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Psychological stress suppresses innate IFN-γ production via glucocorticoid receptor activation: Reversal by the anxiolytic chlordiazepoxide

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Abstract

Studies in humans and in animals indicate that psychological stress can modulate immune responses. Here we demonstrate that exposure to psychological stress (restraint stress) suppresses innate interferon (IFN)-γ production in mice following an *in vivo* lipopolysaccharide (LPS) challenge. IFN-γ signalling was also impaired by stress, as indicated by reduced STAT1 phosphorylation and reduced expression of the IFN-γ-inducible genes, inducible nitric oxide synthase (iNOS) and IFN-γ-inducible protein 10 (IP-10/CXCL10). Furthermore, restraint stress suppressed production of the IFN-γ inducing cytokine interleukin (IL)-12 and increased production of the anti-inflammatory cytokine IL-10, which can inhibit both IL-12 and IFN-γ production. However, using IL-10 knockout mice, we demonstrate that IL-10 does not mediate the suppressive effect of restraint stress on innate IFN-γ production. Restraint stress increased corticosterone concentrations in serum and spleen, and consistent with a role for glucocorticoids in the immunosuppressive actions of stress, pre-treatment with the glucocorticoid receptor antagonist mifepristone completely blocked the stress-related suppression of innate IFN-γ production. Addition of exogenous IL-12 to LPS-stimulated spleen cells reversed the suppressive effect of both restraint stress and corticosterone on IFN-γ production. These data suggest that reduced IL-12 production is a key event in stress-induced suppression of innate IFN-γ production. Finally, we demonstrate that pre-treatment with the anxiolytic drug chlordiazepoxide prevents the suppressive effect action of stress on innate IFN-γ production, and also prevents the stress-induced increase in circulating corticosterone concentrations.

Keywords: Stress, IFN-γ, immunosuppression, glucocorticoid, IL-10, anxiolytic, anxiety

1. Introduction

A number of studies in humans and in animal models have indicated that psychological stress can modulate immune responses (see Glaser and Kiecolt-Glaser, 2005; Kusnecov and Rabin, 1994; Segerstrom and Miller, 2004). Moreover, psychological stress has been associated with an increased incidence of infectious disease, indicating that the immunosuppressive actions of stress translate into significant adverse health effects (Glaser and Kiecolt-Glaser, 2005; Segerstrom and Miller, 2004). Cytokine production appears to be particularly sensitive to modulation by stress (Connor et al., 2005; Curtin et al., 2009; Goujon et al., 1995; Meltzer et al., 2004), and it is suggested that dysregulation of cytokine production could contribute to stress-related increases in disease susceptibility. **In this regard, previous studies have demonstrated that psychological stress suppresses production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (Connor et al., 2005; Goujon et al., 1995; Meltzer et al., 2004), and increases expression of the anti-inflammatory cytokine IL-10 (Connor et al., 2005; Curtin et al., 2009). However on the contrary, there is also evidence indicating that in some instances stress can promote production of inflammatory cytokines such as IL-1β and TNF-α (Johnson et al., 2002).**

In the studies presented we have investigated the impact of a psychological stressor (restraint stress) on IFN-γ production induced by an *in vivo* challenge of mice with lipopolysaccharide (LPS). This *in vivo* LPS challenge model mimics the innate immune response to Gram negative bacterial infection (Latz et al., 2003; Palsson-McDermott and O'Neill, 2004), resulting in production of interleukin (IL)-12 and IL-18; two pro-inflammatory cytokines that drive IFN-γ production (Fehniger et al., 1999; Kim et al., 2000; Salkowski et al., 2000; Varma et al., 2002; Walker et al., 1999; Munder et al., 1998). NK cells are the most potent producers of innate IFN-γ during the early stages of viral, parasitic or bacterial infection (Farrar and Schreiber, 1993), and NK-derived IFN-γ has been shown to play a critical protective role early in bacterial infection (Byrne et al., 2004). Specifically, IFN-γ is the prototypic macrophage-activating factor (James and Nacy, 1993), thus innate IFN-γ produced following LPS is part of a positive back loop which amplifies antimicrobial immune responses by inducing phagocytosis and respiratory burst, antigen presentation, and cytokine secretion by antigen-presenting cells (Boehm et al., 1997). Other functions include enhancement of IgG_{2a} antibody secretion by B cells, promoting cytotoxic T-cell maturation, neutrophil, activation and importantly stimulating T_H1 cell differentiation (Boehm *et al*, 1997; Hasbold *et al.,* 1999). The critical role played by IFN-γ in antimicrobial defense is demonstrated by the increased susceptibilities of IFN-γ and IFN-γ receptor knockout mice to a variety of infectious organisms, particularly to intracellular organisms, such as *Listeria, Mycobacteria* and *Bordetella pertussis* (Shtrichman and Samuel, 2001; Mahon et al., 1997). Of course IFN-γ also plays a key role in anti-viral immunity via its ability to promote IgG_{2a} production; the dominant antibody isotype responsible for complement-mediated lysis reactions (Biron and Brossay, 2001). In addition to its role in host resistance to infection, innate IFN-γ also plays a significant role in anti-tumour immunity (Ikeda et al., 2002; Kim et al., 2000; Tannenbaum and Hamilton, 2000).

In the studies presented here we examine the ability of restraint stress to modulate innate IFN-γ production, and expression of the IFN-γ inducing factors IL-12 and IL-18 following an *in vivo* LPS challenge. In addition, we examine the impact of stressor exposure on IFN-γ signaling as indicated by phosphorylation of the transcription factor signal transducer and activator of transcription-1 (STAT-1) (Rosenzweig and Holland, 2005), and expression of the IFN-γ-inducible genes iNOS and IFN-γ-inducible protein-10 (IP-10/CXCL10) (Gao et al.,

1997; Ohmori and Hamilton, 2001). The study demonstrates that restraint stress suppresses the innate IFN-γ response, thus we examined the mechanisms underlying this effect. **Specifically, we examined the role of the anti-inflammatory cytokine IL-10, and glucocorticoids in mediating the suppressive effect of stress on the innate IFN-γ response. In this regard, previous studies demonstrate that stress augments LPS-induced IL-10 production (Connor al., 2005; Curtin et al., 2009), and that IL-10 suppresses IFN-γ production by suppressing IL-12 (D'Andrea et al., 1993). To assess the role of IL-10 in the suppressive effect of stress in the innate IFN-γ response we compared the ability of stress to suppress the LPS-induced IL-12 and IFN-γ expression in IL-10 knockout mice relative to wild-type controls. With respect to glucocorticoids, a range of psychological stressors are known to activate the hypothalamic pituitary adrenal (HPA) axis resulting in increased circulating concentrations of glucocoticoids (Connor et al., 1997; Laugero and Moberg, 2000; Shanks et al., 1990; Sheridan et al., 1991). Furthermore, *in vitro* exposure of immune cells to glucocorticoids is known to suppress production of IL-12 and IFN-γ via glucocorticoid receptor activation (Ding et al., 1989; Vieira et al., 1998; Visser et al., 1998). Consequently, by pre-treating animals with the glucocorticoid receptor antagonist mifepristone (Lazar and Agarwal, 1986), we evaluated the role of glucocorticoid receptor activation in mediating the suppressive effect of stress on the innate IFN-γ response.**

Considering the deleterious effects of stress on health, a key objective of future research in the field of psychoneuroimmunology is to identify interventions that can ameliorate stress-induced immune modulation and increases in disease susceptibility (see Connor, 2008; Glaser and Kiecolt-Glaser, 2005). Anxiolytic drugs are often prescribed to patients suffering from stress or anxiety in order to alleviate symptoms, consequently, here we determined if administration of the benzodiazepine anxiolytic drug chlordiazepoxide could ameliorate the suppressive effect of stress on the innate IFN-γ response.

2. Material and Methods

2.1 Animals

Male BALB/c or C57BL/6 mice (6-8 weeks) were obtained from Harlan (Oxford, UK) and male IL-10 knockout mice were obtained from a breeding pair in Bioresources unit, Trinity College Dublin, Ireland. BALB/c mice were used for all studies with the exception of the IL-10 knockout study. **IL-10 knockout mice were bread from a mouse strain homozygous for the Il10tm1Cgn targeted mutation as previously described (Kuhn et al., 1993). Genotypes were confirmed using real-time PCR, and absence of IL-10 production was confirmed by stimulation of spleen cells from IL-10 knockout mice with a variety of TLR agonists. Wild-type (C57BL/6) mice and IL-10 knockout mice were housed in separate cages but in similar conditions.** Mice were housed 4-5 per cage and maintained on a 12 hr light: 12 hr dark cycle (lights on at 08.00 am) in a temperature controlled room (22 ± 2^{o}C) and food and water were available *ad libitum* at all times. The experimental protocols were in compliance with the European Communities Council directive (86/609/EEC).

2.2 Drugs

LPS (*E. coli:* serotype 0111:B4), mifepristone and chlordiazepoxide were all obtained from Sigma-Aldrich, Ireland. LPS and chlordiazepoxide were dissolved in **0.9 % NaCl** and administered via the intraperitoneal (i.p.) route in an injection volume of 10 ml/kg, and **0.9 % NaCl** was administered alone as a vehicle to control animals. Mifepristone was dissolved in 20% DMSO administered via the intraperitoneal (i.p.) route in an injection volume of 10 ml/kg, and 20% DMSO was administered alone as a vehicle to control animals.

2.3 Stress protocol

Physical restraint restricts an animal's movement and access to food and water. The restraint stress procedure that was utilized in the studies presented here was based on previous studies using physical restraint (Sheridan *et al*., 1991). However, a 2 h restraint time was chosen as this has been shown to be sufficient to result in immunomodulation (Thaker *et al.,* 2006). For the restraint stress procedure, mice were removed from their home cage and placed in well ventilated 50 ml falcon tubes (Sarstedt Inc, Ireland) for 2 h in an adjacent temperature controlled room (22 ± 2^{o}C) with the lights on. Individual mice were placed in tubes at 9:00 am and removed at 11:00 am. Control mice were food and water deprived during the same time period, however these mice were free to roam in their home cages.

2.4 Experimental design

2.4.1 Experiment I: Effect of restraint stress on the innate IFN-γresponse following an in vivo LPS challenge

(a) Innate IFN-γ production and signalling: Mice were exposed to 2 h of restraint stress or left in their home cage, after which time they were injected immediately with either LPS (250 μg/kg; i.p), or vehicle. Mice were returned to their home cage and 8 h later were sacrificed by cervical dislocation. Trunk blood was obtained by exsanguination, was centrifuged at 800 x g at 4^{o}C for 15 min, and the resultant serum was frozen immediately and stored at -80^{o}C until IFN-γ ELISA was performed. The spleen was dissected, and a portion of tissue was immediately placed in RNA later (Ambion) to ensure integrity of RNA for gene expression analysis. Tissue was subsequently frozen at -80^{o}C until RNA was extracted and real-time PCR for IFN-γ, IL-12p40, IL-12p35, IL-18, iNOS and IP-10 were performed. Another piece of spleen tissue was rapidly frozen on dry ice for analysis of STAT1 phosphorylation using Western immunoblotting.

The dose of LPS used in this study is a sub-septic dose approximately 15-fold lower than the LD_{50} for E. coli LPS in mice (Joshi et al., 2002), and provokes modest stimulation of the immune response characterised by a transient increase in cytokine production. Animals were sacrificed 8 h post LPS-administration for the analysis of the innate IFN-γ response. The 8 h time-point was chosen based on our own pilot studies, and based on previous published studies indicating that IFN-γ production following *in vivo* LPS administration to mice is not readily detectable before 7-8 h post LPS administration (Connor and Boyle, 2007; Fukuda et al., 2005; Varma et al., 2002).

(b) Expression of the IFN-γ-inducing factors IL-12 and IL-18: Mice were exposed to 2 h of restraint stress or left in their home cage, after which time they were injected immediately with LPS (250 μg/kg; i.p), or vehicle. Mice were returned to their home cage and sacrificed 1.5 h later by cervical dislocation. Spleen tissue was taken and stored for analysis of IL-12p40, IL-12p35 and IL-18 mRNA expression by real-time PCR as outlined in Experiment Ia.

2.4.2 Experiment II: A role for IL-10 in mediating the suppressive effect of stress on the innate IFN-γresponse?

Effect of restraint stress on LPS-induced IL-10 production: Mice were either exposed to a 2 h restraint stress or left in their home cage, after which time they were injected immediately with either LPS (250μg/kg; i.p), or vehicle. Mice were returned to their home cage and 45 min later were sacrificed by cervical dislocation. Serum was prepared and stored for analysis of IL-10 concentrations by ELISA as outlined in Experiment Ia.

Effect of restraint stress on the LPS-induced IL-12 and IFN-γ expression in IL-10 knockout mice: Wild type (C57Bl/6) or IL-10 knockout mice were exposed to 2 h restraint stress or left in their home cage, after which time they were injected immediately with either LPS (250μg/kg; i.p). Mice were returned to their home cage and 8 h later were decapitated following cervical dislocation. Spleen tissue was taken and stored for analysis of IL-12 and IFN-γ mRNA expression by real-time PCR as outlined in Experiment Ia.

2.4.3 Experiment III: Effect of stress on corticosterone concentrations in serum and spleen

Mice were exposed to a 2 h restraint stress or left in their home cage, and killed immediately following stressor termination and home cage controls were killed at an equivalent time point. Serum was prepared and stored for analysis of corticosterone concentrations by EIA as outlined in Experiment Ia. Spleen tissue was also stored at -80^{o}C for corticosterone analysis.

2.4.4 Does corticosterone mimic the suppressive effect of stress on IFN-γ and IL-12 production from cultured spleen cells?

To prepare the spleen cells, the spleens were aseptically removed from each mouse and stored in 5ml falcon tubes containing 2ml RPMI 1640 medium. The spleens were mashed through a 40 μm nylon sieve and spleen cells were collected into a petri dish containing RPMI. The solution was titrated to prepare a single cell suspension and placed in a sterile 15ml falcon tube and was centrifuged at 1600 RPM for 15 min at 4 C and the supernatant was discarded. The erythrocytes were removed by hypotonic lysis and the remaining spleen cells were washed twice in RPMI 1640 medium. The spleen cells were resuspended in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin), counted using a veterinary haematology counter (ABC Vet^{TM}; Roche Diagnostics) and adjusted with complete RMPI 1640 medium to a final concentration of 2×10^{6} cells/ml.

475 µl of spleen cells were pipetted into wells of a sterile flat-bottomed 48 well plate (Nunc, Denmark). 25µl of corticosterone (final concentration 0.005-0.5 µM) was added to each well, or RPMI 1640 culture medium alone was added to control wells. Following a 30 min pre-incubation period with corticosterone, 50 µl of LPS (Sigma Chemical Co., Poole, Dorset, U.K.) at a final concentration of 1 µg/ml was added to each well, and cultures were incubated for a further 24hr (to measure IL-12 and IL-10 production) or 72hr (to measure IFN-γ production) at 37°C in a 5% CO_2 atmosphere. At the end of the culture period the supernatants were harvested and stored at -80°C until cytokine assays were performed.

2.4.5 Experiment IV: Is the suppressive effect of stress on the innate IFN-γ response mediated by glucocorticoid receptor activation

Mice were pre-treated with the glucocorticoid receptor antagonist mifepristone (20 mg/kg; i.p.) or vehicle (20% DMSO in 0.89% NaCl) 1 h prior to exposure to a 2 h restraint stress. LPS was administered immediately following stressor termination and mice were killed 8 h later. Serum was prepared and stored for analysis of IFN-γ concentrations by ELISA, and spleen tissue was taken and stored for analysis mRNA expression by real-time PCR as outlined in Experiment Ia.

2.4.6 Experiment V: IL-12 is a key regulator of IFN-γ production following stress and corticosterone

Can IL-12 rescue the stress-induced suppression of IFN-γ production?: Mice were exposed to 2 h restraint stress or left in their home cage, and killed by cervical dislocation followed by decapitation immediately following stressor termination. Home cage controls were killed at an equivalent time point. The spleen was dissected and spleen cells were prepared from freshly isolated spleens as previously described in 2.4.4. 475 µl of the single cell suspension of spleen cells were stimulated with 25 µl LPS (final concentration 1µg/ml). Following LPS administration the cells were incubated at 37°C in 5% CO_2 atmosphere and treated with 25 µl of recombinant mouse IL-12 (final concentration 100 pg/ml) 2 h, 24 h and 48 h after LPS administration. Cell free supernatants were harvested 72 h after LPS administration and stored in at -80°C until the IFN-γ ELISA was performed.

Can IL-12 rescue the corticosterone-induced suppression of IFN-γ production?: Spleen cells were prepared from freshly isolated spleens of adult male BALB/c mice as previously described in 2.4.4. 475 µl of the single cell suspension of spleen cells were pre-treated with 25 µl of corticosterone (final concentration 1 µg/ml) for 30 min prior to stimulation with 50 µl LPS (final concentration 1µg/ml). Following LPS administration the cells were incubated at 37°C in 5% CO_2 atmosphere and treated with 25 µl of recombinant mouse IL-12 (final concentration 100-1000pg/ml) 2 h after LPS administration. Cell free supernatants were harvested 72 h after LPS administration and stored in at -80°C until the IFN-γ ELISA was performed.

2.4.7 Experiment VI: Does the anxyolitic chlordiazepoxide prevent the stress-induced increase in corticosterone and stress-induced suppression of the innate IFN-γ response?

Does chlordiazepoxide prevent the stress-induced suppression of the innate IFN-γ response?: Mice were administered 3 injections of chlordiazepoxide (10 mg/kg; i.p.) 48 h, 24 h and 2 h prior to exposure to a 2 h restraint stress. LPS was administered immediately following stressor termination and mice were killed 8 h later. Serum was prepared and stored for analysis of IFN-γ concentrations by ELISA, and spleen tissue was taken and stored for analysis mRNA expression by real-time PCR as outlined in Experiment Ia.

Does chlordiazepoxide prevent the stress-induced increase in corticosterone?: Mice were administered 3 injections of chlordiazepoxide (10 mg/kg; i.p.) or vehicle, 48 h, 24 h and 2 h prior to being exposed to 2 h restraint stress or being left in their home cage. Mice were killed immediately following stressor termination and home cage controls were killed at an equivalent time point. Serum was prepared as outlined in Experiment Ia and stored at -80^{o}C until analysis of corticosterone concentrations were performed by EIA.

2.5 Real-time PCR analysis of IFN-γ, IL-12, IL-18, iNOS and IP-10 mRNA expression

Total RNA was isolated from spleen tissue or cultured cells using NucleoSpin® RNA II total RNA isolation kit (Macherney Nagel). Any genomic DNA contamination was removed with the addition of DNase to the samples according to the manufacturer's instructions. The yield of the resulting purified RNA was determined by measurement of the absorbance at 260 nm in a spectrophotometer, and RNA samples were subsequently equalized. RNA samples were reverse transcribed into cDNA using a high capacity cDNA archive kit (Applied Biosystems) according to the manufactures protocol.

Real-time PCR was performed using an ABI Prism 7300 instrument (Applied Biosystems, Darmstadt, Germany) as previously described (Boyle and Connor, 2007). Taqman Gene Expression Assays (Applied Biosystems, Darmstadt, Germany) containing primers and a Taqman probe were used to quantify each gene of interest. The assay ID's for the genes examined in this study were as follows: IFN-γ (Mm00801778_m1), IL-12p35 (Mm00434165_m1), IL-12p40 (Mm00434170_m1), IL-18 (Mm00434225_m1), iNOS (Mm00440485_m1) and IP-10 (Mm00445235_m1). PCR was performed in PCR plates in a 20µl reaction volume (9µl of diluted cDNA, 1µl of Taqman Gene expression assay and 10µl of Taqman® Universal PCR Master Mix) and PCR (40 cycles) was run in duplicate using ABIs universal cycling conditions. β-actin was used as endogenous control to normalize gene expression data, and an RQ value (2^{-DDCt}, where Ct is the threshold cycle) was calculated for each sample using Applied Biosystems RQ software (Applied Biosystems, UK). RQ values are presented as fold change in gene expression relative to the control group, which was normalised to 1.

2.6 Analysis of IL-10, IL-12 and IFN-γ concentrations by ELISA

Cytokine concentrations were determined using specific enzyme-linked immunosorbent assays (ELISA) performed using antibodies and standards obtained from Biosource, International. Assays were performed according to the maunfacturer's instructions, and absorbance read at 450 nm using a microplate reader. Absorbance was then recalculated as a concentration (pg/ml) using a standard curve derived using GraphPad Prism Software Version 4.00 (GraphPad software, Inc).

2.7 Analysis of STAT1 phosphorylation by Western immunoblotting

Spleen tissue was homogenised in lysis buffer (50mM Tris Base; 150mM NaCl; 2mM EDTA; 1% NP-40; 0.5% Na^{+} deoxycholate; 0.1% SDS) containing a cocktail of protease and phosphatase inhibitors (Sigma), and were equalised for total protein content following measurement of protein using the BCA protein assay (Pierce). Following protein equalization, tissue samples were diluted with an equal volume of sample buffer (Tris HCl pH 6.8, 0.5M ; SDS 10% w/v ; glycerol 10% v/v ; 2 β-mercaptoethanol, 5% v/v ; bromophenol blue, 0.05% w/v) and were boiled at 100°C for 5 min prior to loading onto a 10% SDS-polyacrylamide gel. Proteins were separated by application of 30 mA constant current for 25–30 min, transferred onto PVDF strips (225 mA for 75 min), and immunoblotted with the appropriate antibody. To assess expression of p-STAT1, nitrocellulose strips were incubated

overnight at 4°C in the presence of a rabbit polyclonal IgG antibody that specifically targets p-STAT1 (Cell Signalling Technologies; Diluted 1:1000); in TBS containing 0.1% BSA, and immunoreactive bands were detected using a peroxidase-linked anti-rabbit IgG (Amersham) in PBS/Tween (0.1% Tween-20) containing 2% nonfat dried milk. Nitrocellulose membranes were stripped using ReBlot Strong Blot Pus (Chemicon) and probed with a rabbit polyclonal IgG antibody that targets total STAT1 (Cell Signalling Technologies; Diluted 1:1000), and immunoreactive bands were detected using a peroxidase-linked anti-rabbit IgG (Amersham) in PBS/Tween (0.1% Tween-20) containing 2% nonfat dried milk.

Protein bands were visualized using Millipore chemiluminescence solution. Immunoblots were exposed to film (Amersham Biosciences) and processed using a Fuji x-ray processor. Bands were quantified by densitometric analysis using Gelworks ID, Version 2.51. Gelworks provides a single value (in arbitrary units) representing the density of each blot.

2.8 Analysis of corticosterone concentrations by EIA

Corticosterone concentrations in serum and spleen tissue was measured using a commercially available EIA corticosterone enzyme immunoassay kit (Immunodiagonstic systems, UK). The assay was performed according to the maunfacturer's instructions, and absorbance read at 450 nm using a microplate reader. Absorbance was then recalculated as a concentration (ng/ml) using a standard curve derived using GraphPad Prism Software Version 4.00 (GraphPad software, Inc).

2.9 Statistical analysis of data

All values are expressed as mean ± standard error of the mean (SEM). Data were analysed using a Students t-test or a one- or two-way analysis of variance (ANOVA), followed by a Newman-Keuls *post hoc* test, where appropriate. A p value less than 0.05 was considered statistically significant.

3. Results

3.1 Exposure to restraint stress suppresses the innate IFN-γ response following an *in vivo* LPS challenge

3.1.1 IFN-γ concentrations and IFN-γ mRNA expression: A two-way ANOVA revealed a significant LPS x restraint stress interaction for serum IFN-γ concentrations [$F_{(1,16)}$=3.99, P<0.05] and splenic IFN-γ mRNA expression [$F_{(1,16)}$=78.08, P<0.0001]. *Post hoc* analysis demonstrated that prior exposure to restraint stress significantly attenuated LPS-induced serum IFN-γ concentrations (P<0.01), and IFN-γ mRNA expression (P<0.01) in spleen tissue (Figure 1).

3.1.2 IFN-γ signaling: A two-way ANOVA revealed a significant LPS x restraint stress for splenic pSTAT-1 expression [$F_{(1,16)}$=4.34, P<0.05]. *Post hoc* analysis demonstrated that prior exposure to restraint stress significantly attenuated LPS-induced STAT-1 phosphorylation (pSTAT1) in mouse spleen (P<0.01) (Figure 2a). Similarly, a two-way ANOVA revealed a significant LPS x restraint stress for expression of the IFN-γ inducible genes iNOS [$F_{(1,16)}$=3.41, P<0.05] and IP-10 [$F_{(1,16)}$=23.68, P<0.001]. *Post hoc* analysis demonstrated that restraint stress significantly attenuated LPS-induced iNOS (P<0.05) and IP-10 (P<0.01) mRNA expression (Figure 2b,c).

----------[Insert Figures 1&2 about here]----------

3.1.3 Expression of IFN-γ inducing factors IL-12 and IL-18: A two-way ANOVA revealed a significant LPS x restraint stress interaction for IL-12p40 mRNA expression 1.5 h [$F_{(1,15)}$=28.50, P<0.0001] and 8 h [$F_{(1,16)}$=8.19, P<0.01] post LPS administration. *Post hoc* analysis demonstrated that prior exposure to restraint stress significantly attenuated LPS-induced IL-12p40 mRNA expression in spleen tissue at both time-points (P<0.01) (Figure 3a,b). In contrast, expression of the IL-12p35 subunit was not altered in mouse spleen by exposure to restraint stress or administration of LPS at either time-point (Figure 3c,d). In addition, whilst IL-18 mRNA expression was significantly increased in mouse spleen 8 h post LPS administration (P<0.05), this increase in IL-18 expression was not altered restraint stress (Figure 3f).

----------[Insert Figure 3 about here]----------

3.2 A role for IL-10 in mediating the suppressive effect of stress on innate IFN-γ production?

3.2.1 Restraint stress augments IL-10 production: A two-way ANOVA exposed a significant LPS x restraint stress interaction for serum IL-10 concentrations [$F_{(1,16)}$=12.93, P<0.001]. *Post hoc* analysis demonstrated that prior exposure to restraint stress significantly (P<0.01) augmented LPS-induced serum IL-10 concentrations (Figure 4a).

3.2.2 ***The stress-induced inhibition of IFN-γ and IL-12p40 expression was not altered in IL-10 knock out mice:*** A two-way ANOVA exposed a significant restraint stress x mouse strain interaction for IFN-γ mRNA expression [$F_{(1,16)}$=78.5, P<0.0001] and IL-12p40 mRNA

expression [$F_{(1,17)}$=28.3, P<0.0001]. As already demonstrated in BALB/c mice (Figure 1a and Figure 3b), restraint stress induced a significant suppression of LPS-induced IFN-γ and IL-12 expression in wild-type C57BL/6 mice relative to non-stressed home cage control animals (P<0.01). Similarly, restraint stress significantly suppressed LPS-induced IFN-γ and IL-12 expression in IL-10 knockout mice (P<0.01). It is noteworthy that IL-10 knockout mice show a significantly greater increase in IFN-γ and IL-12p40 mRNA following LPS challenge relative to wild-type C57BL/6 mice (P<0.01) (Figure 4b,c).

----------[Insert Figure 4 about here]----------

3.3 A role for glucocorticoids in mediating the suppressive effect of stress on the innate IFN-γ response

3.3.1 Restraint stress increases corticosterone concentrations in serum and spleen: The concentrations of corticosterone in mice that were not exposed to stress were very low in the serum and spleen. However, exposure to 2 h of restraint stress significantly increased both serum and splenic corticosterone concentrations (P<0.01) (Figure 5 a, b).

3.3.2 In vitro exposure of spleen cells to corticosterone mimics the ability of stress to inhibit IFN-γ and IL-12 production: A one-way ANOVA revealed a significant effect of corticosterone treatment on LPS-induced IFN-γ [$F_{(5,25)}$=18.71, P<0.0001] and IL-12 [$F_{(5,29)}$=17.43, P<0.0001] production. *Post hoc* analysis demonstrated that pre-treatment with corticosterone (0.005 – 0.5 μM) significantly inhibited LPS-induced IFN-γ and IL-12 production in a dose-dependant manner. **In contrast, corticosterone treatment failed to alter LPS-induced IL-10 production (Figure 5c).**

3.3.3 The GR antagonist mifepristone prevents the ability of stress to suppress the innate IFN-γ response: A two-way ANOVA uncovered a significant mifepristone x stress interaction for serum IFN-γ concentrations [$F_{(1,18)}$=8.98, P<0.01] and mRNA expression of IFN-γ [$F_{(1,18)}$=12.97, P<0.001], IL-12p40 [$F_{(1,18)}$=11.78, P<0.005], IP-10 [$F_{(1,18)}$=5.93, P<0.01] and iNOS [$F_{(1,18)}$=5.85, P<0.01] in spleen tissue. *Post hoc* analysis demonstrated that pre-treatment with the glucocorticoid receptor antagonist mifepristone prevented the stress-induced inhibition of circulating IFN-γ concentrations and splenic IFN-γ and IL-12p40 mRNA expression (Figure 6a-c). Similarly, pre-treatment with mifepristone blocked the ability of stress to inhibit expression of the IFN-γ-inducible genes iNOS and IP-10 (Figure 6d,e).

----------[Insert Figures 5&6 about here]----------

3.4 IL-12 is a key regulator of IFN-γ production following stress and corticosterone

3.4.1 IL-12 restores the stress-induced inhibition of IFN-γ production from spleen cells: Consistent with our findings following *in vivo* LPS challenge, exposure to restraint stress inhibited LPS-induced IFN-γ production from spleen cells *ex vivo*. In order to determine a role of IL-12 in the suppressive effect of stress on IFN-γ production, we determined if treatment of spleen cells from stressed mice with exogenous IL-12 could restore LPS-induced IFN-γ production.

A two-way ANOVA exposed a significant IL-12 treatment x restraint stress interaction for IFN-γ production from spleen cells [$F_{(1,22)}$=420.25, P<0.0001]. *Post hoc* analysis demonstrated that addition of exogenous IL-12 rescued IFN-γ production in spleen cells from stressed mice (P<0.01). It is also noteworthy that administration of exogenous IL-12 to spleen cells resulted in approximately a 4-fold increase in LPS-induced IFN-γ production (Figure 7a).

3.4.2 IL-12 restores IFN-γ production from spleen cells following treatment with corticosterone: Consistent with the data presented in Figure 5c pre-treatment of spleen cells with corticosterone inhibited LPS-induced IFN-γ production. In order to determine a role of IL-12 in the suppressive effect of corticosterone on IFN-γ production, we determined if treatment of spleen cells exogenous IL-12 could prevent the suppressive effect of corticosterone on LPS-induced IFN-γ production. A one-way ANOVA revealed a significant effect treatment on IFN-γ production [$F_{(4,30)}$ = 23.53, P<0.0001]. *Post hoc* analysis demonstrated that addition of exogenous IL-12 rescued IFN-γ production in spleen cells treated with corticosterone (P<0.01) (Figure 7b).

----------[Insert Figure 7 about here]----------

3.5 The anxiolytic chlordiazepoxide prevents the ability of stress to suppress the innate IFN-γ response and to increase circulating corticosterone concentrations

3.5.1 Chlordiazepoxide prevents the ability of stress to suppress the innate IFN-γ response: A two-way ANOVA uncovered a significant treatment x restraint stress interaction for serum IFN-γ concentrations [$F_{(1,20)}$=3.99, P<0.05], IL-12p40 mRNA expression [$F_{(1,18)}$=3.56, P<0.05], and iNOS mRNA expression [$F_{(1,20)}$=4.87, P<0.05]. Post hoc analysis demonstrated that pre-treatment with the anxiolytic chlordiazepoxide prevented the stress-induced suppression of serum IFN-γ concentrations and IL-12p40 mRNA expression (Figure 8a,b). Similarly, pre-treatment with chlordiazepoxide prevented stress-induced inhibition of the IFN-γ inducible gene iNOS (Figure 8c).

3.5.2 Chlordiazepoxide attenuated the increase in circulating corticosterone concentrations induced by stress: A two-way ANOVA uncovered a significant stress x chlordiazepoxide interaction on serum corticosterone concentrations [$F_{(1,15)}$=53.95, P<0.0001]. *Post hoc* analysis demonstrated that pre-treatment with the anxiolytic chlordiazepoxide reduced the stress-related increase in circulating corticosterone concentrations by just over 50% (Figure 8d).

----------[Insert Figure 8 about here]----------

4. Discussion

4.1 Restraint stress suppresses the innate IFN-γ response in vivo: In the present study we demonstrate that restraint stress suppresses innate IFN-γ production induced following an *in vivo* challenge with bacterial LPS. Specifically, LPS increased IFN-γ mRNA expression in spleen, and induced a concomitant increase in circulating IFN-γ concentrations, which was suppressed by stressor exposure. In addition to the ability of stress to suppress innate IFN-γ production, stress also impaired IFN-γ signaling indicated by reduced phosphorylation of the transcription factor STAT1. This finding is of significance as IFN-γ receptor activation results in phosphorylation of STAT1 by janus kinases (JAKs) and homodimers of phosphorylated STAT1 (pSTAT1) translocate to the nucleus, bind to DNA consensus sites and alter gene expression. In this regard, we demonstrated that in tandem with suppressing LPS-induced pSTAT1, stress also reduced expression of the IFN-γ inducible genes iNOS and IP-10. We suggest that this reduction in iNOS and IP-10 expression occurs as a result of the inhibitory action of stress on IFN-γ production and signalling as it has already been established that LPS and IFN-γ-induced iNOS and IP-10 expression are dependent on STAT1 binding to nuclear GAS elements (Gao et al., 1997; Ohmori and Hamilton, 2001). The suppressive effect of stress on iNOS and IP-10 expression could negatively impact upon host defense, as iNOS mediates production of nitric oxide which has bacteriocidal properties (Fierro et al., 1999; Gross et al., 1998) and IP-10 is a chemokine that stimulates migration of T-cells and NK-cells to sites of infection and injury (Dufour et al., 2002; Hsieh et al., 2006; Hyun et al., 2005; Narumi et al., 1992). **Of course innate IFN-γ produced in the early stages of infection is also a trigger for driving an effective adaptive immune response. Specifically, innate IFN-γ enhances MHC class II expression and antigen presentation to T-cells, polarizes T-cells in a Th_1 direction, and drives cytotoxic T-cell maturation; actions that are important for clearing both viral and bacterial infections (see Boehm *et al*, 1997).**

It is well established that IFN-γ production following *in vivo* LPS administration is predominantly derived from NK cells (Fukuda et al., 2005; Kim et al., 2000; Varma et al., 2002). LPS stimulates the innate immune response via activation of TLR4 (Palsson-McDermott and O'Neill, 2004), however, as NK cells do not express functionally responsive TLR4 receptors (O'Connor et al., 2006), LPS-induced IFN-γ production occurs secondary to inducing factors such as IL-12 and IL-18 produced by innate immune cells such as macrophages and dendritic cells. **This explains why it takes 7-8 hours before a measurable IFN-γ response to LPS is observed *in vivo* (Fukuda et al., 2005; Kim et al., 2000; Varma et al., 2002), whereas expression of IFN-γ inducing factors such as IL-12 is observed much earlier at 1-2 hours post LPS administration (Boyle and Connor, 2007; Fukuda et al., 2005; Varma et al., 2002)**. In this study we observed that exposure to restraint suppressed production of IL-12; the major cytokine responsible for driving LPS-induced IFN-γ production *in vivo* (Trinchieri, 2003; Varma et al., 2002), **and this reduction in IL-12 was observed as early as 90 minutes post LPS challenge, thus preceding IFN-γ production.** The reduction in IL-12 was confined to the p40 subunit, and this is in keeping with literature indicating that stringent regulation of IL-12p40 expression governs the biological activity of IL-12 (Trinchieri et al., 2003; Varma et al., 2002). Whilst IL-12 is the major driver of innate IFN-γ production, evidence indicates that IL-18 can also induce IFN-γ production (Fehniger et al., 1999). However, our data demonstrate that stress fails to alter expression IL-18,

suggesting that IL-18 is not a key mediator of IFN-γ production in response to LPS. In accordance with this view, it has been demonstrated that IL-18 does not directly induce IFN-γ production from NK-cells or T-cells, but rather serves to amplify IL-12-induced IFN-γ production (Fehniger et al., 1999; Munder et al., 1998; Strengell et al., 2003). In the context of our results where IL-18 production was unaltered by stressor exposure, it is clear that IL-18 could not compensate or substitute for the reduction in IL-12 induced by stress. In this regard we demonstrate that addition of IL-12 to LPS-stimulated spleen cells from stressed animals completely reverses the stress-related reduction in IFN-γ production. These data suggest that a reduction in IL-12 production **is likely to be** a key event in mediating stress-induced suppression of IFN-γ production**. Of course a limitation of our ex vivo IL-12 stimulation experiment is that the dose of IL-12 employed had a powerful inducing effect on IFN-γ production, resulting in IFN-γ concentrations approximately 4-fold higher than in un-stimulated spleen cells. Thus the possibility exists that the ability of IL-12 to reverse the suppressive effect of stress on IFN-γ production may have occurred due to this powerful IFN-γ stimulating effect of IL-12. Consequently, the ability of a lower dose of IL-12 to rescue ex vivo production of IFN-γ following stress, or the ability of in vivo treatment with IL-12 to reverse stress-induced suppression of IFN-γ production should be assessed in future studies.**

Consistent with our previous findings (Connor et al., 2005; Curtin et al., 2009) restraint stress induced a robust increase in LPS-induced IL-10 production, which preceded the suppressive effect of stress on IFN-γ production. **IL-10 is a cytokine with a broad spectrum of anti-inflammatory actions, and IL-10 production that occurs in response to an infectious stimulus most likely serves to prevent development of an excessive inflammatory response that would be damaging to the host (Moore et al., 2001).** As we have previously demonstrated that IL-10 can suppress IL-12 and IFN-γ production from LPS-stimulated spleen cells (Boyle and Connor, 2007), we hypothesised that IL-10 may be a mediator of the suppressive effect of stress on the innate IFN-γ response. However, the results of our experiments with IL-10 knockout mice **suggest** that IL-10 is not a critical mediator of the stress-induced suppression of IL-12 and IFN-γ production. **In further support of this notion, we have observed that pre-treatment with the β-adrenoceptor antagonist nadolol completely blocked the ability of restraint stress to increase LPS-induced IL-10 production, yet failed to block the suppressive effect of restraint stress on the innate IFN-γ response (Supplementary Figure 1). This finding reinforces the results of the IL-10 knockout study, highlighting a mechanistic dissociation between the ability of restraint stress to increase IL-10, and suppress innate IFN-γ production.** These findings are also is consistent with a previous report where we demonstrated that the ability of swim stress to suppress production of the pro-inflammatory cytokines IL-1β and TNF-α occurred independently of a stress-induced increase in IL-10 (Connor et al., 2005). These data are supported by clinical evidence demonstrating that the suppressive effect of heart surgery on monocyte function (TNF-α production and HLA-DR expression) is not dependent on the increase in circulating IL-10 concentrations that occurs following surgery (Volk et al., 2003).

4.2 Glucocorticoids mediate the suppressive effect of stress on the innate IFN-γ response: Once we established that restraint stress suppressed the innate IFN-γ response, we next examined the role of potential physiological mediators of this response. Stress activates the HPA axis and sympathetic nervous system (Sternberg et al., 2006; McCarty et al., 1981; Connor et al., 1997), and the end products of these pathways (glucocorticoids and catecholamines) are often implicated as mediators of stress-induced immunosuppression.

Our results demonstrate that in vitro exposure of mouse spleen cells to the glucocorticoid corticosterone suppresses LPS-induced IL-12 and IFN-γ production, without altering production of the anti-inflammatory cytokine IL-10. These data are consistent with previous studies conduced in LPS-stimulated human blood (Agarwal and Marshall, 1998; Visser et al., 1998). Moreover, we demonstrate that suppressive action of stress on the innate IFN-γ response was completely blocked by pre-treatment with the glucocorticoid receptor antagonist mifepristone. These data indicate a crucial role for stress-induced corticosterone release and glucocorticoid receptor activation in suppressing the innate IFN-γ response. We suggest that the suppressive effect of stress on IFN-γ production occurs secondary to a glucocortioid mediated reduction in IL-12 production. This contention is supported by the fact that the stress-induced reduction in IFN-γ production is restored by addition of exogenous IL-12, and that the suppressive effect of corticosterone on IFN-γ production is rescued by exogenous administration of IL-12. **This finding is consistent with a previous report where IL-12 was shown to rescue the suppressive effect of hydrocortisone and the synthetic glucocorticoid clobetasol-17-propionate on the ability of dendritic cells to induce IFN-γ production from human $CD4^+$ T-cells (Vieira et al., 2000).**

Whilst pre-treatment with the β-adrenoceptor antagonist nadolol completely blocks the stress related increase in IL-10 production (Curtin et al., 2009), we have found that β-adrenoceptor blockade fails to block the stress-induced suppression of IL-12 and IFN-γ production (Supplementary Figure 1). Thus our findings indicate that stress suppresses innate IFN-γ production via glucocorticoid receptor activation, and via a distinct parallel pathway involving activation of β-adrenoceptors, stress promotes production of the anti-inflammatory cytokine IL-10 (Curtin et al., 2009).

4.3. Pre-treatment with chlordiazepoxide attenuates the suppressive effect of stress on the innate IFN-γ response: The results of this study demonstrate that pre-treatment with chlordiazepoxide attenuated the effect of stress in suppressing innate IFN-γ production. This is a significant finding as chlordiazepoxide is a benzodiazepine anxiolytic commonly used to relieve anxiety (Leonard, 1993). Consistent with a role for glucocorticoids in mediating the suppressive effect of stress on the innate IFN-γ response, we demonstrate that treatment with chlordiazepoxide attenuated the restraint stress-induced increase in circulating corticosterone**.** Based on this finding, and on our finding that glucocorticoid receptor activation is a key event in mediating the suppressive effect of restraint stress on innate IFN-γ production, we suggest that chlordizepoxide prevents the stress-induced suppression of the innate IFN-γ response by blunting stress perception, and therefore blunting the stress-related increase in corticosterone. **In support of this notion, previous studies have shown that chlordiazepoxide reduces stress perception in mice as assessed using the elevated plus maze and light dark box tests of anxiety (Griebel at al. 1996; Lister, 1987). Also, previous reports demonstrate that chlordiazepoxide inhibits stress-induced HPA axis activation and corticosterone release (Degroot et al., 2004; LeFur et al., 1979). However, as chlordiazepoxide only reduced circulating corticosterone concentrations by just over 50%, we can not rule out the possibility that a non-HPA axis mediated action of chlordiazepoxide may have also contributed to its protective effect against stress-induced immunosuppression, however further studies are required to examine this possibility. Nonetheless, irrespective of the underling mechanism involved, t**o our knowledge this is the first demonstration that intervention with benzodiazepines can prevent the suppressive effect of stress on pro-inflammatory cytokine production, and complements our recent finding that chlordiazepoxide

prevents stress-related increases in expression of the anti-inflammatory cytokine IL-10 (Curtin et al., 2009).

4.4 Conclusion: Taken together, our data demonstrate that psychological stress suppresses the innate IFN-γ response via glucocorticoid receptor activation. Considering the critical role played by IFN-γ in host defense to infection (Biron and Brossay, 2001; Rosenzweig and Holland, 2005), and in anti-tumour immunity (Ikeda et al., 2002; Kim et al., 2000; Tannenbaum and Hamilton, 2000), we suggest that suppression of the innate IFN-γ production is a potentially serious consequence of psychological stress. Our data also suggest that treatment with anxiolytic drugs such as chlordiazepoxide could be a useful strategy in combating stress-induced immunosuppression, thereby combating some of the deleterious effects of stress on health.

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Figure legends

Figure 1: Restraint stress suppresses serum IFN-γ concentrations and splenic IFN-γ mRNA expression in response to an *in vivo* LPS challenge

LPS (250µg/kg; i.p.) was administered to **BALB/c** mice immediately following a 2 h restraint stress and the mice were sacrificed 8 h after injection. IFN-γ mRNA expression was measured in spleen (a) and circulating IFN-γ concentrations were measured in serum (b). Data are expressed as mean ± SEM (n=5). **P<0.01 vs. saline-treated control mice, $^{++}$P<0.01 vs. non-stressed LPS-treated control mice (Newman-Keuls post hoc test).

Figure 2: Restraint stress suppresses IFN-γ signaling in response to an *in vivo* LPS challenge

LPS (250µg/kg; i.p.) was administered to **BALB/c** mice immediately following a 2 h restraint stress and the mice were sacrificed 8 h after injection. Phosphorylation of STAT-1 (a) and mRNA expression of the IFN-γ-inducible genes iNOS (b) and IP-10 (c) was measured in spleen tissue. Data are expressed as mean ± SEM (n=5). Values of pSTAT are expressed as the percentage of α + β pSTAT-1 divided by α + β tSTAT-1. **P<0.01 vs. saline treated control mice, $^{++}$P<0.01 vs. non-stressed LPS-treated control mice, $^{+}$P<0.05 vs. non-stressed LPS-treated control mice (Newman-Keuls post hoc test).

Figure 3: Restraint stress suppresses splenic IL-12p40 mRNA expression in response to an *in vivo* LPS challenge

LPS (250µg/kg; i.p.) was administered to **BALB/c** mice immediately following a 2 h restraint stress and the mice were sacrificed either 1.5 h or 8 h after injection. mRNA expression of IL-12p40 (a,b), IL-12p35 (c,d) and IL-18 (e,f) was measured in spleen. Data are expressed as mean ± SEM (n=4-5). **P<0.01 vs. saline control mice, $^{++}$P<0.01 vs. non-stressed LPS control mice (Newman-Keuls post hoc test).

Figure 4: Restraint stress augments LPS-induced IL-10 production, but the suppressive effect of stress on IFN-γ and IL-12 expression is still evident in IL-10 knockout mice

(a) LPS (250µg/kg; i.p.) was administered to mice immediately following a 2 h restraint stress and the **BALB/c** mice were sacrificed 1.5 h after injection. Circulating IL-10 concentrations were measured in the serum. Data are expressed as mean ± SEM (n=5). *P<0.05 vs. saline control mice, **P<0.01 vs. saline control mice, $^{++}$P<0.01 vs. non-stressed LPS control mice (Newman-Keuls post hoc test).

(b-c) mRNA expression of IFN-γ (b) and IL-12 (c) was measured in spleens of IL-10 knock out mice and wild-type controls **(C57/BL6 mice)** 8 h following administration of LPS (250µg/kg). Data are expressed as mean ± SEM (n=5-6). **P<0.01 vs. C57 LPS-treated control mice, $^{++}$P<0.01 vs. C57BL/6-control mice, $^{\#\#}$P<0.01 vs. IL-10 KO LPS-treated control mice (Newman-Keuls post hoc test).

Figure 5: Restraint stress increases corticosterone concentrations in the serum and the spleen, and corticosterone suppresses IFN-γ and IL-12 production from spleen cells

(a-b) **BALB/c** mice were either exposed to a 2 h restraint stress or left in home cages and sacrificed immediately after stress. Corticosterone concentrations were measured in serum (a) and spleen (b). Data are expressed as mean ± SEM (n=6). **P<0.01 vs. non-stressed control mice (Student-t test).

(c) Spleen cells were cultured from freshly isolated spleens from **BALB/c** mice and the cell suspension was adjusted to 2 x 10^6 cells / ml. Spleen cells were pre-treated with corticosterone (0.005 – 0.5 µM) for 30 min prior to simulation with LPS (1 µg/ml). Data are expressed as mean ± SEM (n=5-6). **P<0.01 vs. LPS control (Newman-Keuls post hoc test).

Figure 6: Mifepristone prevents the stress-induced suppression of the innate IFN-γ response

Mifepristone (20 mg/kg; i.p.) was administered to **BALB/c** mice 1 h prior to exposure to a 2 h restraint stress, **and 20% DMSO was administered as a vehicle to control mice.** LPS (250 µg/kg; i.p.) was administered immediately following stressor termination and mice were sacrificed 8 h after LPS administration. Circulating IFN-γ concentrations were measured in the serum (a) and the mRNA expression of IFN-γ (b) and IL-12p40 (c) were measured in spleen. Data are expressed as mean ± SEM (n=5-6). *P<0.05, **P<0.01 vs. non-stressed LPS-treated control mice (Newman-Keuls post hoc test).

Figure 7: IL-12 restores spleen cell IFN-γ production following stress and corticosterone administration

(a) **BALB/c** mice were exposed to restraint stress and sacrificed immediately after stressor termination. Spleen cells were prepared and adjusted to a concentration of 2 x 10^6 cells / ml before stimulation with LPS (1 µg/ml) *ex vivo*. Spleen cells were treated with mouse recombinant IL-12 (100 pg/ml) 2hr, 24hr and 48hr after LPS stimulation and cell-free supernatants were harvested 72 h after LPS stimulation. Data are expressed as mean ± SEM (n=6-7). *P<0.05, **P<0.01 vs. LPS-treated control mice, $^{++}$P<0.01 vs. non-IL-12 treated control mice (Newman-Keuls post hoc test).

(b) Spleen cells prepared from **BALB/c** mice were pre-treated with corticosterone (1µg/ml) for 30 min prior to simulation with LPS (1µg/ml). IL-12 (100-1000 pg/ml) was administered to spleen cells 2hr following LPS stimulation and cell-free supernatants were harvested 72 h following stimulation. Data are expressed as mean ± SEM (n=6-8). **P<0.01 vs. LPS-treated control mice, $^{++}$P<0.01 vs. non IL-12-treated control mice (Newman-Keuls post hoc test).

Figure 8: Chlordiazepoxide prevents the ability of stress to suppress the innate IFN-γ response and increase circulating corticosterone concentrations

(a-c) Chlordiazepoxide (10 mg/kg; i.p.) was administered 3 times to **BALB/c** mice at 48 h, 24 h and 2 h prior to a 2 h restraint stress. LPS (250 µg/kg; i.p.) was administered immediately following stressor termination and mice were sacrificed 8 h after LPS administration.

Data are expressed as mean ± SEM (n=6). **P<0.01 vs. non-stressed LPS-treated control mice, *P<0.05 vs. non-stressed LPS-treated control mice, $^{++}$P<0.01 vs. non-stressed LPS + chlordiazepoxide treated control mice (Newman-Keuls post hoc test).

(d) Chlordiazepoxide (10mg/kg; i.p.) or saline was administered 3 times to **BALB/c** mice at 48 h, 24 h and 2 h prior to exposure to restraint stress for 2 h. Mice were sacrificed immediately following stressor termination. Data are expressed as mean ± SEM (n=4-5). **P<0.01 vs. LPS-treated control mice, $^{++}$P<0.01 vs. non-chlordiazepoxide treated control mice (Newman-Keuls post hoc test).

Figure 1

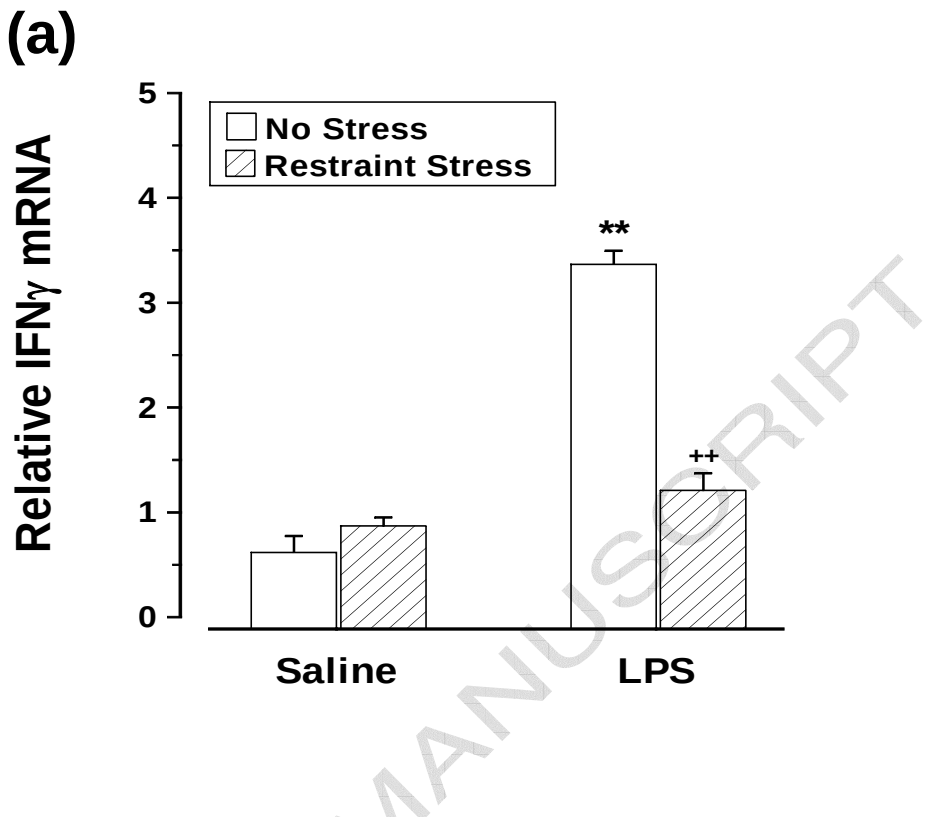
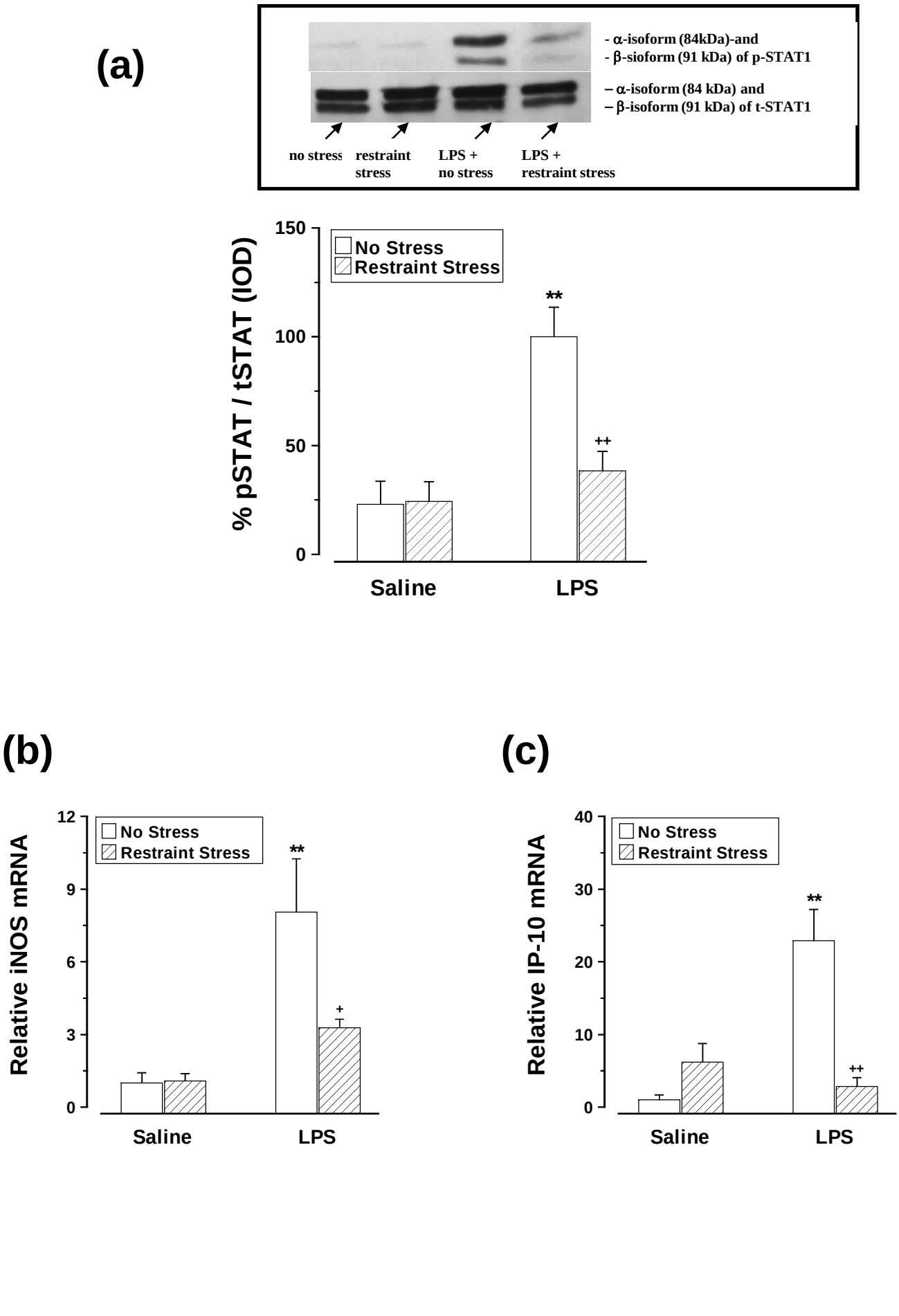


Figure 2



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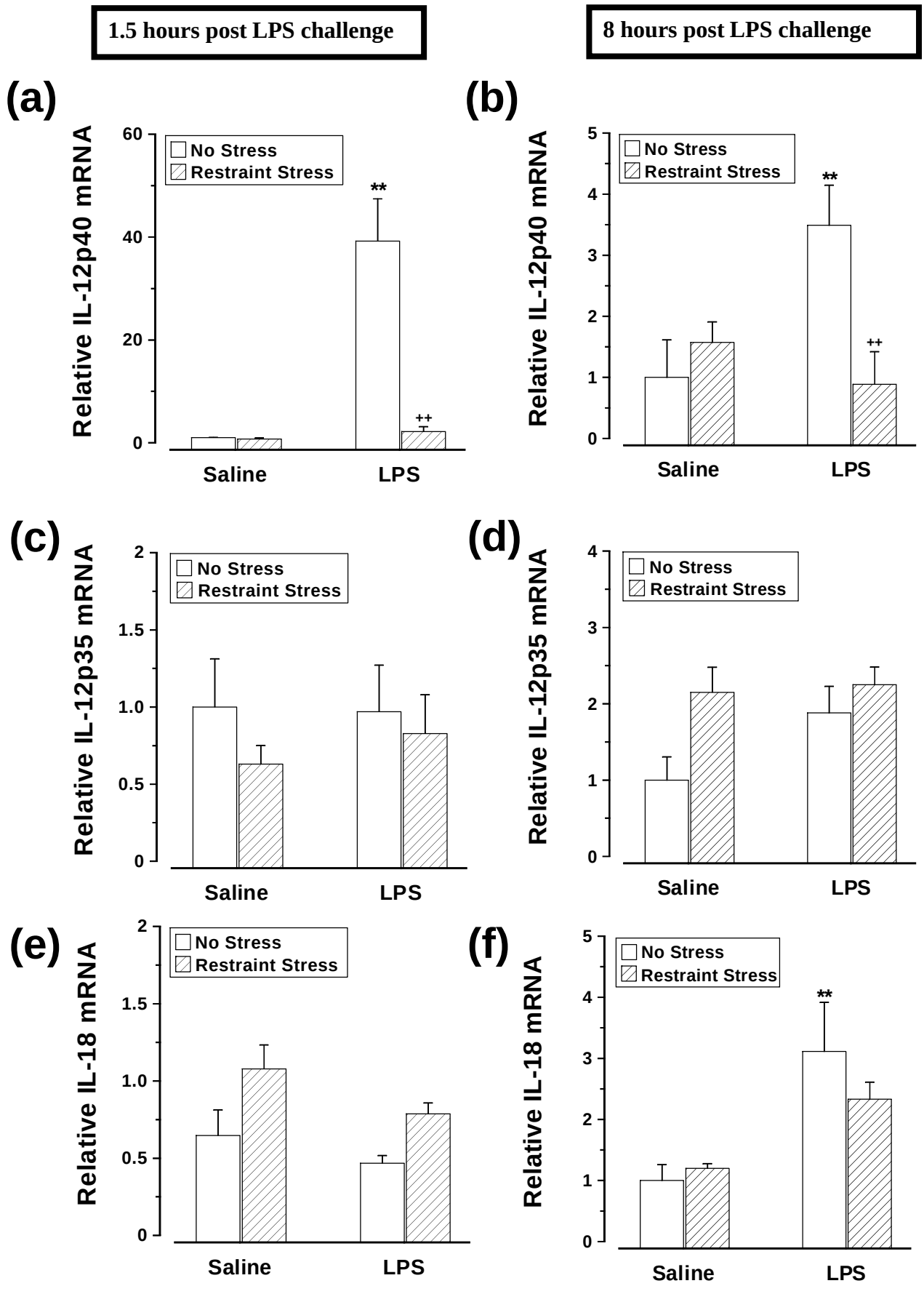

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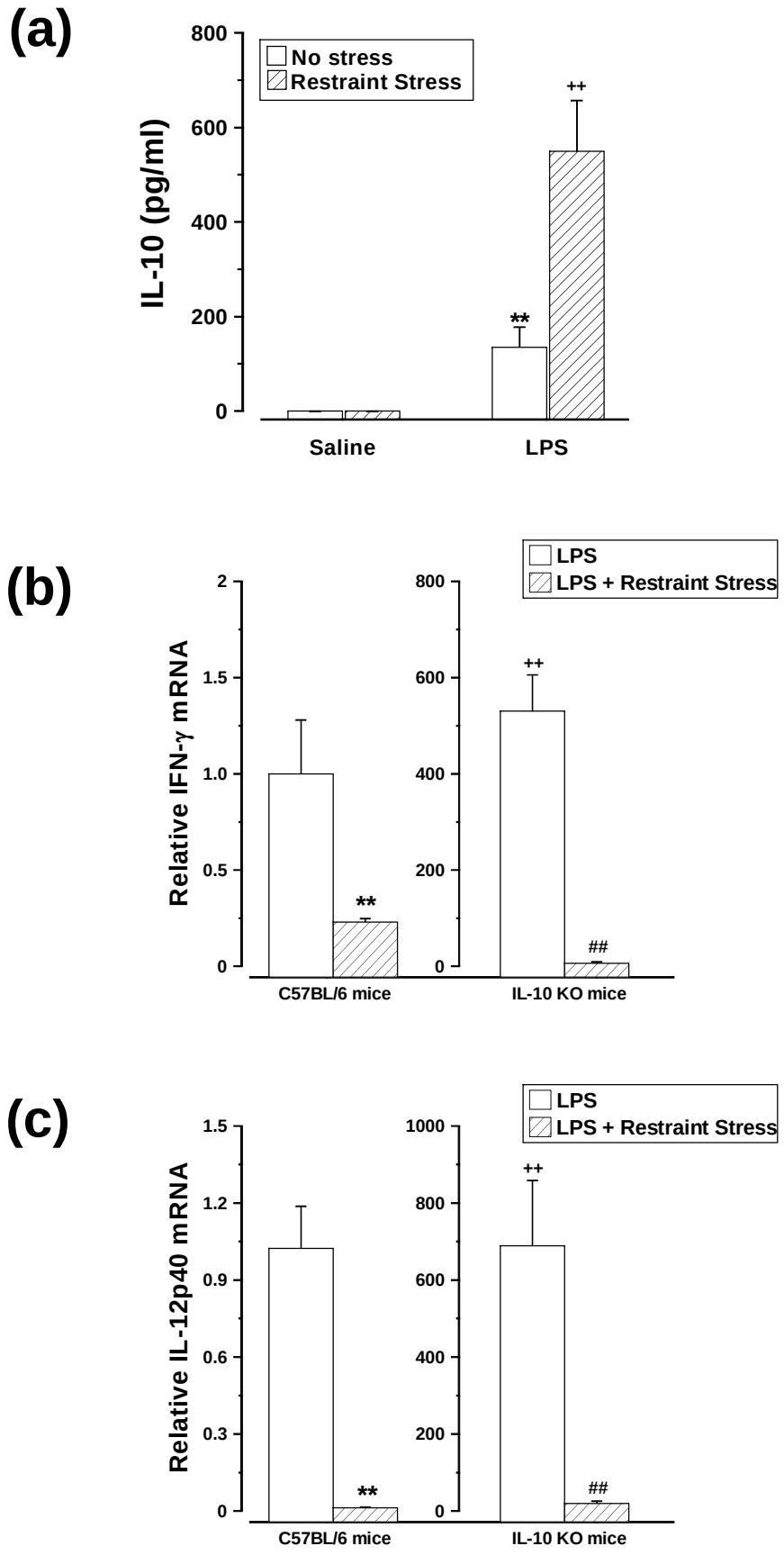

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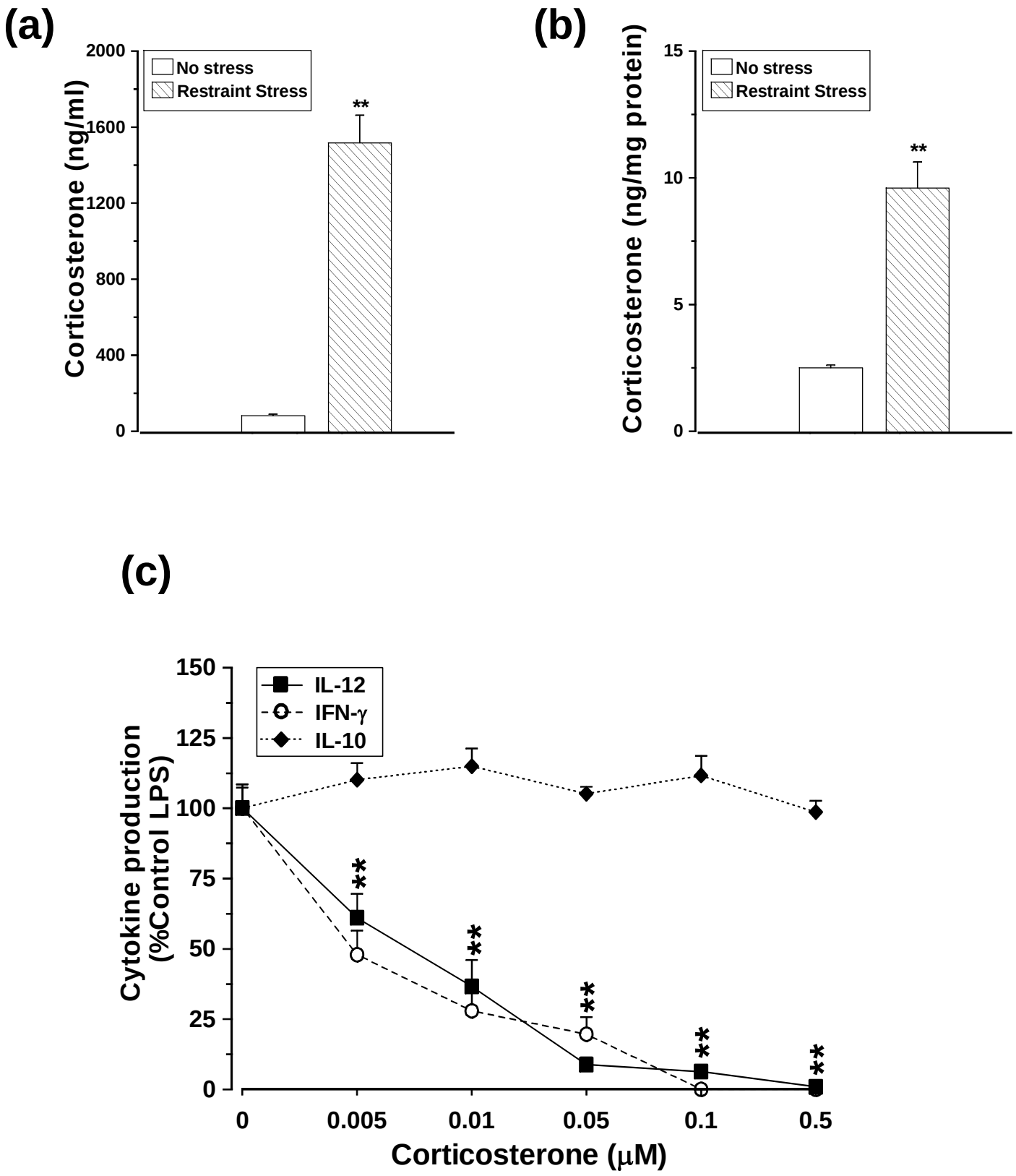

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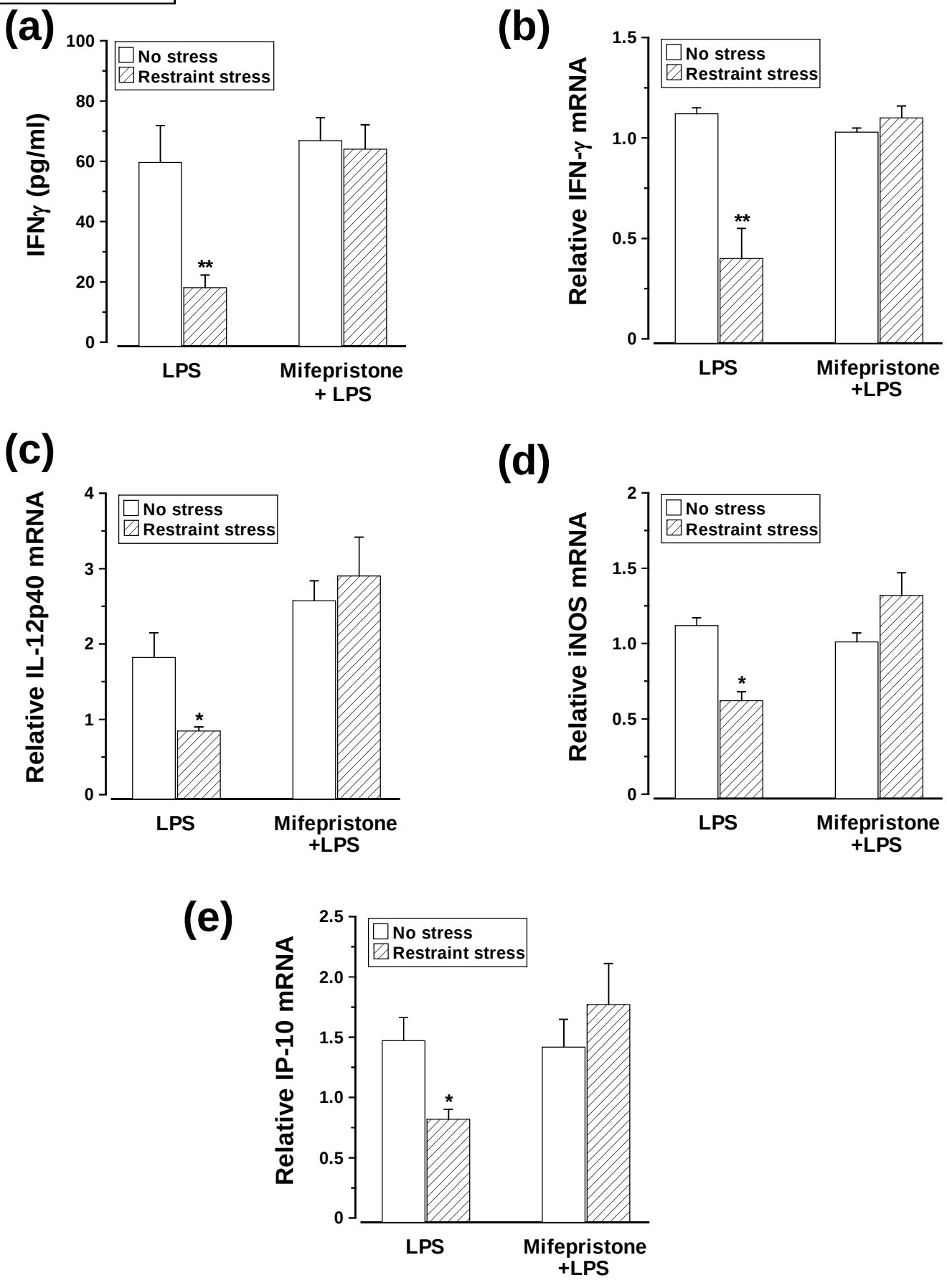
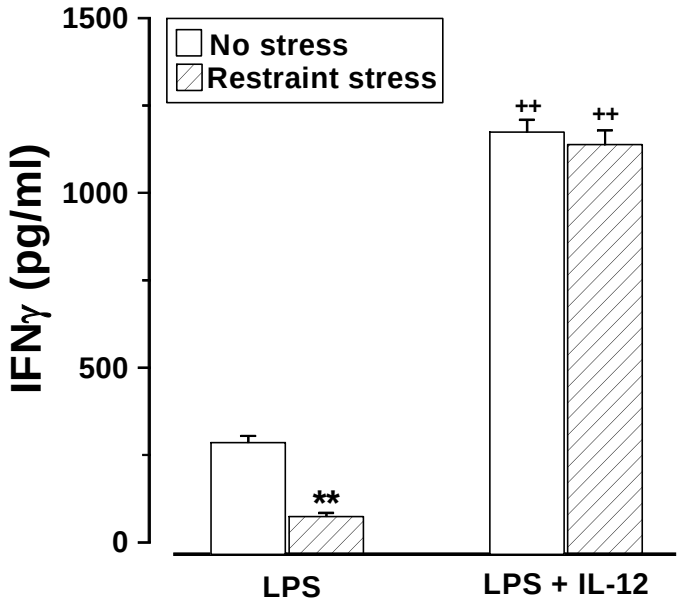


Figure 7

(a)



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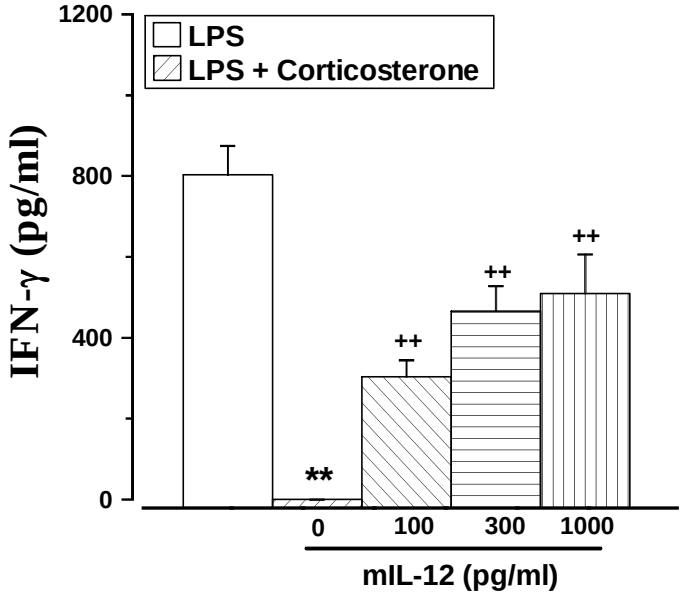

Figure 8