Cocaine Dependence and Acute Cocaine Induce Decreases of Monocyte Proinflammatory Cytokine Expression across the Diurnal Period: Autonomic Mechanisms

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Abstract

Cocaine dependence is associated with an increased risk of infectious diseases. The innate immune system triggers effector pathways to combat microbial pathogens through expression of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). It is not known whether cocaine alters the capacity of monocytes to respond to a bacterial challenge in humans. In cocaine dependent volunteers and controls, this study analyzed monocyte TNF-α and IL-6 expression at rest and in response to the bacterial ligand, lipopolysaccharide (LPS), over a 24-hour period. In addition, the *in vivo* effects of cocaine (40 mg) vs. placebo on monocyte expression of TNF-α and IL-6 were profiled over 48 hours. Cocaine dependent volunteers showed a decrease in the capacity of monocytes to express TNF-α and IL-6 as compared to controls. Moreover, acute infusion of cocaine induced a further decline in the responsiveness of monocytes to LPS, which persisted after cocaine had cleared from the blood. Heart rate variability analyses showed that increases of sympathetic activity along with vagal withdrawal were associated with decreases in monocyte expression of TNF-α. Cocaine alters autonomic activity and induces protracted decreases in innate immune mechanisms. Targeting sympathovagal balance might represent a novel strategy for partial amelioration of impairments of innate immunity in cocaine dependence.

Introduction

Cocaine is the one of most frequently abused drugs in the United States (Mendelson and Mello, 1996), and its use is a significant risk factor for infectious diseases including human immunodeficiency virus (HIV-1), hepatitis C (Thorpe et al., 2000), and bacterial infections (Masi, 1978; Silverman and Smith, 1985; Chaisson et al., 1989; D'Ellia et al., 1991; Caiaffa et al., 1994; Friedman et al., 2003; Dettmeyer et al., 2004; Sierra et al., 2006). Even among cocaine dependent persons who are not engaging in intravenous drug use, cocaine use is associated with a three-fold increase in the incidence of HIV infection (Chiasson et al., 1991) and a three-fold risk for hepatitis C seropositivity (Thorpe et al., 2000). Cocaine is thought to serve as a cofactor for infections as well as HIV-1 progression (Friedman et al., 2003), although the mechanisms mediating cocaine's effects on host resistance to infectious agents are poorly understood. The effects of cocaine dependence and acute cocaine use on the human immune system remain largely unknown.

Endotoxin or lipopolysaccharide (LPS), the main component of the cell wall of gram negative bacteria, initiates Toll-like receptor 4 (*TLR4*) signaling and induces monocyte and macrophage expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)(Miller et al., 2005). Both of these proinflammatory cytokines are important signaling molecules that regulate a wide range of immune responses against invading pathogens (Miller et al., 2005). A decrease in the ability to produce TNF-α and IL-6 in response to bacterial ligands such as LPS serves as a marker of defective host resistance to infection (Pasparakis et al., 1996), which correlates with susceptibility to bacterial pathogens (Miller et al., 2005). It is not known whether cocaine dependence or acute cocaine administration diminishes a functional cellular innate immune responses and attenuates monocyte expression of inflammatory cytokines. Animal data suggest

that *in vitro* doses of cocaine inhibit LPS stimulated production of IL-6 and TNF-α in culture supernatants (Friedman et al., 2003). In an *in vivo* model of inflammatory challenge, an acute dose of cocaine was found to attenuate the release of IL-6 in humans (Halpern et al., 2003).

In this study, we analyzed monocyte proinflammatory cytokine expression at rest and in response to LPS in cocaine dependent men as compared to controls. In addition, the effect of acute cocaine vs. placebo on monocyte expression of TNF-α and IL-6 was tested. Given evidence that proinflammatory cytokines show a marked diurnal variation (Redwine et al., 2000), differences between cocaine dependent men vs. controls were examined over a 24 hour period, whereas the action of acute cocaine was profiled over 48 hours. Finally, we evaluated a potential mechanism for decreases of resting and stimulated monocyte expression of TNF-α and IL-6 in cocaine dependence by assessing variations in sympathetic and parasympathetic nervous system activity as estimated by heart rate variability (1996). Cocaine is reported to induce increases in sympathetic effector mechanisms (Jacobsen et al., 1997), and sympathetic neurotransmitters are known to inhibit proinflammatory cytokine secretion (Friedman and Irwin, 1997; Straub et al., 2000; Meltzer et al., 2004).

Methods

Subjects

The UCLA Institutional Review Board (IRB) and the Research Advisory Panel of California approved all procedures. Non-treatment seeking cocaine dependent volunteers and neighborhood controls were recruited by advertisements jointly placed throughout the greater metropolitan Los Angeles community. Following the guidelines of the UCLA IRB, study purpose (i.e., “to study the effects of cocaine use on health”) was stated in advertisements for

both “cocaine using adults” and for “healthy adults.” Compensation was prorated at $100 per night.

For cocaine dependent persons, screening eligibility criteria were a positive urine screening test for cocaine; a negative urine screening test for other substances; self-reported use of cocaine for 6 months (more than 1 g of cocaine per week); and self report of being unable to sustain abstinence from cocaine for any significant period of time. Research psychiatric diagnoses of cocaine dependent volunteers and controls were made after the administration of a semi-structured interview developed by the multi-site Collaborative Study on the Genetics of Alcoholism (Bucholz et al., 1994), which also provided interview data on alcohol- and other substances including tobacco consumption histories. Data on the presence of other psychiatric disorders (e.g., major depressive disorder) was also ascertained. Trained masters-level clinical psychologists completed all interviews and presented data in a diagnostic consensus meeting that included a board-certified psychologist (SJM) and a board-certified psychiatrist (MRI).

Cocaine dependent volunteers fulfilled Diagnostic and Statistical Manual –IV (*DSM-IV)* criteria for current, primary cocaine dependence. Cocaine dependent volunteers who were co-morbid for current alcohol dependence, current other substance dependence (e.g., opiate dependence) and/or other current Axis I psychiatric disorder were not eligible (American Psychiatric Association, 2000). Controls fulfilled DSM-IV criteria for Never Mentally Ill (American Psychiatric Association, 2000). In addition, all participants underwent additional laboratory blood tests including urine toxicology, as well as a medical history and physical examination, which were performed by a physician (TF).

A total of 55 cocaine dependent men fulfilled screening eligibility criteria, gave informed consent, and entered the research protocol; 24 of these cocaine dependent men were excluded

during the psychiatric diagnostic and medical evaluations because of one or more of the following conditions: co-morbid alcohol or other substance dependence and/or positive urine toxicology for a substance other than cocaine (e.g., opiates); presence of current major depressive disorder or other Axis I psychiatric disorder; presence of a history of cardiovascular disease, inflammatory disorder, or immune compromise secondary to neoplasm or diabetes; or evidence of abnormal screening laboratory examination (e.g., positive HIV or hepatitis C screening) or electrocardiogram. In addition, 6 cocaine dependent men failed to return after the initial evaluation, and 6 subjects withdrew prior to completion of the protocol; 5 of these subjects withdrew after a single night in the laboratory, and one subject withdrew after the first infusion session in which he had received cocaine. Hence, the cocaine dependent group was comprised of 19 men who were healthy and not receiving any prescription or non-prescription medications. A total of 19 neighborhood male controls were identified, matched to the cocaine dependent men on the basis of age $\pm$ 5 years and body mass index.

Procedures

Cocaine dependent volunteers and controls were studied concurrently across the calendar year. Cocaine dependent volunteers and controls were admitted to the UCLA General Clinical Research Center (GCRC) at least 48 hours prior to the onset of the experimental protocol. Both groups followed an identical rest/activity schedule from admission to the end of protocol with lights out at 23:00 and good morning time at 7:00. Beginning on day 3 after admission to the GCRC, serial blood samples were obtained via an intravenous catheter over a 24 hours period. Sampling frequency was every 4 hours from 08:00 h to 20:00 h and every 3 hours from 20:00 h to 08:00 h; there were a total of 7 separate assessments for each cocaine dependent volunteer and control. In addition, subjects were restricted to bed from 22:00 h until 07:00. To be reported

separately, subjects were instrumented for polysomonography recordings during each of the nights in the laboratory. Subjects were not allowed to smoke for the hour preceding daytime blood sampling nor during the nocturnal period.

For evaluation of cocaine effects on monocyte expression of TNF-α and IL-6, cocaine dependent volunteers entered the second phase of the protocol and underwent cocaine vs. placebo infusion, with blood sampling over an additional 96 hours. Given IRB guidelines for the administration of cocaine, control subjects were not included. Human use cocaine HCl was provided by a NIDA contractor. Beginning on day 4 after admission to the GCRC, cocaine (40 mg) or placebo was infused over 60 seconds by an indwelling catheter at 17:00 h in a randomized, double blind, counter-balanced, cross-over design. In balanced 48 hours periods, a randomized block of cocaine dependent volunteers received cocaine first followed by placebo, whereas the other block received placebo first followed by cocaine. Intravenous infusion occurred over 60 seconds by a study physician using a syringe pump with safety features to detect occlusion and/or to prevent unwanted bolus or subcutaneous dosing. Subjects were restricted to bed and a supine horizontal position during the infusion from 16:30 h to 18:00 h and from 22:00 h until 07:00 h during the assessment period. Over two consecutive 48 hours periods, there were a total of 16 blood samples for each cocaine dependent volunteer.

Monocyte intracellular production of TNF-α and IL-6 at rest and in response to LPS stimulation of whole-blood leukoctyes was assessed by flow cytometry using peridinin chlorophyll protein (PerCP)-labeled CD14 mAb, allophycocyanin (APC) labeled anti-TNF-α mAb, and phycoerythrin (PE)-labeled anti-IL-6 mAb as previously described (Prussin and Metcalfe, 1995; Collado-Hidalgo et al., 2006). In brief, heparin-treated blood (1ml) was mixed with or without 100 pg/ml of LPS (Sigma, St Louis, MO) and incubated with 10μg/ml brefeldin

A (Sigma, St Louis, MO) for 4 hrs at 37^{o}C in a platform mixer. Red blood cells were then lysed in FACS lysing solution (BD Biosciences, San Jose, CA), the remaining cells were permeabilized in FACS permeabilizing buffer (BD Biosciences, San Jose, CA) and fluorescence-conjugated antibodies were added for 30 min at room temperature in the dark. Cells were then washed and resuspended in 1% wash buffer for flow cytometry. Three-color flow cytometric analysis was performed on a FACSCaliber (BD) flow cytometer using CELLQuest Pro software. Forward and side scatter were used to gate on the target population (i.e., monocytes). For the monocyte population, the percentage of cytokine-secreting (PE positive and APC positive) cells among CD14-PerCP positive population was determined by counting approximately 12,000 CD14+ cells. Resting levels of monocyte expression of proinflammatory cytokines were determined from unstimulated samples that were incubated in the absence of LPS. Net stimulated cytokine positive events were obtained by subtracting unstimulated percentages from stimulated percentages within constant numbers of monocytes. Results for cytokine positive monocytes in the unstimulated and LPS stimulated conditions were expressed as percentages of CD14+ cells.

Monocytes are the main source of circulating levels of proinflammatory cytokines. To evaluate whether cocaine-induced changes in monocyte expression of proinflammatory cytokines had downstream effects on *in vivo* proinflammatory cytokine activity, circulating levels of soluble TNF-α receptor (TNFrII) were also measured. Levels of TNFrII reflect the action of TNF-α, are highly correlated with plasma levels of TNF and IL-6, and are indicative of systemic immune activation. In addition, TNFrII is more stable that TNF-α and present at higher concentrations; soluble cytokine receptors such as TNFrII are more readily detected in the plasma as compared to cytokines and this latter concern is of methodological importance given the hypothesized effects of cocaine. TNFrII was assessed using commercially available ELISA

(R&D Systems, Minneapolis, MN) following manufacturer specifications. Analyte capture was carried out in 200 μL of plasma incubated 2 hours at room temperature, after which plates were washed and incubated with conjugate antibody 2 hours at room temperature. The plates were then washed and incubated with substrate (20 min in the dark), and then with stop solution at room temperature. Optical density was read on a Multiskan MCC/340 ELISA plate reader (Fisher Scientific, Pittsburgh, PA) at 450 nm wavelength. Optical density values were converted to pg/ml using a standard curve calculation based on recombinant cytokine receptor standards provided by the manufacturer.

Assessment of heart rate variability involved spectral analytic techniques to identify a high frequency power component (HF; 0.15 – 0.4 Hz) that is mediated by parasympathetic activity, and a low frequency power component (LF; 0.04 – 0.15 Hz) that is related to a combination of sympathetic and parasympathetic effects (1996). The ratio of LF/HF refers to the degree of sympathovagal balance, with increases reflecting relative increases sympathetic nervous system activity (1996). As previously described (Irwin et al., 2006), the subject's electrocardiogram (ECG) signal was sampled at a frequency rate of 200 Hz and an interpolation algorithm was used to optimize temporal accuracy of R wave peak detection. The signal was then converted into an RR interval signal, and spectral analyzed utilizing a 12-point autoregressive algorithm (Somnologica, Flaga hf, Medical Devices, Iceland) in accordance with recommended guidelines to generate estimates of high- and low frequency power (1996). Heart rate variability assessment was obtained over 30 minutes during the awake period, prior to sleep onset, with subjects' eyes closed at 22:30; subjects were directed to "rest quietly" and lie supine in bed. The ECG signal was analyzed using two 5-minute segments.

Statistical Analyses

Data were analyzed using SPSS version 12.0 for Windows. Cocaine dependent vs control group differences for age, education, body mass index, alcohol consumption and tobacco smoking indices were tested using *t* – test. To evaluate group differences for monocyte proinflammatory cytokine expression, repeated measures mixed models analysis of variance and covariance (rANOVA, rANCOVA) were performed using a 2 (group: cocaine dependent, *n* =18; control, *n* =18) x 7 (time: 08:00, 12:00, 16:00, 20:00, 23:00, 2:00, 5:00 h) design. One cocaine dependent volunteer and one control pair was missing complete proinflammatory cytokine data across the diurnal period. To evaluate group differences for the measures of heart rate variability, mixed models analysis of variance and covariance (rANOVA, rANCOVA) were performed (cocaine dependent, *n* =19; control, *n* =19). To determine the effects of placebo vs. cocaine on monocyte proinflammatory cytokine expression, mixed models rANOVAs were performed using a 2 (condition: placebo, cocaine) x 15 (time: 08:00, 12:00, 16:00, 17:30, 20:00, 23:00, 2:00, 5:00, 08:00, 12:00, 16:00, 20:00, 23:00, 2:00, 5:00 h) design. One cocaine dependent volunteer was missing a complete set of proinflammatory cytokine data across the infusion protocol; hence a total of 17 cocaine dependent volunteers were included in these analyses. Spearman correlations were used to evaluate the associations between heart rate variability measures and proinflammatory cytokines in the cocaine dependent volunteers and controls; partial correlations were used to evaluate the unique contribution of heart rate variability measures on proinflammatory cytokines independent of the contribution of demographic and clinical variables. For these analyses, a total of 18 cocaine dependent volunteers and 18 controls had complete proinflammatory cytokine and heart rate variability data.

Results

Demographic and clinical characteristics

Cocaine dependent volunteers were similar in age, but more likely to be nonwhite and have less years of education than controls (Table1). BMI was similar in the two groups; none of the subjects were obese (BMI < 32 kg/m^2). Cocaine dependent volunteers were frequent users of cocaine, whereas other substance use was infrequent. Consistent with the exclusion criteria for other current substance dependence, no cocaine dependent volunteer reported use of opiates or other substances in the last 3 months as verified by screening urine and toxicology tests. Marijuana use was reported in a subgroup of the cocaine dependent volunteers (n = 10), and one one control reported a single use of marijuana 21 days prior to study. None of the other controls reported any cocaine or other substance use in the last 3 months. Alcohol use was more frequent and more recent in the cocaine dependent volunteers as compared to the controls, and there was a greater prevalence of tobacco smokers in the cocaine dependent group.

Cocaine dependence and monocyte proinflammatory cytokine expression

After at least 2 days of cocaine abstinence, monocyte expression of proinflammatory cytokines, TNF-α and IL-6, was assessed across a diurnal period in the cocaine dependent volunteers vs. controls. Resting, unstimulated monocyte expression of TNF-α was reduced in the cocaine dependent volunteers as compared to the controls, with a significant group effect ($F(1,33.6)= 4.5$, $p < 0.05$), but no time effect or group x time interaction. Monocyte expression of IL-6 did not differ between the groups, however there was a time effect ($F(6.0, 177.9) = 2.7$, $p<0.02$) .

As shown in Figure 1, LPS stimulated monocyte expression of TNF-α also differed across the diurnal period between the cocaine dependent volunteers and controls, with a time

effect ($F(6.0,179.6) = 2.8$, $p < 0.05$) and a group x time interaction (F (6.0, 179.6)= 2.5, $p < 0.05$) (Figure 1). Controls showed a diurnal increase in stimulated monocyte expression of TNF-α during the late afternoon and night, which was not present in the the cocaine dependent volunteers. Similar results were found for stimulated monocyte expression of IL-6 alone with a strong time effect (F (6.0, 180.3)= 31.1, $p < 0.001$) and a group x time interaction (F (6.0, 180.3)= 5.5, $p < 0.001$).

Additional analyses were performed covarying for the demographic and clinical variables shown in Table 1. Inclusion of covariates for age, ethnicity, marital status, education, tobacco smoking, and alcohol consumption indices did not alter the significant group x time interaction for stimulated monocyte expression TNF-α or for stimulated expression of IL-6. However, with inclusion of the covariates, the group effect for unstimulated TNF-α was reduced to a statistical trend ($p = 0.06$). Furthermore, expresssion of TNF-α and IL-6 did not differ in the cocaine dependent group stratified by marijuana use in the last 3 months.

Stimulated monocyte production of TNF-α and IL-6 is expressed as percentage of total monocyte counts; differences in monocyte numbers do not account for these varying levels of cytokine expression. However, to further evaluate this question, numbers of monocytes (CD14) were determined; there was no group effect nor group x time interaction (all P ' s >0.6).

Acute cocaine infusion and monocyte proinflammatory cytokine expression

After completion of the first phase, cocaine dependent volunteers entered the second phase of the protocol and underwent infusion of placebo vs. cocaine (40 mg), which was administered over 60 seconds in a randomized, counter-balanced, cross-over design. After each infusion, monocyte expression of TNF-α and IL-6 was repeatedly assessed across two diurnal

periods; hence, cocaine dependent volunteers received both infusions and underwent an additional 96 hours of evaluation for the second phase of study.

As compared to placebo condition, cocaine infusion induced a significant change in resting monocyte expression of TNF-α with a significant time effect (F (8.5,127.5)= 3.1, $p <$ 0.005) and a condition x time interaction (F (11.8,177.3)= 2.3, $p < 0.01$). Similar results were found when analyses focused on resting monocyte expression of IL-6 with a signficant time effect (F(7.7,115.6) = 4.6, $p <0.001$) and a condition x time interaction (F (15.5,232.7)= 2.6, $p < 0.001$).

As shown in Figure 2, cocaine admininstration also induced a robust and sustained decrease in LPS stimulated monocyte production of TNF-α, with a significant condition effect (F (1, 15)= 7.8, $p <$ 0.02), time effect (F (10.4, 155.8)= 3.4, $p <$ 0.001), and condition x time interaction (F (8.5, 126.8)= 2.1, $p <$ 0.04). Figure 3 displays representative flow cytometric results at 20:00 h for one cocaine dependent volunteer following placebo vs. cocaine. Cocaine administration did not alter stimulated expression of IL-6 (condition x time, F (9.2,138.0)= 1.1, $p = 0.37$). There was no block effect for the subjects who received cocaine first vs. those who received placebo first.

It is not known whether the decreases in monocyte produciton of TNF-α induced by acute cocaine represent a further decline in this immune response among cocaine dependent volunteers. Hence, a key question is whether responses of stimulated monocyte produciton of TNF-α during the placebo condition differed from responses in controls, as had been found for the cocaine dependent volunteers after two days of abstinence (Figure 1). To address this question, exploratory analyses compared responses of stimulated monocyte produciton of TNF-α between controls and cocaine dependent volunteers during the placebo infusion protocol. Similar

to the findings displayed in Figure 1, ANCOVA showed that responses of stimulated monocyte production of TNF-α in the cocaine dependent volunteers during placebo infusion differed significantly from controls, with significant group x time interactions for both the first- and second diurnal periods of placebo infusion (F (4.2,122.7) = 3.8, $p < 0.01$; F(4.5,131.7)= 4.3 $p < 0.01$). Hence, the altered response in monocyte production of TNF-α found after two days of cocaine abstinence (Figure 1) persists throughout the placebo infusion condition. Together with the findings from the infusion protocol, these data suggest that acute cocaine induces further decreases of monocyte production of TNF-α in cocaine dependence.

Acute cocaine infusion and circulating TNF-rII

Monocytes are the primary souces of circulating proinflammatory cytokines. To assess the effects of acute cocaine on *in vivo* proinflammatory cytokine activity, circulating levels of the TNF-rII were measured following infusion of placebo vs. cocaine. As compared to placebo, cocaine administration induced decreases in circulating levels of TNF-rII with a significant time effect (F (9,252)= 5.0, $p < 0.001$) and a condition x time interaction (F (9,252)= 3.0, $p < 0.01$). As compared to placebo, post-hoc contrasts showed that cocaine induced significant decreases of TNF-rII at 20:00 h and 23:00 h. ($p < 0.05$).

Cocaine blood levels in cocaine dependent volunteers

In vitro doses of cocaine are reported to inhibit LPS stimulated production of TNF-α and IL-6 in culture supernatants (Friedman et al., 2003). Hence, to determine whether the protracted effect of cocaine on monocyte expression of proinflammatory cytokines was associated with cocaine concentrations, circulating levels of cocaine and its metabolites, benzyolecgonine and ecgonine methyl ester were measured over the course of the infusion protocol. Prior to initiation of the blood sampling protocol, levels of cocaine and its metabolites were below the limits of

assay detection (10 ng/ml). Following infusion of cocaine at 17:00 h, levels of cocaine significantly increased to 28.2 ± 7.2 ng/ml at 20:00 h (F (1,17)= 57.9, $p < 0.001$) and returned to baseline (i.e., undetectable) concentrations in all subjects by 9 hours post-infusion. Benzoylecgonine levels increased to 201.3 ± 38.5 ng/ml at 20:00 h (F (1,17)= 102.9, $p < 0.001$) and returned to undetectable concentrations in all subjects by 36 hours post-infusion. Similarly, levels of ecgonine methyl ester increased to 26.4 ± 5.1 ng/ml at 20:00 h (F (1,17)= 80.6, $p < 0.001$) and were not significantly above the limit of detection by 9 hours post-infusion; by 19 hours post-infusion, no subject had detectable concentrations. As shown in Figure 2, a significant decrease of monocyte expression of TNF-α was found to persist for up to 33 hours post-infusion. Circulating levels of cocaine at 20:00 h were not consistently correlated with monocyte expression of TNF-α.

Autonomic mechanisms and proinflammatory cytokine expression in cocaine dependence

Given evidence that sympathetic neurotransmitters suppress secretion of proinflammatory cytokines, we evaluated whether differential levels of resting and LPS-stimulated monocyte expression of TNF-α and IL-6 in cocaine dependence were associated with alterations in sympathetic and parasympathetic nervous system activity. To assess this possibility, heart rate variability was measured and estimates of sympathetic activity (i.e., ratio of LF/HF power) and parasympathetic activity (i.e., HF power) were obtained by spectral analyses. As compared to controls, ANCOVA found that cocaine dependent volunteers had higher levels of the ratio of LF/HF power (3.1 ± 1.3 vs. 4.5 ± 3.3; F (1,30)= 3.7, $p = 0.06$), along with decreases in the HF power (0.15 - 0.4 Hz; 2738.7 ± 1242.7 ms^2 vs. 1895.4 ± 1599.3 ms^2; F (1,30)= 5.1, $p < 0.05$). Similar results were obtained when HF power was expressed as normalized units (F (1,30)= 7.3, $p = 0.01$). Together, these findings indicate a shift toward sympathetic dominance and a

withdrawal of parasympathetic activity at rest in cocaine dependent volunteers as compared to healthy controls.

Correlational analyses were used to determine whether individual differences in autonomic activity were associated with monocyte expression of proinflammatory cytokines. The ratio of LF/HF power was negatively correlated with resting- (Figure 4A) and stimulated monocyte expression of TNF-α (Spearman ρ = -0.54, $p < 0.01$; Spearman ρ = -0.39, $p < 0.05$) in the total sample, with similar results in the groups of cocaine dependent volunteers and controls. In contrast, individual differences in HF power were positively correlated with resting- (Figure 4B) and stimulated monocyte expression of TNF -α (Spearman ρ = 0.38, $p < 0.05$; Spearman ρ = 0.39, $p < 0.05$). No relationship was observed between monocyte expression of IL-6 and LF/HF ratio or HF power.

To determine whether individual differences in autonomic activity were associated with monocyte expression of proinflammatory cytokines, independent of the contribution of age, ethnicity, marital status, education, tobacco smoking and alcohol consumption indices, partial correlations were used. Again, the ratio of LF/HF power remained negatively correlated with resting- and stimulated monocyte expression of TNF-α (pr = -0.55, $p < 0.005$; pr = -0.35, $p < 0.09$). Likewise, individual differences in HF power remained positively correlated with resting- and stimulated monocyte expression of TNF -α (pr = 0.41, $p < 0.05$; pr = 0.33, $p < 0.10$).

Discussion

This study provides the first evidence that cocaine dependence, as well as acute cocaine administration, induce decreases in monocyte expression of proinflammatory cytokines at rest and in response to the bacterial ligand, LPS. During acute abstinence, LPS triggers less

production of TNF-α and IL-6 in cocaine dependent volunteers as compared to controls. Moreover, a single acute dose of cocaine leads to a decline in monocyte expression of TNF-α at rest and in response to LPS challenge; cocaine or a metabolite of cocaine rapidly impairs this functional cellular innate immune response and initiates cellular changes that persist for nearly one day after cocaine is metabolically cleared from the blood. Together these data demonstrate that cocaine use contributes to substantial decreases in innate immune mechanisms, even in cocaine dependent persons who have a history of repeated drug use and show evidence at entry of low monocyte responses to LPS. Moreover, given that the acute effects of cocaine reported here were induced following a single dose of this drug, decreases of monocyte function may be even more profound during binge cocaine use.

The innate immune system triggers effector pathways to combat microbial pathogens through expression of proinflammatory cytokines and upregulation of co-stimulatory molecules to induce adaptive T and B cell responses. The finding that cocaine attenuates the response to LPS has implications for risk of bacterial infectious diseases in cocaine dependent persons who report sustained use, as well as among those who use a single dose of cocaine. Cocaine users are at increased risk for group A streptococci infections (Sierra et al., 2006), cellulits (D'Ellia et al., 1991), abscesses (D'Ellia et al., 1991), and sepsis (Masi, 1978; Silverman and Smith, 1985; D'Ellia et al., 1991; Dettmeyer et al., 2004). LPS binding to *TLR 4* initiates defense against bacteria in which subsequent expression of TNF-α and IL-6 mediates cellular immune responses to recognize and eliminate polymicrobial infections (Miller et al., 2005), especially those due to gram-negative bacteria (Hoshino et al., 1999). Furthermore, the magnitude of decrease in LPS-induced monocyte expression of TNF-α and IL-6 in cocaine dependence is comparable to that found in rheumatoid arthritis patients who are being treated with TNF antagonist medications;

such patients are known to have an increased risk of polymicrobial bacterial infections (Listing et al., 2005).

Heavy crack cocaine use is associated with an over three-fold prevalence of hepatitis C seropositivity (Thorpe et al., 2000), and this increased risk of primary infection might be due to the suppressive effects of cocaine on innate immune mechanisms. IL-6 has antiviral activity and plays a role in hepatitis C clearance; circulating levels of IL-6 correlate with viral clearance during hepatitis C infection, and replication of hepatitis C RNA can be significantly suppressed by IL-6 (Zhu et al., 2004). Moreover, circulating levels of IL-6 are lower in hepatitis C infected persons as compared to hepatitis C negative controls (Lee et al., 2002), and levels IL-6 are higher in patients who respond to interferon-α treatment (Mazur et al., 2001). Similarly, a decrease in the ability of monocytes to express IL-6 may also have implications for primary and early HIV infection (Chaisson et al., 1989; Anthony et al., 1991; Deeks et al., 2004). Cocaine increases HIV replication in PBMCs *in vitro* (Bagasra and Pomerantz, 1993), whereas IL-6 has been shown to suppress HIV-1 replication in mixed brain cell cultures (Lokensgard et al., 1997). In addition, this proinflammatory cytokine is thought to play a critical role in the regulation of host immunity and its ability to eradicate HIV-1 during antiretroviral therapy (Connolly et al., 2005). However, in longitudinal studies of HIV infected persons, cocaine use has not been found to have a substantial influence on progression to AIDS, which was primarily predicted by plasma HIV RNA levels (Rodriguez et al., 2006).

Cocaine is a potent sympathomimetic that potentiates the action of norepinephrine and dopamine by blocking their reuptake. This study identified possible mechanisms that might be involved in the suppression of monocyte expression of TNF-α and IL-6 after cocaine and its metabolites have cleared from the circulation, and found that *in vivo* estimates of sympathetic

activity (i.e., LF/HF ratio) negatively correlated with resting- and LPS stimulated monocyte production of TNF-α, but not IL-6. Moreover, sympathetic activation induces a more robust inhibition of LPS-stimulated production of TNF-α than other proinflammatory cytokines (Elenkov et al. 2000; Meltzer et al., 2004). IL-6 and TNF-α gene expression are modulated by some common signaling pathways (e.g., NF-κB), but each gene is also regulated by distinct transcription control pathways. Previous studies have shown that the protein kinase-A (PKA) signaling pathway in particular can have unique effects on LPS-stimulated production of these two cytokines (Bailly et al., 1990). Given that PKA mediates the effects of catecholamines on gene regulation, it is not surprising that the present data show distinct relationships between heart rate variability indications of *in vivo* sympathetic activity and LPS-induced production of IL-6 vs. TNF-α. Alternatively, *in vivo* estimates of parasympathetic activity were positively correlated with TNF-α production, which suggests that withdrawal of vagal tone may also contribute to the decreases of monocyte production in cocaine dependence. Other effector pathways not measured here could also contribute to the effects of cocaine on proinflammatory cytokine expression. Cocaine induces rapid activation of the HPA axis probably due to enhanced activity of norepinephrine centrally (Halpern et al., 2003). In animals, the cortisol antagonist RU486 blocks the suppressive effects of chronic cocaine administration on lymphocyte responses (Avila et al., 2003).

Use of flow cytometric intracellular cytokine analyses permitted us to conclude that cocaine dependence induces an impaired inflammatory response at the cellular level in monocyte populations. Differences were not identified in simple enumerative analyses of circulating leukocytes subpopulations. Hence, cocaine appears to impair monocyte functional responsiveness to *TLR4* stimulation without altering the total population of monocyte numbers in

circulating blood. Further studies are needed to determine the molecular mechanisms responsible for altered responses of monocytes to LPS; for example, cocaine might alter *TLR4* expression and signaling and/or dysregulate translocation of NF-κB and induction of TNF-α and IL-6 production.

Along with changes in cellular expression of TNF-α and IL-6, acute cocaine was also found to decrease systemic proinflammatory activity as measured by circulating levels of TNF-rII. These data are consistent with a prior study that assessed systemic inflammation by measuring circulating levels of IL-6 as an *in vivo* model for inflammatory challenge (Halpern et al., 2003). Placement of an indwelling intravenous catheter is thought to induce surrounding tissue inflammation, with increases in the local production of IL-6. An acute dose of cocaine (0.4 mg/kg) was found to blunt IL-6 release and attenuate increases in circulating levels of IL-6 four hours after cocaine injection (Halpern et al., 2003). The present study extends these findings by investigating the cellular source that contributes to the suppression of IL-6 following cocaine. Another study in humans found that cocaine administration increased mitogen stimulated production of interferon-γ in mixed PBMC cultures at a single timepoint (i.e, 30 minutes) after infusion (Gan et al., 1998), although such differences using these culture conditions may be due the effects of cocaine on immunoregulatory cell traffic.

There are several limitations to this study. Although sex differences in the immune effects of cocaine have not been found (Halpern et al., 2003), it is not known whether the present findings generalize to women. Second, cocaine dependence is associated with disturbances in sleep-wake activity, and such sleep disruption may contribute to differences in nocturnal monocyte expression of TNF-α. Although the sleep-wake cycle was controlled in this study, additional analyses evaluating sleep quality and EEG sleep measures in relation to

proinflammatory cytokine expression are needed. Third, the subjective experience of cocaine infusion was not formally assessed. However, the intravenous dose and mode of administration of cocaine did not induce any unusual behavioral effects. In addition, it is unlikely that the effects of cocaine infusion on stimulated monocyte production of TNF can be attributed to withdrawal from cocaine during the 96 h infusion protocol, as there were no differences for cocaine-induced changes of cytokine expression between subjects who received cocaine first and those who received placebo first. Fourth, the expression of cocaine was administered intravenously, whereas most cocaine users smoke or insufflate ('snort') the drug. Fifth, changes in the function of monocyte populations may not generalize to altered responses in other cell types, although cocaine administration does not alter the stimulated intracellular production of the anti-inflammatory cytokine, IL-10, in CD4 T cell populations (unpublished data). Finally, cocaine administration induced decreases in circulating levels of soluble TNF-rII, a marker of proinflammatory cytokine activity, but it is not known whether cocaine alters levels of other proinflammatory cytokines. Despite these limitations, our findings show that chronic cocaine dependence, as well as acute cocaine administration, induces a substantial decrease in the capacity of monocytes to initiate an innate immune response to a bacterial stimulus. These data support the rationale for further investigations to determine whether cocaine impairs other clinically relevant immune responses with consequences for infectious disease risk in humans.

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Footnotes

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Legends for Figures

Figure 1: Cocaine dependence and monocyte proinflammatory cytokine expression. Differences in expression of TNF-α in LPS-stimulated CD14+ cells between cocaine dependent (o…o) and control volunteers (•—•) across the diurnal period. Data are represented as mean ± standard error. There was a time effect ($F(6.0,179.6) = 2.8$, $p < 0.05$) and a group x time interaction (F (6.0, 179.6)= 2.5, $p < 0.05$) , with significant differences between groups * $P < 0.01$ at 16:00 h, 20:00 h, 23:00 h, and 5:00 h. The shaded area indicates the nocturnal sleep period.

Figure 2: Cocaine induced decreases in monocyte proinflammatory cytokine expression. Differences in expression of TNF-α in LPS-stimulated CD14+ cells between placebo (•—•) and cocaine infusion (40 mg) (o…o) across 48 hours period. Data are represented as mean ± standard error. There was an overall condition effect (F (1,15)=7.8, $P < 0.02$), with significant differences between conditions * $P < 0.01$ and + $P < 0.05$. The shaded area indicates the nocturnal sleep period.

Figure 3: Representative flow cytometric analyses of monocyte proinflammatory cytokine expression at 20:00 h following placebo and cocaine infusion (40 mg) from one cocaine dependent volunteer. Numbers indicate percentage of the fraction of CD14+ cells that are positive for TNF-α alone (upper left), TNF-α and IL-6 (upper right), or IL-6 alone (lower right), or that are negative for both TNF-α and IL-6 (lower left).

Figure 4: Monocyte expression of TNF at rest (unstimulated) is associated with *in vivo* estimates of sympathetic activity (*A*, LF/HF ratio) and parasympathetic activity (*B*, HF) as measured by heart rate variability. LF/HR ratio negatively correlated with TNF expression (Spearman $\rho = -0.54$, $P < 0.01$), whereas HF power in normalized units positively correlated with TNF expression (Spearman $\rho = 0.38$, $P < 0.05$). Similar correlations were found in cocaine dependent (o) and control volunteers (•). Data are presented for blood samples collected at 23:00 h identical to time for heart rate variability assessment, with similar correlations obtained for other blood sampling timepoints. Similar results were obtained for the relationship between monocyte expression of TNF-α following LPS stimulation and heart rate variability measures (data not shown).

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Table 1:

Demographic and clinical characteristics of control and cocaine dependent volunteers

Characteristic	Controls (*n = 19*)	Cocaine Dependent (*n = 19*)	
Demographics, No. (%)			
Age (years), mean (sd)	39.7 (10.0)	43.2 (5.1)	$t = 1.3$ $P = 0.19$
Ethnicity, No. (%)			
White	8 (42)	2 (11)	$P = 0.06$†
Non-white	11 (58)	17 (89)	
Marital Status, No. (%)			
Married	4 (21)	1 (5)	$P = 0.34$†
Not married	15 (79)	18 (95)	
Education (years), mean (sd)	15.2 (1.7)	12.9 (1.8)	$t = 4.1$ $P < 0.001$
Body mass index	25.2 (3.9)	25.6 (3.0)	$t = 0.4$ $P = 0.7$
Substance Consumption			
Cocaine (last 3 months)			
Days per month, mean (sd)	-	20.7 (6.8)	
Use (grams) per month, mean (sd)	-	122 (117)	
Days since last use, mean (sd)	-	2.9 (3.4)	
Alcohol consumption (last 3 months)			
Days per month, mean (sd)	2.7 (6.8)	13.1 (10.3)	$t = 3.7$ $P = 0.001$
Drinks (1 oz[a]) per month, mean (sd)	4.2 (11.3)	47.5 (57.1)	$t = 3.2$ $P = 0.003$
Days since last use, mean (sd)‡	48.8 (98.7)	4.4 (8.4)	$t = 1.6$ $P = 0.13$
Tobacco consumption (last 3 months)			
Use (cigarettes) per day, mean (sd)	0.8 (2.8)	8.0 (6.5)	$t = 4.4$ $P<0.001$

† Fisher exact test
‡ 13 controls and 19 cocaine dependent subjects reported alcohol use in the last year
[a] = ethanol

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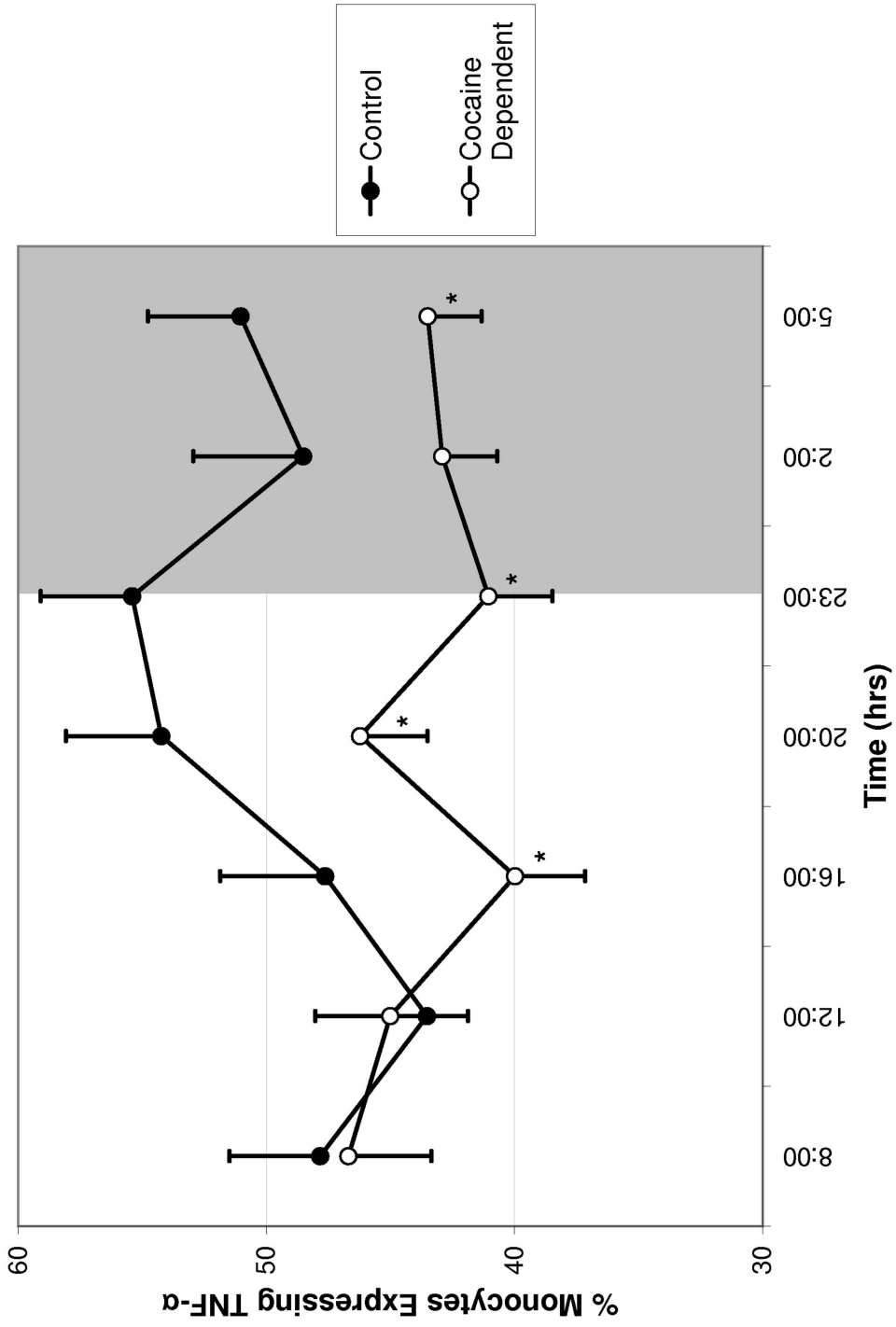

Figure 1

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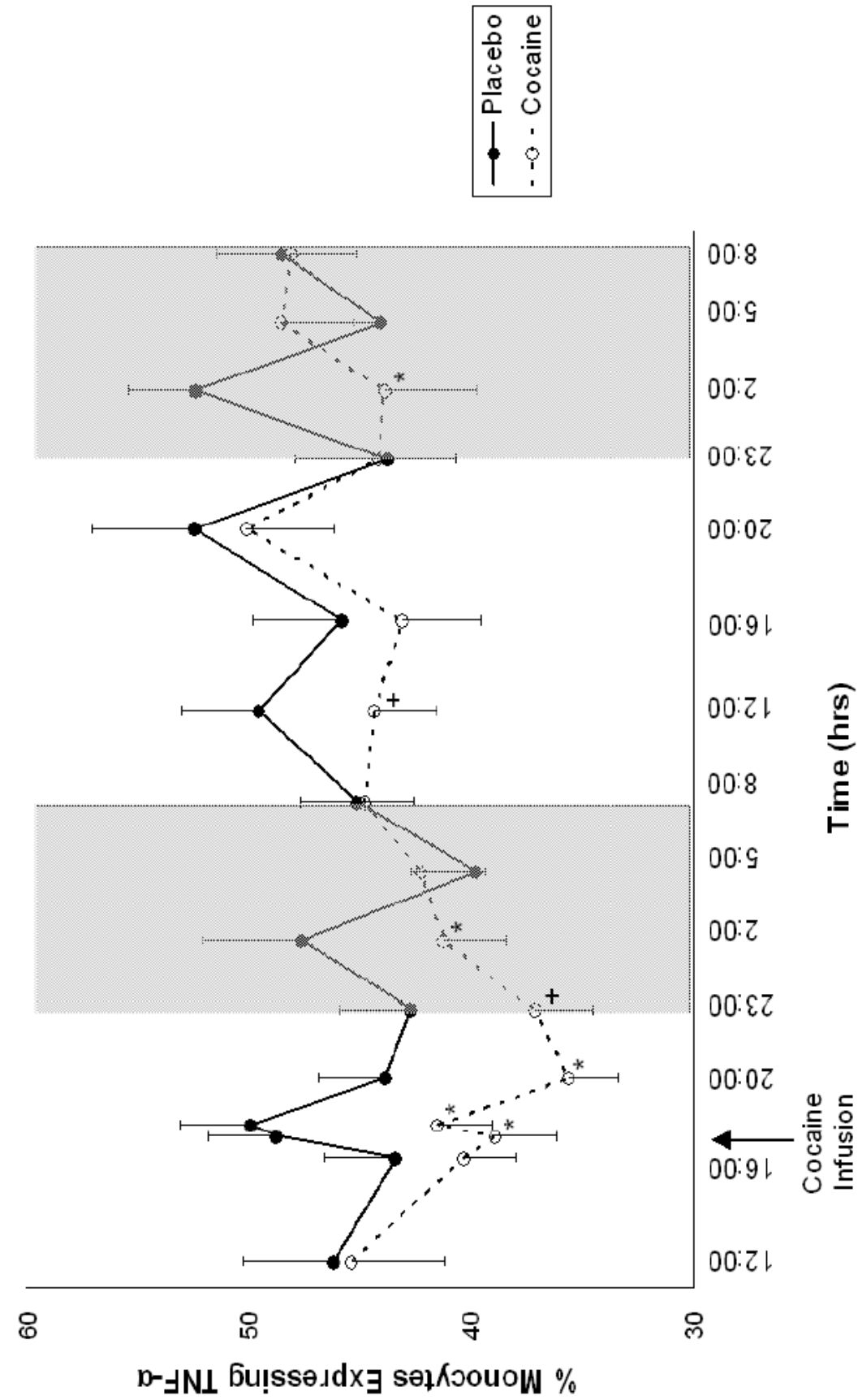

Figure 2

Figure 3

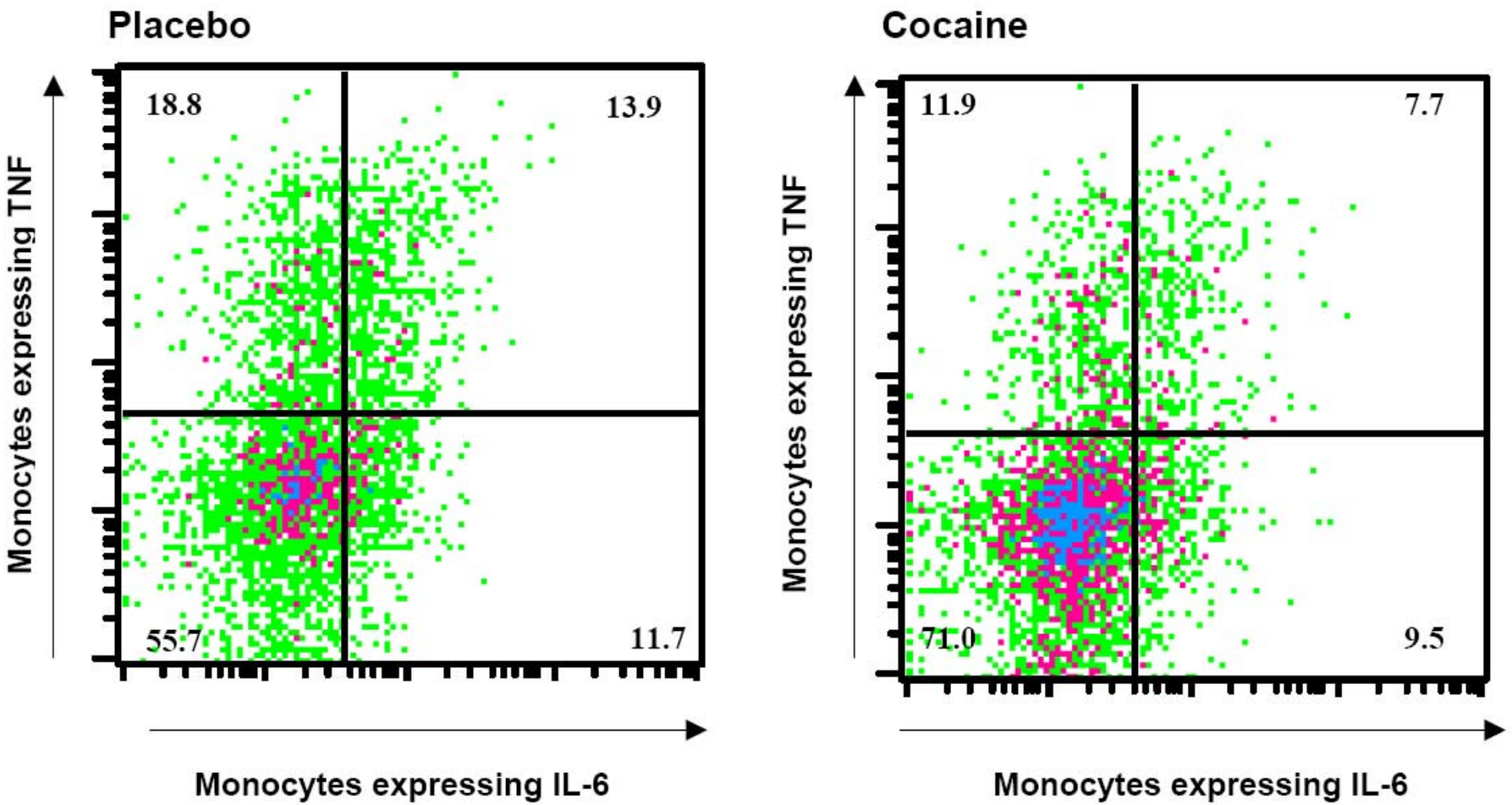

Figure 4