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Protective Effects of Endotoxin in a Rat Model of Chronic Inflammation are Accompanied By Suppressed Secretion of Pro-Inflammatory Cytokines and Biphasic Alteration in Hypothalamo-Pituitary-Adrenal Axis Activity

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This paper is dedicated to the memory of Michael Stephen Harbuz (1959–2006)

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We have previously demonstrated that Gram-negative bacterial endotoxin can exert long-term protective effects against the chronic inflammatory disease adjuvant arthritis in rats. The present study was designed to investigate the mechanisms and time-course of hypothalamo-pituitary-adrenocortical (HPA) axis activity and cytokine secretion underlying this phenomenon. Rats were injected with endotoxin (lipopolysaccharide) and blood was collected either 7 or 21 days later. Priming with endotoxin induced a biphasic alteration in secretion of adrenocorticotrophic hormone and corticosterone in response to a second injection of endotoxin, with decreased secretion observed after 7 days whereas robust secretion was observed at 21 days. Seven days following priming with endotoxin, plasma concentrations of pro-inflammatory cytokines interleukin (IL)-6 and interferon (IFN)-γ were reduced by 90%, and tumour necrosis factor (TNF)-α by 70%, compared to saline-treated rats, whereas robust secretion of the anti-inflammatory cytokine IL-10 was maintained in both groups. A similar net change favouring an anti-inflammatory cytokine secretory milieu was also observed 21 days following priming with endotoxin. This study provides evidence that the long-term protective effects of endotoxin on inflammation are associated with a sustained reduction in secretion of pro-inflammatory cytokines. HPA axis hypoactivity at 7 days suggests that corticosterone is not involved in suppressing IL-6, IFN-γ and TNF-α at this time point. Conversely, hypersecretion of corticosterone at 21 days may underlie synchronous suppression of IL-6 and IFN-γ. These data provide novel insight into interactions between HPA axis activity and cytokine secretion following endotoxin priming prior to induction of inflammatory disease.

Key words: HPA axis, ACTH, corticosterone, endotoxin, cytokines.

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Stress is now recognised as a significant contributory factor in both the aetiology and pathophysiology of many diseases (1–3). The sympathetic nervous system (SNS) and the hypothalamo-pituitary-adrenocortical (HPA) axis, the principal pathways that respond to stress, exert tonic inhibitory control over the immune system through multiple coordinated pathways involving glucocorticoids, catecholamines, neuropeptides and cytokine networks (4, 5). Stress can result in a resetting of immune system parameters with consequent impairment in the ability of the organism to respond to infection and other chronic processes such as inflammatory autoimmune diseases. Much attention has been paid to the deleterious effects of chronic stress on disease processes, but it is also established that acute stress may exert beneficial immunostimulatory effects (6). Relationships between the type of stress, timing of the stressor and selective responses of the SNS, HPA axis and immune system are complex and far from understood in terms of their impact on disease processes.

Lipopolysaccharide (LPS), an endotoxin derived from the cell wall of Gram-negative bacteria, is well-recognised as an activator of the immune system, but LPS can also act as a potent stressor which activates the HPA axis and SNS (7, 8). LPS can elicit acute-phase pro-inflammatory cytokine responses which are inhibited by gluco-

corticoids, in the absence of which a challenge by LPS may prove fatal (9). Glucocorticoids are an essential homeostatic control during inflammation. Several well-characterised animal models of chronic inflammation, such as experimental allergic encephalomyelitis, streptococcal cell wall arthritis and adjuvant-induced arthritis, are characterised by an HPA axis which is reset at a higher level of corticosterone secretion (10–12). Adrenalectomised animals exhibit earlier onset and enhanced severity of inflammation, which rapidly results in death (13, 14), an outcome which can be prevented by exogenous corticosterone replacement.

Activation of the HPA axis in response to an acute stressor has classically been considered to be a finite event, where glucocorticoids return to baseline concentrations within hours following the onset of stress. However, evidence is now emerging for the long-term effects of a single acute stressor on HPA axis activity (15, 16). Long-term sensitisation or desensitisation of HPA axis activity may be dependent on whether the experimental paradigm is composed of homotypic or heterotypic stressors (17).

In addition to the long-term effects of acute stressors on HPA axis activity, we have observed that a single injection of endotoxin several weeks prior to induction of disease in adult rats can completely protect against the onset and severity of inflammation in adjuvant-induced arthritis (18), which is a widely used rat model of rheumatoid arthritis (19). Protection against inflammation in adult animals was observed whether endotoxin was given neonatally (20) or to mature rats (18). In the latter study, footshock, although a potent activator of HPA axis activity, did not protect against development of inflammation, which suggests that the protective effect is not a general consequence of stress but is specific to the stressor endotoxin, and may be determined by the neuroendocrine-immune responses to endotoxin. A single injection of interleukin (IL)-1, a cytokine whose secretion is evoked by endotoxin, attenuated severity of inflammation in experimental allergic encephalomyelitis (21). In the animals injected with endotoxin (18, 20) or IL-1 (21), the HPA axis remained activated even though inflammation was absent. This suggests that the mechanisms involved in mediating the protective effects of endotoxin or IL-1 are not specifically related to increased glucocorticoid secretion.

It has been reported that endotoxins can induce or reactivate experimental arthritis (22, 23), probably through rapid stimulation of secretion of the Th1-type pro-inflammatory cytokines such as IL-1, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, and IL-6 (24). This is counter-intuitive to a protective effect of endotoxin in a disease such as adjuvant-induced arthritis which, like rheumatoid arthritis, is associated with increased secretion of Th1-type cytokines (25). However, long-term patterns of cytokine secretion following endotoxins have received little attention. In the present study, we proposed the hypothesis that endotoxin protects against the onset and severity of inflammation by inducing long-term changes in the blood cytokine milieu which favour secretion of anti-inflammatory cytokines. We also hypothesised that this change in the balance of cytokine secretion is accompanied by increased HPA axis activity leading to increased corticosterone production. We tested this hypothesis by measuring a range of cytokines, and the hormones adrenocorticotrophic hormone (ACTH) and corticosterone, at 7 and 21 days following priming with endotoxin.

Materials and methods

Animals

Adult male Wistar rats (200–225 g; Bantin & Kingman, Hull, UK) were housed under standard conditions of temperature and humidity under a 12 : 12 h light/dark cycle (lights on 07.00 h). Animals were fed laboratory chow and water *ad libitum* throughout the experiments. The care and use of the animals was performed in accordance with the Animals (Scientific Procedures) Act UK 1986 and the Ethical Committee for Animal Experimentation of the School of Medicine of the University of Cadiz, Spain.

Experimental procedures

Rats were injected intraperitoneally (i.p.) with endotoxin-free saline (0.5 ml) or endotoxin (ENDO; Sigma, Poole, UK; serotype *Escherichia coli* 055:B5; 1 mg/kg body weight in 0.5 ml of saline) and returned to their home cages for 7 or 21 days. We have previously demonstrated that doses below 1 mg/100 g body weight of ENDO of the 055:B5 serotype are sufficient to stimulate the HPA axis with no apparent effects on animal behaviour (26). Mean body weights did not differ between groups 21 days after treatment with ENDO or saline. On either day 7 or day 21, separate groups of animals were injected i.p. with either 0.5 ml of saline or endotoxin (ENDO-2) derived from *Salmonella enteritidis* (1 mg/kg body weight, 100K4088; Sigma) in 0.5 ml of saline. This second injection of endotoxin was necessary to stimulate blood cytokine concentrations which under basal conditions would be very low. ENDO-2 was selected from an alternative bacterial source to ENDO to act as a heterotypic stressor, thus avoiding the well-recognised phenomenon of habituation of the HPA axis to repeated homotypic stress (27). Following ENDO-2 injection, groups of animals were returned to their home cages for either 1 h, which is optimal for measurement of TNF-α and IL-10 (28, 29), or 4 h, which is optimal for measurement of IFN-γ and IL-6 (personal observations) before being sacrificed by decapitation. Trunk blood was collected in heparinised tubes, centrifuged, and the plasma was stored at −80 °C prior to measurement of hormones and cytokines.

Measurements

Hormones

Total plasma corticosterone was measured by in-house radioimmunoassay (11). Plasma ACTH was measured by in-house radioimmunoassay following extraction on Sep-Pak columns (30). The intra- and interassay coefficients of variation for ACTH and corticosterone are < 10%. Antisera for corticosterone and ACTH were kindly donated by G. Makara (Institute of Experimental Medicine, Budapest, Hungary).

Cytokines

Cytokines were measured in plasma using commercially available enzyme-linked immunosorbent assay kits specific for rat cytokines (R&D Systems Europe Ltd, Abingdon, UK). The plates were read using a Bio-Rad 550 Microplate reader (Bio-Rad, Hercules, CA, USA) set at 450 nm and the data interpreted using Microplate Manager version 5.1 (Bio-Rad). The limit of detection was 20 pg/ml for IL-6, 5 pg/ml for TNF-α and 10 pg/ml for IFN-γ and IL-10. The intra- and interassay coefficients of variation for all assay kits are < 10%.

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Statistical analysis

All values are expressed as the mean ± SEM. Statistical comparisons were made using the Fisher post-hoc least significant difference test following one-way ANOVA.

Results

Hormones: 7-day timepoint

Basal concentrations of plasma ACTH (Fig. 1A) and corticosterone (Fig. 1B) were similar in both the saline and ENDO-pretreated groups. There were significant increases ($P < 0.0001$) in plasma ACTH and corticosterone 4 h following injection of ENDO-2 (Sal + ENDO-2) compared to the control group (Sal + Sal). These responses were significantly attenuated ($P < 0.0001$) in animals pretreated with ENDO (ENDO + ENDO-2).

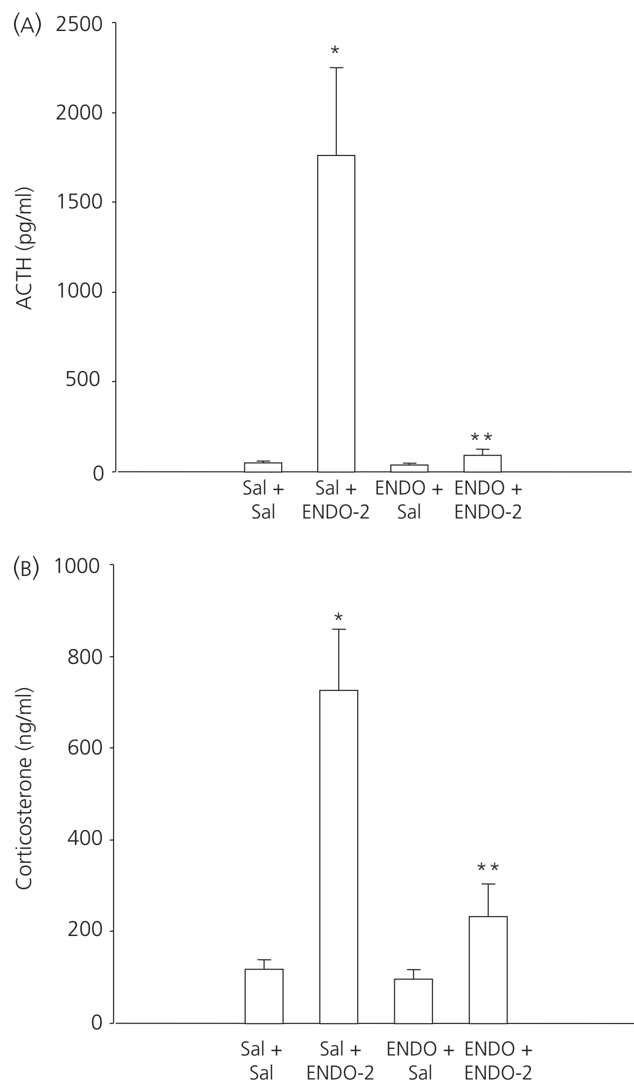

Fig. 1. Plasma adrenocorticotrophic hormone (ACTH) (A) and corticosterone (B) responses to ENDO-2 or saline injection 7 days after an initial ENDO or saline challenge. Blood samples were collected 4 h following ENDO-2. Values are the means ± SEM ($n = 9$ rats per treatment group). *$P < 0.0001$ versus Sal + Sal group; **$P < 0.0001$ versus Sal + ENDO-2 group.

Cytokines: 7-day timepoint

Basal plasma IFN-γ was below the limit of assay detection. There was a significant increase in IFN-γ in the Sal + ENDO-2 group which was significantly attenuated ($P < 0.0001$) in animals pretreated with ENDO (ENDO + ENDO-2) (Fig. 2A).

Basal concentrations of plasma IL-6 (Fig. 2B) and TNF-α (Fig. 2C) were similar in both saline and ENDO-pretreated groups. There were significant increases in plasma IL-6 and TNF-α in the Sal + ENDO-2 group which were significantly attenuated ($P < 0.0001$) in animals pretreated with ENDO (ENDO + ENDO-2).

Basal concentrations of plasma IL-10 (Fig. 2D) were similar in both saline and ENDO-pretreated groups. There were significant increases in plasma IL-10 in the Sal + ENDO-2 group and in the group pretreated with ENDO (ENDO + ENDO-2). Although IL-10 was slightly increased in the latter group, this difference was not statistically significant compared to the Sal + ENDO-2 group

Hormones: 21-day timepoint

Basal concentrations of plasma ACTH (Fig. 3A) and corticosterone (Fig. 3B) were similar in both the saline and ENDO-pretreated groups. Four hours following injection of ENDO-2, there were significant ($P < 0.0001$) ACTH responses to ENDO-2 in both saline and ENDO-pretreated groups, the latter response being slightly but not significantly greater. There were significant ($P < 0.0001$) corticosterone responses to ENDO-2 in both the saline and ENDO-pretreated groups compared to their respective controls.

Cytokines: 21-day timepoint

Basal plasma concentrations of IFN-γ (Fig. 4A) and IL-6 (Fig. 4B) were similar in both the saline and ENDO-pretreated groups. There were significant increases in plasma IFN-γ and IL-6 in response to ENDO-2 in the Sal + ENDO-2 group which were significantly attenuated ($P < 0.0001$) in the group pretreated with ENDO (ENDO + ENDO-2).

Basal TNF-α and IL-10 were not measured. There were no significant differences in plasma TNF-α (Fig. 4C) and IL-10 (Fig. 4D) in animals pretreated with ENDO (ENDO + ENDO-2) compared to the Sal + ENDO-2 group.

Discussion

We have previously observed a fully protective effect of ENDO on inflammation when injected 3 weeks prior to induction of arthritis in a rat model of adjuvant-induced arthritis. ENDO did not merely delay the onset of inflammation but prevented development of disease. Adjuvant-induced arthritis, similar to rheumatoid arthritis, is a disease whose pathology is integrally associated with elevated secretion of pro-inflammatory cytokines such as IFN-γ, IL-6 and

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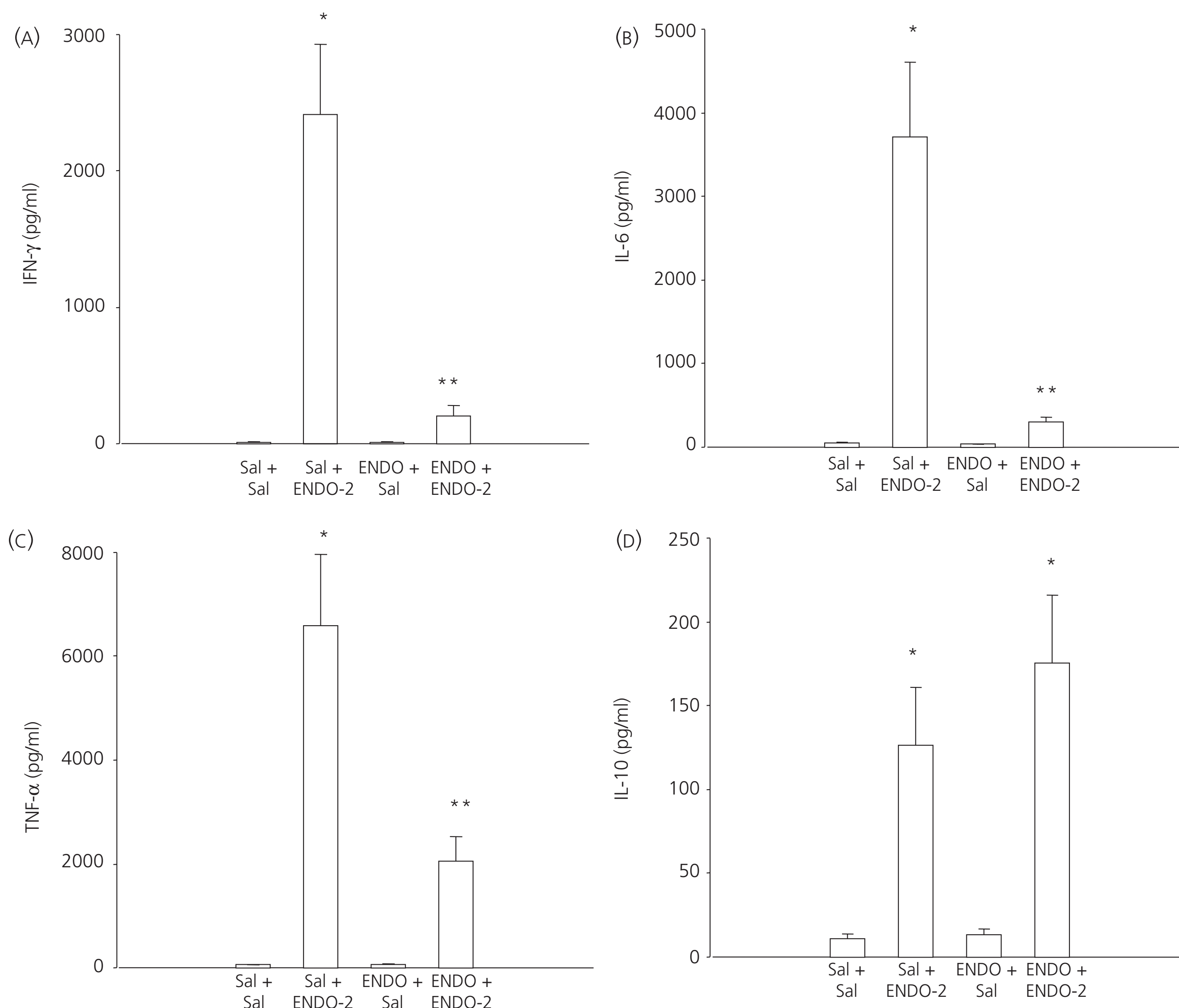

Fig. 2. Plasma interferon (IFN)-γ (A), interleukin (IL)-6 (B), tumour necrosis factor (TNF)-α (C) and IL-10 (D) responses to ENDO-2 or saline injection 7 days after an initial ENDO or saline challenge. Blood samples were collected at 1 h following ENDO-2 for TNF-α and IL-10 and at 4 h for IFN-γ and IL-6. Values are the means ± SEM (n = 5–7 rats per treatment group). *P < 0.0001 versus Sal + Sal group; **P < 0.0001 versus Sal + ENDO-2 group.

TNF-α (25, 31). In the present study, we have established that the protective effects of ENDO are temporally associated with an alteration in the balance of cytokine secretion, favouring the creation of an anti-inflammatory cytokine milieu in the blood. We have also observed that priming with ENDO can exert biphasic effects on HPA axis activity prior to the onset of inflammation. These results demonstrate that the anti-inflammatory effects of ENDO are associated with long-term changes in the blood cytokine and hormonal milieu.

Acute exposure to endotoxins elicits a rapid, largely pro-inflammatory, response characterised by release of many cytokines including TNF-α, IL-1, IL-6 and IL-10 (32). This pattern is quite distinct from the cytokine profile that we observed 7 days and 21 days following ENDO, in which the serum concentrations of TNF-α (at 7 days), also reported elsewhere [23], IFN-γ and IL-6 are much reduced in response to the second injection of endotoxin, whereas concentrations of the strongly anti-inflammatory cytokine IL-10 are essentially unchanged. Therefore, the serum cytokine balance alters from an initial predominantly pro-inflammatory response to an chronic anti-inflammatory milieu during the 3 weeks following initial exposure to ENDO. It has been proposed that activation of the innate response by LPS can also trigger the adaptive response (33), a mechanism now identified as acting through toll-like receptors (34, 35). This linked signalling pathway may underlie the alterations that we observed in cytokine responses to ENDO over a period of days to weeks.

In addition to activating the immune system, endotoxins are also potent stressors since they stimulate the HPA axis and SNS. There have been several reports of protective (and also exacerbatory) effects of stress on inflammation (36–39). Although the underlying pathophysiological mechanisms are poorly well-understood, it is

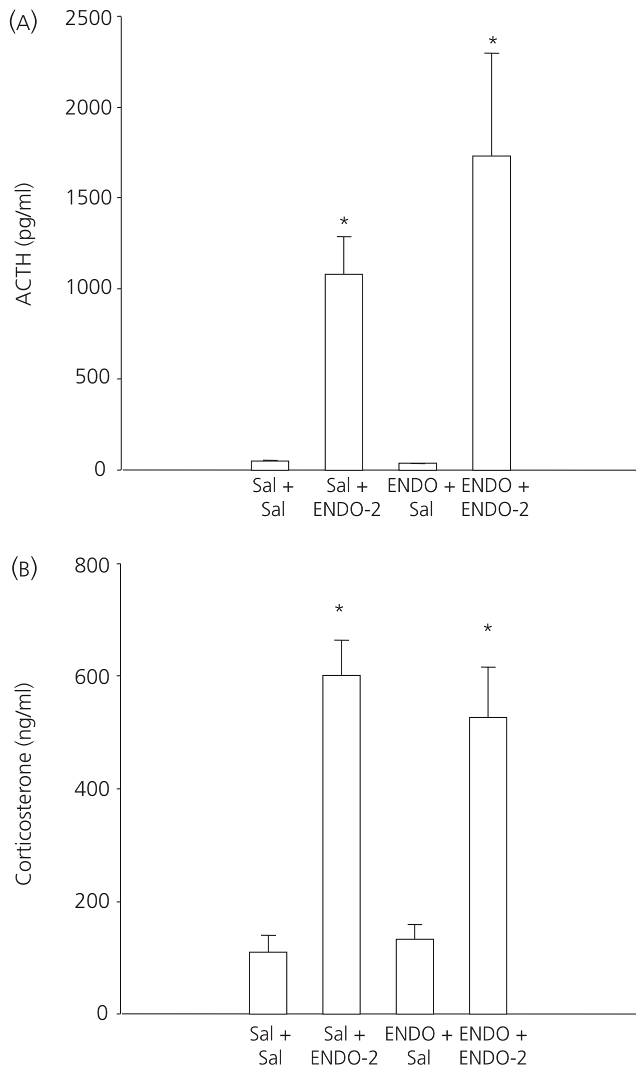

Fig. 3. Plasma adrenocorticotrophic hormone (ACTH) (A) and corticosterone (B) responses to ENDO-2 or saline injection 21 days after an initial ENDO or saline challenge. Blood samples were collected 4 h following ENDO-2. Values are the means ± SEM (n = 9 rats per treatment group). *$P < 0.0001$ versus Sal + Sal group.

clear from these studies that acute stressors can influence the onset and severity of inflammation, and that the effectiveness of the stressor may depend on type, timing and intensity of the stressor, and the nature of the disease. Selective effects of stress in animal models of inflammatory disease may also reflect differences in underlying patterns of pro- and anti-inflammatory cytokine expression and secretion prior to onset of inflammation. It is also apparent from the literature that the protective effects of stress are not necessarily related to increased HPA axis activity and corticosterone release (40, 41).

A single injection of ENDO at the dose which we employed induces rapid ACTH and corticosterone responses which may be sustained for up to 8 h (24, 42). However, in complete contrast to this acute response, we observed that 7 days following ENDO, the HPA axis was hypo-responsive to a second ENDO challenge. At this same timepoint, responses of the major pro-inflammatory cytokines IL-6, TNF-α and IFN-γ to ENDO-2 were attenuated whereas robust secretion of the major anti-inflammatory cytokine IL-10 was sustained. It is well-established that glucocorticoids inhibit secretion of IL-6, TNF-α and IFN-γ (43), and stimulate secretion of IL-10 (44) but, in our model, the alteration in the balance of blood cytokines at the day 7 time point is unlikely to be linked to HPA axis activity because plasma corticosterone was decreased by approximately 70%. Conversely, the rebound in HPA axis activity 21 days following pretreatment with ENDO may underlie the decreased responses of IL-6 and IFN-γ to ENDO-2 at this later timepoint. This is the first observation of long-term biphasic HPA axis activity following an injection of ENDO. Previous work in this field has found that HPA axis and plasma TNF-α responses to LPS (serotype *E. coli* 055:B5) are attenuated at 1 and 4 weeks following initial *E. coli* LPS priming in Sprague-Dawley rats (45). Although the 1-week data are consistent with our own, the attenuated responses at 4 weeks are opposite to those which we observed at 21 days. This raises the possibility that long-term HPA axis and cytokine responses to previous endotoxin exposure are either time-sensitive, dependent on the source of LPS, or may be strain-specific. The dose of LPS used to challenge LPS-primed rats may also be critical (46).

Attenuated cytokine and HPA axis responses to ENDO-2 which we observed on day 7 may represent the phenomenon of endotoxin tolerance (24, 47, 48). However, the robust responses of TNF-α, ACTH and corticosterone to ENDO-2 that we observed on day 21 are not consistent with currently understood kinetics and mechanisms of endotoxin tolerance where blunted responses of cytokines and HPA axis activity from 5–28 days have been reported (49–53). In addition, we did not observe an up-regulation in IL-10, which has been reported in endotoxin tolerance (54). All of these studies used LPS from the same source and it is plausible that our use of endotoxin from two separate sources may explain these discrepancies. We are unaware of any other studies that have investigated this combination of separate types of LPS on this range of hormones and cytokines. Also of interest, when we administered adjuvant to rats 7 days following ENDO, we found that this had no significant effect on hindpaw inflammation (unpublished observations) although a fully protective effect of ENDO on hindpaw inflammation was observed when ENDO was injected 21 days prior to adjuvant. Therefore the attenuated HPA axis and cytokine responses observed on day 7, which may be an example of endotoxin tolerance, had no protective effect on onset of inflammation. Conversely, the altered cytokine and hormone balance observed in response to ENDO-2 on day 21, which is quite atypical of endotoxin tolerance, may represent a genuine underlying anti-inflammatory mechanism protecting against arthritis.

Finally, although our data do not permit conclusions to be drawn with respect to the critical contribution of any one component in the protective effect of ENDO on adjuvant-induced arthritis, we speculate that it is the sustained drive in IL-10 secretion, coupled with robust HPA axis activity at 21 days, which constitutes a potent anti-inflammatory combination. TNF-α and IFN-γ secretion, and Th1 cell proliferation, all of which contribute to the inflammatory pro-

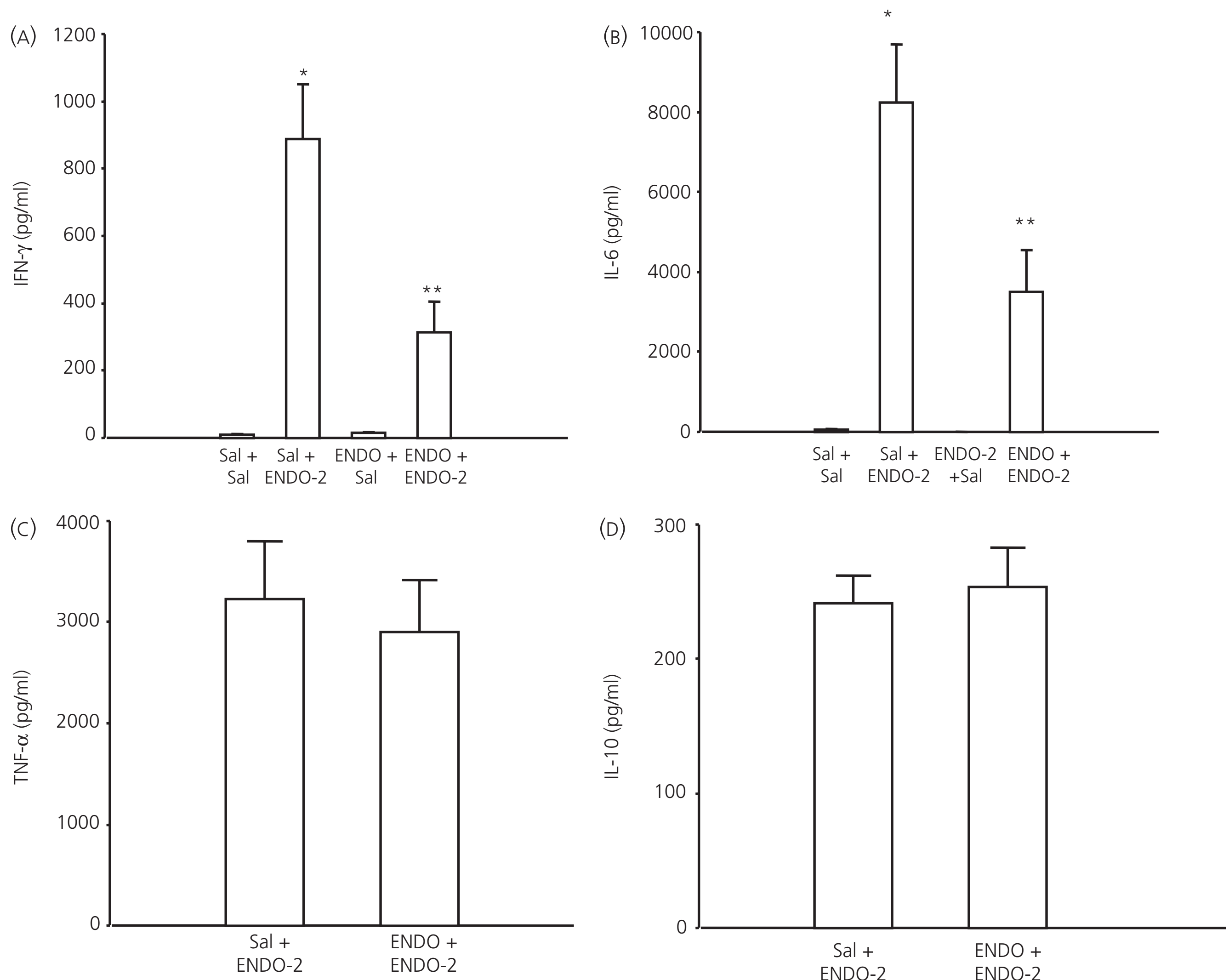

Fig. 4. Plasma interferon (IFN)-γ (A), interleukin (IL)-6 (B), tumour necrosis factor (TNF)-α (C) and IL-10 (D) responses to ENDO-2 or saline injection 21 days after an initial ENDO or saline challenge. Values are the means ± SEM (n = 8 rats per treatment group). *P < 0.0001 versus Sal + Sal group; **P < 0.0001 versus Sal + ENDO-2 group.

cesses in rheumatoid arthritis, are suppressed by IL-10 (55, 56). Further experimental approaches in this animal model to address this issue might include priming with ENDO followed by immunoneutralisation of IL-10 and/or intervention with glucocorticoid antagonists prior to induction of arthritis. Although such experiments will be complex, they may shed valuable light on factors involved in the development of rheumatoid arthritis. There is compelling evidence for an inter-regulatory network of cytokines in rheumatoid arthritis which favours secretion of pro-inflammatory (IL-6, TNF-α and IFN-γ) and anti-inflammatory (IL-10) cytokines in a balance weighted towards a pro-inflammatory Th1-type milieu (57). The ability of ENDO to reverse this bias in adjuvant-induced arthritis and protect against onset of inflammation, as also recently reported in juvenile atopic asthma (58), may provide new insights into the influence of stress or bacterial infection on cytokine and hormonal patterns of secretion underlying the development of chronic inflammatory disease in man.

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