# The effects of macrophage migratory inhibitory factor on acute-phase protein production in primary human hepatocytes

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**Abstract.** Macrophage inhibitory factor (MIF) is a pituitary peptide released during the physiological stress response, a T-cell product secreted during the antigen-specific response and a pro-inflammatory macrophage cytokine secreted after LPS stimulation. It has become apparent that MIF is central to the regulation of the inflammatory response and is implicated in the pathogenesis of a variety of acute and chronic inflammatory conditions. This is, at least in part, due to the apparent counter-regulation of the anti-inflammatory actions of glucocorticoids, including the reversal of glucocorticoid-mediated IL-6 release inhibition. This study examines the effect of recombinant MIF on regulation of the acute phase response in isolated human hepatocytes. MIF alone increased C-reactive protein (CRP) release in a dose-dependent manner ≤0.1 ng/ml after which the effects of MIF were attenuated. In combination with IL-6 both CRP and and α-1-antichymotrypsin (ACT) release were increased above levels found with either IL-6 or MIF treatment alone. Dexamethasone attenuated the effects of MIF upon CRP production but increased the MIF stimulated release of ACT. The study demonstrates that the effects of MIF upon the acute phase response are complex and can differentially modulate the production of acute phase proteins depending on the presence of other factors.

## Introduction

Macrophage migration inhibitory factor (MIF) was first identified in the early 1960s as a T-cell cytokine but has since been found to be a pituitary peptide released during stress (1,2) and a pro-inflammatory cytokine secreted after LPS stimulation (3). MIF appears to be central to the regulation of the innate immune response and, unusually for a cytokine it is constitutively expressed. Administration of LPS *in vivo* results in rapid release of MIF (4,5). MIF-deficient cells are hyporesponsive to LPS administration and it was through this observation that MIF was found to be critical in maintaining the expression of the LPS receptor Toll-like receptor 4 (TLR4) (6). In addition, MIF-deficient mice are unable to mount an effective immune response when infected with *Salmonella typhimurium* (7), the reduced pathogen clearance being associated with attenuated levels of TNF-α and IFN-γ.

Whilst MIF has been shown to be essential in mounting an effective immune response to bacterial pathogens, excessive circulating MIF is a potent mediator of endo- (4) and exotoxemia (8) and has been associated with the pathogenesis of a number of acute and chronic inflammatory conditions, including ARDS (9), arthritis (10), and cancer (11). Neutralisation of MIF activity is associated with reduced lethality of sepsis in mice (12). In a clinical setting increased MIF levels are associated with poor outcomes and plasma concentrations of MIF are significantly elevated in patients with severe sepsis (13).

Central to the role of MIF in regulation of the immune response is an apparent feed-back loop involving anti-inflammatory glucocorticoids. Whilst glucocorticoids inhibit the production of many pro-inflammatory cytokines, low concentrations of dexamethasone induce the secretion of MIF by macrophages (14) and T-cells (15). MIF in turn attenuates the glucocorticoid inhibition of other pro-inflammatory cytokines including interleukin-6 (IL-6) (3). Therefore, some of the pro-inflammatory actions of MIF may actually be attributable to its inhibitory effects on the anti-inflammatory actions of glucocorticoids.

Acute-phase proteins are produced in response to tissue injury and as a result of neoplasia, trauma or infection. High concentrations of C-reactive protein are associated with cachexia and poor survival outcomes in cancer patients. Given the pro-inflammatory role of MIF, we hypothesised that MIF would increase the synthesis of positive acute phase protein in hepatocytes, and would increase the stimulation of acute

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phase proteins by other pro-inflammatory cytokines, IL-6 and IL-8. In apparent contrast to their anti-inflammatory roles, work by us (16), and others (17) have demonstrated that glucocorticoids further up-regulate an acute phase response in primary human hepatocytes in response to IL-6. Moreover, given the apparent inhibitory role of MIF in glucocorticoid counter-regulation of the inflammatory response we further hypothesised that MIF would inhibit the glucocorticoid induction of the acute-phase protein response in primary human hepatocytes.

## Materials and methods

Materials for tissue culture were obtained from Gibco-BRL, Irvine, UK and cytokines from Peprotech, London, UK except where otherwise indicated.

*Isolation of human hepatocytes*. Hepatocytes were isolated as previously described (16,18). Briefly, after obtaining informed consent, hepatocytes were isolated from wedge specimens taken from patients undergoing liver resection for metastatic colorectal cancer. Specimens were taken as distant as possible from the metastasis. Each specimen was immediately flushed with heparinized 0.9% saline through the largest apparent vessels which were then cannulated and attached to a rotary pump (Gilson minipulse 3). The liver specimen was sequentially perfused with Hanks Balanced Salt Solution (HBSS) containing 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), followed by HBSS containing 0.5 mM ethylene glycol-bis[ß-aminoethylether]-N,N,N'N'-tetra acetic acid (EGTA), followed by HBSS only. The liver specimen was subsequently perfused with an enzyme solution for 30 min containing 0.05% collagenase type IV (Boehringer-Mannheim, Lewes, UK), 0.017% hyaluronidase type IV (Sigma, Poole, UK), 0.002% deoxyribonuclease (Boehringer-Mannheim), and 5 mM calcium chloride. All solutions contained penicillin (50 U/ml) and streptomycin (50 $\mu$g/ml), were continuously oxygenated and maintained at a temperature of 37˚C and pH of 7.2-7.4. The liver specimen was then teased apart in William's E solution. The cell suspension was passed through a course mesh to remove large fragments of debris and then through a 200 $\mu$m mesh (Sigma). The cells were then washed 3 times by repeated centrifugation in William's E at 450 rpm for 2 min. Subsequently the cells were layered on Percoll (Sigma) gradients and spun at 2000 rpm for 25 min before harvesting the hepatocytes from the 60-80% interface. The hepatocytes were washed a further 3 times at 1000 rpm for 10 min before counting and their viability assessed by Trypan blue exclusion.

*Culture of hepatocytes*. Hepatocytes were diluted to a concentration of $1.5x10^5$ cells/ml. Cell suspension (200 $\mu$l) were added to each well of 96-well tissue culture plates ($3x10^4$ cells/well). All plates were coated with rat tail collagen (Sigma) at a concentration of 2 $\mu$g/ml. The plates were then incubated for 24 h in humidified air containing 5% $CO_2$ at 37˚C to allow hepatocyte adherence.

*Addition of MIF, IL-6, IL-8 and dexamethasone*. The supernatants from the tissue culture plates were discarded and fresh Williams media containing human leptin (0.01, 0.1, 1, 10 ng/ml) was added to each well. Positive control wells contained IL-6 (1 ng/ml), IL-8 (1 ng/ml) or dexamethasone (1 ng/ml). The tissue culture plates were incubated for 48 h and then the supernatants from the 96-well tissue culture plates were removed and stored at -70˚C. The experiments were repeated 3 times.

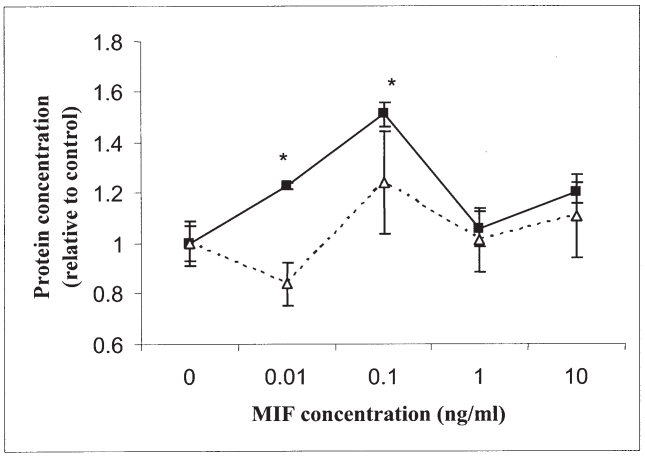


Figure 1. The effects of increasing MIF concentrations upon relative CRP (squares) and ACT (triangles) release in primary human hepatocytes. Values shown are increased protein concentration compared to control values, means ± SEM triplicate cultures; *P<0.05.

*Measurement of acute phase protein production*. The positive acute phase proteins C-reactive protein (CRP) and anti-chymotrypsin (ACT) were measured using sandwich enzyme-linked immunosorbent assay (ELISA). Immunoplates were plated with rabbit antihuman antibody (Dako, Ely, UK) to the specific protein. Sample supernatants were diluted (1:20) and then added to the wells. The plates were incubated for 1 h at room temperature and then washed 3 times. Peroxidase-conjugated rabbit anti-human CRP or ACT antibody (Dako) was added. The plates were incubated for 1 h at room temperature and then washed 3 times. The substrate 3,3',5,5'-tetramethylbenzidine was added and the reaction stopped with 1 M sulphuric acid. The limit of detection for each ELISA taking into account the sample dilutions was 120 pg/ml. The intra-assay variation (%CV) of the ELISA's for CRP and ACT were 3.01 and 0.91 respectively. Inter-assay variation was 7.8 and 9.8 for CRP and ACT respectively.

The plates were read at 450 nm for CRP and ACT using a MR5000 ELISA plate reader (Dynatech, Billinghurst, UK) and the concentrations in the samples were calculated using the AssayZap (Biosoft, Cambridge, UK) computer software. The limit of detection was 0.07 $\mu$g/l for CRP.

*Statistics*. Analysis of significance between variables was performed using the paired two-tailed t-test. A difference was considered significant using 95% confidence intervals ($P<0.05$).

## Results

As far as we are aware no studies have investigated the role of MIF in the synthesis of acute phase proteins from primary

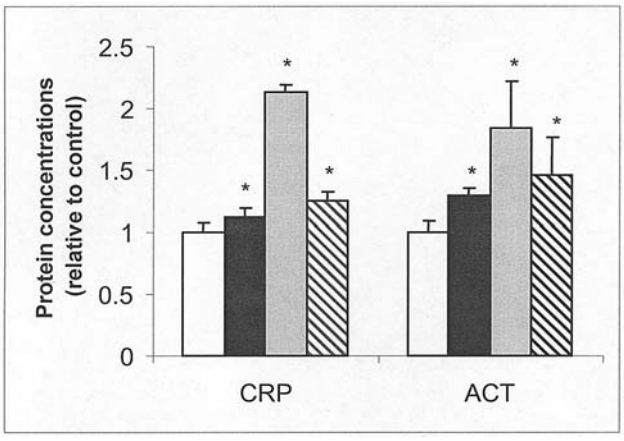


Figure 2. The effects of no treatment (white bars) 1 ng/ml Dex (black bars), 1 ng/ml IL-6 (grey bars) and 1 ng/ml IL-8 (hatched bars), upon relative CRP and ACT release in primary human hepatocytes. Values are means ± SEM triplicate cultures; *P<0.05.

human hepatocytes *in vitro*. Using the model system of primary human hepatocyte culture we demonstrate that MIF increases the synthesis of CRP. The release of the acute phase protein was maximal between 0.01 and 0.1 ng/ml and fell at 1 ng/ml MIF (Fig. 1). MIF alone did not have a significant effect on ACT production.

Interleukin-6 (1 ng/ml) had a significant effect upon CRP and ACT release (Fig. 2) compared with no treatment as previously reported (16). Dexamethasone (1 ng/ml) or IL-8 alone (1 ng/ml) (Fig. 2) also had significant effects on the induction of CRP and ACT production (P<0.05).

Dexamethasone alone (Fig. 3) had a small but significant positive effect upon both CRP (12.3±0.856 control versus 13.8±0.6 ng/ml treated, P=0.001) and ACT (136.4±12.1 ng/ml control versus 175.7±9 ng/ml treated, P=0.000) release. Dexamethasone generally attenuated the effects of MIF upon CRP release. The inhibition of CRP release was maximal at a dose of 0.1 ng/ml MIF (15.8±0.7 ng/ml 0.1 ng/ml MIF/Dex versus 18.5±0.6 ng/ml 0.1 ng/ml MIF alone, P=0.025). In contrast, 1 ng Dex stimulated the MIF-induced increase in ACT release at 0.01 ng and 0.1 ng of MIF.

IL-6 significantly increased production of CRP (Fig. 3) from 12.3±0.9 ng/ml to 26.1±1.2 ng/ml (P=0.022). In the presence of MIF there was an additive effect upon CRP production which was significantly elevated to 35.6±1.5 ng/ml maximally at 0.01 ng/ml compared with IL-6 alone, 26.1±1.2 ng/ml (P=0.000). ACT was stimulated by the addition of IL-6 from 136.4±12.1 ng/ml to 250.3±52.8 ng/ml. Although remaining elevated compared to controls, ACT release was reduced across a range of MIF concentrations compared with IL-6 alone, with a maximal inhibition to 184.7±17.4 ng/ml at 0.1 ng/ml MIF where there was no significant difference (P=0.236) between 0.1 ng MIF and 0.1 ng MIF together with 1 ng IL-6. The inhibitory effect of MIF upon ACT release was ablated at a dose of 10 ng/ml, measurable ACT being 245.3±25.4 ng/ml which was not significantly different from ACT production with IL-6 alone.

Interleukin-8 (Fig. 3) had a small but significant effect upon CRP (12.3±0.9 ng/ml control versus 15.3±0.5 ng/ml treated, P=0.000) as previously described (18), however the increase in ACT release observed in this study did not attain

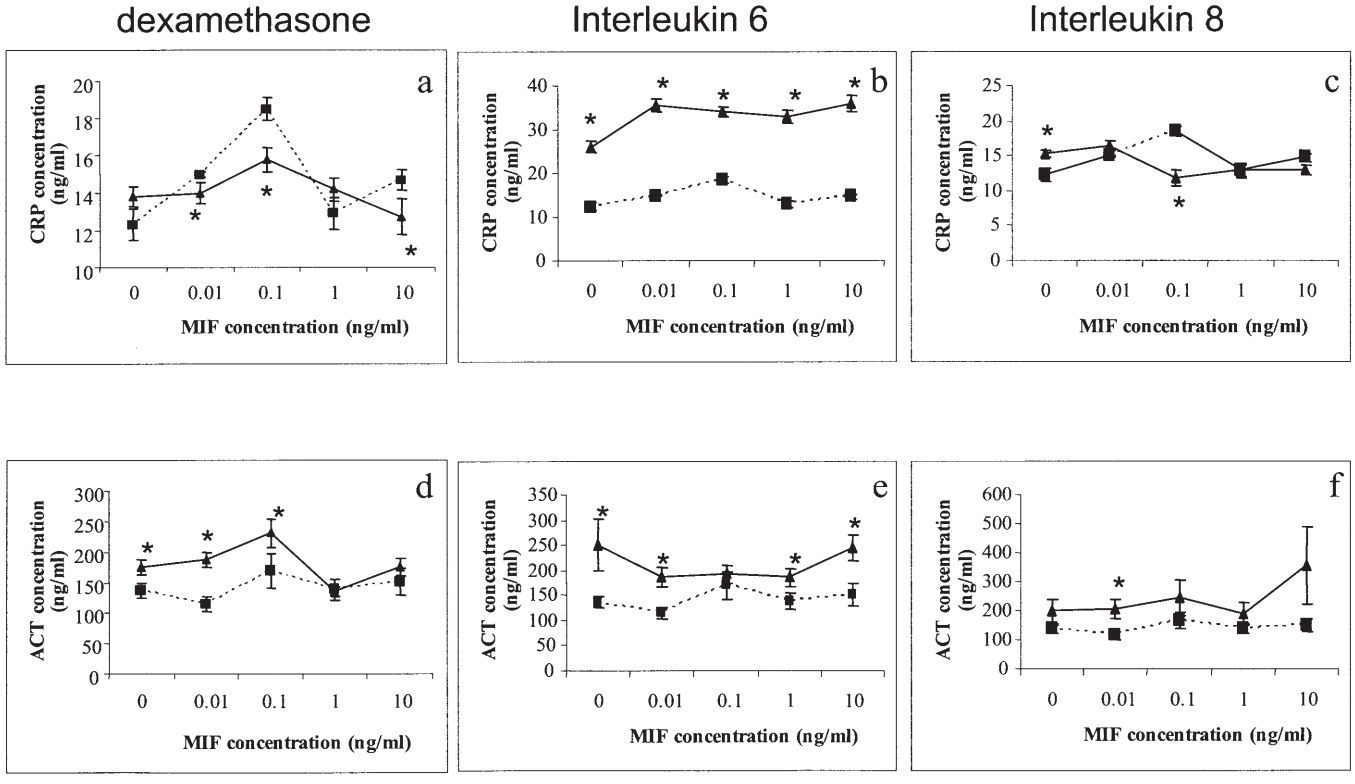


Figure 3. The effects of Dex (a and d), IL-6 (b and e) and IL-8 (c and f) in combination with MIF (triangles) compared with MIF alone (squares) on the induced production of CRP (a-c) and ACT (d-f) from primary human hepatocytes. Values shown are mean levels of protein ± SEM of triplicate cultures. *P<0.05 compared to MIF alone.

significance (136.4±12.1 ng/ml control vs. 199.1±42.3 ng/ml, treated P=0.093). In combination with MIF at concentrations >0.1 ng/ml CRP release was reduced to control levels. The IL-8 mediated increase in ACT release remained unaffected by the addition of MIF at any concentration tested except 0.01 ng.

## Discussion

MIF has emerged as an important regulator of the host immune response to infection, and is essential for efficient clearance of bacterial pathogens (7) and detection of endotoxin (6). One of the primary responses to infection or trauma is the activation of the acute-phase response by the liver upon stimulation by pro-inflammatory cytokines. The present study was undertaken to investigate the effects of MIF upon the production of specific acute phase proteins: CRP which can bind bacteria, activate complement and stimulate expression of pro-inflammatory mediators (19), and ACT, an important protein in the control of the macrophage response (20). These data demonstrate that MIF acts directly upon hepatocytes to amplify the acute-phase response by up-regulation of both CRP and ACT production.

Interleukin-6 is thought to be the principle pro-inflammatory cytokine stimulating the acute-phase response. Serum IL-6 levels are elevated in clinical conditions associated with an acute-phase response (21,22), but although a significant correlation exists between IL-6 and acute-phase proteins, this correlation is weaker than would be expected if IL-6 were alone responsible (23,24). We have previously demonstrated that the effects of IL-6 upon acute phase protein production were influenced by the presence of counter-regulatory hormones (16). The interaction between IL-6 and MIF upon the synthesis of CRP and ACT were examined. Whilst MIF alone significantly increased both CRP and ACT up-regulating both pro- and anti-inflammatory acute phase proteins, the increases were small compared to that achieved with IL-6 treatment. In combination with IL-6 there was a significant increase in CRP but decreased ACT release compared to that stimulated by either IL-6 alone. MIF alone does not regulate IL-6 production, however it is known to reverse glucocorticoid inhibition of IL-6 release (3). It is perhaps through this counter-regulation of glucocorticoid action upon IL-6 production that MIF exerts a more significant effect on the acute phase response.

We have also previously demonstrated that IL-8 elicits an acute-phase response in primary human hepatocytes (18). Given the synergism between MIF and IL-6, and the apparent role of MIF in the regulation of the expression of IL-8, the effects of MIF upon IL-8 actions were investigated. IL-8 increased ACT release however MIF failed to stimulate ACT further. The effects upon CRP were unexpected. Whilst a concentration of 0.1 ng/ml MIF alone gave the most significant increase in CRP, in combination with IL-8, CRP returned to the levels produced by unstimulated hepatocytes.

Glucocorticoids are potent anti-inflammatory mediators and exhibit a complex relationship with MIF. Whilst high concentrations of glucocorticoids inhibit MIF release, low levels stimulate it. Moreover, MIF has been demonstrated to overcome the inhibitory effects of glucocorticoids upon immune cell function. Dexamethasone in combination with MIF decreased the effects of MIF upon CRP production, yet augmented the effects of MIF upon ACT production. By blunting the increase of pro-inflammatory CRP and increasing ACT release, the net effect may lead to a more rapid resolution of the acute-inflammatory response. This study demonstrates that the overall effect of the glucocorticoid-MIF interaction in relation to acute-phase protein secretion appears to be selective, possibly resulting in an overall dampening of the immune response.

In conclusion, the effects of MIF upon the regulation of inflammation are complex. *In vivo* neutralisation of MIF can rescue mice subjected to LPS (12), yet clearance of bacterial pathogens is diminished in MIF-null mice (7). This study has provided evidence that MIF can induce the synthesis of acute phase proteins. Moreover, the actions of MIF upon acute-phase protein synthesis are complex and are governed by the presence of other pro- and anti-inflammatory stimuli. Given the apparent roles of MIF in the pathogenesis of acute and chronic inflammatory disease these complex interactions are worthy of further investigation.

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