A *Brugia malayi* Homolog of Macrophage Migration Inhibitory Factor Reveals an Important Link Between Macrophages and Eosinophil Recruitment During Nematode Infection[1]

Franco H. Falcone,[2,3] P'ng Loke,[2,4] Xingxing Zang,[4] Andrew S. MacDonald,[5] Rick M. Maizels, and Judith E. Allen[6]

Infections with the helminth parasite *Brugia malayi* share many key features with Th2-mediated allergic diseases, including recruitment of eosinophils. We have investigated the dynamics of inflammatory cell recruitment under type 2 cytokine conditions in mice infected with *B. malayi*. Among the cells recruited to the site of infection is a novel population of "alternatively activated" macrophages that ablate cell proliferation and enhance Th2 differentiation. By profiling gene expression in this macrophage population, we found a dramatic up-regulation of a recently described eosinophil chemotactic factor, eosinophil chemotactic factor-L/Ym1, representing over 9% of clones randomly selected from a cDNA library. Because *B. malayi* is known to secrete homologs (*Bm* macrophage migration inhibitory factor (MIF)-1 and -2) of the human cytokine MIF, we chose to investigate the role this cytokine mimic may play in the development of the novel macrophage phenotype observed during infection. Strikingly, administration of soluble recombinant *Bm*-MIF-1 was able to reproduce the effects of live parasites, leading both to the up-regulation of Ym1 by macrophages and a marked recruitment of eosinophils in vivo. Because activity of *Bm*-MIF-1 is dependent upon an amino-terminal proline, this residue was mutated to glycine; the resultant recombinant (*Bm*-MIF-1G) was unable to induce Ym1 transcription in macrophages or to mediate the recruitment of eosinophils. These data suggest that macrophages may provide a crucial link between helminth parasites, their active cytokine mimics, and the recruitment of eosinophils in infection. ***The Journal of Immunology*, 2001, 167: 5348–5354.**

Infection with helminth parasites is associated with elevated IgE, systemic eosinophilia, and mast cell proliferation (1). These features reflect a polarized type 2 T cell response enriched in IL-4, IL-5, and IL-13 production (2, 3), and they are reminiscent of Th2-mediated allergic airway inflammation (4). Helminth infection often induces eosinophilic granulocytes to infiltrate host tissues in intense foci associated with extensive tissue damage (5–7). Although many components and regulatory features of the eosinophil recruitment pathway have been elucidated (5, 7, 8), the molecular basis for the massive infiltration of eosinophils during helminth infection is not fully understood. Further, the role of eosinophils in immunity to helminths remains the subject of considerable debate (9, 10).

Macrophages, activated by pro-inflammatory cytokines such as IFN-γ, are critical in combating infection with intracellular microorganisms (11). In contrast, the type 2 cytokines IL-4 and IL-13 can activate macrophages toward a down-regulatory phenotype (12–14). Such cells, termed alternatively activated macrophages (AAMϕ)[7] may have immunoregulatory functions (12), but their real purpose and range of activities in vivo is not known. One possibility is that AAMϕ dampen Th1- or Th2-mediated tissue-damaging responses during infection with extracellular parasites (15–17) and in allergic disease (18).

In a murine model of filarial infection, the human nematode parasite *Brugia malayi* is surgically implanted into the peritoneal cavity of mice (15, 16, 19, 20) where the recruitment of both AAMϕ and eosinophils occurs. In this paper we present evidence for a link between the differentiation of AAMϕ in response to this nematode parasite and the recruitment of eosinophils to the site of infection. Remarkably, we show that a cytokine homolog secreted by the nematode parasite (*Bm* macrophage migration inhibitory factor (MIF)-1) is involved in activating macrophages and is sufficient for the recruitment of eosinophils.

Mammalian MIF was the first cytokine discovered (21) and is involved in septic shock (22) and counteracting glucocorticoid action (23). Interestingly, homologs of this cytokine have been identified in *B. malayi* (24) that share with human MIF a chemotactic activity for monocyte/macrophages. An unusual feature of MIF proteins is their enzymatic activity (25).[8] In human MIF, mutation of the conserved N-terminal proline to glycine substantially ablates

Institute of Cell, Animal and Population Biology, University of Edinburgh, Edinburgh, United Kingdom

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[2] F.H.F. and P.L. contributed equally to this work.

[3] Current address: ACRC Scientific Office, Domagkstrasse 3, D-48149 Muenster, Germany.

[4] Current address: Department of Molecular and Cell Biology, Howard Hughes Medical Institute, LSA 415, University of California, Berkeley, CA 94720-3200.

[5] Current address: College Of Veterinary Medicine, Cornell University, Ithaca, NY 14850.

[6] Address correspondence and reprint requests to Dr. Judith E. Allen, Institute of Cell, Animal and Population Biology, West Mains Road, University of Edinburgh, Edinburgh EH9 3JT, U.K. E-mail address: j.allen@ed.ac.uk

[7] Abbreviations used in this paper: AAMϕ, alternatively activated macrophages; MIF, macrophage migration inhibitory factor; *Bm*-MIF-1, *Brugia malayi* homolog of MIF; MIF-1G, *Bm*-MIF-1 with N-terminal proline altered to glycine; ECF, eosinophil chemotactic factor; PEC, peritoneal exudate cells; EST, expressed sequence tag.

[8] X. Zang, P. Taylor, J. M. Wang, D. J. Meyer, A. L. Scott, M. D. Walkinshaw, and R. M. Maizels. Functional homologues of human macrophage migration inhibitory factor (MIF) from the parasitic nematode *Brugia malayi*: conservation of activity and crystal structure. *Submitted for publication.*

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both catalytic and cytokine activity (26). We now show that a *B. malayi* homolog of MIF (*Bm*-MIF-1) induces eosinophil recruitment in vivo when injected into the peritoneal cavity of mice and that mutation of the conserved proline residue eliminates this activity. We also find that *Bm*-MIF-1 increases the transcription rate of a gene encoding a novel eosinophil chemotactic factor (ECF-L), also known as Ym1 (27, 28). Gene expression analysis demonstrates that Ym1/ECF-L represents over 9% of the total transcripts in macrophages recruited to the site of *B. malayi* infection. Importantly, Ym1 has been demonstrated to have chemotactic activity for eosinophils both in vitro and in vivo (28). These findings demonstrate an important link between macrophage activation and eosinophil chemotaxis and they suggest that Ym1 may be an important new player in helminth driven inflammatory processes.

Materials and Methods

Mouse strains

Six- to 8-wk-old CBA/Ca or C57BL/6 males were used for *B. malayi* implantation. Both male and female BALB/c mice were used for the injection of *Bm*-MIF-1 or LPS in vivo. C57BL/6 IL-4-deficient (IL-$4^{-/-}$) breeding pairs were purchased from B & K Universal (North Humberside, U.K.) with permission of the Institute of Genetics (University of Cologne, Cologne, Germany). C57BL/6 IL-5-deficient (IL-$5^{-/-}$) mice (29) were the kind gift of Dr. M. Kopf (Basel Institute for Immunology, Basel, Switzerland). All mice were bred in-house.

In vivo mouse models

B. malayi adult parasites were obtained from infected jirds purchased from TRS Laboratories (Athens, GA). Adult worms were removed from the peritoneal cavity of jirds, washed in RPMI, and six live adult *B. malayi* females were surgically implanted into the peritoneal cavity of the mice. After the experimental period, mice were euthanized by cardiac puncture, and peritoneal exudate cells (PEC) were harvested by thorough washing of the peritoneal cavity with 15 ml of RPMI. For analysis of *Bm*-MIF activity in vivo, 1 μg of purified, LPS-free recombinant *Bm*-MIF-1 and *Bm*-MIF-1G were injected i.p. into mice three times a week for 3 wk (nine injections) 3 days after the final injection the PEC cells were harvested as described above.

Characterization of PEC populations

Cytocentrifuge preparations of 1×10^5 cells were made using a Shandon Cytospin (Thermo Shandon, Pittsburgh, PA). Cytospins were air dried, fixed in methanol, and stained with DiffQuik (Dade, Unterschleissheim, Germany) and examined with a Nikon Microphot-FX microscope (Nikon, Melville, NY). Before magnetic bead cell purification, PEC were passed through a 70-μm cell strainer and were purified by centrifugation over Histopaque (Sigma-Aldrich, St. Louis, MO) to remove any microfilariae. PEC were then sorted with MS^+ or VS^+ columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). F4/80^+ cells were purified with biotin-conjugated F4/80 (rat IgG2b; Caltag Laboratories, Burlingame, CA) and streptavidin microbeads (Miltenyi Biotec).

cDNA library construction and express sequence tag analysis

Total RNA was extracted (with RNAstat60; Ambion, Austin, TX) from purified F4/80^+ macrophages from the PEC of *B. malayi*-implanted IL-5-deficient mice. cDNA was synthesized from total RNA and was unidirectionally cloned into the pCMV-Script plasmid vector, using the cDNA library construction kit from Stratagene (La Jolla, CA). Single clones from the unamplified library were randomly picked, and the cDNA inserts were amplified using vector primers T3 (AATTAACCCTCACTAAAGGG) and T7 (CGGGATATCACTCAGCATAATG). Inserts were sequenced using the 5′ vector primer SAC (GGGAACAAAAGCTGGAG) and ABI Big DYE terminators (PerkinElmer/Cetus, Norwalk, CT). Sequencing reactions were analyzed using an ABI 377 automated sequencer (PerkinElmer/Cetus, Norwalk, CT). The sequences were edited manually with vector (SeqEd; Applied Biosystems, Foster City, CA) and poor 3′ sequence removed. The edited sequences were sent to the National Center for Biotechnology Information for Blastn analysis against GenBank sequences (nr) and the expressed sequence tag (EST) database (dbest). Blastx analysis was also conducted against GenBank sequences (nr).

RT-PCR

For RT-PCR, first-strand cDNA was produced with oligo-dT primers from total RNA using the GeneAmp RT-PCR kit (Applied Biosystems). Ym1-specific primers (Ym1-For, *TGG GGG ATC CG* T ACC AGC TGA TGT GCT ACT (64–82); Ym1-Rev, *GTA AAG GAT CC* T CAA TAA GGG CCC TTG CA (1197–1182)) were used to amplify from the first-strand cDNA. The Ym1 primers have an 18-bp overlap with the 5′ and 3′ end of the target gene and an 11-bp overhang containing restriction sites designed for a different purpose (overhanging nucleotides are in italics). As a control, we used primers for β-actin (β-actin-F, TGGAATCCTGTGGCATC CATGAAAC and β-actin-R, TAAAACGCAGCTCAGTAACAGTCCG). PCR conditions were as follows: 94°C for 3 min, 35 cycles of 20 s at 94°C, 30 s 55°C, and 90 s at 72°C resulting in a 1506-bp amplicon for Ym1 and 348-bp for β-actin. For real-time PCR (Light Cycler; Roche Diagnostic Systems, Somerville, NJ), PCR on first-strand cDNA was performed using the SYBR green kit (Roche Diagnostic Systems) with a second pair of Ym1-specific primers (Ym1 -F_{LC}, TCACAGGTCTGGCAATTCTTCTG; Ym1 -R_{LC}, TTTGTCCTTAGGAGGGCTTCCTCG), resulting in a 437-bp product. The β-actin primers shown above were also used for β-actin mRNA determination by real-time PCR.

Bm-MIF-1

Recombinant *Bm*-MIF-1 and *Bm*-MIF-1G were prepared as described elsewhere.[8] Briefly, native and mutant proteins were expressed in *E. coli* using pET29 (Novagen, Madison, WI) with a C-terminal His-tag allowing purification to >97% homogeneity. Endotoxin was removed by phase separation using Triton X-114 (30) and was determined to be LPS free by a commercial assay (E-Toxate; Sigma-Aldrich). Control experiments with LPS were performed with LPS *E. coli* Serotype 026:B6 (Sigma-Aldrich).

Statistical analysis

Statistical analysis was performed using PRISM (GraphPad Software, San Diego, CA). The nonparametric Mann-Whitney test was used to determine measured differences between groups of mice ($n < 30$), and the χ^2 test was used to determine significant differences between the number of mice that up-regulate Ym1 after treatment.

Results

Recruitment of macrophages and eosinophils by B. malayi

Implantation of *B. malayi* filarial parasites into the peritoneal cavity of mice leads to dramatic recruitment of inflammatory cells, with a 5- to 10-fold increase in total cell numbers by 3 wk postinfection (20). Within this population, the predominant cell types are macrophages and eosinophils, the latter having increased by 40-fold in the implanted mice. To determine the kinetics of recruitment, we studied the development of peritoneal cell populations over 21 days after exposure to *B. malayi*. After an initial drop in the first 2 days, the total number of cells increased steadily, reaching maximum levels at 2–3 wk (Fig. 1*A*). Mast cells disappeared within 24 h of implantation by which time there was a striking but short-lived neutrophilia (Fig. 1*B*). In contrast, eosinophil infiltration was observed slightly later, peaking at 7 days after implantation and remaining stable until the experiment was terminated at 21 days. As with total cell numbers, macrophage numbers steadily increased in the first 2 wk (from $1.67 \times 10^6 \pm 0.81 \times 10^6$ on day 0 to $5.12 \times 10^6 \pm 0.86 \times 10^6$ on day 14) and then remained high for the duration of the experiment ($6.57 \times 10^6 \pm 3.54$ on day 21; Fig. 1, *A* and *B*).

To assess whether the recruitment of eosinophils was dependent on the Th2 cytokines IL-4 and IL-5, we implanted parasites into IL-4- and IL-5- deficient mice (Fig. 1, *C* and *D*) for 3 wk before recovering the PEC. Significantly fewer eosinophils were recruited into the peritoneal cavity of IL-$4^{-/-}$,whereas, as expected, eosinophil recruitment was virtually absent in IL-$5^{-/-}$ mice. These data show that eosinophil recruitment in this model is dependent not only on IL-5, but is also partly dependent on host IL-4.

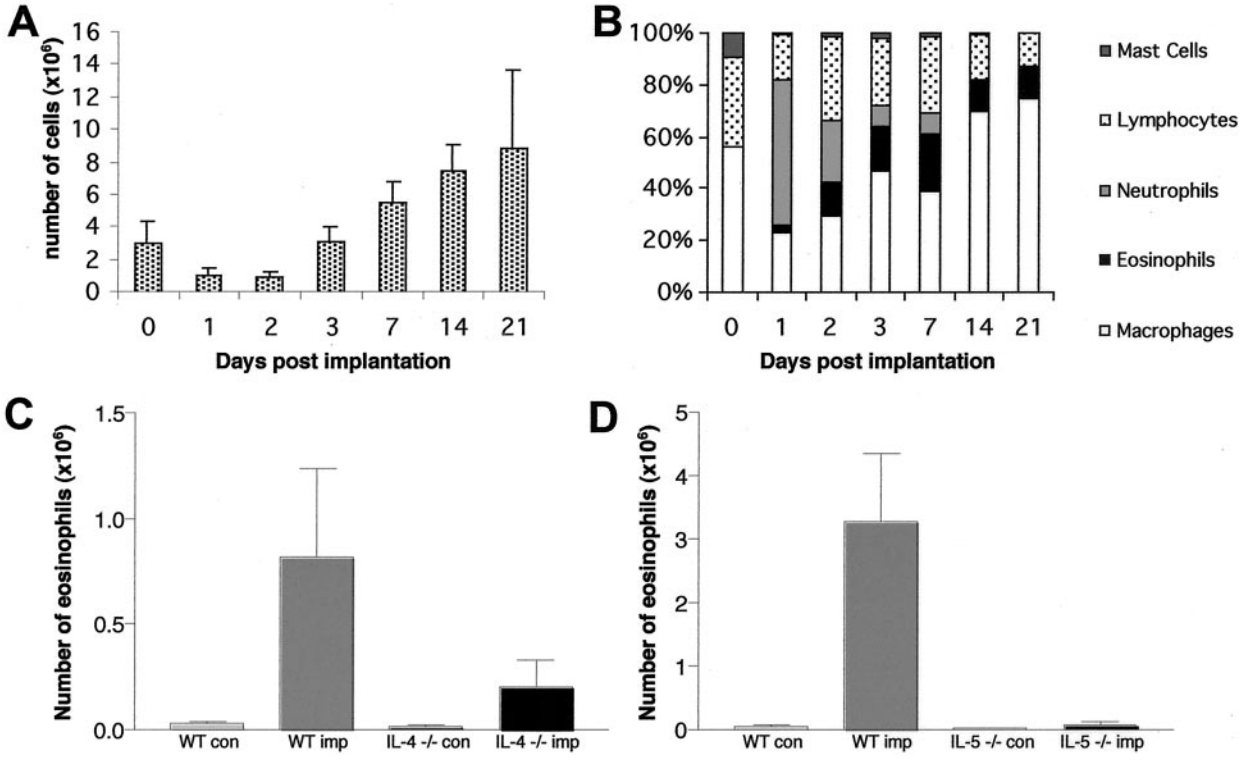

FIGURE 1. Eosinophil recruitment by *B. malayi*. Time course of (*A*) total cell recruitment into the peritoneal cavity and (*B*) changes in the cellular composition after parasite implantation. Cellular composition was determined separately for individual mice and data shown represents the mean for each group. *C*, Eosinophil recruitment into the peritoneal cavity of implanted IL4$^{-/-}$ mice; *D*, IL-5$^{-/-}$ mice and WT mice. All data are shown as mean ± SD for individual mice (usually five per group) assessed separately.

Characterization of gene expression in AAMϕ

Peritoneal macrophages recruited by *B. malayi* profoundly suppress cellular proliferation through a contact-dependent mechanism (16). This suppressive phenotype is entirely dependent on the presence of host IL-4 (20). To explore the molecular basis of the IL-4-dependent phenotype, we constructed a cDNA library from purified F4/80^{+} macrophages, for EST sequencing of randomly selected clones. Macrophages were taken from parasite-implanted IL-5$^{-/-}$ mice, which possess identical suppressive function to implanted wild-type mice (16), as an additional precaution to exclude any eosinophil contribution to the cDNA library.

A total of 252 clones were sequenced from the 5′ end to provide a snapshot of the abundant genes expressed by these suppressive macrophages (Table I). Among the genes highly represented in the library is arginase I, which counteracts the nitric oxide synthesis pathway in macrophages and is induced by Th2 cytokines (31). This finding supports our classification of *B. malayi*-recruited macrophages as AAMϕ. The abundant expression of a novel cysteine-rich protein (PMNG1) by in vivo-derived AAMϕ is of significant interest and is the subject of ongoing investigation in the laboratory. However, the most striking finding was the extremely high representation of a gene of unknown function submitted to the database as Ym1 (27), which accounted for 9.1% of the cDNA clones in this library. In a recent series of studies, Owhashi et al. (32, 33) isolated an ECF produced by CD8^{+} T lymphocytes (ECF-L) after infection with *Schistosoma japonicum* and *Toxocara canis*. This factor was biochemically purified and was shown by direct protein sequencing to be Ym1 (28). These investigators then isolated the cDNA clone and demonstrated that both native and recombinant ECF-L/Ym1 was chemotactic for eosinophils in vitro and in vivo (28).

Table I. *Abundantly expressed transcripts in AAM*Φ *recruited by* B. malayi[a]

No. of Clones	% Transcript	GenBank Match (accession no.)
23	9.1	Ym1/ECF-L (BAA13458)
8	3.2	FIZZ1/PMNG1 (NP_065255)
6	2.4	Serum amyloid A 3 (NP_035445)
5	2.0	Arginase 1 (NP_031508)

[a] A cDNA library was constructed from AAMΦ recruited by *B. malayi*. Randomly selected clones (252) were sequenced from a cDNA library constructed from AAMΦ recruited by *B. malayi*. Data is shown for the transcripts represented by more than 4 clones in this EST analysis.

Ym1 is dramatically up-regulated in macrophages exposed to B. malayi

The discovery that a novel ECF accounted for a startlingly high proportion of the genes expressed in peritoneal macrophages of nematode-implanted mice was highly provocative. We confirmed this finding by an independent RT-PCR analysis (Fig. 2). In resident PECs from control mice, Ym1 was routinely detected at a low, basal level. In comparison, implantation of mice with parasites resulted in the dramatic up-regulation of Ym1 expression in PECs after a period of 3 wk (Fig. 2*A*). This result was observed in every individual mouse of either sex ($n = 10$) analyzed by RT-PCR. Using real-time PCR, we estimated that Ym1 is up-regulated by >10,000-fold as a result of parasite implantation (Fig. 2*B*) and is even more abundantly expressed than β-actin, which is consistent with our preliminary EST analysis.

Because eosinophil recruitment is partly or totally dependent on Th2 cytokines IL-4 and IL-5, we assessed whether Ym1 expression was diminished in mice deficient for these products. Purified F4/80^{+} macrophages from parasite-implanted IL-5$^{-/-}$ mice

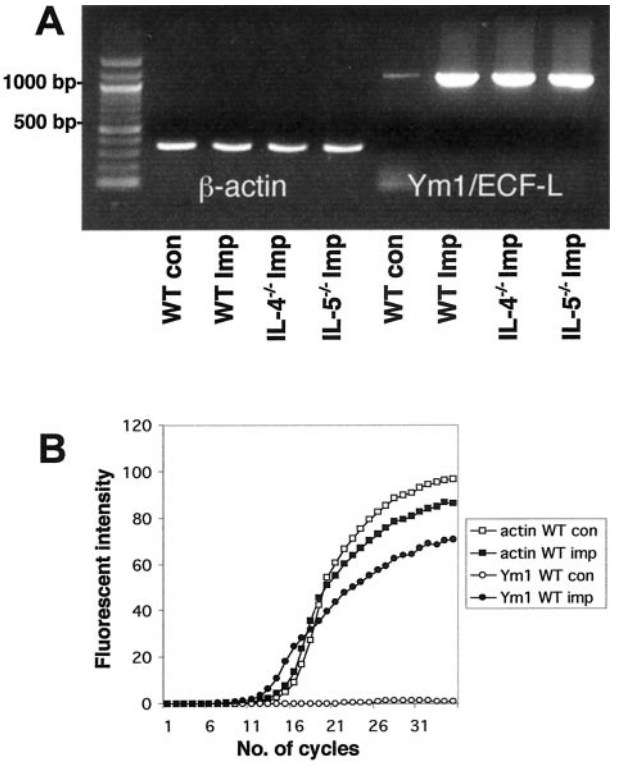

FIGURE 2. Expression of Ym1 in *B. malayi* recruited macrophages. *A*, RT-PCR analysis of Ym1 induction in peritoneal cells derived from non-implanted (WT con) mice, implantation with *B. malayi* (WT imp), and F4/80$^+$-purified macrophages from implanted IL-4$^{-/-}$ (IL-4$^{-/-}$ imp) or IL-5$^{-/-}$ mice (IL-5$^{-/-}$ imp). *B*, Real-time PCR analysis of Ym1 expression in recruited peritoneal cells. Fluorescence of β-actin controls cross-over into a linear expansion phase at 10.41 PCR cycles for WT imp and 11.38 cycles for WT con. Ym1 fluorescence enters the linear phase for WT imp at 8.59 cycles and 24.88 cycles for WT con. Using a standard curve of known quantities of template, we estimate that Ym1 expression is up-regulated by >10,000-fold. Data shown is representative of several independent experiments with individual mice (refer to Table II).

showed the same induction of Ym1 as wild-type mice (Fig. 2*A*). This was expected as Ym1 was originally observed in a cDNA library of IL-5$^{-/-}$ origin. In the absence of IL-5, eosinophils fail to mobilize from the bone marrow and therefore even with abundant Ym1 produced, eosinophils would not be recruited (34). More significantly, we found that F4/80$^+$ macrophages from IL-4$^{-/-}$ mice also showed the same up-regulation of Ym1 (Fig. 2*A*) despite reduced eosinophil recruitment. In IL-4 $^{-/-}$ mice, the failure to make a full-fledged Th2 response may lead to reduced numbers of IL-5-producing Th2 cells and thus reduced eosinophilia. This data demonstrates that induction of Ym1 in this setting is independent of IL-4 or IL-5, but it does not rule out a requirement for type 2 cytokines such as IL-13.

Bm-MIF-1 induces up-regulation of Ym1

B. malayi has been recently shown to secrete homologs of the human cytokine MIF (24) that actively stimulate human monocytes and macrophages. Therefore, we asked whether *Bm*-MIF-1 could be responsible for the Ym1 induction seen in macrophages from implanted mice. To mimic the long-term effect of parasite secretions, we gave nine i.p. injections of endotoxin-free *Bm*-MIF-1 over a period of 3 wk. Both mammalian and parasite MIFs display cytokine activity and an unusual enzyme specificity, dopachrome tautomerase (25, 35, 36). Both activities are dependent upon the N-terminal proline residue. Therefore, we tested a mutant form of *Bm*-MIF-1, in which the proline has been altered to glycine (*Bm*-MIF-1G) and lacks biological activity.[8] We treated two control groups with corresponding volumes of sterile PBS or LPS (0.1 U/injection). Three days after the last injection, the animals were sacrificed and the cells recruited to, or resident in, the peritoneal cavity were processed for RT-PCR analysis.

As shown in Fig. 3*A* and Table II, *Bm*-MIF-1 induced Ym1 (11 of 16 mice), whereas in the PBS-treated mice, Ym1 was only detected at a basal level. Interestingly, *Bm*-MIF-1G only induced Ym1 in 2 of 16 mice. From this result, it appeared that the biological activity, dependent upon an intact N-terminal proline, was responsible for the induction of Ym1. Also shown in Table II, LPS was not effective at inducing Ym1 in treated mice. To ensure that Ym1 was up-regulated in macrophages and that the increased transcript levels were not the result of increased macrophage numbers, PECs were pooled from each group and the F4/80$^+$ cells were purified with immunomagnetic beads for RT-PCR analysis. F4/80$^+$ cells from *Bm*-MIF-1-injected mice expressed high levels of Ym1 (Fig. 3*A*).

Next we sought to perform a reliable quantification of Ym1 induction by *Bm*-MIF-1 using real-time PCR. In an independent experiment, mice were again given nine injections of 1 μg of *Bm*-MIF-1, *Bm*MIF-1G, or PBS over 3 weeks. Equalizing for β-actin mRNA content in all the samples (Fig. 3*B*), we detected a significant increase in the level of Ym1 transcript in mice treated with *Bm*-MIF-1 (mean 610.6 $\pm$ 1088) vs *Bm*-MIF-1G-treated mice (mean 4.498 $\pm$ 2.871) or PBS-treated mice (mean 0.7753 $\pm$ 0.9562; Fig. 3*C*). Nearly identical results were obtained with F4/80$^+$ cells purified from pooled PECs (data not shown). Interestingly, MIF-1G-treated mice showed a slight but significant increase in Ym1 transcription, consistent with the data showing that 2 of 16 MIF-1G-treated mice had up-regulated Ym1 (Table II). This indicates that the 1G mutation does not completely remove all functional activity. We have seen similar residual activity in MIF-1G when analyzing cytokine activity.[8]

It is of interest to note that 100% of parasite-exposed mice induced Ym1, whereas 30% of mice exposed to *Bm*-MIF-1 failed to induce this molecule (Table II). The results obtained with i.p. injection of BmMIF are likely to differ from the live parasite experiments because the live implanted parasites release native *Bm*-MIF continuously over the 3 weeks of the duration of the experiment. The nine injections given to the mice over the same period may only partially mimic the live implant situation. We thus examined whether the 3-wk treatment course with *Bm*-MIF-1 is required for effective induction of Ym1. We treated mice with a single 10-μg dose or three 1-μg doses spread over 1 week. The single injection of a high dose of *Bm*-MIF-1 had no effect, whereas the three consecutive injections did induce Ym1 (data not shown). Again, neither PBS nor *Bm*-MIF-1G induced Ym1. A single high dose of LPS (10 U) also did not induce Ym1 (data not shown). This suggests that Ym1 induction requires the continuous release of *Bm*-MIF-1 as would be found during live infection, and may explain the failure of some *Bm*-MIF-injected mice to induce Ym1 transcription. In addition, other components secreted by the live parasites may reinforce the effects of *Bm*-MIF-1. Unsurprisingly, *Bm*-MIF-1 alone does not reproduce all the effects of parasite implantation (i.e., recruited macrophages are not suppressive), arguing strongly that the parasite is producing other immune modulatory factors.

Bm-MIF-1 induces recruitment of eosinophils

Assuming that Ym1 gene transcription reflects protein levels, and with the knowledge that Ym1 (ECF-L) can recruit eosinophils in vitro as well as in vivo (28), we asked whether there was any

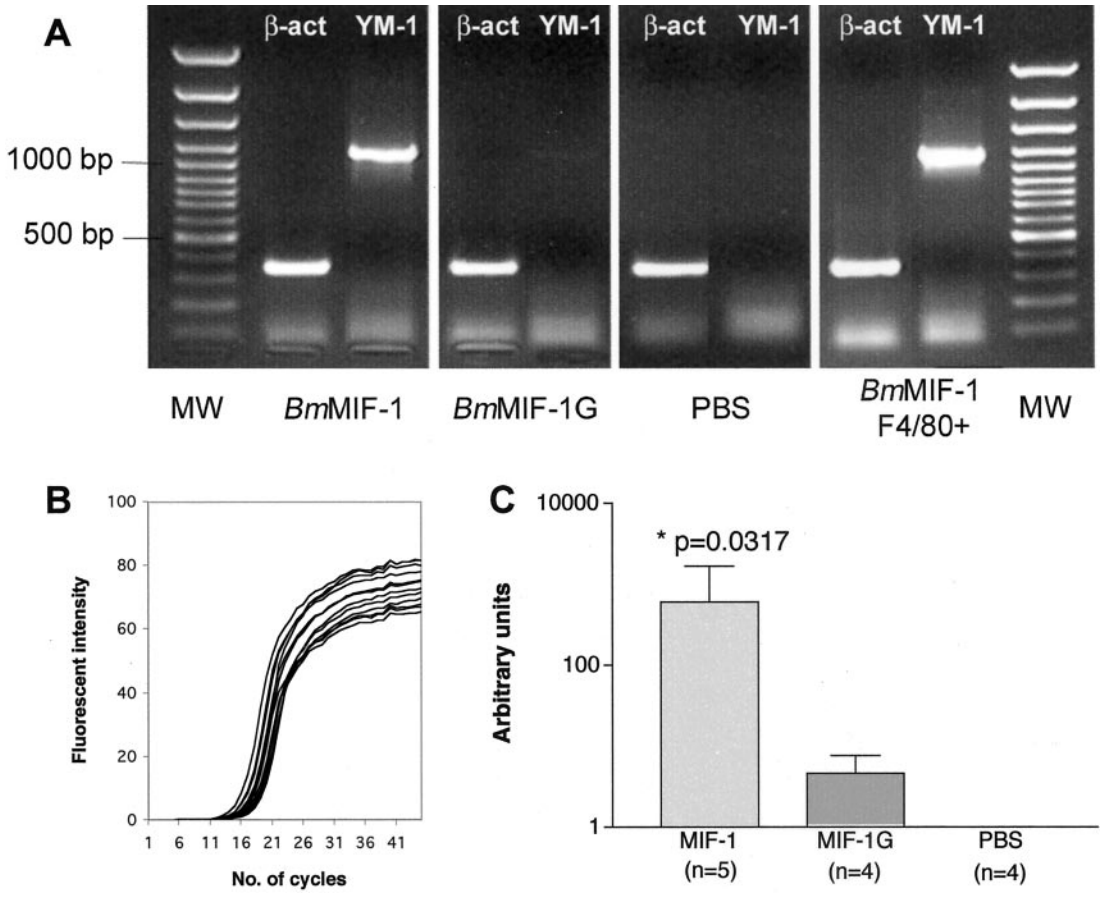

FIGURE 3. Expression of Ym1 after treatment with *Bm*MIF-1. *A*, RT-PCR analysis of β-actin and Ym1 mRNA in peritoneal cells from mice treated with *Bm*MIF-1, *Bm*MIF-1G, or PBS. The right panel shows the same analysis for F4/80$^+$ peritoneal macrophages of *Bm*MIF-1-treated mice. Data shown is representative of several independent experiments with individual mice (refer to Table II). *B* and *C*, Real-time PCR analysis of Ym1 expression in peritoneal cells from *Bm*MIF-1-, *Bm*MIF-1G-, and PBS-treated mice. *B*, β-actin controls showing equal amounts of template in all samples. *C*, Using a standard curve of known quantities of template, Ym1 expression was compared between individual mice. Data shown as mean $\pm$ SD of individual mice.

relationship between recruitment of eosinophils to the peritoneal cavity and the induction of Ym1 gene expression by *Bm*-MIF-1. We thus examined stained cytospins of cells derived from the peritoneal cavity of mice treated with PBS, *Bm*-MIF-1, or *Bm*-MIF-1G, and we assessed the percentage of eosinophil granulocytes. Fig. 4 shows that the 3-wk treatment of mice with *Bm*-MIF-1 led to an average increase of ~3-fold in the number of peritoneal eosinophils compared with PBS- or *Bm*-MIF-1G-treated mice ($p = 0.0001$). It is noteworthy that although the 1-wk treatment with *Bm*-MIF-1 effectively induced Ym1, these mice did not display any increased eosinophil recruitment (data not shown). Without understanding more about both MIF and Ym1 function (and their receptors), it is difficult to directly assess the reasons for this.

Table II. *Summary of RT-PCR studies on Ym1 expression in individual mice implanted with live* B. malayi *or treatment with* Bm*MIF-1 or* Bm*MIF-1G, PBS, or LPS*[a]

	Ym1/ECF-L$^+$	Ym1/ECF-L$^-$	*p* Value
Wild-type implant	10	0	0.0001
Wild-type control	0	5	
*Bm*MIF-1	11	5	0.0012
*Bm*MIF-1G	2	14	
PBS	0	9	
LPS	1	4	

[a] Since Ym1 is expressed at a basal level in resident peritoneal cells, we defined Ym1$^+$ as samples with >50% intensity of β-actin controls.

It may be that early in the inflammatory process there is insufficient expression of Ym1 receptors, or a lack of other key players (cells or soluble mediators) that are essential to the recruitment process.

Discussion

In this study we established that there is an important link between the action of nematode-derived MIF-1, the activation of macrophages to produce Ym1, and the recruitment of eosinophils. We have yet to establish the exact sequence of events as well as the role of other critical players such as IL-13 and eotaxin. Nonetheless, our data suggest that Ym1 may be an important and hitherto unrecognized player in eosinophil-associated inflammatory conditions.

Ym1 is highly homologous to a family of chitinases (37) found in bacteria, plants, and mammals. These molecules may have evolved as a first-line defense against chitin-bearing pathogens such as fungi, but they may also play a role in anti-helminth immunity, as chitin-related carbohydrates are present in larval and egg stages (38). Sequence comparison between Ym1 and active chitinases suggests that Ym1 may no longer possess chitinase activity due to the replacement of an acidic residue in the active site (28, 37). However, even in the absence of chitinase activity, Ym1 may still have the ability to bind carbohydrate structures. Such a carbohydrate-binding activity may be the key to eosinophil chemotaxis, as it has been shown that ecalectin, a lectin with affinity for β-galactosides, is also selectively and potently chemotactic for eosinophils (39). Because neither chitinases nor lectins are related

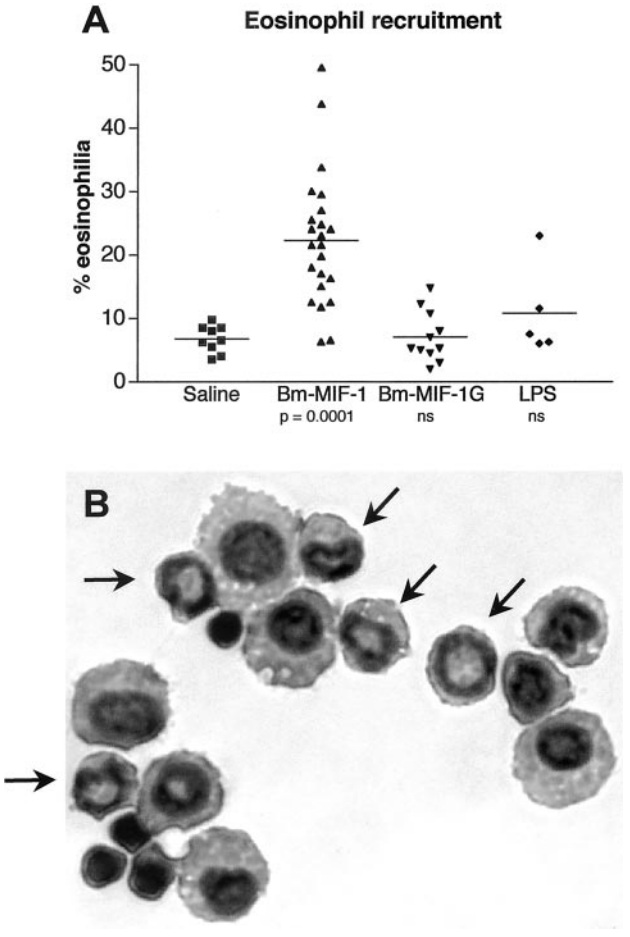

FIGURE 4. Eosinophil recruitment into the peritoneal cavity after *Bm*-MIF-1 treatment. *A*, The cell composition of PEC (from i.p. injected mice) was determined by counting ~500 cells from randomly selected fields per DiffQuik-stained cytospin. Data presented is from several independent experiments. Each data point represents an individual animal, with the horizontal bar representing the mean of the group. *B*, Example of the cellular composition of PEC after *Bm*MIF-1 injection. Eosinophils are indicated with arrows. The other cells are macrophages (large) and lymphocytes (small).

to other known ECFs, it will be interesting to study the molecular mechanism of chemoattraction in detail.

Ym1 has also been described in three other contexts, each associated with pathological conditions in the lung. Guo et al. (40) found that crystals in the lungs of viable moth-eaten mice (me^v/me^v) are composed of Ym1 protein. These crystals were found in the cytoplasm of alveolar macrophages and are thought to be similar to the Charcot-Leyden crystals in humans associated with eosinophil-rich inflammation of the lungs. In a model system of vaccination with the helminth parasite, *Schistosoma mansoni*, protein crystals were found in the pulmonary macrophages of infected mice along with significant numbers of eosinophils (41). By amino-terminal sequencing, these protein crystals were recently identified as Ym1 (R. A. Wilson, unpublished observations). The potential role for Ym1 in the pathology of pulmonary diseases is further highlighted by a recent study of murine pulmonary *Cryptococcus neoformans* infection that suggests that Ym1-containing protein crystals are responsible for significant cellular damage (42). Interestingly, in both schistosome vaccination and cryptococcal infection, significant eosinophilia can occur in the absence of a dominant type 2 immune response. This is consistent with our finding that Ym1 up-regulation is IL-4 independent.

Taken together, the findings thus far suggest that Ym1 is an abundant molecule associated with eosinophil recruitment, lung pathology, and type 2 cytokine environments. Interestingly, two human homologs of Ym1 with unknown function, chitotriosidase and human cartilage gp39, are expressed in activated macrophages (43). Chitotriosidase is highly induced in patients with Gaucher's disease (44), whereas both chitotriosidase and human cartilage gp39 are expressed in macrophages from atherosclerotic lesions (45). It is particularly interesting to note that human chitotriosidase may be relevant to lymphatic filariasis. In a recent study in South India (46), filarial-infected individuals were significantly more likely than uninfected individuals to have the HH variant of the CHIT1 gene—a genotype that leads to decreased activity and levels of chitotriosidase, suggesting this molecule may be involved in host protection. In the context of lung pathology, the most closely related Ym1 homolog (68% identity) is a gene, TSA1902, which is expressed specifically in the lung (47). Functions for these human homologs remain unknown and thus our studies may provide insight into the role of these highly expressed human genes.

The relationship of MIF to eosinophil recruitment is highly provocative. Mammalian MIF is strongly associated with type 1 proinflammatory conditions (22), whereas the role of nematode MIF in parasite infection remains to be elucidated. Mammalian MIF has not been reported to have eosinophil recruitment activity (directly or indirectly), although interestingly it is produced by human eosinophils (48), raising the possibility of a positive feedback loop. We are currently investigating whether this is a yet undiscovered function for MIF, which we have identified by studying the parasite homolog. Our data strongly suggest that macrophages provide a crucial link between parasitic infections and eosinophil chemotaxis. Because the production of MIF-like enzymatic activity has been demonstrated in a variety of nematode parasites (35), Ym1 induction by parasite-secreted MIF and subsequent eosinophil recruitment to the tissues could be a widely spread mechanism accounting at least partially for the well-known phenomenon of tissue eosinophilia in parasitic infections. Although each of these events has been demonstrated in isolation, we have not yet shown a direct link between BmMIF, YM1 induction, and recruitment of eosinophils. Future studies with neutralizing Abs will be required to find out whether these are the critical cascade of events that occur during infection. Further, to understand the sequence of events more thoroughly, we will need a far clearer picture of both MIF and Ym1 function. What is the receptor for Ym1? What factors, in addition to MIF, induce Ym1 expression? What levels of Ym1 protein are required for eosinophil recruitment? Are Ym1 crystals purely a pathological outcome or are they part of the normal inflammatory processes?

This work raises the possibility that MIF and/or Ym1 are important components in Th2-mediated pathology in general and, as such, could be relevant to chronic inflammatory conditions such as asthma. Finally, as the role of eosinophils in parasite infection remains unresolved, it is intriguing to consider the evolutionary rationale behind the production of MIF by filarial parasites. In secreting this cytokine mimic, the parasite may be directly responsible for high-level eosinophil recruitment, suggesting that eosinophils may, under some circumstances, function to benefit rather than destroy the parasite.

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