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Methylenedioxymethamphetamine (MDMA; "Ecstasy") suppresses production of the pro-inflammatory cytokine tumor necrosis factor–α independent of a β-adrenoceptor-mediated increase in interleukin-10

Thomas J. Connor, Andrew Harkin*, and John P. Kelly

Department of Physiology, Trinity College Institute of Neuroscience, Trinity College, Dublin 2, Ireland (T.J.C.).

Department of Pharmacology, National University of Ireland, Galway, Ireland (T.J.C., A.H., J.P.K.)

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Correspondence should be addressed to:

Dr. Thomas Connor, Department of Physiology, Trinity College, Dublin 2, Ireland

Tel: +353-1-6083095; Fax: +353-1-6793545; E-mail: connort@tcd.ie

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Abbreviations: ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; ELISA, Enzyme-linked immunosorbent assay; IL, Interleukin; LPS, Bacterial Lipopolysaccharide; MDMA, Methylenedioxymethamphetamine; MHC, Major histocompatability complex; TNF, Tumor necrosis factor.

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Abstract

Recent data suggest that 3,4-methylenedioxymethamphetamine (MDMA; "Ecstasy") has marked immunosuppressive properties. In this study we investigate the effect of MDMA on production of the anti-inflammatory cytokine interleukin (IL)-10 in response to an *in vivo* challenge with bacterial lipopolysaccharide (LPS). Our data demonstrate that both acute and repeated administration of MDMA increases production of LPS-induced IL-10 *in vivo*, and this increase correlates inversely with the ability of MDMA to suppress the pro-inflammatory cytokine tumor necrosis factor (TNF)-α. Despite this correlation, immunoneutralisation of IL-10 does not reverse the suppressive effect of MDMA on LPS-induced TNF-α production, indicating that suppression of this pro-inflammatory cytokine is not mediated by IL-10. *In vitro* exposure to MDMA does not mimic the immunosuppressive cytokine phenotype induced *in vivo*, suggesting that these immunosuppressive effects are not mediated by a direct action on monocytes *per se*. As MDMA activates that hypothalamic pituitary adrenal axis and sympathetic nervous system, we examined the role of glucocorticoids and catecholamines in its immunosuppressive actions. However, the immunosuppressive cytokine phenotype induced by MDMA was not altered by adrenalectomy, by sympathetic denervation or by ganglionic blockade, indicating that neither glucocorticoids nor adrenal/sympathetic-derived catecholamines mediate these immunosuppressive effects of MDMA. Interestingly, β-adrenoceptor blockade completely inhibited the increase in IL-10 induced by MDMA without altering the suppression of TNF-α. Taken together, these data demonstrate a role for β-adrenoceptor activation in the ability of MDMA to increase LPS-induced IL-10, and highlight a mechanistic dissociation between the ability of MDMA to increase IL-10 and suppress production of the pro-inflammatory cytokine TNF-α.

Introduction

Methylenedioxymethamphetamine (MDMA; "Ecstasy") is a widely abused drug, and is associated with a number of acute adverse reactions (Green et al., 2003). Over the last number of years we have demonstrated that MDMA suppresses aspects of innate and adaptive immunity in rats (see Connor, 2004). Moreover, recent studies demonstrate that MDMA elicits immunosuppressive effects in humans that are qualitatively similar to those observed in rats (Pacifici et al., 2001a,b; 2004).

We previously demonstrated that MDMA suppresses production of pro-inflammatory cytokines, particularly tumor necrosis factor-α (TNF-α) following an *in vivo* challenge with a sub-septic dose of bacterial lipopolysaccharide (LPS) (Connor et al., 2000; 2001). This LPS challenge model essentially mimics the initial immune response to bacterial infection, and enables us to examine the impact of pharmacological treatments on the immune response. Without stimulation with LPS, basal concentrations of cytokines are low and often undetectable, thus an immunological challenge is required to stimulate cytokine production. For instance, LPS stimulates production of pro-inflammatory cytokines including TNF-α, from innate immune cells such as monocytes, macrophages and dendritic cells (see Suzuki et al., 2003). TNF-α is an important signalling molecule in initiating and co-ordinating a range of immune responses against invading pathogens (Hamblin, 1994), and consequently an inability to produce TNF-α can result in defective host resistance to infection (Pasparakis et al., 1996).

In addition to producing pro-inflammatory cytokines when stimulated with LPS, innate immune cells also produce interleukin-10 (IL-10), an anti-inflammatory or immunosuppressive cytokine that inhibits several macrophage functions including pro-

inflammatory cytokine production (Bogdan et al., 1992**;** Gerard et al., 1993). Thus we suggest that an increase in IL-10 production in response to LPS has the propensity to mediate the suppression of TNF-α induced by MDMA. Although the ability of MDMA to alter IL-10 production from cells of the innate immune system has not been examined to date, there is evidence that MDMA increases T-cell derived IL-10 production (Pacifici et al., 2001a). In addition, another drug of abuse namely THC increases innate IL-10 production in a sepsis model (Smith et al., 2000). Consequently, in this study we examined the impact of MDMA on LPS-induced IL-10 production, and once we established that MDMA increased IL-10, we examined the possibility that the suppression of TNF-α induced by MDMA was mediated by IL-10.

We previously demonstrated that the suppressive effect of MDMA on LPS-induced pro-inflammatory cytokine production observed *in vivo* could not be mimicked by *in vitro* exposure to the drug (Connor et al., 2000). Similarly in this study we observed that *in vitro* exposure to MDMA did not alter LPS-induced IL-10 production. In all, these data indicate that the ability of MDMA to promote an anti-inflammatory cytokine phenotype is not due to a direct action on immune cells *per se*, and is likely to be due to the release of endogenous immunomodulatory substances. In this regard, MDMA activates the sympathetic nervous system and hypothalamic pituitary adrenal axis (Grob et al., 1996; Nash et al., 1988), and we suggest that the end products of these pathways (catecholamines and glucocorticoids) could mediate MDMA-induced immunosuppression. A number of studies demonstrate that catecholamines increase IL-10 production in response to LPS, and suppress LPS-induced TNF-α production (Elenkov et al., 1995; Siegmund et al., 1998; Zinyama et al., 2001), and that these effects occur due

to β-adrenoceptor activation and increased intracellular cAMP production (Siegmund et al., 1998). For instance, in murine macrophages elevated cAMP suppresses LPS-induced TNF-α production by increasing the production of IL-10 (Arai et al., 1995). Therefore, catecholamines present themselves as likely mediators of MDMA-induced suppression of TNF-α either directly, or via increasing production of IL-10. A vast literature also indicates that glucocorticoids have immunosuppressive properties, including the ability to suppress production of pro-inflammatory cytokines (Zuckerman et al., 1989). Thus we suggest that glucocorticoids could potentially mediate the immunosuppressive actions of MDMA. It is also noteworthy that activation of the sympathetic nervous system has been shown to mediate immunosuppressive effects of other drugs of abuse such as morphine and d-amphetamine (Bencsics et al., 1997; Pezzone et al., 1992), and it has been recently demonstrated that glucocorticoids mediate the immunosuppressive actions of cocaine withdrawal (Avila et al., 2003). Consequently, in this study we examined the role of glucocorticoids and catecholamines in the ability of MDMA to promote an immunosuppressive cytokine phenotype in our model. Whilst previous studies have examined the central drivers of MDMA induced immunosuppression (Connor et al., 2001; Pacifici et al., 2004), to our knowledge this is the first study to investigate peripheral mediators of MDMA-induced immunosuppression.

Materials and methods

Animals

Male Sprague-Dawley rats weighing approximately 250-350g were obtained either from a breeding colony in the Department of Pharmacology, NUI, Galway, or from Harlan UK, and were housed four per cage. Rats were maintained on a 12h light:12h dark cycle (lights on at 08.00 h) 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).

Drugs

MDMA was obtained from NIDA, Research Triangle Park, NC, USA. Chlorisondamine was obtained from Novartis, Basel, Switzerland. Lipopolysaccharide (E.coli: serotype 0111:B4), 6-hydroxydopamine and Nadolol were all obtained from Sigma-Aldrich, Ireland. All drugs were dissolved in 0.89 % NaCl and administered via the intraperitoneal (i.p.) route in an injection volume of 1ml/kg and 0.89 % NaCl was administered alone as a vehicle to control animals.

Normal sheep serum and anti-IL-10 anti-serum was obtained from Dr. Steve Poole, NIBSC, Potters Bar, Hertsfordshire, UK.

Experimental design

Effect of MDMA on production of the anti-inflammatory cytokine IL-10

Timecourse: Rats received either Vehicle or MDMA (5 mg/kg; i.p.) co-administered with LPS (100 μg/kg; i.p.), and were killed 30, 60, 120 or 240 min later by decapitation. Following decapitation, trunk blood was collected from each animal, centrifuged (800 x g

at 4^{o}C for 15 min), and the resultant serum was frozen immediately and stored at -80^{o}C until the IL-10 assay was performed.

We have previously found that this dose and route of administration of LPS produces quantifiable increases in circulating IL-10 and TNF-α concentrations, and that 1hr post LPS is the optimal time-point for simultaneous sampling of these two cytokines (Connor and Kelly, 2002). The dose of LPS used in this study, and in our previous studies (Connor et al., 2000, 2001; Connor and Kelly, 2002) is a sub-septic dose that is approximately 25-fold lower than the LD_{50} (Hawes et al., 1992), and provokes modest stimulation of the immune response in rats characterised by a transient increase in cytokine production.

***Dose-response*:** Vehicle or MDMA (1.25 – 10 mg/kg; i.p.) was co-administered with LPS, and animals killed by decapitation 60 min later. Serum was prepared and stored for IL-10 analysis.

In vitro effect of MDMA: A diluted whole blood method was used for the assessment of cytokine production ex vivo as previously described (Connor and Kelly, 2002). In diluted whole blood the natural cell-cell interactions are preserved, whereas the methods used to isolate peripheral blood mononuclear cells modify the lymphocyte/monocyte ratio and eliminate endogenous immunomodulatory agents. Thus in vivo conditions are better represented using whole blood culture methods. 900 µl aliquots of diluted whole blood obtained from naïve rats were pipetted into wells of a sterile flat-bottomed 48 well plate (Nunc, Denmark). To each well was added either 100 µl of RPMI 1640 culture medium alone (control) or 100 µl of MDMA at a final concentration of either 0.1, 0.5, 1, 2.5, 5 or

10 μg/ml dissolved in RPMI 1640. Following a 1 h pre-incubation period with MDMA, 100 μl of LPS (Sigma Chemical Co., Poole, Dorset, U.K.) at a final concentration of 10 μg/ml was added to each well, and cultures were incubated for a further 24 h at 37°C in a 5% CO_2 atmosphere. At the end of the culture period the supernatants were harvested and stored at -80^{o}C until cytokine assays were performed.

Examination of the role of increased IL-10 production in the suppressive effect of MDMA on TNF-α

Correlation between increased IL-10 and reduced TNF-α: Rats were treated with either an acute (1 dose), or repeated (9 dose) regimen of MDMA (2 mg/kg; i.p). Rats in the control group received 9 injections of 0.89 % saline, rats in the acute MDMA group received 8 injections of 0.89% saline and a final injection of MDMA (2mg/kg; i.p.), whereas rats in the repeated MDMA group received 9 injections of MDMA (2mg/kg; i.p.). Each animal received three injections per day with an interval of 3hrs between each injection, and this treatment regimen continued for three consecutive days. The last dose of vehicle or MDMA was co-administered with LPS (100 μg/kg; i.p.), and animals were killed by decapitation 60 min later. Serum was prepared and stored as described in Study I prior to IL-10 and TNF-α analysis.

Immunoneutralization: In this study rats were pre-treated with either anti-IL-10 anti-sera or normal sheep serum (1ml per rat; s.c.) immediately prior to administration of MDMA (5 mg/kg; i.p.) and LPS (100 μg/kg; i.p.), and blood samples were obtained by cardiac puncture 1hr later under halothane anaesthesia. Serum was prepared and stored for analysis of IL-10 and TNF-α.

The anti-IL-10 anti-serum used in the present study was previously shown to neutralize IL-10 in the rat (Cartmell et al., 2003; Souza et al., 2003).

Examination of the role of glucocorticoids and catecholamines in the ability of MDMA to increase IL-10 and suppress TNF-α

Adrenalectomy: Sham operated and adrenalectomised rats were purchased from Charles River, UK. Following arrival in the laboratory adrenalectomised rats were provided with saline solution (0.5 %) supplemented with corticosterone (25 μg/ml in 0.2% ethanol) in order to restore basal corticosterone concentrations (see Houghtling and Bayer, 2002). Sham operated rats were provided with drinking water containing 0.2 % ethanol. Rats were given 10 days to acclimate to the laboratory prior to experimentation. In this experiment vehicle or MDMA (2 mg/kg; i.p.) was co-administered with LPS, and animals were killed 60 min later by decapitaion. Serum was prepared and stored for analysis of IL-10 and TNF-α analysis.

Adrenalectomy was verified at the end of the experiment by measuring serum corticosterone concentrations using a commercially available OCTEIA corticosterone enzyme immunoassay kit (IDS, UK) and was performed as per manufacturers instructions.

Chemical sympathectomy: 6-hydroxydopamine was dissolved in PBS supplemented with 0.01% Ascorbic acid. On the first day animals received 6-hydroxydopamine at a dose of 40mg/kg. The following 2 days, animals received an 80mg/kg dose of 6-hydroxydopamine, thus over a 3 day period animals received a total of 200mg/kg 6-hydroxydopamine. The regime of 6-hydroxydopamine treatment used in the present study

was previously shown to induce a 90% depletion in splenic noradrenaline concentrations (see Exton et al., 2002). Three days after the last 6-hydroxydopamine treatment, vehicle or MDMA (2 mg/kg; i.p.) were co-administered with LPS, and animals killed 60 min later by decapitation. Serum was prepared and stored for IL-10 and TNF-α analysis.

The degree of sympathectomy was determined by measuring splenic noradrenaline concentrations. Noradrenaline was measured using a HPLC system coupled with electrochemical detection as previously described. A piece of spleen tissue was homogenised in 1 ml of HPLC mobile phase spiked with 20ng/20µl of N-methyl serotonin as internal standard. Mobile phase contained 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.1 M EDTA, 1.4 mM octane-1-sulphonic acid and 10 % (v/v) methanol, and was adjusted to pH 2.8 using 4 N Sodium hydroxide. Homogenates were centrifuged at 15,000 x g for 15 min, and a 15 µl sample of the resultant supernatant was injected onto a reverse phase column (LI Chrosorb RP-18, 25 cm x 4 mm internal diameter, particle size 5 µm) for separation of the neurotransmitters (flow rate 1 ml/minute). Noradrenaline concentrations were quantified by electrochemical detection (Shimadzu), and chromatograms were generated using a Merck-Hitachi D-2000 integrator. Protein concentrations were determined using the Bradford Dye-binding assay, and noradrenaline concentrations were expressed as ng/mg protein.

Ganglionic blockade: In this study rats were pre-treated with either vehicle or chlorisondamine (0.5 or 1 mg/kg; i.p.) 30 min prior to administration of MDMA (2 mg/kg; i.p.) and LPS (100 µg/kg; i.p.), and were killed 60 min later by decapitation. Serum was prepared and stored for IL-10 and TNF-α analysis.

β-adrenoceptor antagonism: In this study rats were pre-treated with either vehicle or nadolol (0.25 mg/kg; i.p.) 30 min prior to administration of MDMA (2 mg/kg; i.p.) and LPS (100 μg/kg; i.p.), and were killed 60 min later by decapitation. Serum was prepared and stored for IL-10 and TNF-α analysis.

Cytokine determinations

TNF-α concentrations were determined using specific rat enzyme-linked immunosorbent assays (ELISA) performed using antibodies and standards obtained from Dr. S. Poole (NIBSC, UK) as previously described (Connor and Kelly, 2002). Similarly IL-10 concentrations were determined by ELISA performed using antibodies and standards (Rat IL-10 cytoset) obtained from Biosource, International as previously described (Connor and Kelly, 2002).

Statistical analysis of data

Data were analysed using either a Student's t-test, or a one-way or two-way analysis of variance (ANOVA) followed by *post hoc* comparisons using Newman-Keuls test. Data were deemed significant when $P < 0.05$., and expressed as group means with standard errors.

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Results

Effect of MDMA on production of the anti-inflammatory cytokine IL-10

In order to determine if MDMA could alter production of the anti-inflammatory cytokine IL-10, we examined the effect of *in vivo* and *in vitro* exposure to MDMA on LPS-induced IL-10 production.

In vivo administration of MDMA: Administration of MDMA (5 mg/kg) to rats significantly increased LPS-induced IL-10 production 30 min - 2 hr following LPS challenge. When the area under the curves were analysed, it was revealed that the overall IL-10 response was increased almost 3-fold by treatment with MDMA (Figure 1a). In the dose response study it was observed that MDMA (1.25-10 mg/kg) produced a significant increase in circulating IL-10 concentrations following the *in vivo* LPS challenge (Figure 1b). A maximal response was induced by MDMA (2.5 mg/kg), with no additional increase observed following administration of MDMA (5-10 mg/kg). We also examined the ability of MDMA to alter circulating IL-10 concentrations under basal conditions (ie) in the absence of an immunological challenge. When MDMA (5 mg/kg) was administered to rats in the absence of LPS, it produced a modest but statistically insignificant ($P = 0.082$; Student's t-test) increase in circulating IL-10 concentrations 1hr later: ***Vehicle:*** 7.5 ± 4.2 pg/ml; ***MDMA:*** 17.4 ± 3.2 pg/ml (n=7).

In vitro administration of MDMA: In contrast to the robust increased in LPS-induced IL-10 production observed *in vivo, in vitro* exposure to MDMA (0.1 – 10 μg/ml) failed to alter LPS-induced IL-10 production in diluted whole blood culture. (Figure 2)

Examination of the role of increased IL-10 production in the suppressive effect of MDMA on TNF-α

In order to determine if the observed increase in IL-10 could account for the ability of MDMA to suppress production of the pro-inflammatory cytokine TNF-α (Connor et al., 2000, 2001), we first determined if there was a correlation between the effect of MDMA on IL-10 and TNF-α production following both acute and repeated treatment. The results indicate that acute and repeated treatment with MDMA produced a significant increase in IL-10 that was accompanied by a suppression of TNF-α production. Moreover, correlational analysis demonstrated a significant ($P < 0.01$) inverse correlation ($R = -0.62$) between LPS-induced IL-10 and TNF-α production (Figure 3).

As a strong inverse correlation was observed, we then determined if immunoneutralization of IL-10 could inhibit the suppressive effect of acute MDMA administration on LPS-induced TNF-α production. The results demonstrate that MDMA induced a significant increase in LPS-induced IL-10 production, and this was abrogated in those animals treated with the anti-IL-10 anti-serum (Figure 4a). However, MDMA induced a significant decrease in TNF-α production in both control and IL-10-depleted animals (Figure 4b). Thus immunoneutralisation of IL-10 fails to block the suppressive effect of MDMA on LPS-induced TNF-α production. The data presented in Figure 4a demonstrate that the ELISA could not detect IL-10 in control animals pre-treated with the anti-IL-10 antiserum. In contrast, in the MDMA challenged rats treated with the anti-IL-10 antiserum, IL-10 was clearly detectable (360 ± 180 pg/ml). These data indicate that the anti-IL-10 anti-sera did not interfere with the IL-10 assay *per se*, as the assay could detect an increase in circulating IL-10 following MDMA administration.

Examination of the role of glucocorticoids and catecholamines in the ability of MDMA to increase IL-10 and suppress TNF-α

Adrenalectomy: In order to test the hypothesis that MDMA promotes an immunosuppressive cytokine phenotype (characterized by increased IL-10 and reduced TNF-α production) via release of glucocorticoids or catecholamines from the adrenal gland, we examined the effect of MDMA on LPS-induced IL-10 and TNF-α production in adrenalectomised rats. The results demonstrate that MDMA-induced a significant increase in serum IL-10 and a significant decrease in TNF-α levels in both sham and adrenalectomized animals (Figure 5a&b). Thus adrenalectomy failed to block the ability of MDMA to promote an immunosuppressive cytokine phenotype.

Adrenalectomy was verified by measuring serum corticosterone concentrations. The results show that adrenalectomy drastically reduced circulating corticosterone concentrations in both vehicle and MDMA-treated rats, in comparison to their sham-operated counterparts. There was also a modest but non-significant elevation in circulating corticosterone concentrations in the MDMA-treated sham group, in comparison to their vehicle-treated counterparts (Figure 5c).

Chemical sympathectomy: The results revealed that MDMA-induced a significant increase in serum IL-10 levels in both control (non-sympathectomized), and 6-hydroxydopamine treated (sympathectomized), animals (Figure 6a). MDMA provoked a characteristic decrease in serum TNF-α concentrations in non-sympathectomized animals. However pre-treatment with 6-OHDA alone also significantly suppressed LPS-induced TNF-α production. Whilst MDMA also suppressed TNF-α in sympathectomized animals, this effect was not significant due to the suppression of TNF-

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α production induced by 6-hydroxydopamine alone (Figure 6b). Therefore chemical sympathectomy using 6-hydroxydopamine fails to block the ability of MDMA to promote an immunosuppressive cytokine phenotype.

Sympathectomy was verified by measuring splenic noradrenaline concentrations. The results demonstrate that 6-hydroxydopamine dramatically reduced splenic noradrenaline concentrations in both vehicle and MDMA-treated rats. There was also a modest but non-significant reduction in splenic noradrenaline concentrations in the Vehicle/MDMA group when compared to the Vehicle/Vehicle group (Figure 6c).

Ganglionic blockade: The results demonstrate that MDMA-induced a significant increase in serum IL-10 levels and this effect was not altered by pre-treatment with either dose of chlorisondamine (Figure 7a). MDMA also provoked a characteristic decrease in serum TNF-α concentrations, which was not altered by pre-treatment with chlorisondamine (0.5mg/kg). Pre-treatment with chlorisondamine (1mg/kg) significantly suppressed LPS-induced TNF-α production in a similar manner to MDMA, and therefore no significant difference was seen between these two treatment groups (Figure 7b). Thus ganglionic blockade with chlorisondamine fails to block the ability of MDMA to promote an immunosuppressive cytokine phenotype.

β-adrenoceptor blockade: Pre-treatment with the peripherally acting β-adrenoceptor antagonist nadolol completely blocked the increase in serum IL-10 levels induced by MDMA (Figure 8a). However, whilst MDMA provoked a characteristic decrease in serum TNF-α concentrations, this was not altered by pre-treatment nadolol (Figure 8b). Thus peripheral β-adrenoceptor blockade abolished the MDMA-induced increase in IL-10 production, without altering the suppression of TNF-α.

Discussion

Effect of MDMA on production of the anti-inflammatory cytokine IL-10: We examined the effect MDMA on production of the endogenous immunosuppressive agent IL-10, an anti-inflammatory cytokine that inhibits several macrophage functions (see Moore et al., 2001). We observed that MDMA at a dose as low as 1.25 mg/kg induced a rapid increase in LPS-induced IL-10 production. A limited dose response relationship was observed, as the MDMA-induced increase in IL-10 was maximal at 2.5 mg/kg. Reports indicate that a wide range of doses of MDMA (from 1 to 30 mg/kg) are ingested by humans (see Connor et al., 2000), indicating that the dose-range of MDMA used in the present series of experiments is of relevance to human consumption. In contrast to the ability of MDMA to stimulate LPS-induced IL-10 production in vivo, we clearly demonstrate that MDMA fails to increase IL-10 following in vitro exposure, indicating that the increase in IL-10 induced by MDMA in vivo is not due to a direct action on monocytes *per se*. We are satisfied that the concentration range of MDMA examined *in vitro* is reflective of *in vivo* conditions, as it was based on the results of a previous study where we measured plasma MDMA concentrations following *in vivo* administration in rats (Connor et al., 2000). Specifically, in current study we employed *in vitro* concentrations that were both below, and well in excess, of peak plasma MDMA concentrations observed following *in vivo* administration of MDMA (5-20 mg/kg).

The capacity of MDMA to rapidly increase production of an endogenous immunosuppressive factor such as IL-10 could serve to elicit a broad spectrum of immunosuppressive actions. For instance IL-10 has a multitude of immunoregulatory effects such as the ability to suppress pro-inflammatory cytokine production, and down-regulate antigen presenting and the co-stimulatory molecules on antigen presenting cells

(Ding et al., 1993; Gerard et al., 1993). Therefore it is possible that the large increase in IL-10 induced by MDMA *in vivo* will have a negative impact on the antigen presenting and/or co-stimulatory capacity of macrophages or dendritic cells, and a consequential downstream effect on T-cell mediated immunity (see DeWaal Malefyt et al., 1991).

Examination of the role of IL-10 in the suppressive effect of MDMA on production of the pro-inflammatory cytokine TNF-α: In parallel to the MDMA-induced increase in IL-10 production, a concomitant suppression of the pro-inflammatory cytokine TNF-α was observed. This is consistent with previous studies in rats (Connor et al., 2000; 2001), and also with studies in humans (Pacifici et al., 2001a; 2004), and demonstrates that acute treatment with MDMA promotes an overall immunosuppressive cytokine phenotype. A strong inverse correlation was observed between the MDMA–induced increase in IL-10 and suppression of TNF-α, suggesting that the ability of MDMA to suppress TNF-α production may be mediated by IL-10. Despite this correlation, immunoneutralisation of IL-10 did not reverse the suppressive effect of MDMA on TNF-α production. Therefore whilst IL-10 has a well-established inhibitory action on TNF-α production (Bogdan et al., 1992; Gerard et al., 1993), it is not the key factor in mediating the suppression of this pro-inflammatory cytokine in response to MDMA. Similarly, other pharmacological agents have recently been shown to suppress pro-inflammatory cytokine production independently of a concomitant increase in IL-10 (Connor and Kelly, 2002; Shames et al., 2001).

As recreational drug users often repeatedly ingest MDMA over the course of a weekend, and as repeated administration can result in sensitization to its immunosuppressive effects (Pacifici et al., 2001b), we assessed ability of both acute and repeated MDMA

administration to promote an immunosuppressive cytokine phenotype. The results demonstrate that acute and repeated administration of MDMA had equivalent effects on IL-10 and TNF-α production, indicating that neither tolerance nor sensitization developed to its immunosuppressive actions. These data are at variance with a recent study in humans reporting that repeated administration of MDMA increased the severity of MDMA-induced decrements in circulating $CD4^+$ cell numbers and T-cell proliferation (Pacifici et al., 2001b), and may indicate that a differential response to acute vs. repeated treatment with MDMA could be dependent on the immune parameter(s) being studied, or the species under investigation.

Examination of the role of glucocorticoids and catecholamines in the ability of MDMA to increase IL-10 and suppress TNF-α: Based on the in vitro data presented in this study (Figure 2) and in our previous study (Connor et al., 2000), we conclude that the ability of MDMA to increase IL-10 and suppresses TNF-α following *in vivo* administration is not due to a direct action on monocytes *per se*. In addition to having a direct effect on immune cells, MDMA has the propensity to elicit its immunosuppressive actions by releasing endogenous immunoregulatory substances. For instance**,** MDMA activates both the sympathetic nervous system and hypothalamic pituitary adrenal axis (Connor et al., 1998; Grob et al., 1996; Nash et al., 1988). In contrast to their *in vitro* counterpart, *in vivo* immunopharmacological studies take into account the contribution of circulating endogenous immunomodulatory substances such as glucocorticoids and catecholamines; the end products of the hypothalamic pituitary adrenal-axis and sympathetic nervous system respectively**.** As it is well established that glucocorticoids and catecholamines have the ability to promote an immunosuppressive cytokine phenotype (Elenkov et al., 1995; Siegmund et al., 1998; Zuckerman et al., 1989), we tested the hypothesis that

increased circulating glucocorticoid or catecholamine concentrations mediated the immunosuppressive cytokine phenotype induced by MDMA. To this end we examined the effect of MDMA on LPS-induced IL-10 and TNF-α production in adrenalectomised rats. However, adrenalecotomy failed to attenuate the MDMA-induced suppression of TNF-α and increase in IL-10, indicating that neither glucocorticoids nor adrenal-derived catecholamines mediated these responses. In addition to the ability of circulating catecholamines to alter immune cell function, sympathetic innervation and local release of noradrenaline within immune organs such as the spleen is known to have immunomodulatory properties (see Elenkov et al., 2000). Therefore, we examined the ability of sympathetic denervation induced by peripheral administration of the noradrenergic neurotoxin 6-hydroxydopamine to block the immunosuppressive cytokine phenotype induced by MDMA. In this regard, we hypothesised that following an *in vivo* LPS challenge, that splenic cytokine outflow would be a significant contributor to circulating cytokine concentrations. However, although 6-hydroxydopamine caused an 83% depletion of splenic noradrenaline, it failed to block MDMA induced-increase in IL-10 and suppression of TNF-α, indicating that its ability to alter production of these cytokines is independent of sympathetic innervation. This assertion is reinforced by the fact that pre-treatment with the ganglionic blocker chlorisondamine failed to block the immunosuppressive cytokine phenotype induced by MDMA. The dose range of chlorisondamine used in the present study blocks sympathetic outflow, and has previously been found to block the immunomodulatory actions of morphine (Houghtling and Bayer, 2002). Interestingly, animals treated with 6-hydroxydopamine alone, or with chlorisondamine (1mg/kg) also, had a reduced capacity to produce TNF-α, indicating that a basal level of noradrenaline is required to mount a TNF-α response to LPS.

Whilst the results of the adrenalectomy, sympathectomy, and ganglionic blockade studies argue against a role for catecholamines in the immunosuppressive cytokine phenotype induced by MDMA, we observed that pre-treatment with nadolol, a β-adrenoceptor antagonist that does not cross the blood brain barrier, completely blocked the increase in IL-10 induced by MDMA, yet failed to alter its suppressive actions on TNF-α. Moreover, the ability of nadolol to block the MDMA-induced increase in IL-10 production is shared by the related β-adrenoceptor antagonist propranolol (Unpublished data). The fact that β-adrenoceptor blockade inhibits the MDMA-induced increase in IL-10 production without altering its suppressive effects on TNF-α production reinforces the immunoneutralization studies, further highlighting a mechanistic dissociation between the ability of MDMA to increase IL-10 and suppress TNF-α production. These data also point towards an alternative source of catecholamines in mediating this response. One possibility is that peripheral dopamine may play a role, for instance a recent study demonstrated that dopamine increased IL-10 and suppressed IL-12 production via β-adrenoceptor activation (Hasko et al., 2002). This is not an entirely surprising finding, as it had been previously demonstrated that dopamine has both affinity for, and efficacy at, β-adrenoceptors (Ruffolo et al., 1984). In addition, a recent report indicates that plasma dopamine levels are elevated in a cohort of human MDMA users (Stuerenburg et al., 2002). Another possibility is that metabolism of MDMA could yield agents that have activity at β-adrenoceptors. For instance, a major metabolite of MDMA is α-methyldopamine (see Monks et al., 2004), and evidence suggests that this compound can be transformed into a β-adrenergic effector following metabolism by monoamine oxidase (Langeneckert & Palm, 1968). Nevertheless, whilst the precise identity and source of the effector that mediates the MDMA-induced enhancement of IL-10 production is not known, these data clearly demonstrate that it is a β-adrenoceptor mediated event.

Conclusion: MDMA increases LPS-induced IL-10 production via β-adrenoceptor activation. However, neither MDMA-induced IL-10 production, nor MDMA-induced glucocorticoid or catecholamine secretion mediates the suppressive effect of MDMA on production of the pro-inflammatory cytokine TNF-α. Further studies are needed in order to elucidate the exact mechanisms that underlie MDMA-induced suppression of TNF-α, and the functional consequences of MDMA-induced increases in IL-10.

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Requests for reprints: Requests for reprints should be addressed to Dr. Thomas Connor, Department of Physiology, Trinity College, Dublin 2, Ireland.
E-mail: connort@tcd.ie

*** Present address:** Department of Pharmacology & Therapeutics, School of Pharmacy, University College Cork, Ireland.

Figure legends

Figure 1: MDMA stimulates a time-related and dose-dependent increase in IL-10 production in response to an *in vivo* LPS challenge.

(a) MDMA (5 mg/kg) was co-administered with LPS (100 μg/kg), and animals killed 30, 60, 120 and 240 min following LPS challenge for the measurement of IL-10. ANOVA demonstrated a significant MDMA x time interaction [$F(4,65) = 27.81$, $P < 0.0001$].

(b) MDMA (1.25 – 10 mg/kg) was co-administered with LPS, and animals killed 60 min following LPS challenge for the measurement of IL-10. ANOVA demonstrated a significant effect of MDMA treatment [$F(4,34) = 22.10$, $P < 0.0001$]. Data expressed as means ± SEM (n = 6-8). * $P < 0.05$, ** $P < 0.01$ vs. Vehicle (Newman-Keuls test).

Figure 2: *In vitro* exposure of diluted whole blood to MDMA fails to alter LPS-induced IL-10 production.

Diluted whole blood cultures were pre-incubated with MDMA (1-10 μg/ml) for 1 h prior to stimulation with LPS (10μg/ml), and cell culture supernatants were harvested 24hr later for the measurement of IL-10. Data expressed as means ± SEM (n = 7-8).

Figure 3: The increase in IL-10 induced by MDMA is observed following both acute and repeated treatment, and is correlated with a suppression of TNF-α production.

Rats were treated with either an acute (1 dose), or repeated (9 doses) regimen of MDMA (2 mg/kg/dose). The last dose of MDMA was co-administered with LPS (100 μg/kg) and animals were killed 60 min following LPS challenge. Concentrations of IL-10 (a) and TNF-α (b) were determined in serum, and a correlational analysis was performed (c). ANOVA demonstrated a significant effect of MDMA on LPS-induced IL-10 [$F(2,15) = 24.51$, $P < 0.001$] and TNF-α [$F(2,15) = 7.26$, $P < 0.01$] production. Data expressed as means ± SEM ($n = 6$). ** $P < 0.01$ vs. Vehicle (Newman-Keuls test).

Figure 4: Immunoneutralization of IL-10 fails to block the suppressive effect of MDMA on LPS-induced TNF-α production.

Rats were treated with either normal sheep serum or anti-IL-10 anti-serum immediately before MDMA (5 mg/kg) and LPS (100 μg/kg). Blood was collected 60 min following LPS challenge and concentrations of IL-10 (a) and TNF-α (b) were determined in the serum. ANOVA demonstrated a significant effect of MDMA [$F(1,16) = 14.36$, $P < 0.01$], and a significant effect of the anti-IL-10 antiserum [$F(1,16) = 11.12$, $P < 0.01$] on IL-10 production. There was also a significant effect of MDMA treatment on LPS-induced TNF-α production [$F(1,16) = 49.15$, $P < 0.001$]. Data expressed as means ± SEM ($n = 5$). * $P < 0.05$ vs. Saline-treated counterparts (Newman-Keuls test).

Figure 5: Adrenalectomy fails to block the ability of MDMA to increase IL-10 and suppress TNF-α production.

Either sham or adrenalectomized rats were treated with MDMA (2 mg/kg) co-administered with LPS (100 μg/kg), and were killed 60 min following LPS challenge. Concentrations of IL-10 (a) and TNF-α (b) were determined in the serum. Adrenalectomy was verified by measuring plasma corticosterone (c). ANOVA demonstrated a significant effect of MDMA on serum IL-10 [$F(1,25) = 97.61$, $P < 0.0001$], and on serum TNF-α [$F(1,25) = 14.55$, $P < 0.001$] concentrations, and a significant effect of adrenalectomy on circulating corticosterone concentrations [$F(1,25) = 99.48$, $P < 0.0001$]. Data expressed as means ± SEM (n = 6-8). ** $P < 0.01$ vs. Vehicle, ++ $P < 0.01$ vs. Sham-operated counterparts (Newman-Keuls test).

Figure 6: Chemical sympathectomy fails to block the ability of MDMA to increase IL-10 and suppress TNF-α production.

Either vehicle or 6-hydroxydopamine treated rats were treated with MDMA (2 mg/kg) co-administered with LPS (100 μg/kg), and were killed 60 min following LPS challenge. Concentrations of IL-10 (a) and TNF-α (b) were determined in the serum, and sympathectomy was verified by measuring splenic noradrenaline concentrations (c). ANOVA a significant effect of MDMA on serum IL-10 concentrations [$F(1,20) = 19.36$, $P < 0.001$], and a significant effect of MDMA [$F(1,20) = 10.16$, $P < 0.01$] and 6-OHDA [$F(1,20) = 9.46$, $P < 0.01$] on serum TNF-α concentrations. There was also significant effect of 6-hydroxydopamine treatment on splenic noradrenaline concentrations [$F(1,20) = 15.73$, $P < 0.001$]. Data expressed as means ± SEM (n = 5-7). ** $P < 0.01$ vs. Saline-challenged counterparts, + $P < 0.05$ vs. Vehicle + Saline, ++ $P < 0.01$ vs. non-sympathectomised counterparts (Newman-Keuls test).

Figure 7: Ganglionic blockade fails to block the ability of MDMA to increase IL-10 and suppress TNF-α production.

Vehicle or chlorisondamine treated rats were treated with MDMA (2 mg/kg) co-administered with LPS (100 μg/kg), and were killed 60 min following LPS challenge. Concentrations of IL-10 (a) and TNF-α (b) were determined in serum. ANOVA demonstrated a significant effect of MDMA on serum IL-10 concentration [$F(1,24) = 24.34$, $P < 0.0001$], and a significant chlorisondamine x MDMA interaction on serum TNF-α concentrations [$F(2,24) = 5.84$, $P < 0.01$]. Data expressed as means ± SEM (n = 5). ** $P < 0.01$ vs. Vehicle-treated counterparts (Newman-Keuls test).

Figure 8: β-adrenoceptor antagonism blocks the ability of MDMA to increase IL-10, but fails to block the suppressive effect of MDMA on TNF-α production.

Rats were pre-treated with either saline or Nadolol (0.25 mg/kg) 30 min prior to administration of MDMA (2 mg/kg) and LPS (100 μg/kg), and were killed 60 min following LPS challenge. Concentrations of IL-10 (a), TNF-α (b) were determined in the serum. ANOVA demonstrated a significant nadolol x MDMA interaction on serum IL-10 concentrations [$F(1,24) = 36.28$, $P < 0.0001$], and a significant effect of MDMA on serum TNF-α concentrations [$F(1,24) = 27.37$, $P < 0.0001$]. Data expressed as means ± SEM (n = 6-8). ** $P < 0.01$ vs. Vehicle, ++ $P < 0.01$ vs. Saline + MDMA (Newman-Keuls test).

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Figure 1

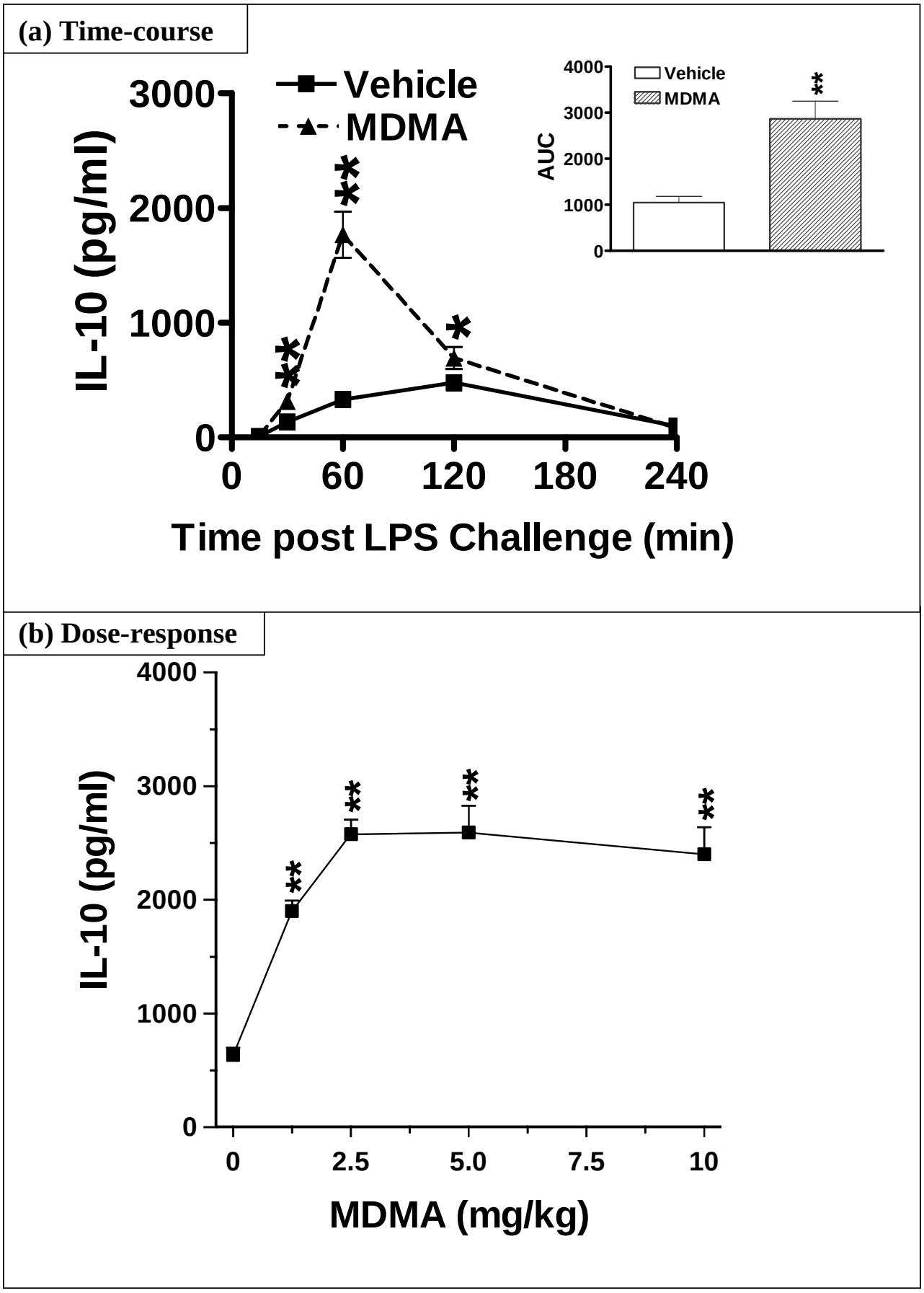

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Figure 2

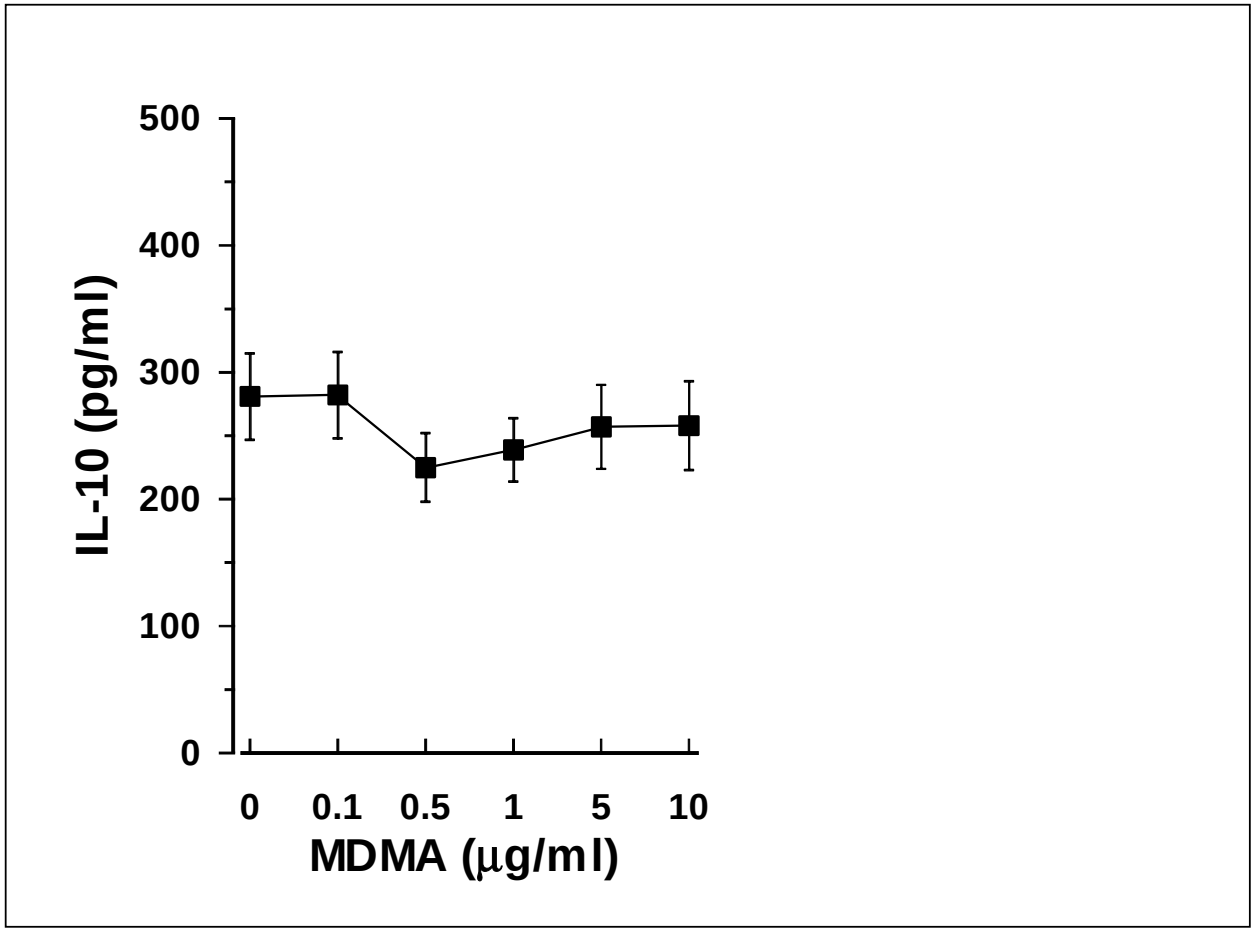

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Figure 3

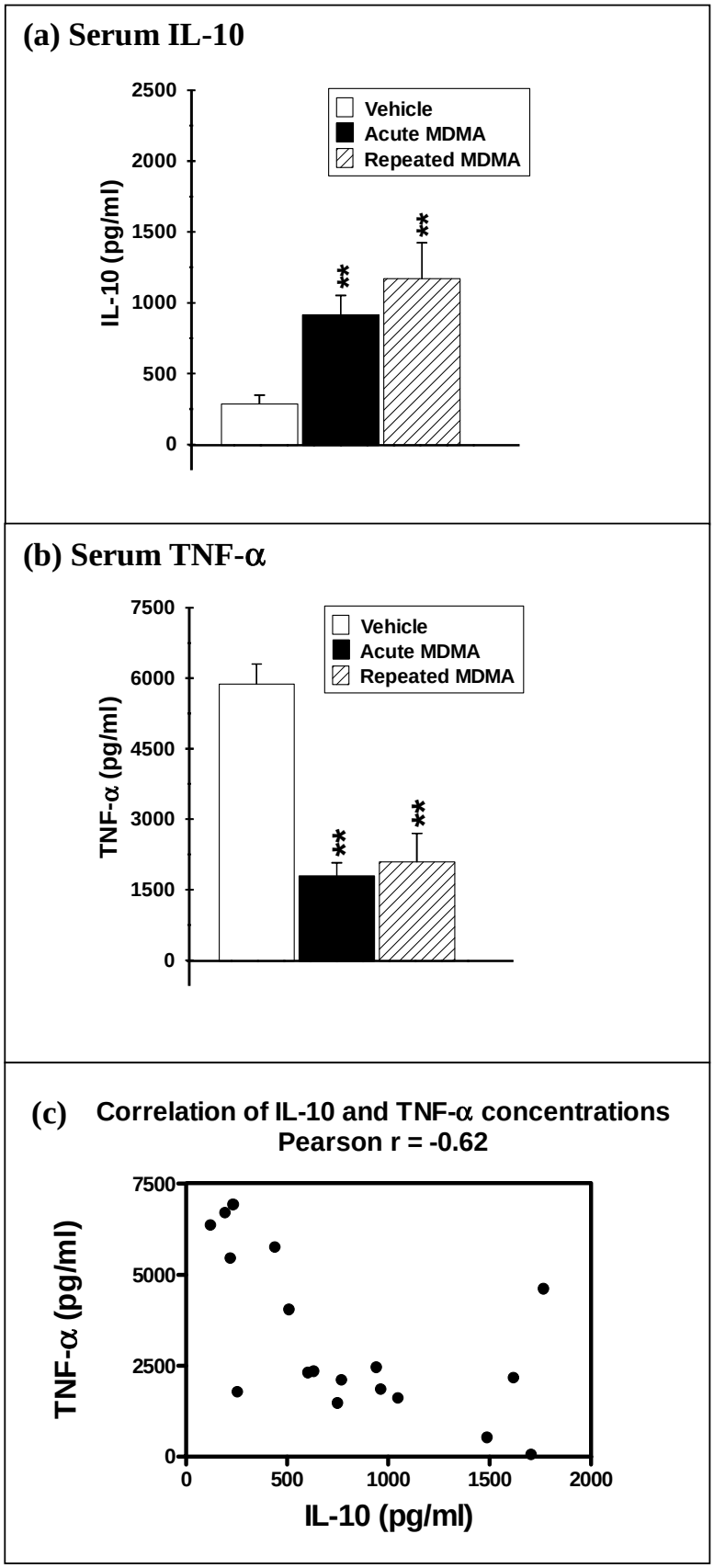

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Figure 4

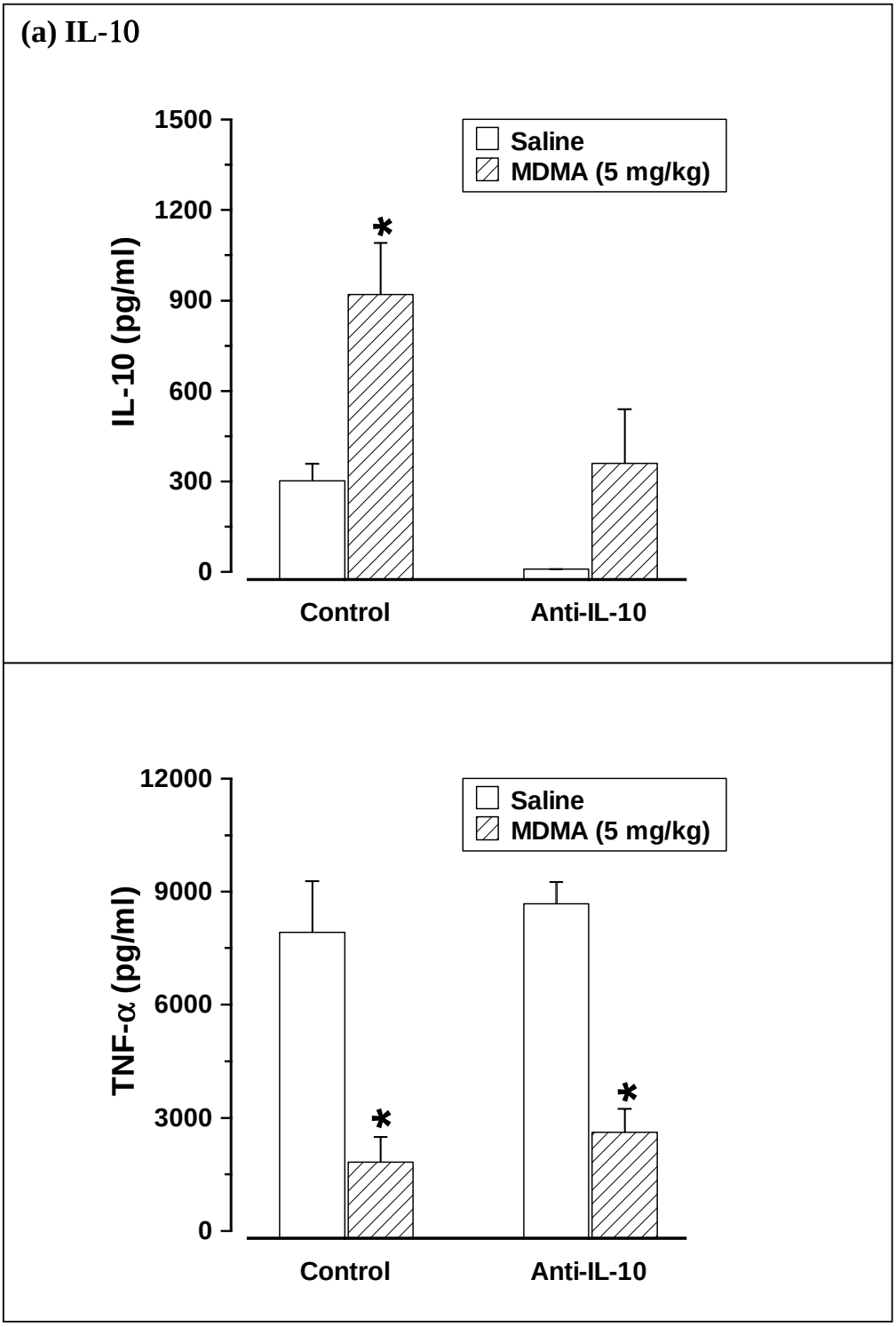

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Figure 5

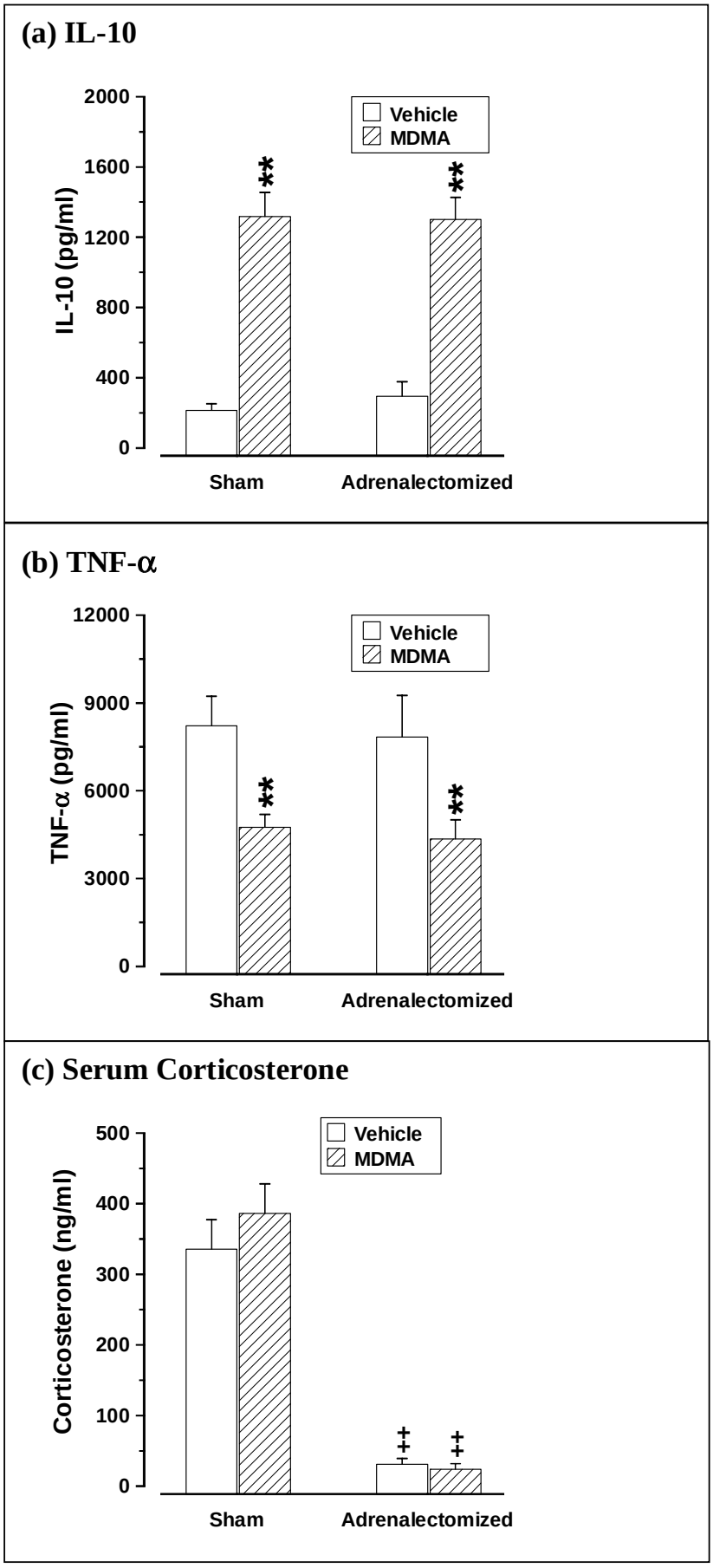

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Figure 6

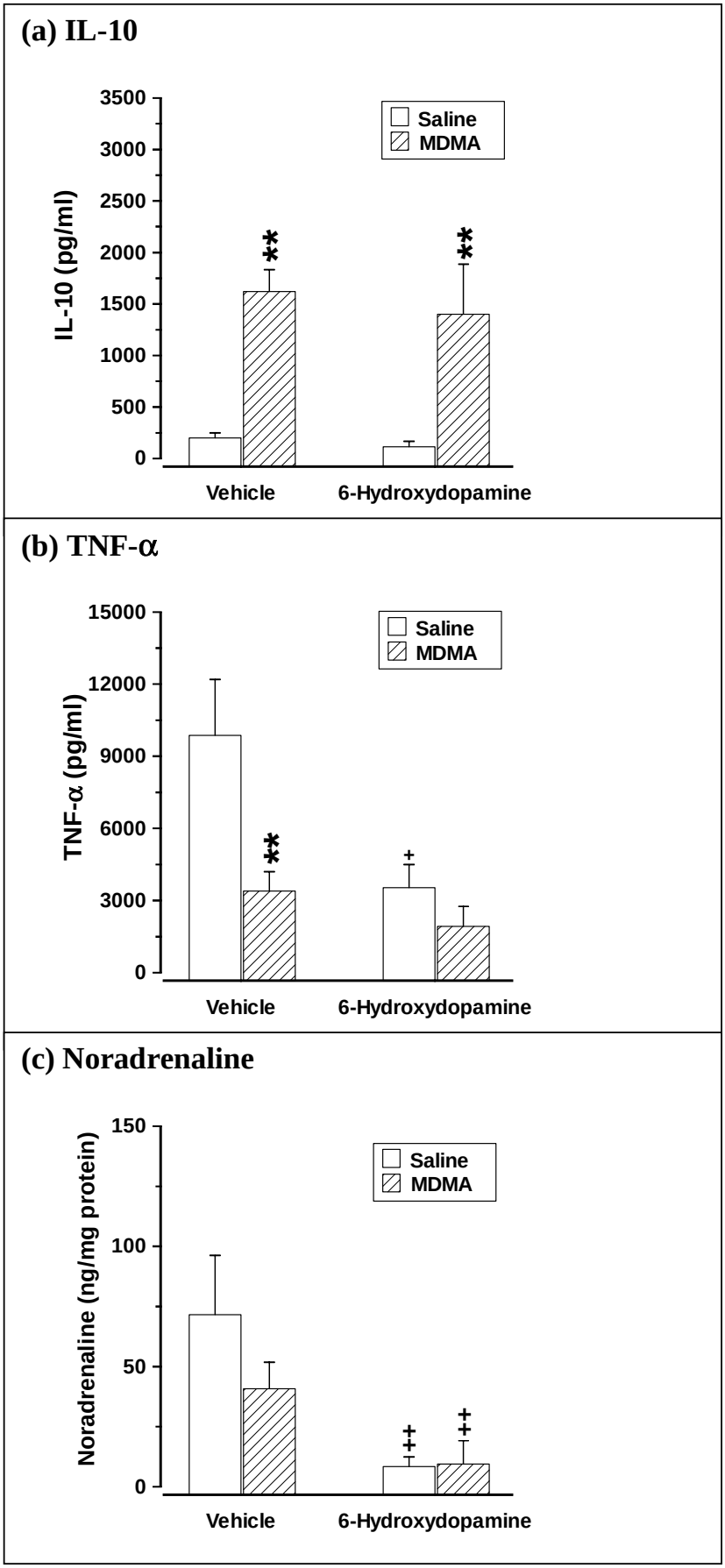

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Figure 7

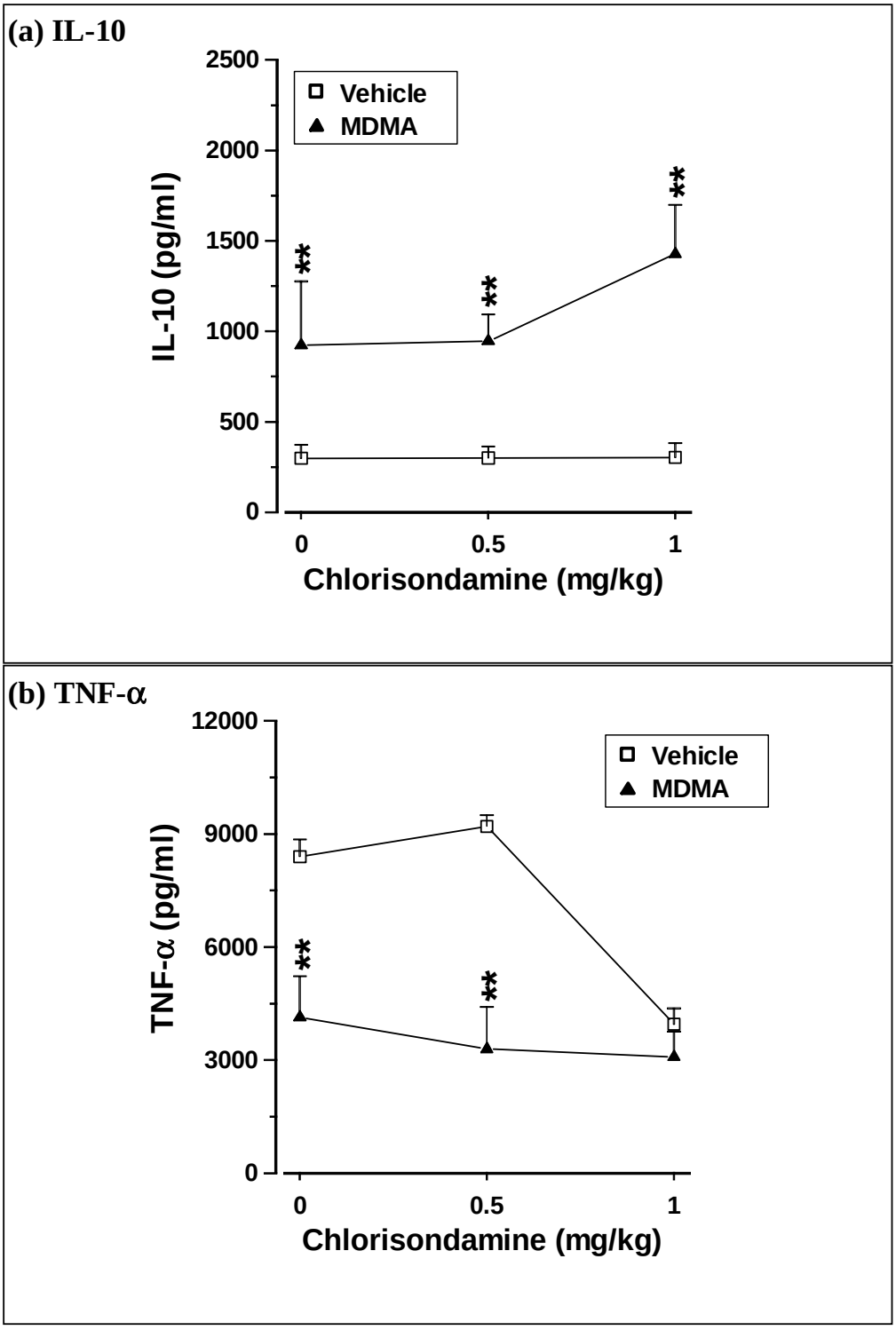

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Figure 8