0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(1):35–43

doi: 10.1210/en.2004-0946

Macrophage Migration Inhibitory Factor Is Released from Pituitary Folliculo-Stellate-Like Cells by Endotoxin and Dexamethasone and Attenuates the Steroid-Induced Inhibition of Interleukin 6 Release

Tanya Tierney, Reshma Patel, Caroline A. S. Stead, Lin Leng, Richard Bucala, and Julia C. Buckingham

Department of Cellular and Molecular Neuroscience (T.T., R.P., C.A.S.S., J.C.B.), Division of Neuroscience and Psychological Medicine, Faculty of Medicine, Imperial College London, London W12 0NN, United Kingdom; and Department of Internal Medicine and Pathology (L.L., R.B.), Yale University School of Medicine, New Haven, Connecticut 06520

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine produced by peripheral immune cells and also by endocrine cells in the anterior pituitary gland. MIF exerts its proinflammatory actions in the host-defense system by blocking the inhibitory effects of glucocorticoids on the release of other proinflammatory cytokines (*e.g.* IL-1, IL-6, TNFα). Reports that pituitary folliculo-stellate (FS) cells share many characteristics with immune cells led us to propose that these cells may serve as an additional source of MIF in the pituitary and that pituitary-derived MIF may act in an autocrine or paracrine manner to modulate endotoxin-induced cytokine release from FS cells. In the present study we addressed this hypothesis by using 1) immunohistochemistry to localize MIF in primary pituitary tissue and 2) well-characterized FS (TtT/GF), corticotroph (AtT20), and macrophage/monocyte (RAW 264.7) cell lines to explore the effects of CRH, endotoxin, and dexamethasone on MIF release and to examine the effects of MIF on IL-6 release. Our immunohistochemical study showed that MIF is expressed in abundance in S100-positive FS cells and also in other pituitary cell types. All three cell lines expressed MIF protein and responded to endotoxin (10–1000 ng/ml, 24 h) and dexamethasone (100 pM to 10 nM, 24 h) with concentration-dependent increases in MIF release. CRH (10–100 nM) also stimulated MIF release from AtT20 cells but, unlike endotoxin and dexamethasone, it had no effect on MIF release from TtT/GF or RAW cells. Recombinant MIF did not affect the basal release of IL-6 from TtT/GF cells; however, it effectively reversed the inhibitory effects of dexamethasone (1 nM) on the endotoxin-induced release of IL-6 from these cells. The results suggest that the FS cells are both a source of and a target for MIF and raise the possibility that MIF serves as a paracrine/autocrine factor in the pituitary gland that contributes to the protective neuroendocrine response to endotoxin. (*Endocrinology* 146: 35–43, 2005)

RECIPROCAL COMMUNICATION BETWEEN the neuroendocrine and immune systems is of fundamental importance to homeostasis. Not surprisingly, in the light of the powerful antiinflammatory and immunosuppressive properties of glucocorticoids, particular attention has focused on the interface between the hypothalamo-pituitary-adrenocortical (HPA) axis and the host-defense system. It is now well established that proinflammatory cytokines released in response to an immune challenge cause marked activation of the HPA axis (reviewed in Refs. 1 and 2) and that the resulting rise in glucocorticoid secretion plays a critical role in tempering the immune response (2). This is amply illustrated by reports that adrenalectomized mice (3) and rats (4) are highly susceptible to bacterial endotoxins (lipopolysaccharide, LPS) and that animals with compromised HPA function show a more severe, and frequently lethal, course of inflammatory disease after induction of *e.g.* experimental arthritis or autoimmune encephalomyelitis (1, 2). Communication between the neuroendocrine and immune systems is effected in part by signaling molecules that are released and recognized by both systems (reviewed in Ref. 5). The common usage of these molecules has blurred the distinction between cytokines and hormones because it has become apparent, for instance, that the receptors for the pituitary hormones, growth hormone and prolactin, belong to the cytokine receptor superfamily and that proinflammatory cytokines such as IL-1 and IL-6 are produced in the neuroendocrine system and act at multiple levels to stimulate glucocorticoid secretion (2). In particular, IL-6 has been shown to enhance and prolong the HPA response to stress (6).

The positive effects of peripherally produced cytokines (*e.g.* IL-1, IL-6, tumor necrosis factor-α, TNFα) on ACTH release are largely dependent on the secretion of CRH and vasopressin by the hypothalamus (reviewed in Refs. 1 and 2). However, cytokines produced within the pituitary and adrenal glands themselves also play a role in activating the HPA axis. Within the anterior pituitary gland, the folliculo-stellate (FS) cells, first identified by Rhinehart and Farquar (7), are an important source of cytokines (*e.g.* IL-1, IL-6, leukemia inhibitory factor) and growth or angiogenic factors (*e.g.* vascular endothelial growth factor and basic fibroblast growth factor, reviewed in Ref. 8), which they

First Published Online September 23, 2004

Abbreviations: FCS, Fetal calf serum; FS, folliculo-stellate; HPA, hypothalamo-pituitary-adrenocortical; LPS, lipopolysaccharide; MIF, macrophage migration inhibitory factor; NF, nuclear factor; rMIF, recombinant MIF.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

use to communicate with the surrounding endocrine and vascular cells. FS cells are also a target for endotoxin (9, 10). Reports that LPS administration *in vivo* increases pituitary IL-6 expression (9) together with evidence that pituitary reaggregate cultures respond to LPS with an increase in ACTH release that is dependent upon the release of IL-6 from FS cells (10), point to an important role of FS cells in the manifestation of the HPA responses to endotoxemia.

The biology of the FS cells is poorly understood. The origin of the cells is disputed and may differ from that of the surrounding endocrine cells, which are derived during development from the oral ectoderm. FS cells stain for S100, a neuroectodermal marker; however, reports that FS cells show characteristics such as phagocytotic activity (11), cytokine production (12–15), and CD14 positivity (16) have fueled a premise that FS cells are of lymphoid rather than neuroectodermal origin. On the other hand, whereas FS cells undoubtedly show some characteristics of macrophages, no cells have been identified in the anterior pituitary gland that stain positively for both FS (S100) and macrophage (OX-42) markers (17). However, Allaerts *et al.* (18) demonstrated cells staining positively for both FS (S100) and the dendritic cell marker, OX-6, suggesting a relationship between these cell types. Interestingly, peripheral cells of a dendritic lineage are also S100 positive (19).

Macrophage migration inhibitory factor (MIF) is a potentially interesting protein with regard to neuroendocrine-immune interactions because it is produced by both immune cells and endocrine tissues (20). MIF was first described as a T cell-derived cytokine that inhibits the random migration of macrophages (21). It has since also been shown to be produced by monocytes and macrophages and to have an important role in the development of Gram-negative sepsis (22). Administration of MIF thus potentiates the lethal effects of LPS (23), whereas neutralizing anti-MIF antibodies have a protective effect (24) and MIF knockout mice are relatively resistant to LPS (25). Interestingly, low levels of glucocorticoids promote MIF release from monocytes and macrophages (26), an apparent paradox given that MIF is a proinflammatory cytokine and glucocorticoids usually exert powerful anti-inflammatory actions. MIF then acts in an autocrine or paracrine manner within the host-defense system to block the effects of glucocorticoids (another paradox) on LPS-induced cytokine release (26). The interplay between the pro- and anti-inflammatory actions of MIF and glucocorticoids thus appears to act as a counterregulatory system that aids the maintenance of homeostasis (20).

The anterior pituitary gland is another important source of MIF. Immunohistochemical analysis at the electron microscopic level has localized MIF to granules in the corticotrophs and thyrotrophs and shown that in corticotrophs some granules contain MIF only, whereas others contain both MIF and ACTH (27). Functional studies on the AtT20 murine corticotroph cell line showed that MIF is secreted in response to LPS and CRH, with a peak response occurring at 3 h (27), compared with 20 h in monocytes, macrophages, and T cells (22). Interestingly, these studies also revealed that the concentration of CRH required to release MIF is lower than that needed to release ACTH, indicating the presence of a mechanism for differential release.

Much attention has focused on the release of MIF from endocrine cells, and the possibility that MIF is also produced by FS cells has not been addressed. Because FS cells have much in common with immune cells, they represent a potential source of and also a possible target for MIF. Our review of the literature has led us to propose that 1) FS cells contribute to the pituitary-derived pool of MIF, 2) MIF is released from FS cells in response to an LPS challenge, and 3) MIF modulates the release of cytokines (*e.g.* IL-6) from FS cells, which normally play a critical role in effecting the neuroendocrine responses to endotoxemia, in particular the activation of the HPA axis. In the present study, we addressed this hypothesis by using 1) immunohistochemistry to determine whether MIF is expressed by primary FS cells and 2) well-characterized FS [TtT/GF (28)], corticotroph [AtT20 D16:16 (29)], and macrophage/monocyte [RAW 264.7 (30)] cell lines to explore the effects of CRH, endotoxin, and dexamethasone on MIF release and to examine the effects of MIF on IL-6 release.

Materials and Methods

Double immunofluorescence staining for S100 and MIF

Pituitary glands were removed postmortem from adult male Sprague-Dawley rats (200–250 g), fixed overnight in 4% paraformaldehyde (Sigma, Poole, UK) in PBS (0.1 M PBS, pH 7.4; Sigma), washed in PBS, and cryopreserved for 24 h in 20% sucrose (VWR International, Poole, UK) in PBS. Cryostat sections (15 μm) were cut with a rotary microtome with a fixed blade (Bright, Cambridge, UK) and mounted on gelatin-coated slides. The sections were air-dried before immunofluorescence staining for S100 (a marker of FS cells) and MIF, using a modification of the method of Chapman *et al.* (31). Briefly, the sections were subjected sequentially to the following procedures with PBS washes between each stage: antigen retrieval [1 mg/ml trypsin (Sigma), 1 mg/ml $CaCl_2$ (VWR International) in PBS for 15 min at 37 C], permeabilization [0.2% Triton X-100 (Sigma) for 5 min], reduction of autofluorescence [75 mM NH_4Cl (VWR International), 20 mM glycine (Sigma) for 10 min], and blocking of nonspecific binding [3% BSA (Sigma) for 1 h]. The sections were then stained for S100 or MIF using the following antibody systems (all made up in 3% BSA in PBS): 1) rabbit anticow S100 (1:100, 4 C, overnight; DakoCytomation Ltd., Ely, Cambridgeshire, UK) followed by biotinylated swine antirabbit IgG (1:200, room temperature, 30 min; DakoCytomation Ltd.) and streptavidin-Texas Red (10 μg/ml, room temperature, 30 min; Pierce, Rockford, IL) and 2) affinity-purified goat antihuman MIF (1:100, 4 C, overnight; R&D Systems, Abington, Oxon, UK) followed by fluorescein isothiocyanate-conjugated rabbit antigoat IgG (1:200, room temperature, 30 min; Sigma). Antibody dilutions were selected on the basis of published observations and the supplier's data sheet (31, 32). Negative controls were performed by replacing the primary antibody with nonimmune rabbit or goat serum at the same concentration as the primary antibody. (Further details of antibody specificity are provided in Refs. 31 and 32.) The slides were mounted in Mowiol (Sigma) and visualized using a LSM5 Pascal laser-scanning confocal microscope (Zeiss, Thornwood, NY).

Cell lines

Murine FS-like (TtT/GF; a gift from Prof. John Morris, University of Oxford, UK), corticotroph (AtT20 D16:16; a gift from Dr. Simon Guild, University of St. Andrews, Fife, UK), and macrophage (RAW 264.7; purchased from American Type Culture Collection, Manassas, VA, purchased via LGC Promochem, Teddington, Middlesex, UK) cell lines were maintained as described previously (33) at 37 C in DMEM (Invitrogen Ltd., Paisley, UK) enriched with 10% fetal calf serum (FCS; PAA

Laboratories Ltd., Yeovil, UK), penicillin (100 U/ml, Sigma), and streptomycin (0.1 mg/ml, Sigma) in a 5% CO_2 humidified atmosphere.

Cell line extracts

Total cell lysates of TtT/GF, AtT20 and RAW cells were prepared by extraction in lysis buffer [1ml/T-75 flask: 250 mM Tris (Sigma), 1 mM EDTA (Sigma), 1 mM EGTA (Sigma) 0.1% Triton X-100 (Sigma), pH 7.5 + protease inhibitors (Complete EDTA free; Roche Diagnostics, Lewes, UK)]. The lysates were sonicated on ice, subjected to one freeze-thaw cycle before being cleared by centrifugation (13,000 × *g*, 5 min), and stored at −20 C. The total protein content of the extracts was determined by the Bradford assay (34).

Cell line incubations

Cells were plated at 250,000 (TtT/GF) or 500,000 (AtT20 and RAW) cells per well in 24-well plates and maintained for 2 d in DMEM and 1% FCS before treatments. They were then incubated for 24 h in DMEM + 1% FCS in the presence and absence of graded concentrations of CRH (0.1–1000 nM; Bachem, St. Helen's, Merseyside, UK), LPS (0.1–1000ng/ml, *Escherichia coli* 0.111.B4, activity not <500,000 EU/mg; Sigma), or dexamethasone (10 fM to 1 μM; Faulding Pharmaceutical plc, Leamington Spa, UK). In some experiments, the TtT/GF cells were preincubated with dexamethasone (0.1–1000 nM) and/or murine recombinant MIF (rMIF, 100 ng/ml) for 1 h before the addition of LPS (100 ng/ml). At the end of the 24-h incubation period, the medium was collected and centrifuged at 10,000 × *g* for 5 min to pellet any floating cells. The supernatant fluid was removed and either assayed immediately for MIF or IL-6 or stored at −20 C for subsequent assay.

MIF and IL-6 ELISA

MIF and IL-6 were quantified by commercial ELISAs (R&D Systems) in accordance with the manufacturer's instructions. Briefly, 96-well high-binding plates (VWR International, Lutterworth, UK) were coated overnight with capture antibody (2 μg/ml monoclonal anti-MIF or monoclonal anti-IL-6 in PBS). After washing three times with wash buffer [Dulbecco's PBS (Oxoid Ltd., Basingstoke, UK) plus 0.05% Tween 20 (Sigma)], the plates were blocked for 1 h [5% sucrose (VWR International), 1% BSA (Sigma), and 0.05% sodium azide (VWR International)] in PBS. After further washes, standards (8–2000 pg/ml MIF or 4–1000 pg/ml IL-6) or samples were added to the wells in duplicate and incubated at room temperature for 2 h. With further washes between each stage, the plates were incubated sequentially with the detection antibody [2 h; 100 μl 200ng/ml biotinylated goat anti-MIF or biotinylated goat anti-IL-6 in reagent diluent (PBS + 1% BSA)], 100 μl streptavidin-HRP (20 min; 1:200 in reagent diluent) and substrate solution (100 μl, 20 min; R&D Systems). The color reaction was stopped by addition of 50 μl 2 N H_2SO_4 and the intensity read at 450 nm with background correction at 540 nm. Samples from each experiment were always analyzed in single assays to avoid interassay variance.

Statistical analysis

The data are presented as mean ± SEM of triplicate wells. Because there was some variation between experiments in basal MIF and IL-6 release and in the magnitude (but not the pattern) of the secretory responses to stimulation, statistical analysis was applied to data from individual experiments rather than pooled data from two or more experiments. The analysis was carried out using one-way ANOVA followed by Dunnett's post hoc test (Instat 3; GraphPad Software Inc., San Diego, CA) or two-way ANOVA followed by Holm-Sidak post hoc test (SigmaStat 3.0; SPSS, Chicago, IL). Data were considered statistically significant when $P < 0.05$. The quantitative data shown are representative of at least two separate experiments.

Results

Figure 1 shows immunofluorescence staining of S100 and MIF in rat pituitary sections, both individually (A and B) and overlaid (C). Cells staining positively for S100 (*red*, A) and MIF (*green*, B) were abundant in the tissue. There was a high degree of colocalization of S100 and MIF (*yellow staining*, C), indicating that rat FS cells contain MIF protein. Other areas stained for MIF only, an observation that is consistent with reports that MIF is also expressed by endocrine cells in the anterior pituitary gland (27). No staining was evident when the primary antibodies were replaced with nonimmune serum (Fig. 1, D and E), indicating the staining was specific.

To study the regulation of MIF release from FS cells, we used the murine TtT/GF FS cell line as a model and compared the responses of these cells with those of murine corticotroph (AtT20) and macrophage (RAW) cell lines. Preliminary experiments on protein extracts of the cells demonstrated by ELISA that all three cell lines express MIF protein, with a concentration of approximately 700 pg/mg protein in the TtT/GF cells, compared with 200 and 275 pg/mg in AtT20 and RAW cells, respectively.

Figures 2–4 demonstrate the effects of graded concentrations of CRH, dexamethasone, and LPS on the secretion of MIF from the three cell lines. The AtT20 cells responded to CRH with a concentration-dependent increase in MIF release. Maximal effects were observed at 10 nM ($P < 0.01$ *vs.* basal), and at higher concentrations, the effects were reduced (Fig. 2A). In contrast, CRH had no significant effects ($P > 0.05$ *vs.* basal) on the release of MIF from TtT/GF (Fig. 2B) or RAW (Fig. 2C) cells. Unlike CRH, dexamethasone stimulated the release of MIF from all three cell lines (Fig. 3). Thus, at a concentration of 100 pM, dexamethasone had a highly significant effect in AtT20 cells ($P < 0.01$ *vs.* basal, Fig. 3A), although lower (10 fM and 1 pM) and higher (10 nM and 1 μM) concentrations of the steroid were without effect ($P > 0.05$ *vs.* basal), whereas in the TtT/GF (Fig. 3B) and RAW (Fig. 3C) cells, dexamethasone produced significant increases in MIF release at 100 pM and 10 nM ($P < 0.01$ or $P < 0.05$), but not at the other concentrations tested. LPS also stimulated the release of MIF from all three cell lines (Fig. 4). In the AtT20 cells, a maximal response ($P < 0.01$ *vs.* basal) was attained at 10 ng/ml LPS (Fig. 4A), and the response to a higher concentration appeared less pronounced. By contrast, in TtT/GF (Fig. 4B) and RAW (Fig. 4C) cells, a maximal response was evident at 1000 ng/ml LPS. The magnitude of the response of the AtT20 cells to LPS was considerably greater than that of either the TtT/GF or RAW cells. In addition, the magnitude of response of AtT20 cells to LPS far exceeded that of the responses to CRH (see Fig. 2A) or dexamethasone (see Fig. 3A).

Subsequent experiments aimed to determine whether TtT/GF cells, as well as being a source of MIF, are a target for MIF. This was done by assessing the ability of human rMIF to reverse the inhibitory actions of dexamethasone on LPS-stimulated IL-6 release. Figure 5A shows that LPS caused a concentration-dependent increase in IL-6 release over 24 h, with significant elevations in IL-6 release evident at concentrations of 10 ng/ml LPS and above ($P < 0.01$ *vs.* basal). A separate experiment showed that the ability of LPS (100 ng/ml) to induce the release of IL-6 release is reduced in a concentration-dependent manner by preincubation of the cells with dexamethasone (0.1–1000 nM, Fig. 5B). In the absence of dexamethasone, preincubation of the cells with rMIF (100 ng/ml) had no significant effect on LPS-stimulated

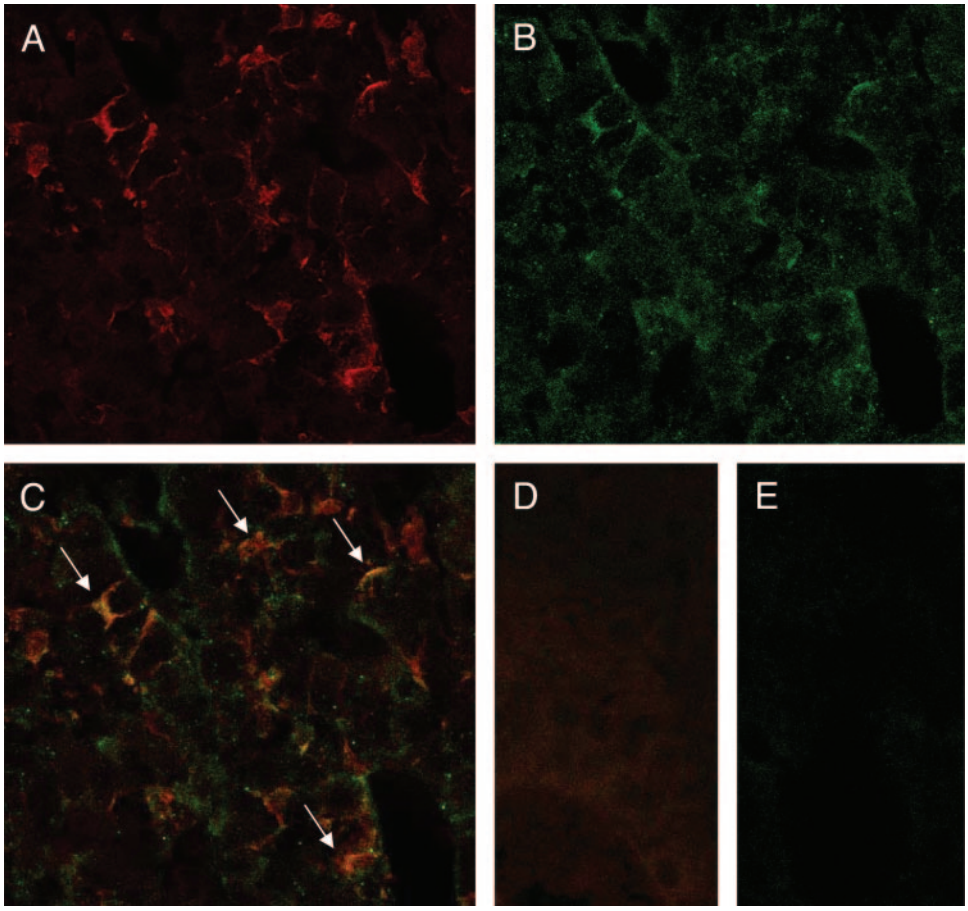

FIG. 1. Confocal images of 15-μm sections of adult male rat pituitary tissue stained for S100 (marker of FS cells, Texas Red; A), MIF (fluorescein isothiocyanate; B), and S100 and MIF (C). Double-positive cells (*orange*) in panel C are indicated with *arrows*. Note that not all cells stain positively for both antigens, suggesting that MIF is also expressed by cells other than FS cells. Negative controls (secondary antibody alone) for S100 and MIF are shown in panels D and E, respectively.

IL-6 release (Fig. 6). However, rMIF (10 ng/ml) partially reversed the inhibitory actions of dexamethasone (1 nM) on LPS-stimulated (100 ng/ml) MIF release ($P < 0.01$), although it failed to modify the inhibitory effects of a higher concentration of the steroid (100 nM, $P > 0.05$). Neither dexamethasone nor rMIF had any effect on basal IL-6 release (data not shown).

Discussion

This study shows for the first time that MIF, a proinflammatory cytokine, is found in abundance in the S100-positive FS cells of the anterior pituitary gland. It thus suggests that the FS cells are an important source of MIF and raises the possibility that FS cell-derived MIF plays a role in mediating the pituitary responses to endotoxemia and other immune insults. Interestingly, a small number of S100-positive FS cells appeared not to stain for MIF (red cells, Fig. 3C). This could be because some FS cells express relatively low levels of the protein, possibly because of their stage of the cell cycle, which defy detection with our staining methodology; alternatively, it is feasible that a subpopulation of FS cells exists that does not stain positively for the protein. Our immunohistochemical analysis also revealed other MIF-positive cells in the pituitary gland; as MIF protein has been identified previously in corticotrophs and thyrotrophs (27), it seems likely that these were endocrine cells.

To provide some insight into the physiological relevance of FS-derived MIF, we compared the effects of three putative MIF secretagogues on MIF release from FS cells and corticotrophs to determine whether the two cell types respond similarly to different stimuli. Because purified populations of primary FS cells and corticotrophs are difficult to prepare and require substantive amounts of pituitary tissue, we based our study on mouse pituitary FS (TtT/GF) and corticotroph (AtT20 D16:16) cell lines after first confirming by ELISA that both cell lines, like their parent cells, express MIF. In addition, as FS cells have been described as "glial-like" and show some characteristics of macrophages (11), we also performed parallel studies on a MIF-expressing murine macrophage/monocyte cell line, RAW 264.7. Although we appreciate that cell lines are not necessarily representative of their parent cell types, we reasoned that these clonal populations would provide useful models in which to explore fundamental mechanisms.

Our data show clearly that CRH stimulates the release of MIF from AtT20 cells but not from TtT/GF and RAW cells. In line with these findings, Nishino *et al.* (27) also demonstrated positive concentration-dependent effects of

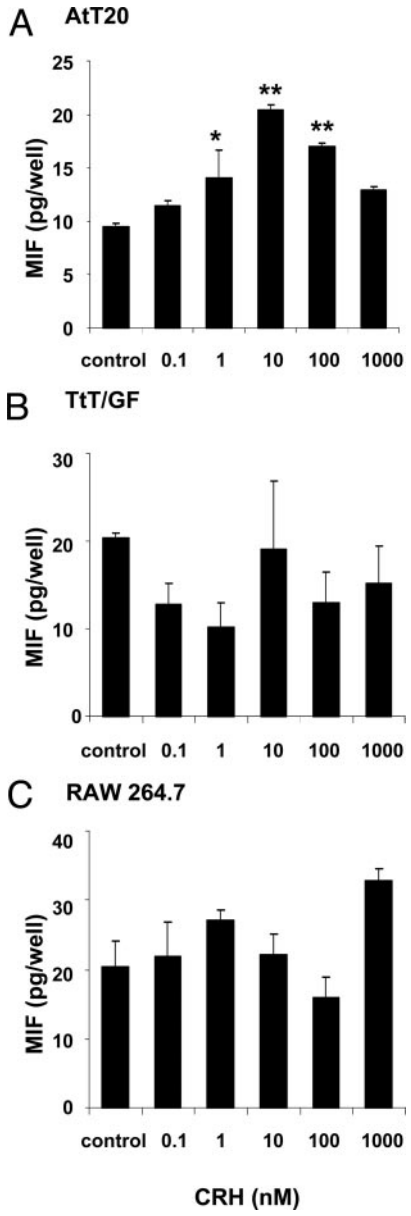

FIG. 2. Effects of graded concentrations of CRH on MIF release over 24 h from AtT20 cells (A), TtT/GF cells (B), and RAW 264.7 cells (C). *, $P < 0.05$, and **, $P < 0.01$, CRH-stimulated *vs.* control. Statistical analysis was carried out by ANOVA and Dunnett's *post hoc* test. The data shown (mean ± SEM, n = 3) are derived from a single experiment and are representative of two separate experiments.

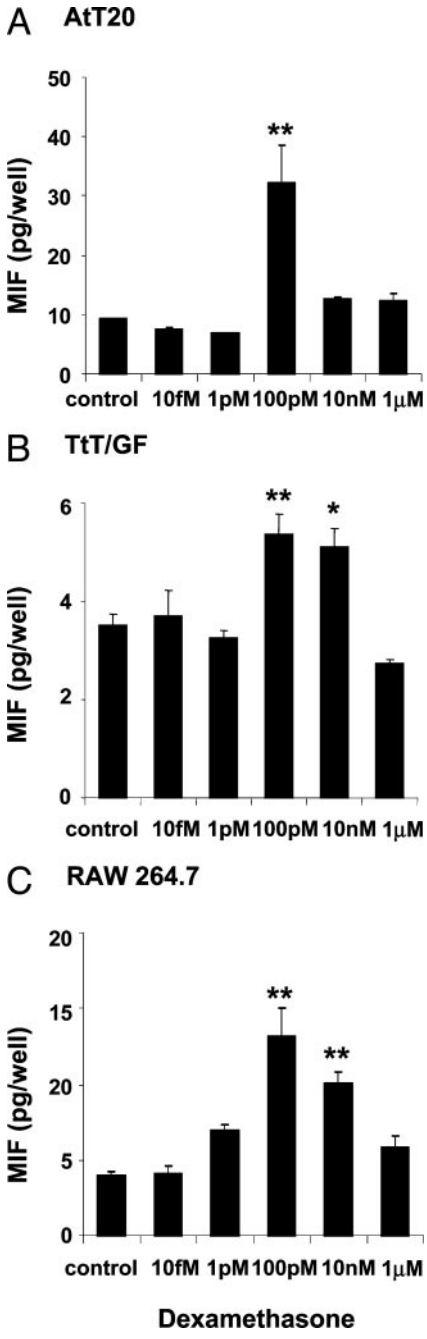

FIG. 3. Effects of graded concentrations of dexamethasone on MIF release over 24 h from AtT20 cells (A), TtT/GF cells (B), and RAW 264.7 cells (C). *, $P < 0.05$, and **, $P < 0.01$, dexamethasone-stimulated *vs.* control. Statistical analysis was carried out by ANOVA and Dunnett's *post hoc* test. The data shown (mean ± SEM, n = 3, from a single experiment) are representative of two separate experiments.

CRH on MIF release from AtT20 cells, although in their study maximal effects of CRH were observed at only 0.1 *vs.* 10 nM in the present study. This potency difference may reflect differences in contact time (16 *vs.* 24 h), culture conditions (particularly the presence or absence of FCS) or, possibly, phenotypic drift of the cell lines. Irrespective of this difference, both studies showed bell-shaped concentration-response curves, with maximal effects occurring at CRH concentrations well below those required for maximal release of ACTH (27, 33), a phenomenon that has been attributed to the presence of two populations of secretory granules, *viz.* those containing MIF only and those containing both MIF and ACTH (27). The failure of CRH to cause MIF release from the TtT/GF and RAW cells is interesting and supports the hypothesis that FS cells behave more like immune cells than endocrine cells. To the best of our knowledge, CRH receptor expression has not been examined in either primary FS cells or the TtT/GF cell line and, thus, we cannot exclude the possibility that the apparent inactivity of CRH in the TtT/GF cells reflects a

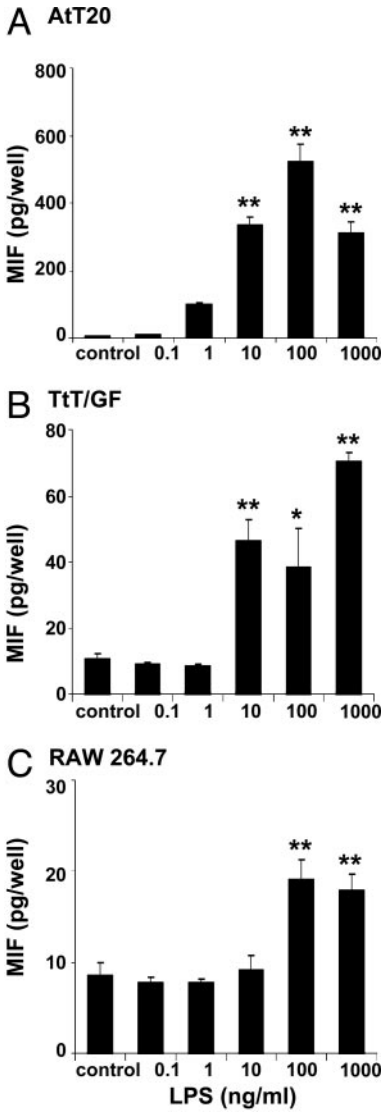

FIG. 4. Effects of graded concentrations of LPS on MIF release over 24 h from AtT20 cells (A), TtT/GF cells (B), and RAW 264.7cells (C). *, $P < 0.05$, and **, $P < 0.01$ LPS-stimulated *vs.* control. Statistical analysis was carried out by ANOVA and Dunnett's *post hoc* test. The data shown (mean ± SEM, n = 3) are derived from a single experiment and are representative of two separate experiments.

lack of functional CRH receptors. CRH receptors are, however, expressed by RAW 264.7 cells and, when activated, they augment LPS-induced TNFα, IL-1β, and IL-6 release (35). Because TNFα stimulates MIF release from macrophages (22), these findings raise the possibility that the reported proinflammatory effects of CRH (2) are mediated in part by potentiation of LPS-stimulated MIF release. If a similar synergistic mechanism operates in FS cells, the LPS-induced release of proinflammatory cytokines would be enhanced; as these cytokines act locally on the corticotrophs to increase ACTH release (reviewed in Ref. 2), this would provide a mechanism for enhancing the HPA response to endotoxemia.

Previous studies have shown that LPS induces MIF release from RAW (22) and AtT20 (23) cells and from primary pituitary tissue (27). In accord with these findings,

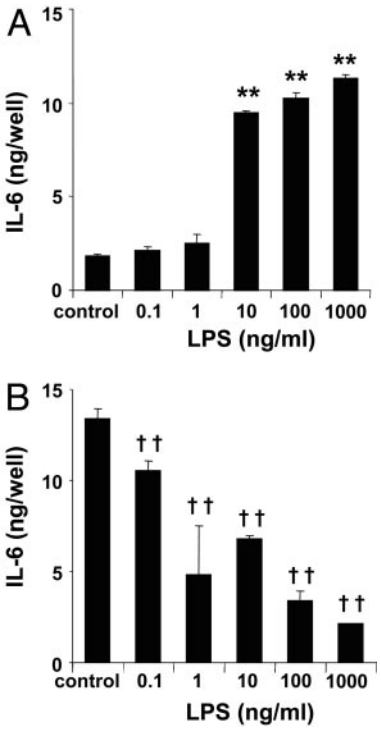

FIG. 5. Effects of LPS on the release of IL-6 from TtT/GF cells over 24 h. A, Concentration response curve. B, Effects of graded concentrations of dexamethasone (added to the medium 1 h before LPS) on the release of MIF induced by LPS (100 ng/ml). **, $P < 0.01$ LPS-stimulated *vs.* control. ††, $P < 0.01$ LPS alone *vs.* LPS + dexamethasone. Statistical analysis was carried out by ANOVA and Dunnett's *post hoc* test. The data shown (mean ± SEM, n = 3, from a single experiment) are representative of two separate experiments.

we also demonstrated positive, concentration-dependent effects of LPS on MIF release from both cell lines; in addition, we showed for the first time that LPS produces comparable effects on TtT/GF cells. LPS signaling is enhanced by a se-rum LPS-binding protein (36) and transduced, in a cell-specific manner, by complex interactions between various membrane-bound and intracellular proteins; these proteins include CD-14, MD-2, and the Toll-like receptors, Tlr-4 and, possibly, Tlr-2 (reviewed in Ref. 37). Although the roles of these proteins are well defined in immune cells (37), their expression and function in the pituitary gland is less well understood, although there is evidence that TtT/GF cells express CD14 and Tlr4 (16). Our laboratory has recently shown by RT-PCR and Western blot analysis that the cell lines used in this study express CD14, Tlr4, Tlr2, and MD-2 mRNAs and protein (Mehet, D., E. Solito, C. John, and J. Buckingham, unpublished data), observations that support a role for the Toll-like receptor family in LPS signal transduction in these cells. Although it is not yet known whether the primary corticotrophs are targets for LPS, several lines of evidence suggest that primary FS cells are. Indeed, LPS-induced ACTH release *in vivo* appears to be effected in part via paracrine actions of IL-6 (10) and possibly other glycoprotein 130 cytokines (2) that are released from the FS cells in response to the toxin.

Like LPS, dexamethasone also stimulated the release of

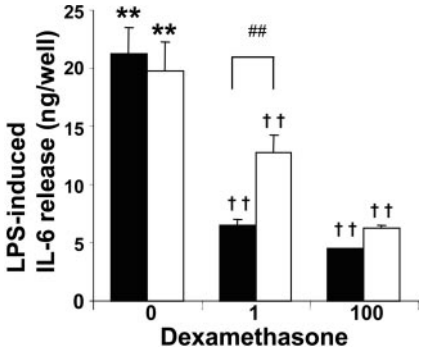

FIG. 6. Effects of rMIF on the inhibitory effects of dexamethasone on the release of IL-6 induced over 24 h by LPS (100ng/ml) from TtT/GF cells. Dexamethasone and rMIF were added to the medium 1 h before LPS. Closed columns, Controls; open columns, rMIF. **, $P < 0.01$, LPS-stimulated *vs.* control. In the absence of LPS, basal IL-6 release was <5.0 pg/ml and near the limit of detection of the assay. Neither dexamethasone nor rMIF affects basal IL-6 release. ††, $P < 0.01$ LPS + dexamethasone *vs.* corresponding LPS alone; ##, $P < 0.01$ LPS + dexamethasone + rMIF *vs.* LPS + dexamethasone. Statistical analysis was carried out by two-way ANOVA and Holm-Sidak *post hoc* test. The data shown (mean ± SEM, n = 3, from a single experiment) are representative of two separate experiments.

MIF from all three cell lines studied. In the case of the RAW cells, our data confirm those of Calandra *et al.* (26), who also showed a bell-shaped concentration-response curve to the steroid. However, whereas Calandra *et al.* (26) reported maximal effects at steroid concentrations of 10 fM to 100 pM, we required concentrations of 100 pM to 10 nM to evoke maximal release. This discrepancy may be attributable to differences between 1) batches of cell lines that are prone to phenotypic drift, 2) culture conditions, or 3) steroid preparations (free steroid *vs.* sodium salt). Although the magnitude of response was lower, the profile of LPS-induced MIF release from TtT/GF cells bore a striking resemblance to that of the RAW cells, with peak responses occurring at concentrations of 100 pM to 10 nM dexamethasone. As in classical immune cells (26), this finding poses a paradox as the release of proinflammatory cytokine from FS cells is normally inhibited by glucocorticoids (*e.g.* Ref. 38). The finding that dexamethasone stimulates the release of MIF from AtT20 cells was particularly surprising, although glucocorticoids have recently been shown to augment MIF protein expression in endocrine tissues, apparently via a posttranslational mechanism (39). CRH and glucocorticoids typically have opposing actions on corticotrophs, and the steroids exert powerful inhibitory effects on ACTH secretion *in vivo* (40) and *in vitro* by both primary pituitary tissue (41) and AtT20 cells (42). However, there are examples of CRH and glucocorticoids acting in concert on AtT20 cells, for example in the regulation of type 2 iodothyronine deiodinase (43). Furthermore, although the colocalization of ACTH and MIF in some corticotroph secretory granules predicts that dexamethasone would suppress MIF release, it is possible that the steroid promotes exocytosis of MIF-only-containing granules; such a view is supported by our recent observation (unpublished) that the prosecretory effect of dexamethasone on MIF is dependent upon the presence of extracellular Ca^{2+}.

The functional relevance of our finding that MIF is localized to FS cells as well as endocrine cells (27) in the anterior pituitary gland requires further investigation. In general, substances secreted from the endocrine cells (*e.g.* corticotrophs) are destined for the circulation and act as endocrine factors. In accord with this premise, previous studies have shown that pituitary-derived MIF has important roles in the periphery (23), hence its classification as a hormone as well as a cytokine. However, unlike substances derived from the endocrine cells, FS cell-derived factors (*e.g.* cytokines and growth factors) generally have more local actions and are likely to be present in high concentrations in the extracellular fluid surrounding the pituitary cells *vs.* the systemic circulation (8). FS cell-derived MIF may thus act as a local paracrine or autocrine factor. Because macrophages are both a source of and a target for MIF (22) and FS cells show macrophage-like characteristics (11), we explored the possibility that FS cells may be a target for MIF, using the TtT/GF cells as a model. Our data show clearly that, like RAW cells (22), TtT/GF cells respond to LPS with a dexamethasone-reversible increase in IL-6 release. They also show that the inhibitory effects of a low, but not a higher, concentration of dexamethasone, on IL-6 release are attenuated by rMIF and, thus, demonstrate for the first time that TtT/GF cells are a target for MIF. In human peripheral blood mononuclear cells, MIF inhibits glucocorticoid action by inhibiting the steroid-induced synthesis of inhibitory protein κB (IκB) and thereby preventing IκB binding to nuclear factor (NF)-κB. This facilitates the translocation of NF-κB to the nucleus and, thereby, allows LPS-stimulated cytokine production to continue in the presence of glucocorticoids (44). Because the LPS-induced IL-6 production by TtT/GF cells is also mediated by the NF-κB pathway and subject to glucocorticoid inhibition (16), it is possible that effects of MIF on TtT/GF cells reported here are effected via a similar mechanism.

Increasing evidence supports the view that direct actions of endotoxin on the pituitary gland play an important part in mediating the HPA responses to endotoxemia. Particular attention has focused on the role of FS cell-derived IL-6 (reviewed in Ref. 2). If the present data can be translated to primary cells, they raise the possibility that MIF may also be important in this regard. Our data predict that MIF would be released from pituitary cells in endotoxemia through the combined actions of LPS, glucocorticoids, and CRH on both FS cells and corticotrophs. In addition to acting systemically, MIF would act locally within the pituitary gland to augment and sustain ACTH release by opposing the suppressive effects of endogenous glucocorticoids on IL-6 release. The bell-shaped concentration-response curves of the steroid predict that this effect would be self-limiting and that once the concentration of endogenous glucocorticoids reached a threshold, both MIF release and the ability of MIF to overcome the inhibitory effects of the steroids on IL-6 release would be diminished. This "escape mechanism" would allow the negative feedback regulation of the release of IL-6, and possibly other pituitary cytokines, to continue. It would

thereby check the ACTH response to ensure that glucocorticoid levels were maintained within appropriate limits.

In conclusion, the present data suggest that FS cell-derived MIF is an important intrapituitary mediator of neuroendocrine communication in conditions of infection and inflammatory disease. Such a view is supported by the recent discovery of a functional polymorphism in the promoter region of the human *Mif* gene, especially given that this polymorphism lies within the transcription binding site for the pituitary-specific Pit-1 transcription factor (45, 46), and warrants further investigation.

Acknowledgments

We are grateful to Mr. Colin Rantle and Mr. Simon McArthur for their help in the laboratory.

Received July 22, 2004. Accepted September 13, 2004.

Address all correspondence and requests for reprints to: Prof. Julia Buckingham, Department of Cellular and Molecular Neuroscience, Division of Neuroscience and Psychological Medicine, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, United Kingdom. E-mail: j.buckingham@imperial.ac.uk.

We acknowledge the Wellcome Trust (Grant 69234/B/02/Z, to J.C.B.) and National Institutes of Health (Grants A142310 and AR050498, to R.B.) for financial support.

References

1. **Eskandari F, Webster JI, Sternberg EM** 2003 Neural immune pathways and their connection to inflammatory disease. Arthritis Res Ther 5:251–261
2. **John CD, Buckingham JC** 2003 Cytokines: regulation of the hypothalamo-pituitary-adrenocortical axis. Curr Opin Pharmacol 3:78–84
3. **Bertini R, Bianchi M, Ghezzi P** 1988 Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. J Exp Med 167: 1708–1712
4. **Coelho MM, Souza GE, Pela IR** 1992 Endotoxin-induced fever is modulated by endogenous glucocorticoids in rats. Am J Physiol 263:R423–R427
5. **Blalock JE** 1994 The syntax of immune-neuroendocrine communication. Immunol Today 15:504–511
6. **Zhou D, Shanks N, Riechman SE, Liang R, Kusnecov AW, Rabin BS** 1996 Interleukin 6 modulates interleukin-1-and stress-induced activation of the hypothalamic-pituitary-adrenal axis in male rats. Neuroendocrinology 63: 227–236
7. **Rinehart JF, Farquar MG** 1953 Electron microscopic studies of the anterior pituitary gland. J Histochem Cytochem 1:93–113
8. **Inoue K, Couch EF, Takano K, Ogawa S** 1999 The structure and function of folliculo-stellate cells in the anterior pituitary gland. Arch Histol Cytol 62: 205–218
9. **Muramami N, Fukata J, Tsukada T, Kobayashi H, Ebisui O, Segawa H, Muro S, Imura H, Nakao K** 1993 Bacterial lipopolysaccharide-induced expression of interleukin-6 messenger ribonucleic acid in the rat hypothalamus, pituitary, adrenal gland, and spleen. Endocrinology 133:2574–2578
10. **Gloddek J, Lohrer P, Stalla J, Arzt E, Stalla GK, Renner U** 2001 The intrapituitary stimulatory effect of lipopolysaccharide on ACTH secretion is mediated by paracrine-acting IL-6. Exp Clin Endocrinol Diabetes 109: 410–415
11. **Stokreef JC, Reifel CW, Shin SH** 1986 A possible phagocytic role for folliculo-stellate cells of anterior pituitary following estrogen withdrawal from primed male rats. Cell Tissue Res 243:255–261
12. **Vankelecom H, Carmeliet P, Van Damme J, Billiau A, Denef C** 1989 Production of interleukin-6 by folliculo-stellate cells of the anterior pituitary gland in a histiotypic cell aggregate culture system. Neuroendocrinology 49:102–106
13. **Zhang ZX, Koike K, Sakamoto Y, Jikihara H, Kanda Y, Inoue K, Hirota K, Milake A** 1997 Pituitary folliculo-stellate-like cell line produces a cytokine-induced neutrophil chemoattractant. Neuropeptides 31:46–51
14. **Gospodarowicz D, Lau K** 1989 Pituitary follicular cells secrete both vascular endothelial growth factor and follistatin. Biochem Biophys Res Commun 165: 292–298
15. **Ferrara N, Winer J, Henzel WJ** 1992 Pituitary follicular cells secrete an inhibitor of aortic endothelial cell growth: identification as leukemia inhibitory factor. Proc Natl Acad Sci USA 89:698–702
16. **Lohrer P, Gloddek J, Nagashima AC, Korali Z, Hopfner U, Pereda MP, Arzt E, Stalla GK, Renner U** 2000 Lipopolysaccharide directly stimulates the intrapituitary interleukin-6 production by folliculostellate cells via specific receptors and the p38α mitogen-activated protein kinase/nuclear factor-κB pathway. Endocrinology 141:4457–4465
17. **Sato T, Inoue K** 2000 Dendritic cells in the rat pituitary gland evaluated by the use of monoclonal antibodies and electron microscopy. Arch Histol Cytol 63:291–303
18. **Allaerts W, Fluitsma DM, Hoefsmit EC, Jeucken PH, Morreau H, Bosman FT, Drexhage HA** 1996 Immunohistochemical, morphological and ultrastructural resemblance between dendritic cells and folliculo-stellate cells in normal human and rat anterior pituitaries. J Neuroendocrinol 8:17–29
19. **Uccini S, Vitolo D, Stoppacciaro A, Paliotta D, Cassano AM, Barsotti P, Ruco LP, Baroni CD** 1986 Immunoreactivity for S-100 protein in dendritic and in lymphocyte-like cells in human lymphoid tissues. Virchows Arch B Cell Pathol Incl Mol Pathol 52:129–141
20. **Bucala R** 1996 MIF re-discovered: pituitary hormone and glucocorticoid-induced regulator of cytokine production. Cytokine Growth Factor Rev 7:19–24
21. **Bloom BR, Bennett B** 1966 Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. Science 153:80–82
22. **Calandra T, Bernhagen J, Mitchell RA, Bucala R** 1994 The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. J Exp Med 179:1895–1902
23. **Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, Manogue KR, Cerami A, Bucala R** 1993 MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365:756–759
24. **Calandra T, Echtenacher B, Roy DL, Pugin J, Metz CN, Hultner L, Heumann D, Mannel D, Bucala R, Glauser MP** 2000 Protection from septic shock by neutralization of macrophage migration inhibitory factor. Nat Med 6:164–170
25. **Bozza M, Satoskar AR, Lin G, Lu B, Humbles AA, Gerard C, David JR** 1999 Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J Exp Med 189:341–346
26. **Calandra T, Bernhagen J, Metz CN, Spiegel LA, Bacher M, Donnelly T, Cerami A, Bucala R** 1995 MIF as a glucocorticoid-induced modulator of cytokine production. Nature 377:68–71
27. **Nishino T, Bernhagen J, Shiiki H, Calandra T, Dohi K, Bucala R** 1995 Localization of macrophage migration inhibitory factor (MIF) to secretory granules within the corticotrophic and thyrotrophic cells of the pituitary gland. Mol Med 1:781–788
28. **Inoue K, Matsumoto H, Koyama C, Shibata K, Nakazato Y, Ito A** 1992 Establishment of a folliculo-stellate-like cell line from a murine thyrotropic pituitary tumor. Endocrinology 131:3110–3116
29. **Furth J, Gadsen EL, Upton AC** 1953 ACTH secreting transplantable pituitary tumors. Proc Soc Exp Biol Med 84:253–254
30. **Raschke WC, Baird S, Ralph P, Nakoinz I** 1978 Functional macrophage cell lines transformed by Abelson leukemia virus. Cell 15:261–267
31. **Chapman LP, Epton MJ, Buckingham JC, Morris JF, Christian HC** 2003 Evidence for a role of the adenosine 5′ triphosphate binding cassette transporter A1 in the externalization of annexin 1 from pituitary folliculostellate cells. Endocrinology 144:1062–1073
32. **Arcuri F, Ricci C, Ietta F, Cintorino M, Tripodi SA, Cetin I, Garzia E, Schatz F, Klemi P, Santopietro R, Paulesu L** 2001 Macrophage inhibitory factor in human endometrium: expression and localization during the menstrual cycle and early pregnancy. Biol Reprod 64:1200–1205
33. **Tierney T, Christian HC, Morris JF, Solito E, Buckingham JC** 2003 Evidence from studies on co-cultures of TtT/GF and AtT20 cells that annexin 1 acts as a paracrine or juxtacrine mediator of the early inhibitory effects of glucocorticoids on ACTH release. J Neuroendocrinol 15:1134–1143
34. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
35. **Agelaki S, Tsatsanis C, Gravanis A, Margioris AN** 2002 Corticotropin-releasing hormone augments proinflammatory cytokine production from macrophages in vitro and in lipopolysaccharide-induced endotoxin shock in mice. Infect Immun 70:6068–6074
36. **Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ** 1990 Structure and function of lipopolysaccharide binding protein. Science 249:1429–1431
37. **Triantafilou M, Triantafilou K** 2002 Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol 23:301–304
38. **Pereda MP, Lohrer P, Kovalovsky D, Perez Castro C, Goldberg V, Losa M, Chervin A, Berner S, Molina H, Stalla GK, Renner U, Arzt E** 2000 Interleukin-6 is inhibited by glucocorticoids and stimulates ACTH secretion and POMC expression in human corticotroph pituitary adenomas. Exp Clin Endocrinol Diabetes 108:202–207
39. **Fingerle-Rowson G, Koch P, Bikoff R, Lin X, Metz CN, Dhabhar FS, Meinhardt A, Bucala R** 2003 Regulation of macrophage migration inhibitory factor expression by glucocorticoids in vivo. Am J Pathol 162:47–56
40. **Keller-Wood ME, Dallman MF** 1984 Corticosteroid inhibition of ACTH secretion. Endocr Rev 5:1–24
41. **John C, Cover P, Solito E, Morris JF, Flower RJ, Buckingham JC** 2002

Annexin 1-dependent actions of glucocorticoids in the anterior pituitary gland: roles of the N-terminal domain and protein kinase C. Endocrinology 143:3060–3070

42. **Woods MD, Shipston MJ, Mullens EL, Antoni FA** 1992 Pituitary corticotrope tumor (AtT20) cells as a model system for the study of early inhibition by glucocorticoids. Endocrinology 131:2873–2880
43. **Araki O, Morimura T, Ogiwara T, Mizuma H, Mori M, Murakami M** 2003 Expression of type 2 iodothyronine deiodinase in corticotropin-secreting mouse pituitary tumor cells is stimulated by glucocorticoid and corticotropin-releasing hormone. Endocrinology 144:4459–4465
44. **Daun JM, Cannon JG** 2000 Macrophage migration inhibitory factor antagonizes hydrocortisone-induced increases in cytosolic IκBa. Am J Physiol Regul Integr Comp Physiol 279:R1043–R1049
45. **Baugh JA, Chitnis S, Donnelly SC, Monteiro J, Lin X, Plant BJ, Wolfe F, Gregersen PK, Bucala R** 2002 A functional promoter polymorphism in the macrophage migration inhibitory factor (MIF) gene associated with disease severity in rheumatoid arthritis. Genes Immun 3:170–176
46. **Gregersen PK, Bucala R** 2003 Macrophage migration inhibitory factor, MIF alleles, and the genetics of inflammatory disorders: incorporating disease outcome into the definition of phenotype. Arthritis Rheum 48:1171–1176

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**