

# MIF homologues from a filarial nematode parasite synergize with IL-4 to induce alternative activation of host macrophages

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**Abstract:** Macrophage migration inhibitory factor (MIF) is a highly conserved cytokine considered to exert wide-ranging, proinflammatory effects on the immune system. Recently, members of this gene family have been discovered in a number of invertebrate species, including parasitic helminths. However, chronic helminth infections are typically associated with a Th2-dominated, counter-inflammatory phenotype, in which alternatively activated macrophages (AAMs) are prominent. To resolve this apparent paradox, we have analyzed the activity of two helminth MIF homologues from the filarial nematode *Brugia malayi*, in comparison with the canonical MIF from the mouse. We report that murine MIF (mMIF) and *Brugia* MIF proteins induce broadly similar effects on bone marrow-derived mouse macrophages, eliciting a measured release of proinflammatory cytokines. In parallel, MIF was found to induce up-regulation of IL-4R on macrophages, which when treated in vitro with MIF in combination with IL-4, expressed markers of alternative activation [arginase, resistin-like molecule  $\alpha$  (RELM- $\alpha$ ) or found in inflammatory zone 1, Ym-1, murine macrophage mannose receptor] and differentiated into functional AAMs with in vitro-suppressive ability. Consistent with this finding, repeated in vivo administration of *Brugia* MIF induced expression of alternative macrophage activation markers. As mMIF did not induce RELM- $\alpha$  or Ym-1 in vivo, alternative activation may require components of the adaptive immune response to *Brugia* MIF, such as the production of IL-4. Hence, MIF may accentuate macrophage activation according to the polarity of the environment, thus promoting AAM differentiation in the presence of IL-4-inducing parasitic helminths. *J. Leukoc. Biol.* 85: 844–854; 2009.

**Key Words:** infection · cytokines

## INTRODUCTION

Mammalian migration inhibitory factor (MIF) was among the first immune system cytokines to be discovered as a soluble factor released from activated lymphocytes that inhibited ran-

dom migration of macrophages [1, 2]. The designation of macrophage MIF has endured, although MIF is now recognized as being highly pleiotropic in regulation of innate and adaptive [3, 4] immune cell populations, as well as cells of the neuroendocrine system. Equally, MIF is notable for its production by a wide range of cell types, locally by macrophages [5], T cells [3], and eosinophils [6], as well as systemically by the anterior pituitary gland [7].

There are compelling data from a variety of systems that MIF acts in a strongly proinflammatory manner [8, 9]. In vitro, exogenous MIF elicits the release of IL-6, IL-8, TNF- $\alpha$ , and endogenous MIF secretion from human monocytes and mouse macrophages. In vivo, MIF synergizes with LPS to accentuate toxic shock [7]. Most strikingly, MIF deficient (MIF<sup>0/0</sup>) mice survive the normally lethal administration of LPS or staphylococcal enterotoxin [10]. Similarly, MIF<sup>0/0</sup> animals do not succumb to 2,4,6-trinitrobenzene sulfonic acid-induced colitis [11], demonstrating that MIF is required in sustained inflammatory disease as well as in the acute setting of septic shock. Not only Th1-type but also Th2-dominated allergic responses are diminished in the absence of MIF [12, 13], which may be essential in resistance to bacterial infection. Not only does it up-regulate innate pattern receptors such as TLR4 [14], but MIF<sup>0/0</sup> mice die following low-dose *Salmonella typhimurium* infection [15]. The same MIF-deficient animals are more susceptible to a protozoan (*Leishmania major* [16]) and helminth (*Taenia crassiceps* [17]) infection, albeit a helminth infection where parasite clearance is mediated by proinflammatory cytokines [18]. Against this background of a spectrum of immune reactivities promoted by MIF, it is perhaps surprising that many tumors also produce this cytokine [19], and in the setting of cancer, MIF may act in an immunosuppressive capacity [20–24].

MIF has two unusual biochemical properties. First, it encodes no signal sequence [25] but is secreted by an alternative

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route requiring an ABC-containing transporter protein [26]. Second, MIF is unique in combining cell-stimulatory activity (cytokine) with two enzymatic activities within a relatively small protein [25], namely a tautomerase, which is dependent on Pro-2 [27], and an oxidoreductase, dependent on cysteine residues 57 and 60, forming a thioredoxin-like motif [28]. The signaling pathway for MIF-induced effects has proven more enigmatic [4, 29, 30]. CD74 (invariant chain) is one receptor for MIF that can, via CD44, activate intracellular MAPK pathways [31–33]. MIF can also bind the chemokine receptors CXCR2 and CXCR4, which are expressed on many CD74-negative cell types, linking it more closely to chronic inflammation such as atherosclerosis [34]. However, in the cytoplasm, MIF also binds c-Jun activation domain-binding protein-1 (Jab1), preventing the latter from activating the proinflammatory transcription factor AP-1 [35]. Hence, in some circumstances, MIF can exert an anti-inflammatory effect. Although this activity may require relatively high MIF concentrations [30], studies on tumors have suggested that immune evasion results, directly or indirectly, from high levels of MIF production [22].

In this context, the discovery of MIF secreted by parasites, which establish a broadly counter-inflammatory environment [36, 37], is particularly intriguing. Thus, in the filarial nematode parasite *Brugia malayi*, two MIF homologues are present, *Brugia* (*Bm*)-MIF-1 and -2, with, respectively, 40% and 27% identity with the mammalian proteins [38, 39]. As these levels of amino acid identity were relatively low, it was possible that the parasite homologues were functionally different from the mammalian ones. We therefore compared *Brugia* and mammalian MIFs, to test whether parasite proteins would antagonize the mammalian ones or even stimulate host cells in a different way altogether. Surprisingly, tests with human monocytes showed that like human MIF (hMIF), parasite MIF proteins induce a proinflammatory profile of cytokines such as TNF- $\alpha$ , IL-8, and even hMIF [39]. Thus, parasite MIF induces host cells to release more MIF of endogenous origin. Moreover, *Brugia* MIFs were found to be biochemically and structurally similar to mammalian MIFs. *Bm*-MIF-1/2 have high levels of dopachrome tautomerase enzyme activity, although some significant differences in substrate preference were observed [39]. The three-dimensional crystal structure of *Bm*-MIF-2 [39], like that of hookworm MIF [40], is remarkably similar between host and parasite molecules. It should also be noted that *Bm*-MIF-2 has neither the Cys-57 nor -60 associated with oxidoreductase activity but retains similar *in vitro* cytokine activity to *Bm*-MIF-1 in which these residues are conserved [39].

The synergy between MIF and LPS in the induction of TNF- $\alpha$  and IL-6 suggests a role in the classical activation of macrophages by microbial products and proinflammatory cytokines. Yet, over recent years, it has become apparent that in helminth infection, macrophages develop a distinct activation state that is dependent on the Th2 cytokines, IL-4, and IL-13 [36, 41–44]. These “alternatively activated macrophages” (AAMs) are characterized by high-level transcription of arginase type I, the chitinase-like molecule Ym-1 and resistin family member, resistin-like molecule  $\alpha$  {RELM- $\alpha$ ; or found in inflammatory zone 1 (Fizz1) [45–48]}, and surface expression of the C-type lectin family member mannose receptor (MR)

[49]. *Brugia* infection of mice has proved a valuable model for the *in vivo* study of alternative macrophage activation, as large numbers of AAMs arise within 7 days of live adult *Brugia* transplantation into the peritoneal cavity. These macrophages suppress proliferation of a range of target cells through a contact-dependent mechanism that is dependent on IL-4 [46, 47, 50–52].

Because of the prominence of these novel macrophages in the response to *Brugia* and the known activity of MIF for macrophages, we injected *Bm*-MIF-1 (free of detectable LPS) nine times over 3 weeks into the peritoneal cavity [45]. The infiltrating population of cells was then tested: Although the macrophages were not directly suppressive, they expressed Ym-1, and a threefold rise in eosinophils was also observed, consistent with the description of Ym-1, as an eosinophil chemotactic factor [53] and the recent evidence that AAMs are involved in recruiting eosinophils to peripheral sites [54]. Significantly, such changes were not observed with *Bm*-MIF-1G mutant recombinant protein nor with LPS at a dose corresponding to that which would have been undetectable in the original MIF-1 preparation.

These findings have posed a central paradox: Why should parasites that appear masterful at avoiding inflammatory attack [36, 55, 56] secrete molecules that are thought to amplify inflammation? In this study, we questioned whether parasite MIFs do in fact differ functionally from mammalian cytokines and in particular, how in the context of a parasite-induced Th2 response, MIF exposure can promote the generation of AAMs.

## MATERIALS AND METHODS

### Bone marrow-derived macrophages (BMDM)

BM cells were taken from femoral bones of C57BL/6 mice and cultured in DMEM supplemented with 20% FCS, 2 mM L-glutamine, 0.25 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 20% L929 cell-conditioned medium as a source of M-CSF. At Day 6, culture plates were washed once with PBS at 37°C to remove nonadherent cells before detaching adherent macrophages with 3 mM EDTA and 10 mM glucose in PBS. Macrophages were then replated in 24- or 96-well plates at the appropriate concentration and allowed to adhere overnight prior to treatment. Cell preparations were typically >85% F4/80-positive.

### Recombinant MIFs (rMIFs)

The pET29 system (Novagen, San Diego, CA, USA) was used for bacterial expression of recombinant 6-His-tagged proteins as described previously [39]. Mouse MIF (mMIF) was produced in the same system, using a coding insert provided generously by Dr. Patrick Skelly (Harvard University, Cambridge, MA, USA). mMIF-G was constructed by altering the codon for Pro-2 within a PCR primer as described [39]. Plasmid DNA was transformed into *Escherichia coli* BL21(DE3), single colonies were grown at 37°C, and recombinant protein expression induced in the presence of 1 mM isopropylthiogalactoside. Recombinant proteins were recovered by sonication in 20 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 0.5 M NaCl, 10 mM imidazole, followed by nickel-chelating affinity chromatography on an ÄKTAprime system (Amersham Biosciences, Piscataway, NJ, USA). To avoid effects as a result of any endotoxin contamination, recombinant proteins were treated with 20  $\mu$ g/ml Polymixin B sulfate (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 37°C prior to use in cellular assays.

### *In vitro* stimulation

For *in vitro* stimulation, 10<sup>6</sup> macrophages were incubated with 10  $\mu$ g/ml each recombinant protein. For some experiments, macrophages were cotreated with IL-4 at different concentrations (0.2–100 ng/ml), as indicated in the text.

TABLE 1. Primers Used for Real-Time PCR

Primer	Position (nt, exon)	Sequence
$\beta$ -actin-for		5'-TGGAATCCTGTGGCATCCATGAAAC-3'
$\beta$ -actin-rev		5'-TAAAACGCAGCTCAGTAACAGTCCG-3'
ym-1-for		5'-TCACAGGTCTGGCAATTCTCTG-3'
ym-1-rev		5'-TTTGTCCCTTAGGAGGGCTTCCTC-3'
RELM $\alpha$ -for		5'-GGTCCCAGTGCATATGGATGAGACCATAGA-3'
RELM $\alpha$ -rev		5'-CACCTCTTCACTCGAGGGACAGTTGGCAGC-3'
arginase-1-for		5'-CAGAAGAAATGGAAGAGTCAG-3'
arginase-1-rev		5'-CAGATATGCAGGGAGTCACC-3'
inos-for		5'-GCATTTGGGAATGTAGACTG-3'
inos-rev		5'-GTTGCATTGGAAGTGAAGCGTTTC-3'
IL-4R $\alpha$ -for		5'-GCTACAAGCCCTTCCAGAAATCC-3'
IL-4R $\alpha$ -rev		5'-ACAATACCAAAGCCCAGGTGATC-3'
IFN $\gamma$ R1-for		5'-ATGTGGAGCATAACCCGGAGT-3'
IFN $\gamma$ R1-rev		5'-GGTAAGAGGAGCAACCACCA-3'
GAPDH-for		5'-TTCCAGTATGACTCCACTCACGG-3'
GAPDH-rev		5'-GCCCTTCCACAATGCCAAAG-3'
mMR-for		5'-CTCGTGGATCTCCGTACAC-3'
mMR-rev		5'-GCAATGGAGCCGTCTGTGC-3'

for, Forward; rev, reverse.

## In vivo stimulation

BALB/c mice or IL-4R $\alpha^{-/-}$  mice on the BALB/c background were injected i.p. with 1  $\mu$ g/ml each recombinant protein, diluted in PBS (pyrogen-free, Sigma Chemical Co., D8537). Control animals were injected with PBS alone. Injections were given twice a week for a total of nine injections.

## Flow cytometry for surface markers

Cells were stained at 24 h post-treatment with the following reagents for surface activation markers: MHC-II (clone 2G9, rat IgG2a), CD40 (clone 3/23, rat IgG2a), CD80 (clone 16-10A1, Armenian hamster IgG2K), and CD86 (clone GL1, rat IgG2a; all from Becton Dickinson, San Jose, CA, USA; used at 1:200 dilution) and F4/80 (clone BM8, rat IgG2a, Caltag, South San Francisco, CA, USA). Appropriate isotype controls with same fluorophore were used (Becton Dickinson and Caltag). Cells were analyzed using a FACSCaliber (Becton Dickinson) and FlowJo software, Version 4.5.9.

## Cytokine ELISAs

Supernatants were collected for cytokine measurement by sandwich ELISA; IL-6 (capture: MP5-20F3; detection: MP5-32C11; top standard: 100 ng/ml), IL-10 (capture: JES5-2A5; detection: SXC-1; top standard: 10 ng/ml), IL-12p40 (capture: C15.6; detection: C17.8; top standard: 200 ng/ml), and IL-12p70 (capture: 9A5; detection: C17.8; top standard: 10 ng/ml) were from PharMingen (San Diego, CA, USA), and TNF- $\alpha$  (DY410; top standard: 2 ng/ml) was from R&D Systems (Minneapolis, MN, USA).

## Western blot

Samples were mixed with 4 $\times$  SDS sample buffer (Invitrogen, Carlsbad, CA, USA) containing 2-ME. Samples were heat-denatured and resolved using precast 10% gradient Bis-Tris-NuPAGE gels (Invitrogen), which were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), blocked with 5% milk in TBS-0.1% Triton X-100-0.05% Tween 20 (TBSTT), followed by incubation with the appropriate antibodies. Anti-rMIF mouse polyclonal serum, raised by immunization of MIF-deficient mice with recombinant protein, was used at a 1:200 dilution in 5% milk TBSTT, followed by total anti-mouse IgG-HRP conjugate (Dako, Carpinteria, CA, USA) at 1:2000 dilution. ECL substrate (Amersham Biosciences) was used for detection.

## RNA extraction and real-time PCR

Following treatment, 1  $\times$  10<sup>6</sup> macrophages were collected in 1 ml TRIzol (Invitrogen), and RNA extraction was performed following the manufacturer's protocol, transcribing 1  $\mu$ g RNA using Moloney murine leukemia virus RT

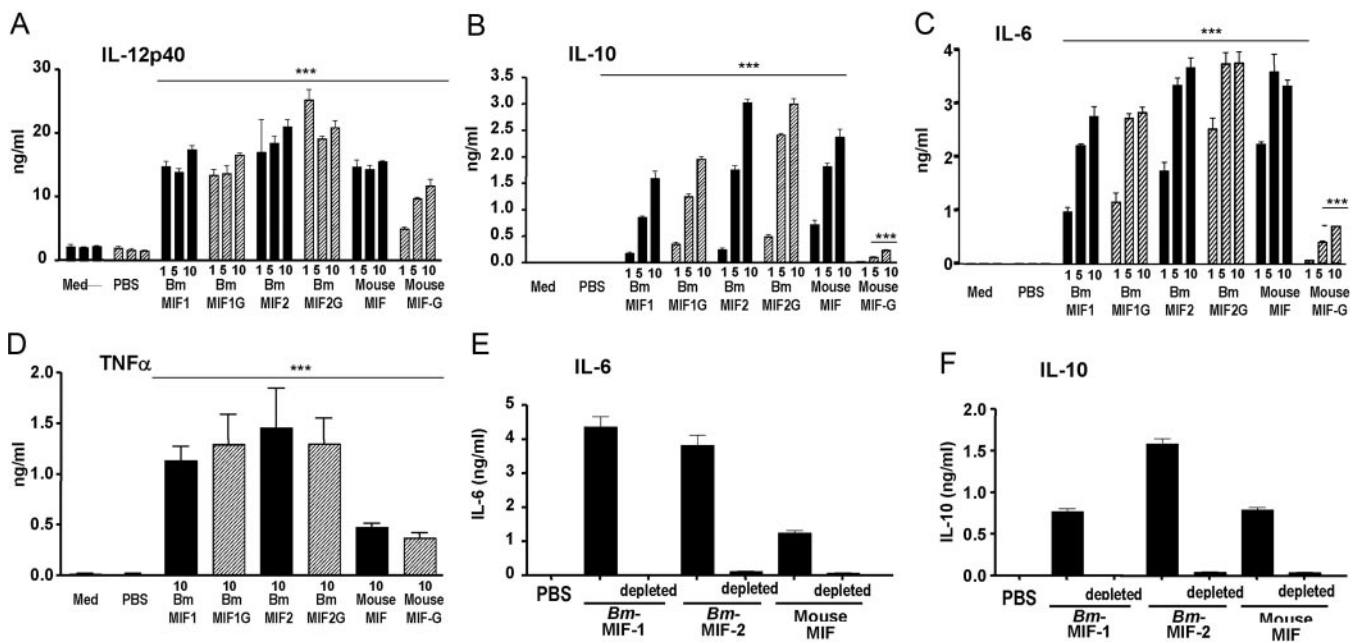
(Stratagene, La Jolla, CA, USA). Ym-1, RELM $\alpha$ , Arginase-1, inducible NO synthase (iNOS), murine macrophage MR (mMR), IL-4R $\alpha$ , and IFN- $\gamma$ R1 RNA levels were measured by real-time PCR using LightCycler (Roche Molecular Biochemicals, Indianapolis, IN, USA) or Chromo4 (Genetic Research Instrumentation Ltd., UK) real-time PCR machines.  $\beta$ -Actin or GAPDH were used as reference genes. Primer sequences are given in **Table 1**. LightCycler PCR amplifications were carried out in 10  $\mu$ l, containing 1  $\mu$ l cDNA, 4 mM MgCl<sub>2</sub>, 0.3  $\mu$ M primers, and the LightCycler-DNA SYBR Green I mix (Roche Molecular Biochemicals). *Ym-1* was amplified using the following conditions: 30 s denaturation at 95°C, 5 s annealing of primers at 63°C, and 12 s elongation at 72°C for 40–60 cycles. The fluorescent DNA-binding dye SYBR Green (Roche Molecular Biochemicals) was monitored after each cycle at 85°C. For all other primer pairs, the annealing temperature was 55°C, and SYBR Green fluorescence was monitored at 86°C. Chromo4 PCR reactions were carried in 10  $\mu$ l containing 1  $\mu$ l cDNA, 0.3  $\mu$ M primers, and SYBR Green Supermix-uracil DNA glycosylase (Invitrogen). The following conditions were used: 15 min hot start at 95°C, followed by denaturation at 95°C for 20 s, primer annealing at 55°C for 20 s, and elongation at 72°C 20 s for 50 cycles. The fluorescent DNA-binding dye SYBR Green was monitored after each cycle at 80°C. Expression levels were estimated using the absolute quantitation method by comparison with a standard curve generated from a pool of all samples diluted appropriately. Relative expression of the gene of interest was then calculated as the ratio to a housekeeping gene that remained unaltered after treatment. For some experiments, fold-increase over untreated macrophages was calculated.

## Suppression assay

BMDM were treated with different MIF proteins in the absence or presence of 20 ng/ml IL-4 for 24 h. After this time, supernatants were removed, and EL4 cells were then added at a ratio of 1:10 macrophages, and cells were cocultured for a further 72 h when proliferation was measured by tritiated-thymidine incorporation.

## Anti-MIF mAb production

BALB/c mice were immunized on Day 0 with 100  $\mu$ g alum-precipitated *Bm*-MIF-1 and subsequently challenged i.v. with 1  $\mu$ g protein on Days 28–30. On Day 31, splenocytes were recovered and fused with SP2 myeloma cells. On Day 7 after fusion, cells were screened against recombinant protein by ELISA. Cells from positive wells were cloned by limiting dilution in 96-well plates. Cells were screened and cloned a second time, and selected clones expanded for antibody production. For antibody purification, cells were grown in 500 ml RPMI supplemented with low IgG FCS in disposable bioreactors (VectraCell single-use bioreactor system, BioVectra, Canada). Cells were cultured for 3–4



**Fig. 1.** In vitro cytokine release from MIF-stimulated macrophages. (A–C) Seven-day C57BL/6 BMDM were treated with 1, 5, or 10 µg/ml rMIF proteins overnight, and supernatants were harvested for IL-12p40 (A), IL-10 (B), and IL-6 (C) analysis by ELISA. “G” proteins represent P2G site-directed mutants, in which tautomerase enzyme activity has been ablated by replacement of Pro-2 with Gly. In direct comparisons of macrophages stimulated with 100 ng/ml LPS, MIF proteins elicited similar or lower responses (e.g., IL-12p40, LPS stimulation  $9.0 \pm 2.9$  ng/ml and 10 µg/ml *Bm*-MIF-1  $7.7 \pm 3.1$  ng/ml; IL-6, LPS stimulation  $8.8 \pm 3.0$  ng/ml and *Bm*-MIF-1  $4.3 \pm 1.1$  ng/ml), and responses to MIF were not inhibited by Polymixin B treatment, which ablated responses to LPS completely. (D) TNF-α cytokine release from BMDM stimulated with 10 µg/ml rMIF proteins, which (E and F) were removed from the indicated preparations with a specific mAb (clone 2F11C9), and BMDM were treated with 10 µg/ml rMIF or the equivalent volume of the depleted fraction. IL-6 (E) and IL-10 (F) release was measured after 24 h. Data were analyzed by one-way ANOVA. \*\*\*,  $P < 0.001$ , compared with PBS treatment.

weeks to allow maximum antibody production. Supernatants were harvested and spun down to remove cells. mAb were purified using a protein-G sepharose column and the ÄKTAprime system (Amersham Biosciences). Antibody was eluted using 1 M glycine, pH 2.9, into neutralizing buffer (Tris, pH 9). Purified antibodies were dialyzed against PBS, filtered under sterile conditions, and frozen for storage.

### MIF mAb depletion

MIF recombinant proteins were incubated with 10× excess (w/w) 2F11C9 mAb that recognizes *Brugia* and mouse homologues for 2.5 h at 4°C. Immunocomplexes were precipitated using protein-G agarose/sepharose beads (Upstate, Lake Placid, NY, USA), which were spun down, and supernatants were subjected to a second round of immunoprecipitation to ensure complete protein removal.

### Statistical tests

One-way ANOVA was performed on log-transformed replicate data, and different treatments were compared by Bonferroni post-test using GraphPad Prism 4 software.

## RESULTS

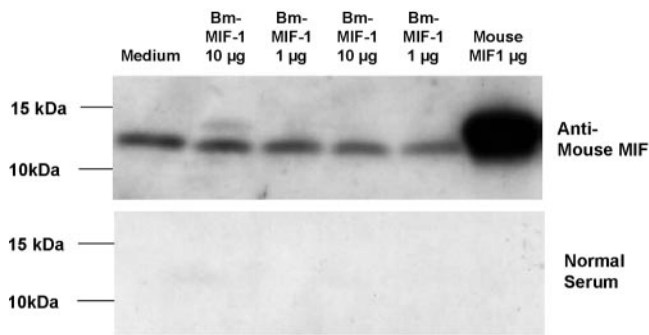
### Stimulation of different macrophage populations by MIFs

To compare the functions of mouse and helminth MIF homologues, we first treated murine BMDM with rMIF proteins for 24 h and assayed cytokine release as a measure of activation. The three MIF proteins (*Bm*-MIF-1, *Bm*-MIF-2, and mMIF) exerted similar effects in this regard, with IL-12p40 release at all doses tested (Fig. 1A) and additional IL-10 production at

higher MIF concentrations (Fig. 1B). It was noted that the tautomerase-deficient mutants *Bm*-MIF-1G and -2G acted with similar potency to the wild-type proteins, and the mutant mMIF-G was greatly attenuated in effect. All MIF products tested also induced IL-6 (Fig. 1C) production and as has been noted previously for *Bm*-MIF-1 and -2 with human monocytes [39], TNF-α (Fig. 1D). Neither IL-12p70 nor IL-23 was produced at any detectable level (data not shown). Quantitatively, levels of IL-12p40, IL-10, and IL-6 released were of a similar magnitude to, but lower than, those elicited by LPS stimulation and resistant to Polymixin B inhibition. A similar pattern of cytokine release was observed with thioglycollate-elicited peritoneal and purified splenic macrophage populations (data not shown). Activation was also assessed by flow cytometry, and although neither mouse nor parasite MIF altered expression of the costimulatory molecules CD40, CD80, or CD86, a reduction in MHC-II expression was observed after treatment with mMIF or *Bm*-MIF-2 (data not shown). MIF-stimulated macrophages were also tested for production of NO by assay of culture supernatants by the Greiss reaction, but levels were not found to be significantly above background amounts (data not shown).

### MIF depletion from the recombinant preparations abolishes cytokine production

To verify that macrophage stimulation was specific for the MIF proteins, we depleted MIF from each preparation with a mAb (2F11C9) specific for, and cross-reactive between, mammalian and parasite MIF proteins (see Materials and Methods). Ali-



**Fig. 2.** Macrophages were treated with the indicated proteins and supernatants collected after 24 h and separated on SDS-PAGE for Western blot analysis probed with polyclonal antibody to mMIF, which does not cross-react with parasite MIF (upper panel) or control normal serum (lower panel). Note that the strong band in mMIF-treated supernatant is a result of residual recombinant protein in the supernatant reacting with the antibody.

quots were subjected to two rounds of incubation with an excess of antibody for 2 h at 4°C, following which, immunocomplexes were removed on protein-G sepharose beads. SDS-PAGE analysis confirmed that all detectable MIF protein had been removed by this procedure, and samples no longer containing MIF were unable to stimulate macrophages, as judged by the abolition of IL-6 (Fig. 1E) and IL-10 (Fig. 1F) responses.

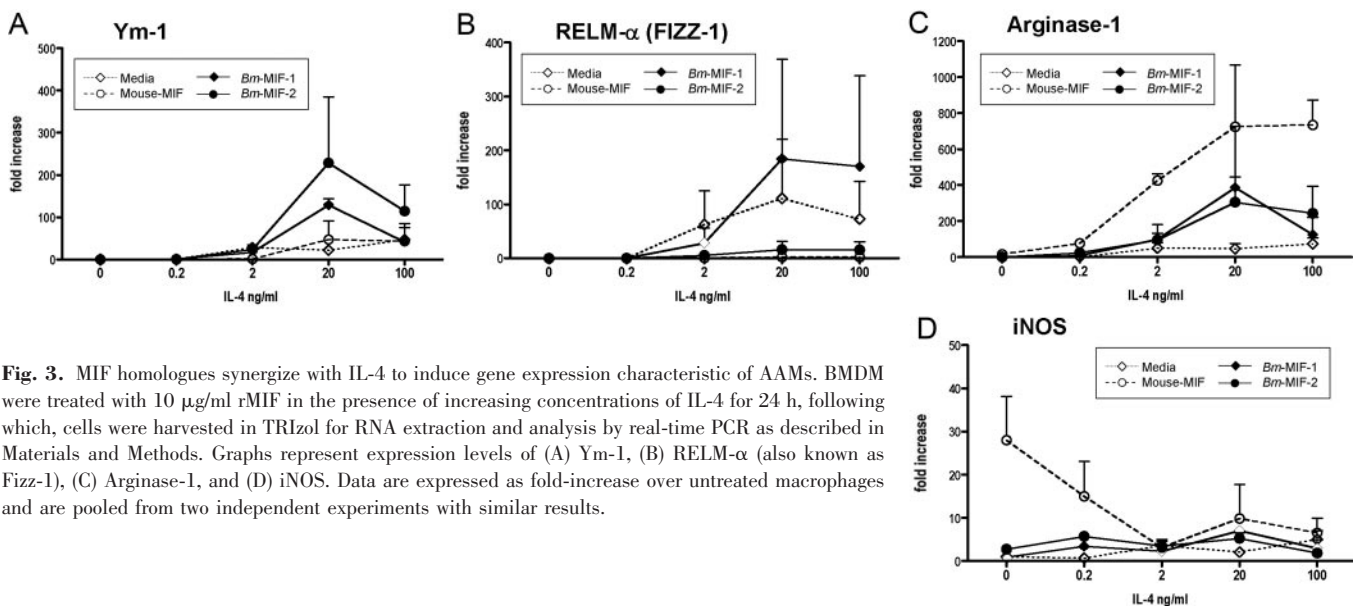
### Filarial MIF homologues do not induce production of endogenous mMIF

As exposure of macrophages to MIF homologues from parasites might elicit secretion of endogenous mMIF, thereby acting indirectly in these experiments, we probed culture supernatants from stimulated macrophages with an antibody specific for mMIF. We noted a constitutive level of MIF secretion from unstimulated cells, which was not increased in the presence of parasite MIF proteins (Fig. 2), indicating that the effects observed can be attributed directly to parasite MIF homologues rather than through the murine cytokine.

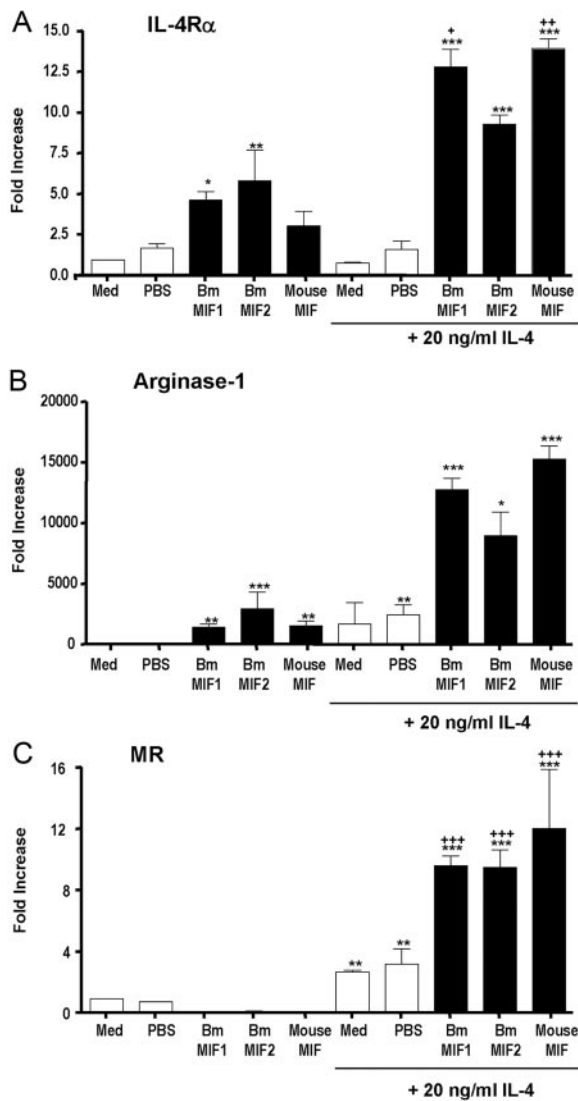
## MIF homologues enhance alternative activation of macrophages induced by IL-4

We have described previously the ability of *Bm*-MIF-1, when administered in vivo, to promote the alternative activation of macrophages [45]. We first attempted to reproduce this effect in vitro by incubating MIF homologues with BMDM. As markers for alternative activation, we measured expression of Ym-1, RELM- $\alpha$ , Arginase-1 [45–48], and mMMR [57] by real-time PCR. In these experiments, however, no up-regulation of alternative activation marker genes was detected when incubating macrophages with increasing concentration of parasite MIF (data not shown), raising the question of whether other factors present in vivo might be required in our in vitro system. As macrophage expression of Ym-1, RELM- $\alpha$ , and Arginase-1 is induced by IL-4 in vivo and in vitro [47, 58, 59], we tested the response of macrophages cultured with MIF in the presence of increasing concentrations of IL-4. After overnight treatment, cells were collected in TRIzol for RNA extraction, and expression levels of Ym-1, RELM- $\alpha$ , and Arginase-1 were measured by real-time PCR. In addition, we assayed iNOS as a marker of classical macrophage activation.  $\beta$ -Actin and GAPDH were used as reference genes and showed equivalent results.

**Figure 3, A–C,** shows that although MIF homologues alone were unable to induce expression of alternative activation markers, a marked synergy occurred in the presence of IL-4, reaching an optimal level at 10  $\mu$ g/ml rMIF with 20 ng/ml IL-4. Interestingly the synergistic effect of the different MIF homologues differed between mammalian and filarial proteins. Although *Bm*-MIF-1 and -2 preferentially enhanced Ym-1 expression (6.5- and 14.8-fold over 20 ng/ml IL-4), mMIF with IL-4 induced higher Arginase-1 levels (17.2-fold increase over 20 ng/ml IL-4 compared with 6.1 and 7.4 induced by *Bm*-MIF-1 and *Bm*-MIF-2, respectively). Perhaps most strikingly, mMIF alone induced iNOS in macrophages, which in the presence of IL-4, was abolished in favor of the production of arginase transcripts (Fig. 3D). Only RELM- $\alpha$  showed no clear



**Fig. 3.** MIF homologues synergize with IL-4 to induce gene expression characteristic of AAMs. BMDM were treated with 10  $\mu$ g/ml rMIF in the presence of increasing concentrations of IL-4 for 24 h, following which, cells were harvested in TRIzol for RNA extraction and analysis by real-time PCR as described in Materials and Methods. Graphs represent expression levels of (A) Ym-1, (B) RELM- $\alpha$  (also known as Fizz-1), (C) Arginase-1, and (D) iNOS. Data are expressed as fold-increase over untreated macrophages and are pooled from two independent experiments with similar results.



**Fig. 4.** MIF homologues up-regulate expression of IL-4R and MR. BMDM were treated with 10  $\mu\text{g/ml}$  rMIF in the presence or absence of 20 ng/ml IL-4 for 24 h, following which, cells were harvested and analyzed as above for expression levels of (A) IL-4R $\alpha$ , (B) arginase, and (C) MR. Expression levels are calculated in arbitrary units relative to a housekeeping gene control (GAPDH). Statistical significances were assessed by one-way ANOVA of transformed (log) data, including the Bonferroni post-test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with medium alone; +,  $P < 0.05$ ; ++,  $P < 0.01$ ; +++,  $P < 0.001$ , compared with corresponding group without IL-4.

synergies with MIF, as this was induced strongly by IL-4 alone (black line in Fig. 3B).

### MIF homologues induce up-regulation of IL-4R $\alpha$

Macrophage alternative activation is dependent on IL-4R $\alpha$  [42, 43]. To elucidate whether the mechanism by which MIF enhances IL-4 activation involves IL-4R $\alpha$  up-regulation, BMDM were cotreated with 20 ng/ml IL-4 and 10  $\mu\text{g/ml}$  MIF and IL-4R $\alpha$  expression measured by RT-PCR. All three MIF homologues induced IL-4R $\alpha$  expression in the absence of IL-4, although in the case of mMIF, the change did not attain statistical significance (Fig. 4A). Importantly, treatment of macrophages with IL-4 alone did not raise IL-4R $\alpha$  mRNA levels, and it required the combination of both ligands to

achieve a substantial increase in expression levels; this was true for all MIF homologues (Fig. 4A). IL-4R $\alpha$  induction was observed in the presence of wild-type and tautomerase-deficient proteins. The synergy between MIF and IL-4 was again evident in the induction of arginase (Fig. 4B) and of the FMR (Fig. 4C), which is an additional marker of alternative activation of macrophages [49]. Arginase and MR levels were elevated in the presence of IL-4 alone but were greatly enhanced on addition of any of the MIF proteins tested.

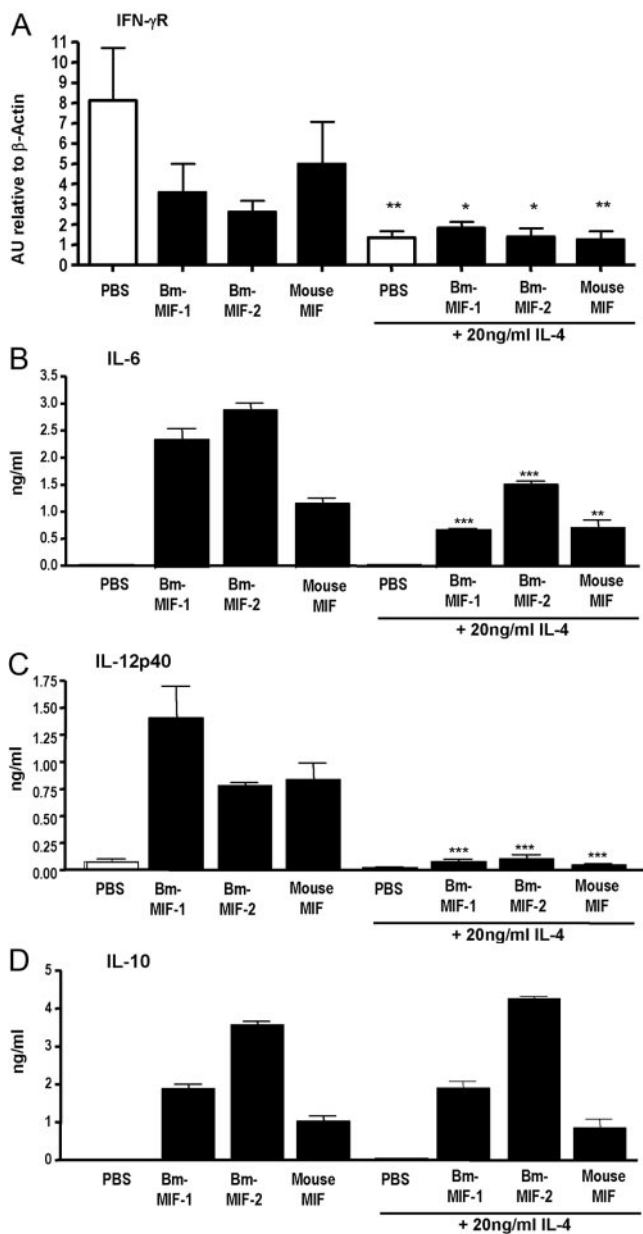
In contrast to the strongly synergistic enhancement of IL-4R $\alpha$  in macrophages by MIF proteins, similarly treated cells did not up-regulate the IFN- $\gamma$ R (Fig. 5A). Indeed, a small reduction in transcript levels appears to accompany MIF exposure (although this did not reach statistical significance), and IL-4 itself was necessary and sufficient to suppress transcript levels. IL-4 was also found to inhibit the ability of MIF to induce proinflammatory IL-6 and IL-12p40 (Fig. 5, B and C), while having no effect on the macrophage IL-10 response to MIF proteins (Fig. 5D). As IL-4 alone cannot stimulate IL-10 production, the combination of MIF stimulation and selective inhibition by IL-4 results in a strongly polarized, counter-inflammatory outcome.

### MIF homologues synergize with IL-4 to suppress EL4 proliferation

A notable feature of AAMs is their ability to suppress T cell proliferation, an important characteristic during helminth infection [44, 52, 60, 61]. To test whether MIF homologues are involved in the induction of this key functional characteristic, BMDM were treated as before with MIF in the presence or absence of 20 ng/ml IL-4 overnight. After this time, EL4 cells were added to cultures at a 1:10 ratio. Cells were cocultured for 72 h, following which, proliferation was measured by thymidine incorporation (Fig. 6A). Macrophages that had been treated with any of the MIF proteins alone exerted a small reduction in the proliferation of EL4 cells in vitro, which did not reach statistical significance. Similarly, IL-4-treated macrophages were able to effect only a small reduction in EL4 cell-proliferative activity. However, in the presence of exogenous IL-4, MIF homologues induced macrophages to be strongly suppressive, effectively blocking EL4 proliferation (Fig. 6B). Up to 75% suppression was observed from macrophages exposed to *Bm*-MIF-1, *Bm*-MIF-2, or mMIF. Suppression was also observed, albeit at a more modest level, when macrophages were treated with tautomerase-deficient MIF mutants (Fig. 6C).

### *Brugia* MIF but not mMIF induces alternative activation in vivo

We had demonstrated previously that repeated injection of *Bm*-MIF-1 but not *Bm*-MIF-1G induced macrophage and eosinophil recruitment to the peritoneal cavity, with induction of Ym-1 as a marker of alternative activation [45]. We chose to repeat and extend these in vivo experiments to determine whether the activity of mMIF was comparable with that of parasite MIF. BALB/c mice were injected i.p. with recombinant protein nine times over a 3-week period. As described previously, MIF-1 but not MIF-1G induced Ym-1 expression in macrophages recruited to the peritoneal cavity (Fig. 7A).



**Fig. 5.** Counter-inflammatory effects of MIF and IL-4 combinations. (A) Macrophages were incubated for 24 h with 10  $\mu$ g/ml of the indicated proteins in the absence or presence of IL-4 and levels of expression of IFN- $\gamma$ R measured by real-time PCR relative to a control gene. AU, Arbitrary units. (B–D) BMDM were treated with 10  $\mu$ g/ml rMIF in the presence or absence of 20 ng/ml IL-4 for 24 h, following which, supernatants were tested for IL-6 (B), IL-12p40 (C), and IL-10 (D). Data were analyzed by one-way ANOVA. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with treatment with PBS.

Expression of RELM- $\alpha$  (Fig. 7B) and Arginase-1 (Fig. 7C) by *Bm*-MIF-1-elicited macrophages further supported the conclusion that these cells were alternatively activated. Interestingly, mMIF showed a tendency to induce a similar expression profile, although not statistically significant, in a pattern consistent with the in vitro data, in which mMIF-1 was able to promote Ym-1 and Arginase-1 expression in the presence of IL-4. Moreover, *Bm*-MIF-1 and mMIF injections stimulated a peritoneal eosinophilia (Fig. 7D), which has previously been associated with the presence of Ym-1 [53, 62]. As *Bm*-MIF-1

induces a more robust expression of key macrophage genes in vivo than does mMIF, we hypothesize that the parasite proteins also stimulate an adaptive immune response in vivo, including the enhancement of IL-4, which amplifies the alternative activation pathway. Consistent with this proposition, we also noted IL-4R $\alpha$ -deficient mice are unable to produce Ym-1 or RELM- $\alpha$  in response to *Bm*-MIF-1 challenge in vivo (data not shown).

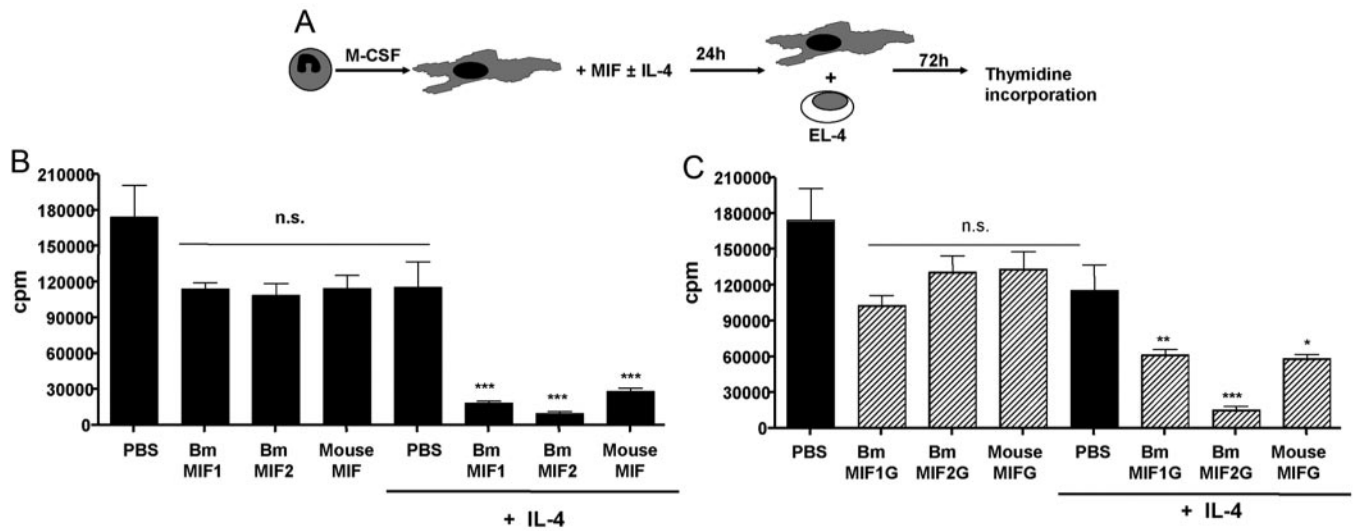
## DISCUSSION

MIF is an important, if incompletely understood, cytokine with deep, evolutionary roots [30, 63, 64]. In recent years, expression of MIF homologues has been reported in a range of parasitic organisms infective to man, including the protozoa *Plasmodium falciparum* [65, 66] and *L. major* [67], and among the helminth worms, in the nematodes *Brugia* [38, 39] and *Trichinella spiralis* [68]. In characterizing MIF from helminth parasites of man, the question arises of whether parasite molecules mimic or counteract the host cytokine and if the study of parasite MIF may reveal a hitherto unknown facet of the mammalian molecule. In now linking *Brugia* MIF with the alternative activation of macrophages, we have also shown that mammalian MIF can, in the context of a type-2 environment, promote a similar phenotype in host macrophage populations.

MIFs from the filarial nematode *Brugia* were first characterized by their homology with the mammalian protein [38, 39]. Functional studies revealed that *Bm*-MIF-1 has the ability to inhibit random migration of human macrophages [38] and that *Bm*-MIF-1 and -2 induce cytokine production by these cells [39]. In addition, the potential role of *Bm*-MIF-1 in alternative activation of macrophages during filarial infection was suggested by the observation that in vivo injection of *Bm*-MIF-1 induced a population of macrophages that expressed Ym-1 [45]. These different observations lead us to hypothesize that although mammalian MIF is a potent proinflammatory factor, filarial homologues will activate macrophages to an alternative phenotype that contributes to counter-inflammation during *Brugia* infection.

We first analyzed the direct effect of each parasite MIF homologue on macrophages in comparison with the host protein. Neither *Brugia* nor mMIF significantly altered macrophage surface phenotype but were able to stimulate cytokine production by in vitro-differentiated and ex vivo-purified macrophage populations. We then demonstrated a new function for MIF in synergizing with IL-4 for the induction of AAM function and associated gene expression. In addition, the synergy we report between MIF and IL-4 appears at several levels. For example, by increasing IL-4R $\alpha$  expression, MIF can prime macrophages to respond to Th2 conditions, and the ability of MIF to elicit proinflammatory cytokines is ablated in the presence of IL-4.

Helminth parasites, in common with allergenic stimuli, are powerful initiators of the type-2 response with prominent IL-4 and IL-13 production [69–71]. These cytokines act through the IL-4R $\alpha$  pathway and STAT-6 phosphorylation to induce an alternatively activated phenotype characterized by the production of Ym-1 [47] and Arginase-1 [43, 58], expression of

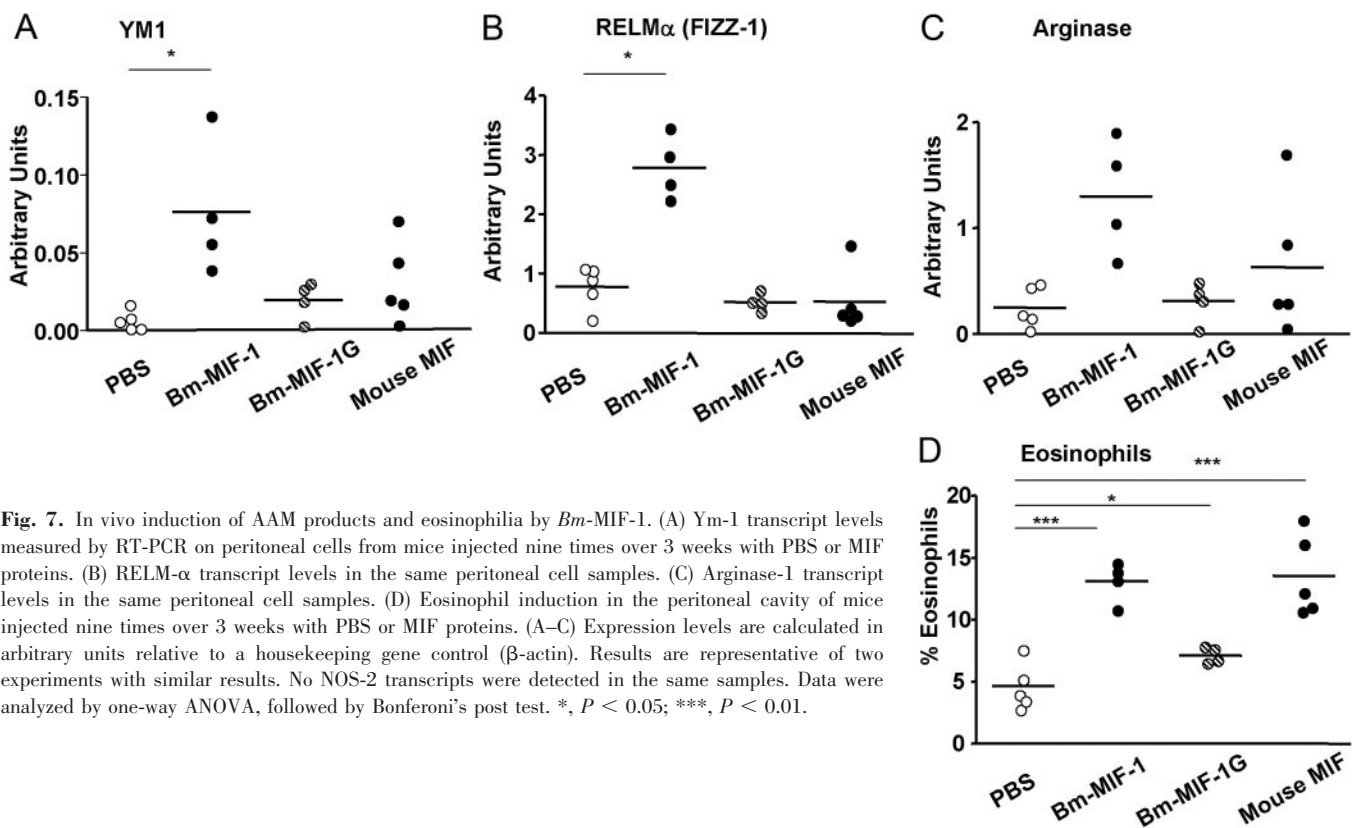


**Fig. 6.** Suppression of proliferation by AAMs treated with MIF and IL-4. (A) Schematic of experimental assay; wells containing  $1 \times 10^5$  BMDM were treated (for 24 h) with  $10 \mu\text{g/ml}$  each rMIF in the presence or absence of  $20 \text{ ng/ml}$  IL-4. After this time,  $1 \times 10^4$  EL4 cells were added to each well. Cells were cocultured for a further 72 h, following which, proliferation was measured by thymidine incorporation. (B and C) Proliferative responses of EL4 cells cocultured with macrophages exposed to MIF proteins with or without exogenous IL-4. (B) Wild-type MIF proteins; (C) MIF-G mutants. Statistical significances were assessed by one-way ANOVA of transformed (log) data, including the Bonferroni post-test. n.s., Not significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , compared with medium (PBS) alone.

surface MRs, and the elaboration of functional cell-suppressor capacity [52]. Our recent work has demonstrated the importance of IL-4R signaling in the innate induction of Ym-1 and other alternative activation markers [72]. The present work now suggests that in *Brugia* infection, parasite-released Bm-MIF may be the first stimulus for macrophages to begin alternative

differentiation but that host IL-4 is necessary for this process to complete.

Although the two *Brugia* homologues, MIF-1 and -2, are more potent than mMIF at inducing Ym-1 and RELM- $\alpha$  in vitro and at driving alternative macrophage activation in vivo, the homologues are broadly equivalent in stimulation of in vitro



**Fig. 7.** In vivo induction of AAM products and eosinophilia by Bm-MIF-1. (A) Ym-1 transcript levels measured by RT-PCR on peritoneal cells from mice injected nine times over 3 weeks with PBS or MIF proteins. (B) RELM- $\alpha$  transcript levels in the same peritoneal cell samples. (C) Arginase-1 transcript levels in the same peritoneal cell samples. (D) Eosinophil induction in the peritoneal cavity of mice injected nine times over 3 weeks with PBS or MIF proteins. (A–C) Expression levels are calculated in arbitrary units relative to a housekeeping gene control ( $\beta$ -actin). Results are representative of two experiments with similar results. No NOS-2 transcripts were detected in the same samples. Data were analyzed by one-way ANOVA, followed by Bonferroni's post test. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.01$ .

cytokine production from macrophages. Moreover, mutants lacking tautomerase enzyme activity are able to induce cytokines and alternative activation *in vitro*. However, these mutants are inactive *in vivo*, indicating a lower potency in a system that we have determined requires nine injections to achieve a significant effect (W. F. Gregory, unpublished observations). As MIF-2 lacks the conserved oxidoreductase site with three conserved cysteine residues in identical positions (57, 60, and 81), this activity is evidently not required for immunological effects. Hence, the induction of AAM-related transcripts is likely to be independent of ERK1/2 phosphorylation events, as discussed below, or the production of inflammatory cytokines.

A major unresolved issue is how parasite MIF molecules may signal to host macrophages. It is known that mMIF activates the MAPK pathway, particularly through ERK1/2 and p38 phosphorylation [32], in a manner that can be mediated through CD74 and is tautomerase-dependent [31]. However, our studies to date do not indicate that an identical signaling pathway is in play with respect to the parasite MIFs, which retain activity in the tautomerase-deficient mutants, are markedly less potent in activating MAPK phosphorylation (unpublished results), and can stimulate CD74-deficient macrophages (L. Prieto-Lafuente and Elizabeth Bikoff, unpublished data). Interestingly, it has been shown recently that a MIF homologue from the hookworm *Ancylostoma caninum* is able to bind to the CD74 receptor [40], and similar experiments with the *Brugia* molecules would advance our knowledge in this area.

The concept that MIF can exert anti-inflammatory effects is certainly not new, as inactivation of Jab1 at the intracellular level has been predicted to have a down-modulatory impact on the immune response [30, 35]. At the systemic level, some striking examples of MIF acting to suppress immunity have emerged from tumor studies, in which neuroblastoma [22, 24], melanoma [21], and ovarian cancer [23] cells have each been found to overexpress MIF, with suppressive consequences for NK and T cell functions. Mechanistic parallels between immune evasion by tumors and parasites are rarely explored, and it is possible that further research into MIF in this regard would be well worthwhile.

In conclusion, our data support the hypothesis that filarial MIF homologues promote alternative activation of macrophages and further argue that mammalian MIF can exert the same effect under similar circumstances of a strong Th2 environment. The link between *Bm*-MIF-1 and alternative activation has been indicated previously [45], and we now show that *Bm*-MIF-2 and mouse-MIF can, in the presence of IL-4, induce macrophage expression of alternative activation markers *in vivo* or *in vitro*. Critically, MIF showed marked synergy with IL-4, enhancing the expression of alternative activation markers induced by IL-4 alone, as well as synergizing to render macrophages suppressive. Moreover, MIF alone induced IL-4R $\alpha$  expression, and IL-4R $\alpha$  levels were strongly up-regulated when IL-4 was present in the media. Taken together, these data suggest that MIF is not a unidirectional mediator that always promotes classical, type-1 inflammation but acts on macrophages according to the prevailing cytokine environment. In a set-

ting in which innate inflammatory mediators are induced, such as septic shock, MIF is certainly a powerful amplifier of inflammation. Our results suggest that in other contexts—in the Th2 milieu of helminth infection, as well as, evidently, allergic inflammation [12, 13]—MIF may accelerate the development of type-2 reactivity, including AAMs and eosinophilia.

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