recover to normal levels when the PBLs are cultured in normal growth medium in vitro. Accordingly, the reduced NKG2D expression levels observed in vivo do not seem to be due to T or NK cell-intrinsic deficiencies in OvCA patients but rather to the presence of OvCA-derived factors including MIF. Not surprisingly, however, immune cells that are treated with tumor ascites or serum still show a moderate down-regulation of NKG2D when MIF is antagonized which indicates that further tumor-associated immunosuppressive cytokines also contribute to the observed effect. Because TGF-β has so far been the only cytokine known to affect NKG2D expression, we confirmed the presence of low to moderate levels of TGF-β1 by ELISA (median concentration, 0.5 ng/ml) and sought to differentiate between effects caused by TGF-β and MIF.

One difference appears to be a much higher specificity of MIF for NKG2D. Whereas NKGA2, NKGC2, Nkp30, Nkp44, Nkp46, and Nkp80 expression levels on NK cells were not affected by MIF, TGF-β also induces a strong down-regulation of Nkp30 (37) as well as an up-regulation of NKGA2 (Fig. 5A and data not shown). The recovery of NKG2D levels on cytokine-treated cells also seems to follow a different kinetics: Whereas NKG2D expression on MIF-treated cells is restored to control levels within 48 h after withdrawal of the cytokine, TGF-β shows a more persistent effect (Fig. 5C). Mechanistically, we show that MIF inhibits the transcription of NKG2D mRNA, whereas TGF-β appears to exert its effect on NKG2D mainly via posttranscriptional mechanisms (Fig. 5D). In accordance with these data, both a blocking anti-MIF Ab and the TGF-β receptor kinase inhibitor SD-208 partly prevented the down-regulation of NKG2D by tumor cell SN, with additive effects (Fig. 4B) (46). SD-208, however, did not prevent
the reduction of NKG2D levels when PBLs were treated with rh-MIF (Fig. 5B). Thus, MIF and TGF-β must use different receptor mechanisms to suppress NKG2D expression. Although we have not identified the 5D-208-insensitive receptor involved in the MIF-dependent down-regulation of NKG2D, CD74 can be excluded because it is not found on NK or CD8+ T cells. This leaves as primary candidates the noncognate MIF receptors CXCR2 and CXCR4 which are expressed by resting and activated NK and CD8+ T cells. In fact, a blocking Ab against CXCR2 could partly prevent the effect of MIF on NKG2D expression (refs. 14, 21, and 22 and data not shown).

To test whether NKG2D expression on NK cells really correlates with lytic activity against OvCA targets, we performed killing assays using MIF-treated IL-2-activated NK cells either immediately after 48 h of exposure to the cytokine or after an additional 48 h of recovery in medium with IL-2 (100 IU/ml). As predicted, the initially reduced killing capacity of MIF-treated NK cells was largely restored after the recovery period (Fig. 5E). When (instead of the recombiant cytokine) MIF-containing tumor cell SN was used, addition of anti-MIF Ab also attenuated the inhibition of NK cell-lytic activity at 48 h. Again, the MIF-dependent differences in target cell lysis lost their significance after additional 48 h of culture in fully supplemented RPMI 1640 + IL-2. However, OvCA SN also induced MIF-independent long-term effects including significant cell death and reduced lytic capacity (data not shown).

To further confirm the functional importance of MIF-induced down-regulation of NKG2D, we treated purified NK cells with MIF and performed a lysis assay in the presence of either a control or a blocking anti-NKG2D Ab (Fig. 5F). This showed that the blocking of NKG2D by Ab represses the cytolytic activity of NK cells even more efficiently than treatment with MIF, suggesting that the low levels of NKG2D remaining on the surface of MIF-treated cells are still sufficient to mediate some activation and ensuing killing activity. An important observation, however, was that there was no remaining MIF-dependent effect when NKG2D was blocked by neutralizing Abs. Accordingly, MIF does not seem to affect the residual NKG2D-independent lytic activity of NKG2D-blocked NK cells which illustrates that the effect of MIF on lytic activity is mediated by the induced loss of NKG2D.

Taken together, our study is the first to demonstrate that MIF expression in OvCa increases with the grade of malignancy. Functionally, we demonstrate that MIF inhibits antitumor immunity by down-regulating NKG2D on NK and CD8+ T cells. Autocrine effects of MIF on the progression of OvCa have not been investigated, but are likely. Thus, MIF inhibitors that are currently being developed for the treatment of rheumatoid arthritis (39) may become promising agents also for the treatment of OvCa.

Disclosures
The authors have no financial conflict of interest.

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