

***Brief Definitive Report*****An Essential Role for Macrophage Migration Inhibitory Factor in the Tuberculin Delayed-type Hypersensitivity Reaction**

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**Summary**

30 years ago, investigations into the molecular basis of the delayed-type hypersensitivity reaction (DTH) provided evidence for the first lymphokine activity: a lymphocyte-derived mediator called macrophage migration inhibitory factor (MIF), which inhibited the random migration of peritoneal macrophages. Despite the long-standing association of MIF with the DTH reaction and the cloning of a human protein with macrophage migration inhibitory activity, the precise role of MIF in this classic cell-mediated immune response has remained undefined. This situation has been further complicated by the fact that two other cytokines, interferon  $\gamma$  and IL-4, similarly inhibit macrophage migration and by the identification of mitogenic contaminants in some preparations of cloned human MIF. Using recently developed molecular probes for mouse MIF, we have examined the role of this protein in a classical model of DTH, the tuberculin reaction in mice. Both MIF messenger RNA and protein were expressed prominently in DTH lesions, as assessed by reverse transcription polymerase chain reaction, in situ hybridization, and immunostaining with anti-MIF antibody. The predominant cellular origin of MIF appeared to be the monocyte/macrophage, a cell type identified recently to be a major source of MIF release in vivo. The administration of neutralizing anti-MIF antibodies to mice inhibited significantly the development of DTH, thus affirming the central role of MIF in this classic immunological response.

Cell-mediated immunity is the principle host response to a variety of viral, bacterial, and parasitic infections, and it plays a critical role in controlling invasion by intracellular pathogens such as *Mycobacterium tuberculosis* (1). The delayed-type hypersensitivity reaction (DTH) exhibits many features of this response and is characterized histologically by the infiltration and accumulation of mononuclear cells (i.e., macrophages and T cells) into regions of infection or antigen deposition. DTH is readily demonstrated by the classic tuberculin reaction in which the intradermal injection of mycobacterial purified protein derivative (PPD) into previously sensitized hosts produces erythema and induration that persists for 24–72 h (2). Thirty years ago, investigations into the molecular basis of DTH provided evidence for the first lymphokine or cytokine “activity,” a nondialyzable substance present in lymphoid cell supernatants that inhibited the random migration of peritoneal exudate cells (3, 4). A protein designated macrophage migration inhibitory factor (MIF) was proposed to account for this activity and was ultimately cloned from a human T cell line in 1989 (5).

Numerous studies over the years have implicated various T cell- and macrophage-derived cytokines in the induction of the DTH response (6–9). Yet, despite the long-standing association of MIF “activity” with the DTH reaction, the precise role of the MIF “protein” in this immunological response has remained undefined. This situation has been further confounded by the description of two other cytokines: IFN- $\gamma$  and IL-4, which exhibit MIF activity (10, 11), and by the unavailability both of purified, mitogen-free MIF protein and of specific, neutralizing anti-MIF antibodies (12, 13). In a series of recent studies, however, the mouse homologue of human MIF has been isolated, cloned, and identified to be a major secretory product of anterior pituitary cells and activated macrophages (14, 15). The mouse and human MIF proteins also have been expressed in high yield, purified, and have become available for biological studies (16). MIF, for example, has been found to potentiate endotoxin (LPS) lethality and neutralizing anti-MIF antibodies fully protect mice in experimentally-induced septic shock (14). MIF induces the production of TNF- $\alpha$  and acts together with IFN- $\gamma$  to promote

nitric oxide release (15, 16). MIF also has been identified recently to be released from immune cells by glucocorticoid stimulation and, once secreted, acts to counterregulate glucocorticoid inhibition of cytokine production (17).

In the present study, we used recently developed molecular probes for mouse MIF to reexamine the role of this protein in a classical model of DTH, the tuberculin reaction. Both MIF protein and RNA were expressed in DTH lesions and were localized predominantly to cells of the monocyte/macrophage lineage. The administration of neutralizing anti-MIF antibodies to previously sensitized mice inhibited significantly the development of DTH, affirming the central role of MIF in this classic immunological response.

## Materials and Methods

**Reagents.** CFA containing 1 mg/ml *M. tuberculosis* H37Ra was obtained from Sigma Chemical Co. (St. Louis, MO). Tuberculin purified protein derivative (PPD) used for in vivo immunizations was from Cooper's Animal Health Inc. (Kansas City, KS). Tubersol<sup>®</sup> tuberculin purified protein derivative (Connaught Laboratories Ltd., Toronto, Canada) was used for the in vitro cytokine production assays and was dissolved in sterile PBS (final concentration of 1 mg/ml) and stored at 4°C. The PPD solution contained negligible quantities of endotoxin (10.6 pg LPS/ $\mu$ g PPD) as determined by the *Limulus* amoebocyte assay (BioWhittaker, Inc., Walkersville, MD).

**Induction and Quantitation of the DTH Response.** Female BALB/c mice ( $n = 5-6$  per group) were primed by subcutaneously injecting 25  $\mu$ l of CFA into the plantar aspect of a distal hind paw and 50  $\mu$ l of CFA into the dorsal aspect of the neck. 12 d later, the mice were injected with 25  $\mu$ l of PPD into the contralateral hind paw. Control animals received an injection of pyrogen-free saline instead of the PPD challenge. After 48 h, the mice were killed by CO<sub>2</sub> asphyxiation and the hind paws were examined for footpad swelling before histological and RNA expression studies.

Footpad thickness was measured in a blinded fashion with an electronic caliper (code 500-133; Mitutoyo Corporation, Tokyo, Japan) both before and 48 h after PPD challenge (18, 19). The precision of this caliper was  $\pm 5 \mu$ m. Each thickness measurement was obtained in triplicate for each animal and time point, and care was taken to avoid exerting tension upon the skin and compressing edema fluid (20). Values are expressed as the difference in footpad thickness for each mouse measured before and after challenge ( $\Delta_{\text{thickness}} [\mu\text{m}]$ ).

A polyclonal rabbit antiserum raised to purified, recombinant mouse MIF was used for the antibody neutralization studies and was established previously to neutralize MIF activity in vivo (14). For antibody inhibition of DTH, 200  $\mu$ l of polyclonal rabbit anti-mouse MIF serum or control, normal rabbit serum (NRS), was injected intraperitoneally 2 h before injecting 50  $\mu$ l of a 1:1 mixture of PPD and either anti-MIF serum or NRS into the contralateral hind paw of the previously sensitized mice. 12 mice were studied per group and footpad swelling was assessed and quantitated as described above. For histological studies, the distal hind limbs were detached mid-tibiotarsally immediately after euthanasia and fixed in 10% neutral-buffered formalin for 48 h. The tissues were decalcified, embedded in paraffin, and 0.5–0.6- $\mu$ m sections cut through the mid-longitudinal section of each specimen (21).

**Reverse Transcription PCR (RT-PCR) Analysis.** DTH reac-

tions were induced in mice ( $n = 4$  per group) as described above and the footpads removed and rapidly transferred into a solution of RNazol-B (Tel-Test Inc., Friendswood, TX) for RNA extraction. RT-PCR for MIF was performed as described previously (15), except that the annealing step was performed at 60°C and the DNA amplification reactions were cycled 26 times. The intron spanning mouse MIF primers were 5'-CCATGCCTATGTCATCGTG-3' and 5'-GAACAGCGGTGCAGGTAAGTG-3' and the  $\beta$ -actin primers were 5'-GTGGGCCGCTCTAGG-CACCA-3' and 5'-TGGCCTTAGGGTTCAGGGGG-3'.

**In Situ Hybridization Studies.** BALB/c mice ( $n = 2$ ) were immunized with CFA as described above and 12 d later injected subcutaneously with 30  $\mu$ l of PPD into the contralateral hind paw. The in situ hybridization procedures followed methods described previously and were performed on paraffin-embedded tissue sections that were subjected to limited proteinase K digestion (21, 22). The MIF mRNA-specific probe was obtained as follows. The plasmid pET11b (Novagen, Madison, WI) containing the full-length mouse MIF cDNA (16) was digested with the restriction enzymes XbaI and BamHI, and the 348-bp insert was isolated and ligated into the XbaI/BamHI-digested phagemid Bluescript SK<sup>+</sup> (Stratagene, La Jolla, CA). The antisense riboprobe was obtained by linearizing the resulting construct with BamHI and synthesizing the complementary RNA from the T3 promoter using an RNA synthesis kit (Promega, Madison, WI). The sense riboprobe was obtained by linearizing the phagemid construct with XbaI and using the T7 promoter for RNA synthesis. Serial sections also were stained for macrophage/monocyte-specific (Ram11 mAb [diluted 1:30]; DAKO, Carpinteria, CA) and T cell-specific (anti-Thy-1.2 mAb [diluted 1:10]; Boehringer Mannheim Biochemicals, Indianapolis, IN) cell surface markers (23).

**Immunohistochemical Analysis of MIF Protein.** Control or DTH footpads were obtained from two mice per group as described above, immersed in OCT embedding medium (Tissue-Tek; Miles Inc., West Haven, CT), and snap frozen in isopentane/liquid N<sub>2</sub>. 5- $\mu$ m frozen sections then were cut and placed on poly-L-lysine-coated slides. Immunohistochemical staining of MIF was performed following a modification of the avidin-biotin complex-immunostaining procedure (23), except that the slides were incubated with the primary antibody for 16 h at 4°C. Rabbit anti-MIF serum and NRS were used at a dilution of 1:500. Negative staining controls used PBS or NRS instead of the primary antibody solution. In separate experiments, preincubation of anti-MIF antiserum with recombinant MIF was found to abolish positive immunostaining.

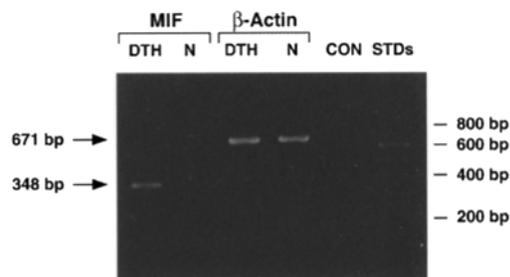
**Analysis of PPD-induced Cytokine Production.** Cytokine production was analyzed in cell culture supernatants of RAW 264.7 mouse macrophages, thioglycollate-elicited mouse peritoneal exudate cells (PECs), and purified mouse T cells. T cells were prepared from the spleens of unprimed BALB/c mice by passage over a mouse T cell enrichment column (R&D Systems, Minneapolis, MN) and were >95% pure by flow cytometry analysis after staining with an anti-CD3 antibody (PharMingen, San Diego, CA). Macrophages ( $3 \times 10^6$  cells/ml) or T cells ( $2 \times 10^6$  cells/ml) were cultured in RPMI 1640/10% FBS containing 2 mM glutamine and 50  $\mu$ g/ml gentamicin (GIBCO BRL, Gaithersburg, MD) and were incubated for 16 h with various concentrations of PPD (10 pg/ml–10  $\mu$ g/ml). Cell-conditioned media were removed at intervals, and the MIF content was analyzed by SDS-PAGE/Western blotting (15) and ELISA (Metz, C. N., J. Bernhagen, M. Bacher, T. Calandra, S. B. Doty, T. Donnelly, and R. B. Bucala, manuscript in preparation). Western blotting

analysis of PEC- and T cell-derived MIF was performed in combination with enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). TNF- $\alpha$  activity was quantitated by L929 cell cytotoxicity (15).

## Results

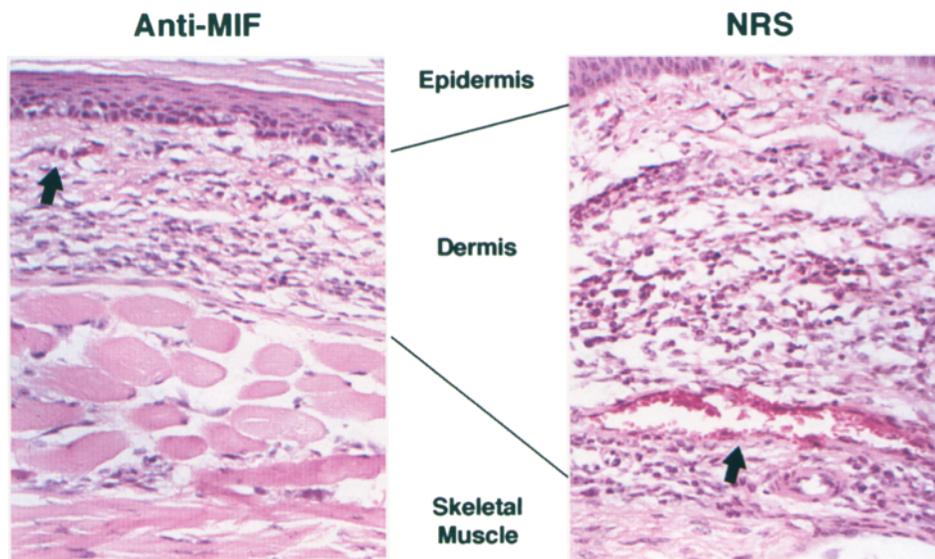
The injection of PPD into the footpads of CFA-sensitized mice produced within 48 h a characteristic DTH response: erythema, induration, and mononuclear cell infiltration. Evidence for the expression of MIF in this response was first obtained by RT-PCR analysis. Total tissue RNA was prepared from four PPD-injected and four saline-(control) injected footpads, reverse transcribed, and the resulting cDNA products were amplified with intron-spanning MIF or  $\beta$ -actin primers. DNA amplification for 26 cycles demonstrated prominent MIF expression in the DTH, but not in the control mouse footpads (Fig. 1).

To assess the importance of MIF production in this model of the DTH response, we then treated CFA-primed mice ( $n = 6$  per group) with either a neutralizing antiserum raised to recombinant mouse MIF or a control (nonimmune) serum. After PPD challenge, mice that were treated with anti-MIF antiserum showed a significantly reduced footpad inflammatory reaction at 48 h when compared to mice treated with control serum. The mean footpad thickness ( $\Delta_{\text{thickness}}$ ) was  $170 \pm 20 \mu\text{m}$  in the anti-MIF-treated mice versus  $240 \pm 30 \mu\text{m}$  in the control mice (mean  $\pm$  SEM,  $P < 0.05$ , by Student's  $t$  test, independent variable). Histological examination showed a mixed mononuclear cell infiltrate, a few scattered neutrophils, and dermal edema in both groups of footpads. However, the degree of infiltration and dermal expansion was approximately two-fold less in the anti-MIF-treated mice than in the control serum-treated group (Fig. 2). There was also a marked degree of vascular dilatation in the control DTH lesions that was not present in the footpads of the anti-MIF-treated mice.

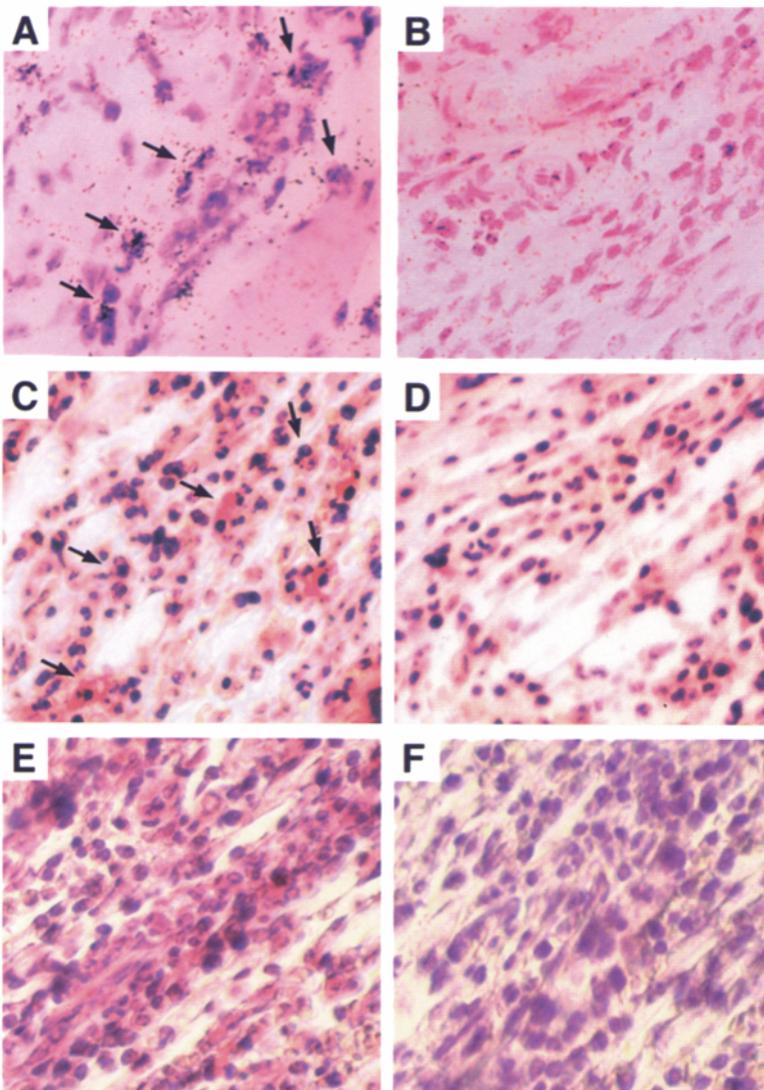


**Figure 1.** RT-PCR analysis of MIF expression in the tuberculin DTH reaction. RNA was isolated from the footpads of CFA-sensitized BALB/c mice 48 h after injection of PPD or saline ( $n = 4$  mice per group). The DNA amplification products shown were obtained from a representative PPD-injected (DTH), and a normal saline-injected (N) footpad. CON, PCR control performed without added cDNA template. STDs, DNA bp standards.

Although MIF activity has been historically considered to be a product of activated T cells (3, 4), recent studies have identified the macrophage to be an important source of MIF production in vivo (15). To investigate the cell source(s) of MIF production in the DTH response, we performed in situ hybridization analysis of MIF gene expression in DTH lesions obtained from the PPD-injected, CFA-sensitized mice. Cells that were positive for MIF mRNA expression were identified to be present in tissue sections that were hybridized to an MIF antisense probe (Fig. 3 A), but not in tissue sections that were hybridized to an MIF sense probe (Fig. 3 B). The cells that expressed MIF were grouped in clusters and appeared overall to display a morphology that was more consistent with that of monocytes/macrophages (i.e., abundant cytoplasm and a large round or oval nucleus) than with T lymphocytes. This impression was confirmed by costaining studies performed with antibodies directed against the monocyte/macrophage-specific marker Ram11 and the T cell-specific marker Thy-1.2. The Ram11-positive cells were clustered and colocalized with the MIF mRNA-positive cells



**Figure 2.** Effect of anti-MIF antiserum on DTH. Representative footpad sections from an anti-MIF serum-treated mouse (Anti-MIF) and from a control NRS-treated mouse (NRS).  $\times 150$ . Arrows indicate blood vessels.

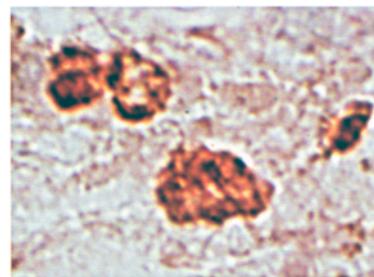


**Figure 3.** In situ hybridization of MIF mRNA in DTH. Footpads of CFA-sensitized BALB/c mice were injected with PPD 48 h before analysis by in situ hybridization with MIF-specific antisense (A) and sense (B) riboprobes. Cell types were determined by immunostaining serial sections for the macrophage-specific marker Ram11 (C) and the T cell-specific marker Thy-1.2 (E). Antibody specificity was established by staining studies performed in the absence of the respective primary antibodies (D and F).  $\times 300$ .

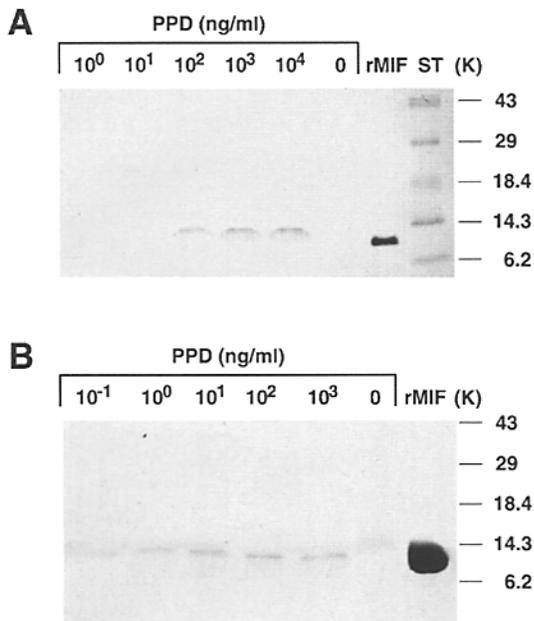
(Fig. 3, C and D), whereas the Thy-1.2-positive T cells were distributed in a more diffuse and homogenous pattern (Fig. 3, E and F). The Thy-1.2 positive cells displayed a morphology that was smaller and more round than the Ram11-positive monocytes/macrophages. Immunohistochemical staining of DTH footpads with anti-MIF antibody also showed the MIF-positive cells to be more consistent morphologically with cells of the monocyte/macrophage phenotype (Fig. 4).

The role of the macrophage in the DTH response was investigated further by examining the ability of PPD to directly induce MIF production in vitro. PPD, a protein-enriched fraction of *M. tuberculosis* culture filtrate, is a known macrophage activator that has been reported to directly induce the release of a number of proinflammatory mediators such as TNF- $\alpha$  and IL-1 (24–26). Supernatants obtained from PPD-stimulated (100 pg/ml–10  $\mu$ g/ml) RAW 264.7 mouse macrophages were found to contain significant quantities of TNF- $\alpha$ , as measured by L929 cytotoxicity (0.02 ng/ml–10.4 ng/ml), thereby confirming

these previous observations (data not shown). Of note, incubation of RAW 264.7 mouse macrophages with PPD (100 pg/ml–10  $\mu$ g/ml) for 16 h also produced a specific, concentration-dependent release of MIF that was detected by SDS-PAGE/Western blotting (Fig. 5 A). By ELISA



**Figure 4.** Immunohistochemical staining of representative DTH lesions with anti-MIF antibody ( $\times 750$ ). Control studies performed with NRS or PBS in place of the primary antibody solution were uniformly negative (not shown).



**Figure 5.** PPD-induced MIF production by RAW 264.7 cells (A) and PECs (B). Macrophages were incubated for 16 h with PPD at the indicated concentrations, and the MIF secreted into the medium were analyzed by Western blotting. 10 ng of rMIF was electrophoresed and transferred as a standard. ST, molecular weight markers.

analysis, the MIF-positive supernatants were determined to contain immunoreactive MIF that was in the range of 1.8–7.4 ng/ml. A similar PPD-induced secretion of MIF was observed in thioglycollate-elicited, peritoneal macrophages (Fig. 5 B), although ELISA analysis showed that lower amounts of MIF were produced by these primary macrophages ( $0.05\text{--}0.2 \pm 0.1$  ng/ml; mean  $\pm$  SD of four experiments). By contrast, MIF release was not detected after stimulation of primary mouse T cells or the LBRM T cell line with PPD (100 pg/ml–10  $\mu$ g/ml for 16 h), as assessed by SDS-PAGE/Western blotting using a sensitive enhanced chemiluminescence staining method or by MIF-specific ELISA analysis (not shown).

## Discussion

The tuberculin skin reaction is a classical model of the DTH response. It is specific to the sensitizing stimulus, is “delayed” in onset, and is mediated strictly by a cellular im-

mune response and not by humoral antibodies. That the administration of neutralizing anti-MIF antibodies to sensitized mice inhibits the development of DTH affirms the many observations over the years that have linked MIF “activity” with soluble product(s) elaborated by the infiltrating mononuclear cells (3, 4). While MIF has been considered originally to be a product of activated T cells (3, 4), however, the present data indicate that the macrophage, rather than the T cell, is the predominant source of the MIF expressed in the tuberculin-induced DTH reaction. The macrophage has been shown recently to be responsible for much of the MIF which is produced *in vivo* (15). MIF is present in preformed intracellular stores and is readily released from macrophages by stimulation with Gram-negative endotoxin, Gram-positive exotoxins, or by the proinflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  (15, Calandra, T., unpublished observations).

Although the present data indicate that PPD can directly elicit MIF production from macrophages, the precise signals necessary to induce the production of MIF from macrophages *in vivo* may vary with the nature of the particular invasive stimulus. Thus, in the absence of microbial products such as PPD, the macrophage release of MIF in DTH may be induced by IFN- $\gamma$  secreted from antigen-stimulated T cells and/or by TNF- $\alpha$  released in an autocrine fashion from infiltrating monocytes/macrophages. While MIF expression was not detected in T cells by either *in situ* hybridization or immunostaining, the present data do not exclude a role for T cell MIF in the elaboration of the DTH response. Like the macrophage, T cells have been identified recently to contain MIF protein in preformed intracellular pools (15). MIF can be released from T cells by both mitogen- and antigen-specific stimuli, and T cell-derived MIF also may contribute significantly to the evolution of the DTH response.

Macrophage and T cell MIF is secreted in direct response to glucocorticoid stimulation and acts to “override” the inhibitory effect of steroids on proinflammatory cytokine production (i.e., TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8) (17). Locally produced MIF thus may act to balance the antiinflammatory effects of stress- or infection-related increases in systemic glucocorticoids that might prevent local inflammatory responses from working to eliminate the source of tissue invasion.

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