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PRO-INFLAMMATORY CYTOKINE MODULATION IN RESPONSE TO CHRONIC FENTANYL SELF-ADMINISTRATION IN RATS

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A thesis submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Psychology

Written under the direction of

Mark West

And approved by

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New Brunswick, New Jersey

October 2020

ABSTRACT OF THE THESIS

Pro-inflammatory cytokine modulation in response to chronic fentanyl self-administration in rats

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Opioid abuse is associated with an increased prevalence of blood borne viruses and opportunistic infections due to specific immunomodulatory effects of opioid drugs that can influence this susceptibility. Fentanyl, a schedule II opioid, has gained prominence on the illicit market contributing to the rising number of opioid-related deaths, but little information is available regarding 1) the immune effects of fentanyl and 2) how chronic self-administration (SA) of fentanyl impacts central and systemic inflammation. In the current project, a rat model was used to examine the effects of voluntary chronic, long-access fentanyl SA on *in vivo* cytokine production in response to the endotoxin lipopolysaccharide (LPS). The specific cytokines of interest were IL-1β, TNF-α, and IL-6, which are the most prominent neuromodulatory cytokines associated with inflammation. Following 30 days of fentanyl SA, IL-1β and TNF-α levels (IL-6 data not yet available) were suppressed after 7 days of abstinence compared to Saline/SA control

concentrations; this suppression recovered to that of Saline/SA control concentrations after 30 days of abstinence. The results of this study show for the first time that chronic fentanyl SA significantly suppresses the *in vivo* cytokine response to endotoxin challenge with possible implications for opioid relapse. Results also show for the first time that recovery from the expected opioid-mediated immunosuppression is possible after an extended period of abstinence.

ACKNOWLEDGEMENTS

I would like to thank the members of this thesis committee, Dr. Alexander Kusnecov and Dr. Ben Samuels. I would also like to thank both current and past members of West Lab for their support and assistance. Additionally, I would like to thank my friends and family for their love and support throughout this process. Lastly, I would like to thank Dr. West for being an excellent mentor and providing assistance and guidance through the completion of this project.

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INTRODUCTION

Opioid addiction is a chronic, relapsing, psychiatric disorder marked by compulsive opioid abuse, spontaneous withdrawal, and frequent relapse. Substance abuse and addiction incur an annual economic burden of $740 billion (NIDA, 2017), reaching epidemic proportions. Of the 72,000 drug overdose deaths reported in 2017, the sharpest increase was related to fentanyl which is estimated to be associated with 115 deaths per day for 2018 (NIDA, 2018; Glaser & Keicolt-Glaser, 2005; NIDA, 2017). Adding to this concern is the characteristic immunosuppression associated with opioid abuse which leaves users more susceptible to opportunistic infections and exacerbates public health risks. Recently, synthetic opioids have been involved in nearly half of all opioid related fatalities (an increase of nearly 16,500 deaths between 2010 and 2016) and have passed prescription opioids as the most common drugs involved in overdose deaths in the United States. In particular, fentanyl, a synthetic opioid painkiller 50 to 200 times more potent than morphine, is the primary synthetic opioid associated with these overdose deaths (NIDA, 2018).

Fentanyl, a μ-opioid receptor (MOR) agonist, is a prescription painkiller used for the treatment of chronic and severe pain. It is also used as an anesthetic known to induce less stress on the body and cardiovascular system than other anesthetics (Stanley, 2014; Stanley, Philbin, & Coggins, 1979). It has a high lipophilicity which allows it to cross the blood-brain barrier and impart is pharmacologic effects on the central nervous system, and its rapid onset, short half-life, and inactive metabolites make it a desirable choice for

analgesia and anesthesia (Anderson, 2011). Fentanyl produces the same adverse side effects as similar prescription opioids, but less severe adverse effects are reported because its high potency requires generally lower doses to reach an effective or therapeutic dose (Belzarena, 1992; White et al., 1992). Human brain imaging during fentanyl administration revealed its effects are highly localized to areas of the brain involved in nociception, pain-related affective behaviors, attention, reward, and addiction such as the medial prefrontal cortex and orbitofrontal cortex (Firestone et al., 1996). Studies suggest that repeated opioid exposure leads to impairments in the prefrontal cortex and cognitive functioning which may play a role in the development of compulsive substance abuse and addiction (Jentsch & Taylor, 1999; Kalivas, Volkow, & Seamans, 2005; Badiani et al., 2011).

Susceptibility to opioid dependence relies on genetic and environmental factors specific to individual drug users (Kreek et al., 2005; Tsuang et al., 1998) as well as the pharmacological effects of opioids and their neurobiological impacts. The pharmacokinetic properties of opioid drugs allow for a quick onset of a dose-dependent drug effect which is correlated with the rewarding effects of opioid use (Marsch et al., 2001), while the negative symptoms of opioid withdrawal, such as agitation, anxiety, nausea, and vomiting, are the most powerful factors driving opioid dependence (Kosten & George, 2002). Human studies report that compulsive drug use and withdrawal are associated with negative affect, which can be readily modeled in laboratory rats by analyzing ultrasonic vocalizations (Barker et al., 2014; Klein et al., 2020).

Addiction research has primarily focused on the mesocorticolimbic dopamine system which consists of dopaminergic cell bodies originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAcc). This system has been strongly implicated in the reinforcement of drug taking because an increase in dopamine (DA) in this system during drug use is thought to help drive addiction and drug seeking behavior (Koob & Volkow, 2010; Wise & Bozarth, 1987). This system has numerous feedback loops to prefrontal cortex areas and has been implicated as a link between limbic and motor systems, integrating emotion and movement (Mogenson, Jones, & Yim, 1980). In the absence of drug during normal functioning, dopaminergic VTA neurons release DA into areas of the limbic forebrain where it binds to receptors on post-synaptic neurons and is eventually recycled during re-uptake. This is the same biochemical process triggered during feelings of natural reward and pleasure but is highjacked by chronic drug use.

The main target for prescription opioids is the MOR which is distributed in high numbers throughout the central nervous system, predominately in areas modulating pain and mesocorticolimbic reward areas (Marsch et al., 2001; McDonald & Lambert, 2005; Maldonado et al., 1992). MORs in the spinal cord are found on the presynaptic surface of afferent neurons in the spinothalamic tract of the dorsal horn of the spinal cord and are involved in the transmission of nociceptive stimuli via inhibition of glutamate and substance P release (Jessell & Iverson, 1977). Activation of these G-protein-coupled receptors eventually leads to decreased cell excitability which results in inhibition of neurotransmitter release, ultimately producing analgesia by reducing the transmission of

nerve impulses, as well as respiratory suppression, euphoria, addiction, and other drug effects (McDonald & Lambert, 2005; Roy et al., 2011).

During opioid abuse, MORs in these limbic regions dose-dependently hyperpolarize and inhibit GABAergic interneurons which disinhibits VTA neurons, increasing the release of DA projecting to the NAcc, resulting in feelings of pleasure and euphoria (Johnson & North, 1992; Gysling & Wang, 1983). Administration of a MOR antagonist reverses this GABAergic inhibition of interneurons, decreasing DA levels in this system (Gysling & Wang, 1983). Both prescription opioids and endogenous opioid peptides are readily self-administered when intracranially delivered to the VTA, augmenting dopamine levels in the mesocortiocolimbic dopamine system and producing the same rewarding effects (Koob, 1992; Bozarth & Wise, 1981). Repeated exposure to these drugs alters brain functioning such that it cannot function normally in the absence of drug, ultimately leading to tolerance where more drug is needed for the VTA to release the same amount of DA into the NAcc, contributing to the development and maintenance of opioid addiction (Kosten & George, 2002). The resulting tolerance and escalation of drug intake are major criteria for assessing drug addiction in humans and in animal models of addiction (Ahmed, 2011).

Immunomodulation by Opioids

Immune cells express a range of neurotransmitter, neuropeptide, and hormonal receptors including those for endogenous opioids (Borner et al., 2008; Kraus, 2009; Sharp, 2006), identifying a link between neural, endocrine, and immune system activity. This allows

the immune system to be influenced by neural and endocrine factors secreted by the autonomic nervous system and various endocrine axes served by the pituitary (e.g., hypothalamic-pituitary-adrenal [HPA] axis) (Glaser & Kiecolt-Glaser, 2005; Steinman, 2004; Sternberg, 2006). There is abundant evidence that chronic opioid abuse is associated with increased incidence of viral and bacterial infections (Cherubin & Shapira, 1993; Contoreggi et al., 1998; Mathers et al., 2008; Nelson et al., 2011), and opioids can dose-dependently directly affect immune cells and their responses thus affecting immune competence in opioid abusers (Al-Hashimi et al., 2013; Plein & Ritter, 2017). Activation by opioids of MORs on the surface of immune cells suppresses immune cell activity by inhibiting NF-κB activation, the transcription factor responsible for properly activating immune cells and initiating a pro-inflammatory state (Welters et al.,. 2000). This opioid-mediated immunomodulation, which is reversible by administration of a MOR antagonist (Roy & Loh, 1998), includes suppression of pro-inflammatory cytokines, microglial activation, cell mediated cytotoxicity, and leukocyte proliferation (Roy et al., 2011; Martin et al., 2010; Wang et al., 2005) which ultimately results in a weakened immune response incapable of adequately addressing antigenic challenge.

In animals, this evidence is based on non-contingent, experimenter administration of morphine or heroin, which does not adequately model the manner or self-dosing of human drug abuse. This model is most often coupled with *in vitro* measures of immune system activity. Moreover, less is known regarding specific immune effects of fentanyl. Immunomodulation by fentanyl has largely been demonstrated after *in vitro* incubation of the drug with leukocytes (Bastami et al., 2001; Brand et al., 2001; Declue et al., 2014;

Hyejin et al., 2013). In two *in vivo* studies using mice, non-contingent injections of fentanyl showed inhibition of antibody (Filipczak-Bryniarska et al., 2012) and suppression of the cytokine tumor necrosis factor (TNF) in response to the bacterial endotoxin lipopolysaccharide (LPS; Molina-Martinez et al., 2014). Additionally, drug use, withdrawal, and endotoxin exposure are stressors for both the immune system and CNS, activating the HPA axis and increasing glucocorticoid secretion which in turn further suppresses immune system activity (Al-Hashimi et al., 2013). During opioid withdrawal, specifically, a profound immunosuppressive effect is reported within 24 hours of abstinence from experimenter-administered drug and persists for at least a week (Rahim et al., 2002). During this time, subjects are particularly susceptible to infection (Roy et al., 2011; Feng et al., 2014), even though behavioral, somatic, and affective symptoms of withdrawal have ceased (Liu et al., 2008; Bruijnzeel et al., 2006).

Thus, a critical gap in the literature is our lack of knowledge whether similar results obtain in animal models employing opioid *self-administration.* Another issue that has not been documented is whether the severe suppression of immune system activity seen during withdrawal following opioid abuse is able to recover after a period of abstinence from drug. To test this idea, controlled studies not possible in humans require an appropriate self-administration (SA) animal model that enables chronic, extended access to drug to allow for adequate modeling of human drug abuse along with *in vivo* measures of immune system activity after a period of abstinence following spontaneous withdrawal. The present experiment was conducted to test whether similar

immunosuppression obtains in a self-administration model, and whether this effect recovers after a period of abstinence.

Cytokines and the Brain

Cytokines are the regulatory molecules of the immune system that control the magnitude and duration of an immune response (e.g., antibody production), but also exert endocrine actions, whereby they can impact biological functions of non-immune organs. They function in the CNS to recruit other immune processes as well as physiological, behavioral, and endocrine mechanisms to aid in immune functioning via their autocrine and paracrine influences across different areas of the CNS where cytokine receptors are widespread (Vezzani & Viviani, 2015; Brebner et al, 2000). Three prominent centrally released cytokines implicated in inflammation both systemically and within the CNS are interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6). These are considered the prototypical pro-inflammatory cytokines, generated and secreted mainly by macrophages of the innate immune system, including microglial cells in the brain. They also act synergistically to dose-dependently increase plasma corticosterone concentrations and adrenocorticotropic hormone (ACTH) levels (Brebner et al., 2000; Matta et al., 1992), 1) indicating that the immune system interacts with and influences the HPA axis and modulates the body's stress response and 2) identifying pro-inflammatory cytokines as activators of the neuroendocrine system (Dunn, 1998).

Indeed, these cytokines activate the CNS to initiate various behavioral adjustments (e.g., lethargy, anorexia, and anhedonia; Dantzer et al., 2008; Anisman et al., 2005),

with associated stress-like neurochemical and neuroendocrine alterations, and anxiety-like and depression-like behaviors (i.e., sickness behavior; Dantzer et al.., 2008; Anisman et al., 2005). The comorbidity of depressive and anxiety disorders and substance use disorders is well documented (Volkow, 2004; Rao, Hammen, & Poland, 2009) and this cytokine-mediated modulation of stress and affective state may play a role in predisposing individuals to begin using drug or to prompt addicted individuals to relapse in an attempt to self-medicate, escaping or avoiding a negative affective state (Volkow, 2004). Increased innate immune signaling also leads to cognitive and emotive dysfunctions that may further contribute to drug use and the development of addiction (Crews et al., 2017).

During normal physiological conditions, circulating cytokine levels in plasma are relatively low or hard to detect but increase markedly in response to trauma or antigenic challenge, such as bacterial endotoxins (Feng et al., 2005). Suppression and inactivation of macrophages associated with opioid abuse results in decreased generation and secretion of these pro-inflammatory molecules resulting in a general inactivation of other aspects of the immune system. As such, circulating cytokine levels can be used as a measurement of immune system activity.

Objectives

With the wide range of immunomodulatory effects of opioids and the ongoing opioid epidemic, it is imperative to have a better understanding of the immunosuppressive effects of opioid abuse. The methods of testing these effects in animal models are further

complicated by the stress-inducing methods often employed (e.g., non-contingent drug administration, aversive doses). Thus, more extensive research is required to further understand the role opioid withdrawal and abstinence play in modulating immune system activity. Therefore, the current experiment aims to address the immunomodulatory effects of chronic, long-access fentanyl SA on *in vivo* cytokine production after periods of abstinence. Specifically, the study will identify the degree of opioid-mediated immunosuppression using a SA model, and examine whether immune recovery is possible.

There is an absence of research regarding how *in vivo* immune responses are affected by chronic opioid self-administration and, although signs indicate the possibility of recovery after a week of abstinence (Rahim et al., 2002), no research exists examining the long-term persistence of this immunosuppression. The current experiment will address this by 1) using a SA protocol that models chronic opioid intake and abstinence and 2) determining whether recovery of *in vivo* pro-inflammatory responses to a bacterial endotoxin is possible. Preliminary data indicate that chronic, long-access fentanyl SA initially suppresses systemic parameters of immune activation and inflammation in male rats. Once physical dependence and tolerance to opioids set in, abrupt withdrawal results in significant suppression of immune system activity that persists for at least 7 days, though other measures (e.g., body and spleen weight) show recovery equal to pre-abstinence levels (Rahim et al., 2002) suggesting that full recovery of immune system activity may be possible after an extended period of abstinence. We predict that 30 days of abstinence will be a long enough period for full recovery to occur.

Results from this experiment will address a gap in knowledge regarding the effects of voluntary chronic fentanyl exposure on *in vivo* cytokine production, addressing 3 specific hypotheses:

Hypothesis 1: Rats will readily acquire self-administration of fentanyl over 30 days in a manner consistent with animal models of addiction, but rats will not readily self-administer saline.

Hypothesis 2: After 1 week of abstinence, Fentanyl/SA rats will exhibit a suppressed response of the inflammatory cytokines IL-1β, TNF-α, and IL-6 relative to Saline/SA rats.

Hypothesis 3: Suppressed immune responses of Fentanyl/SA rats at one week will recover after 1 month of abstinence to be no different from that of Saline/SA rats.

The interaction of opioid abuse and immune system functioning is not yet fully understood, partially due to the current techniques used to study opioid-induced immunomodulation such as non-contingent experimenter-administered opioid administration and *in vitro* measures of immune system activity. Compounding these concerns, it is known that individual subjects differ with respect to their preferred drug level such that too much or too little drug can be aversive (Barker et al., 2014) and, in turn, act as a significant stressor on the CNS and immune system. This project addresses

these concerns by focusing on opioid-immune interactions in a model with substantial translational potential, employing a well-established model of SA that more accurately depicts human addiction and allows each animal to achieve and maintain its individual preferred drug level.

METHODS

A two-group (Drug/Vehicle) × 2 time point (1 week/1 month) design will be employed. The general experimental design will involve self-administration of fentanyl or saline, followed by an abstinence period of 1 week (7 days) or 1 month (30 days). At the end of these abstinence periods, rats will be treated with LPS for assessment of plasma and spleen cytokine levels. *Figure 1* summarizes the overall design and experimental protocol.

Animals and Group Allocation

Twelve male and 2 female Sprague Dawley rats (Charles River, Wilmington, MA) were trained to self-administer the opiate receptor agonist fentanyl HCL (dose = 2.5 μg/kg per i.v. infusion) for 30 consecutive days. Eleven age matched control rats (10 male and 1 female) were catheterized and self-administered saline throughout this 30-day phase of the experiment. These animals served as controls for surgery and environmental manipulation. Rats were singly housed on a 12:12 light:dark cycle. Prior to surgery, rats were allowed to reach adult weight (350g for males, 250g for females) and maintained at this weight thereafter, to avoid addition of fat tissue.

Catheterization Surgery

Animals were anesthetized with a ketamine/xylazine (K/Xyl) mixture (50mg/kg; I.P) and given an injection of atropine (10mg/kg; I.P.) to decrease fluid buildup in lungs and prevent respiratory arrest. Anesthesia was monitored and maintained throughout

surgery by intermittent K/Xyl injections. During surgery, all animals were chronically implanted with an intravenous catheter in the right jugular vein. This catheter was threaded subcutaneously and exited at the scalp where it was secured to the skull via a j-shaped stainless-steel cannula attached to the skull using dental cement and jewelers screws. Following surgery, animals were housed in a self-administration operant chamber at all times for the entirety of the SA experiment. Animals were given one week to recover from surgery before starting SA, during which time they received once daily IV infusions of antibiotics and NSAID pain reliever (Rimadyl and Baytril). Patency of catheter lines was maintained with a saline infusion every 25 minutes via a mechanical pump and timer. This saline infusion persisted every 25 minutes from the day of surgery until the end of the experiment, during overnight hours when drug was not available. Animals received water ad libitum and received enough food to keep males at 350g and females at 250g throughout the duration of the experiment.

Self-administration Apparatus

The clear Plexiglas chamber in which animals were housed included a corner with a fixed 6-photocell device used to monitor and record head movements (Root et al., 2011). Drug or saline was administered only when a correct operant response was made in this corner. A correct operant response consisted of breaking photocells 2 and 3 in succession within 1 second. All rewarded responses (RR) and unrewarded responses (UR) were recorded. The Plexiglas chamber was housed within a ventilated, sound-attenuating outer shell.

SA: Fentanyl or Saline

The SA session (6 hours/day, 7 days/week) was conducted using the long-access model of Ahmed and Koob (1998), which models human addiction, including escalation of intake and persistent increase in the motivation for drug taking (Ahmed, 2000; Lenoir et al., 2012). SA sessions ran each day for 30 consecutive days, starting at light onset of a 12:12 light:dark cycle. Sessions automatically ended upon completion of 6 hours. During the session, drug was available during the entire 6 hours on a FR1 reinforcement schedule. A correct response turned on the pump and automatically dispensed a 0.8μg per 0.075mL solution of intravenous fentanyl through the surgically implanted catheter over 2.5 seconds (males) or 0.56 μg fentanyl per 0.0525mL infusion over 1.75 seconds (females), for an average infusion dose of 2.4 μg/kg. This correct response was defined as a RR. A RR immediately triggered a 40 second inter-trial-interval timeout as a precautionary measure to prevent overdose, but all responses made during this time were recorded as URs. Both RRs and URs were considered drug-seeking responses. Both fentanyl and saline SA followed this same procedure. Animals were randomly assigned to SA fentanyl or saline. Saline/SA animals received an equivalent volume of 0.075mL saline over 2.5 seconds for males or 0.0525mL over 1.75 seconds for females, on the same FR1 schedule.

LPS Challenge Injection and Sample Collection

Following 30 days of either fentanyl or saline SA, animals from each group were randomly assigned an abstinence period of 7 days or 30 days. These time points were chosen based on data from previous studies that suggest overt withdrawal signs occur and

conclude within 4 days, and immune suppression persists for at least 6 days (Rahim et al., 2002; Bruijnzeel et al., 2007; Bruijnzeel et al., 2006). The choice of the 7-day sampling time point ensured that the animals were no longer experiencing withdrawal at the time of sample collection to eliminate any withdrawal-related stressors. The 7-day abstinence group remained in the experimental chamber throughout the course of abstinence during which time they continued to receive around the clock saline infusions every 25 minutes to preserve catheter patency. The 30-day abstinence group remained in the experimental chamber for 1 day of abstinence (to allow for recordings of ultrasonic vocalizations during abstinence); they were then moved to a colony room where they remained singly housed in a home cage for the remainder of the 30 days. On the last day of abstinence, all animals received an injection of LPS (1.5mg/kg; I.P.). No saline I.P. injection groups were necessary because saline does not readily produce generation and secretion of cytokines from immune cells.

Six hours after LPS injection, all animals from both 7- and 30-day abstinence groups were deeply anesthetized with a K/Xyl mixture at which time 1- 1.5mL of cardiac blood was collected immediately prior to perfusion and fixation of the brain. The spleen was collected at this time as well. These time points were chosen because plasma TNF- α levels peak 2 hours after antigenic challenge, while plasma IL-1ß and IL-6 levels peak 6 hours after antigenic challenge. Anesthesia used for perfusion did not affect circulating cytokine levels because animals were quickly sacrificed after being anesthetized; detectable cytokine levels generated by LPS-activated macrophages require hours to accumulate in plasma. Blood samples were immediately centrifuged after collection at

2000 rpm for 10 minutes in a refrigerated centrifuge. The resulting supernatant plasma was collected and stored for later cytokine measurement. The spleen was immediately frozen and later homogenized for cytokine measurement as well. Following spleen and plasma collection, animals were transcardially perfused using 0.9% PBS followed by 4% paraformaldehyde. Brains were harvested and post-fixed in 4% paraformaldehyde for 48 hours and subsequently submerged in a 30% sucrose solution for additional analyses.

ELISA Cytokine Assessment

Blood and homogenized spleens were centrifuged at 2000 rpm for 10 minutes in a refrigerated centrifuge. The resulting plasma supernatant was collected and aliquoted for cytokine assay by sandwich ELISA which assayed for IL-1ß and TNF-α according to manufacturer's instructions (R&D Systems).

Analyses

Data were analyzed using Prism-GraphPad and SPSS software. Behavioral Measures included (i)number of infusions/session (*Figure 2*), (ii) average drug level (mg/kg) maintained during SA (*Figure 4)* and (iii) slope of escalation of intake (*Figure 2).* Immune measures included concentration of IL-1ß and TNF-α in collected spleen samples (*Figure 3)*. Saline/SA and Fentanyl/SA groups were compared with respect to all measures except drug level.

Drug Level Calculation: Assuming first order kinetics and a one compartment model of drug absorption, individual second-by-second drug levels (mg/kg) were determined using the equation $Bn = (Bn\text{-}1 + D)e^{-KTn}$ (Yokel & Pickens, 1974), where Tn = elapsed time since previous fentanyl infusion (mins), D = infusion dose (mg/kg), Bn-1 = calculated fentanyl level at the time of last infusion (mg/kg), and K = a rate constant (0.693/half-life) representing a plasma half-life for fentanyl of 7.9mins (Hug & Murphy, 1981) (*Figure 4).*

Escalation of Intake: For both Fentanyl SA and Saline SA, total number of reinforced responses (RR) was regressed over session (1-30). The analysis was conducted using a simple linear regression, where RR was defined as the dependent variable and session was defined as a continuous independent variable. For Fentanyl/SA only, an additional linear regression was performed where total fentanyl intake was regressed over session (1-30), where intake was defined as a dependent variable and session was defined as a continuous independent variable. To incorporate body weight into the calculation of total fentanyl intake, the following equation was used: intake = (#RR × ug fentanyl per infusion) / body weight (*Figure 2).*

Cytokine Concentration: Two separate multivariate analyses of variance (ANOVA) were used to analyze cytokine concentrations where SA group and abstinence period were the 2 factors, and IL-1ß and TNF-α concentrations were the dependent variables. Due to small sample sizes (see next paragraph), partial eta^2 values were calculated and used in place of statistical significance to estimate the effect the factors have on the

analysis and to predict what results will be significant once the full data set can be assayed. The guidelines for partial eta^2 and effect size are: small = .01, medium = .09, large = .25.

RESULTS

Given the circumstances surrounding the COVID-19 pandemic, a full data set was not available to conduct the originally proposed analyses. Prior to laboratory shutdowns, 25 animals completed 30 days of either fentanyl or saline self-administration and a subsequent abstinence period, and plasma and spleen samples were collected upon perfusion, frozen, and stored for future assay; this constitutes a full dataset that would have been properly powered to reach statistical significance. It was fully intended to assay all the collected samples and conduct a full analysis of the results for completion of this thesis. However, laboratory shutdowns interrupted the ongoing cytokine assays resulting in insufficient sample sizes for the planned statistical analyses investigating opioid-induced modulation of immune activity. We were not able to perform the IL-6 assay before shutdown and thus no data were obtained to analyze this cytokine. However, concentrations of the 3 pro-inflammatory cytokines examined in this study correlate to one another (Neta et al., 1992) and it is expected that any suppression and recovery of IL-1ß and TNF-α identified in our data would correspond to a similar suppression and recovery among IL-6 levels. Therefore, the cytokine results presented in this document are representative of what is expected to be found once laboratory work can be resumed and the remaining samples can be assayed.

Behavioral Results

Acquisition of fentanyl SA: increased fentanyl intake, but not saline, over days

Animals self-administered fentanyl in a manner consistent with animal models of substance use disorder, in which escalation of intake is a key marker of addiction (Ahmed, 2011). Average reinforced responses (#RR) was plotted against SA session (session, 1-30) for both Fentanyl/SA and Saline/SA animals (*Figure 2)*. A simple linear regression revealed that the slope of the line for Fentanyl/SA (0.2947) was significantly different from 0, $F(1, 519)=3.935$, $p=0.0478$, identifying escalation of RRs over time. Saline/SA intake did not escalate over time as indicated by a slope that did not differ from 0, ($F(1, 355)=1.775$, $p>0.05$. The slope of Fentanyl/SA escalation was also significantly different from the slope of Saline/SA intake, $F(1,874)=4.270$, $p=0.0391$. Accounting for body weight, a linear regression similarly identified escalation of fentanyl intake over days, $F(1,28)=6.397$, $=0.0173$ (*Figure 2)*.

Cytokine Results

IL-1B and TNF-α concentrations were initially suppressed by fentanyl SA, but recovered after a period of abstinence

Two separate multivariate ANOVAs were conducted to investigate whether fentanyl SA suppressed immune activity (as measured by IL-1ß and TNF-α concentrations) after 7 days of abstinence and if this suppression would recover after 30 days of abstinence. Analyses revealed a large effect size for the interaction of SA

Treatment x Abstinence Period for both IL-1ß concentrations (partial eta^2=0.277; *Fig. 3a*) and TNF-α concentrations (partial eta^2=0.445; *Fig. 3b*). These results suggest that the interaction between SA Treatment x Abstinence Period for both IL-1ß and TNF-α accounted for 27.7% and 44.5% of the group differences plus associated error variance, respectively. Thus, it is predicted that these same analyses performed on the full data set would yield significant results showing an initial suppression of IL-1ß and TNF-α at 7 days of abstinence for Fentanyl/SA animals that would recover after 30 days of abstinence.

Fentanyl dose-dependently suppressed immune system activity

Animals maintain individual preferred drug levels to avoid aversive drug effects and withdrawal symptoms (Barker et al., 2014). It is also widely reported that a drug can dose-dependently affect immune activity. Drug levels were calculated throughout the duration of each SA session for all Fentanyl/SA animals revealing differences in each animal's average drug level maintained over 30 sessions (*Figure 4).* Once all samples can be assayed, it is intended to use these drug level differences to investigate what effect, if any, drug level has on cytokine concentration. Using the one high-intake Fentanyl/SA animal and one low intake Fentanyl/SA animal available in our dataset, it appears that there may be a dose-dependent effect for IL-1ß, but not TNF-α, concentrations after 7 days of abstinence from drug (*Figure 5).* Saline/SA animals do not show this same suppression.

DISCUSSION

The results presented here suggest that chronic fentanyl SA initially suppresses the pro-inflammatory cytokines IL-1ß and TNF-α after 7 days of abstinence from drug. This initial suppression appears to recover after a period of 30 days of abstinence. These results, gathered for the first time using a translatable SA model of addiction and *in vivo* measures of immune system functioning, are consistent with and support previous reports that find immune suppression using experimenter-administered drug and *in vitro* methods (Molina-Martinez et al., 2014; Filipczek-Bryniarska et al., 2012; Rahim et al., 2002), reinforcing their validity.

Addiction is characterized by compulsive drug seeking, escalation of intake over days, and the emergence of a negative affect during withdrawal. Escalation of intake has widely been accepted as the most suitable characteristic for modeling drug addiction in nonhuman animals due to its objectivity, presence among all drugs of abuse, and ability to be operationalized in laboratory animals (Ahmed, 2011). This was used as a measure to validate the present model and, as expected, it was found that escalation occurs for fentanyl SA, but not saline SA, identifying a key behavioral difference between the two SA groups. Fentanyl/SA animals readily self-administered drug in an escalating manner, consistent with the presence of addiction (Ahmed, 2011), as well as with other opioid SA studies (Ahmed & Koob, 2000; Lenoir et al., 20120).

The long-access model of Ahmed and Koob (1998) used here best replicates human drug abuse because it allows for escalation of intake over time (compared to the short access model which does not develop escalation) and is a highly replicable phenomenon. Additionally, it enables each individual animal to maintain their preferred drug level (*Figure 4),* consistent with human drug use. Allowing animals to titrate their preferred drug level avoids the excessive doses often employed with experimenter-administered drug studies that are above the animal's preferred level, which can be highly aversive (Barker et al., 2014). This is particularly critical in studies investigating drug-mediated immune effects because an aversive drug level would likely be stressful to the animal. HPA axis activation during times of stress releases increased levels of glucocorticoids into circulation which directly suppresses immune system activity (Al-Hashimi et al., 2013). For this same reason, the present samples were collected after the conclusion of withdrawal, eliminating conflicting variables and ensuring that any change in immune activity was a result of chronic fentanyl use.

The pro-inflammatory cytokines presented here were initially suppressed in response to the endotoxin LPS after 7 days of abstinence, consistent with previous literature reporting suppression of immune activity lasting at least 6 days after the onset of spontaneous withdrawal from opioids (Rahim et al., 2002). Extending this line of evidence, we found that 30 days of abstinence is enough time to allow for recovery such that IL-1ß and TNF-α levels of Fentanyl/SA animals were similar to those of Saline/SA controls. This suggests that although chronic opioid abuse initially suppresses neuroimmune activity, recovery is eventually possible. These results are the first reports of cytokine activity

fully recovering after initial opioid-mediated suppression. Though sample sizes were low, the large effect sizes found indicate that the variance present in the data is likely attributable to the factors of SA Treatment and Abstinence Period, and not just attributable to chance. Thus, it is expected that performing the same analyses with a full sample will produce statistically significant results, i.e., an initial suppression of IL-1ß and TNF-α concentrations in Fentanyl/SA animals eventually recovers to levels similar to Saline/SA control concentrations. Though no IL-6 data were analyzed, it is also expected that IL-6 concentrations would similarly be suppressed after 7 days of abstinence and then recover after 30 days of abstinence because this cytokine tends to be consistent with the changing concentrations of IL-1ß and TNF-α (Neta et al., 1992). Analyzing the full sample will also allow for post-hoc analyses and examination of whether immune suppression correlates with drug level differences.

Previous literature has found that opioid mediated effects on neuroimmune functioning are largely dose dependent (Brebner et al., 2000; Plein & Rittner, 2018), and we expect to observe this when the full data set is analyzed and fentanyl intake differences can be identified. When considering each animal's individual average drug level across all 30 sessions (*Figure 3*), there is a wide range of different drug levels consumed between animals that is consistent with experimenter-administered studies that identify "high" and "low" levels of systemic fentanyl (Liu et al., 2008; Bruijnzeel et al., 2007). Our SA model generated drug levels that are consistent with these experimenter-administered doses, allowing our ongoing analyses to have a reference for designating certain DLs as high or low. Applying these drug levels to the present samples available for analysis, it

appears that a high DL over the course of 30 days of SA may correlate with increased IL-1ß (but not TNF-α) concentrations compared to low DL at the 7 day abstinence timepoint. This is a preliminary observation that may not hold true when the entire data set can be analyzed; proper drug level analyses on a complete data set will yield more insights into whether a dose-dependent fentanyl-mediated modulation of neuroimmune activity obtains.

A compromised immune system introduces the risk of chronic infections and resulting persistent inflammation, both in the periphery as well as in the CNS (Skaper et al., 2018). Chronic drug exposure results in neuroadaptations that induce deficits in reward processing and lower the threshold for resilience to stress (Koob, 2013; Koob & Volkow, 2016). Therefore, it is conceivable that immunologic stressors (viz., endogenous cytokine elevations) and subsequent immune activation may increase susceptibility for relapse to drug-seeking behavior. It is expected that there will be recovery of the peripheral cytokine response after prolonged abstinence from fentanyl SA, and this may render animals increasingly vulnerable to relapse induced by LPS. Proinflammatory cytokines are neuromodulatory and induce anxiety-like and depressive-like symptoms in both animals and humans known as sickness behaviors (Anisman et al., 2005; Kiecolt-Glaser et al., 2015). These conditions, produced by the behavioral impact of peripheral and central inflammation, may induce a negative affective state. In an addicted individual, this may engender a predisposition for relapse to opioid use as a form of self-medication (Volkow, 2004) due to heightened vulnerability to stressors during withdrawal and abstinence, despite immune recovery. Thus, the need to understand the contribution of

pro-inflammatory cytokines to relapse is imperative and may pose as a therapeutic target for treatment with anti-inflammatory drugs at appropriate stages of abstinence, representing a viable approach to minimizing relapse rate.

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APPENDICES

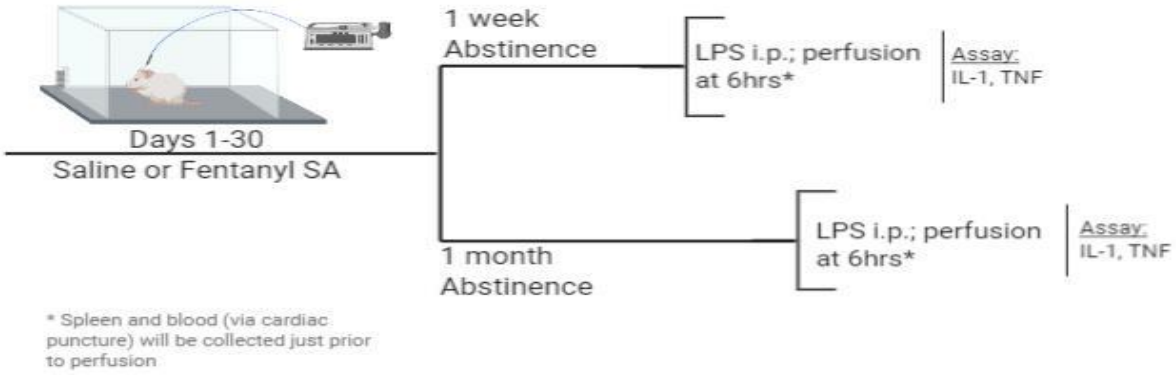

Figure 1: The overall design and experimental protocol for this experiment.

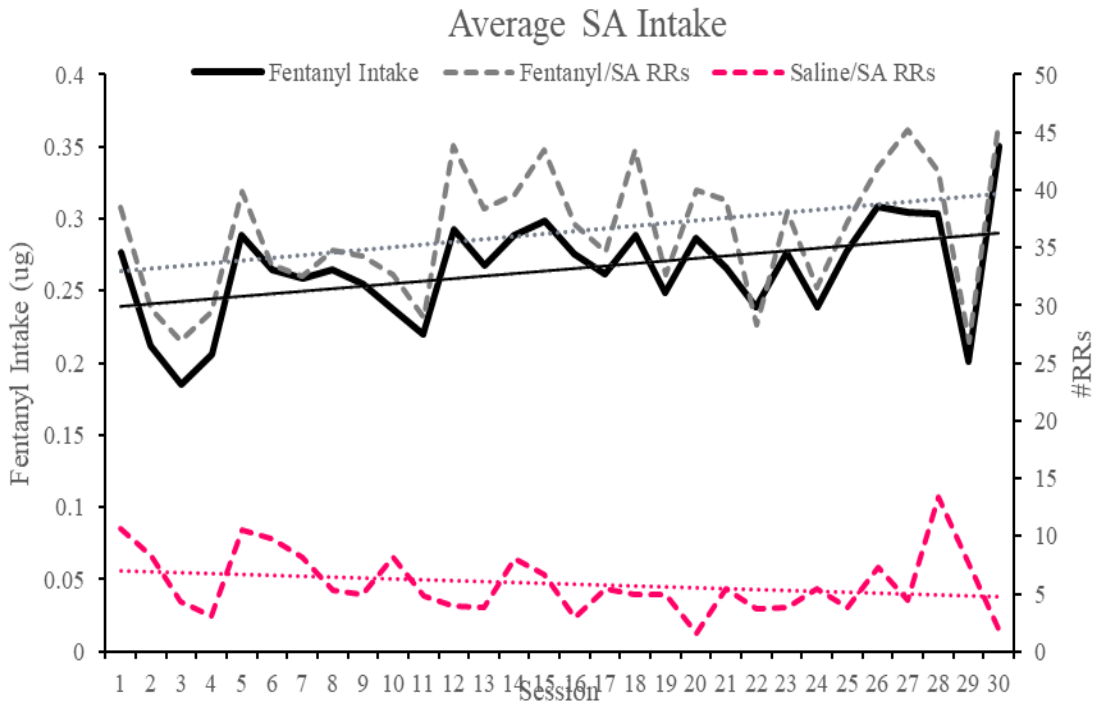

Figure 2: Escalation of intake was identified for Fentanyl/SA but not Saline/SA.

Fentanyl intake could be predicted by session using the following formula:

Intake=0.0.001772(session)+0.2373, R^2=0.1860.

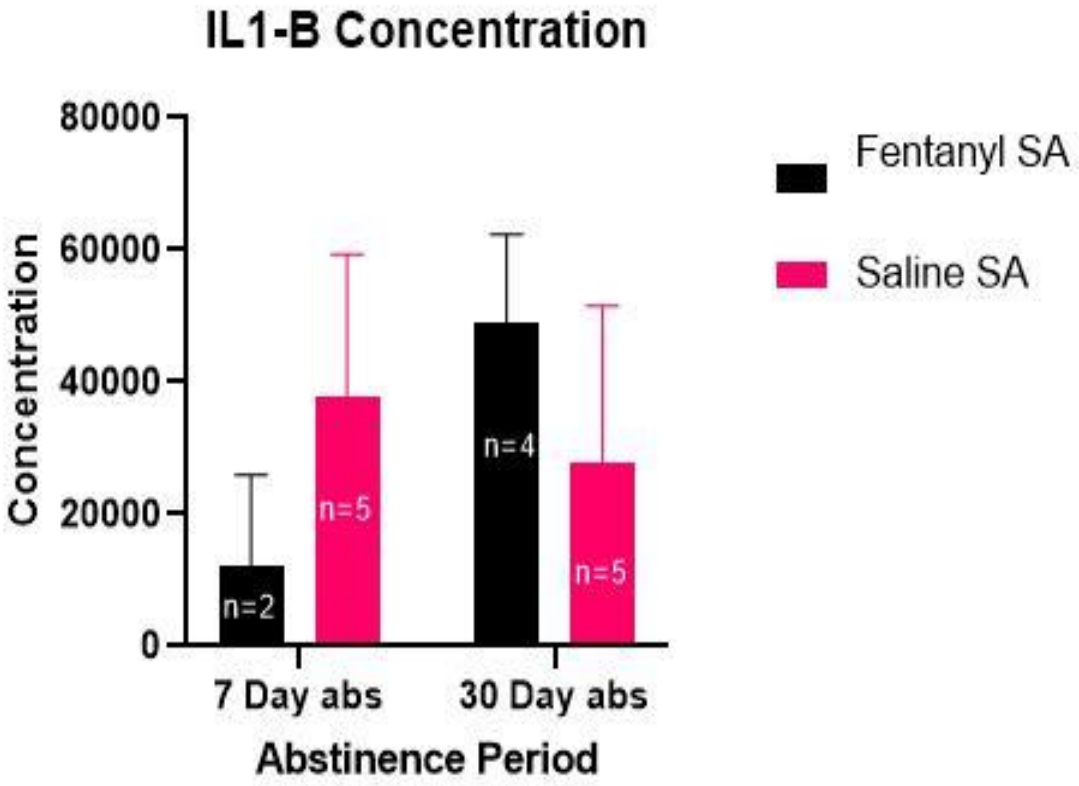

Figure 3a: IL-1B concentrations were initially suppressed following 7 days of abstinence from Fentanyl SA; this suppression recovered to Saline/SA control levels after 30 days of abstinence. Partial eta^2=0.227.

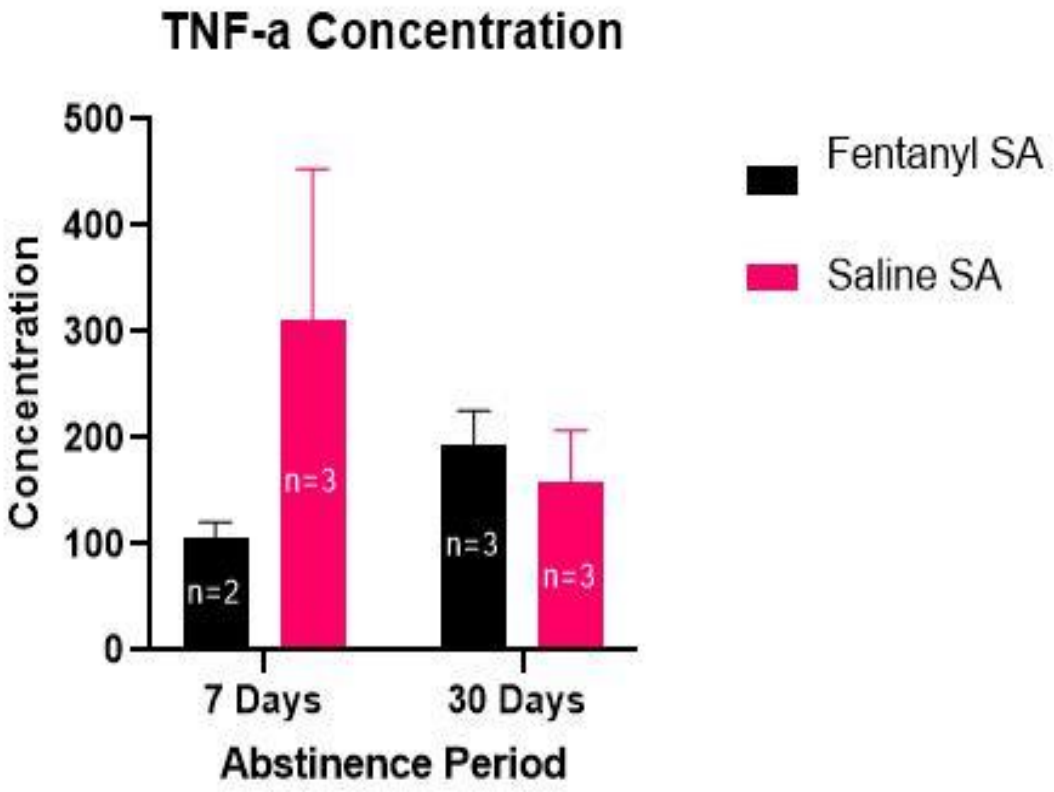

Figure 3b: TNF-α concentrations were initially suppressed following 7 days of abstinence from Fentanyl SA; this suppression recovered to Saline/SA control levels after 30 days of abstinence. Partial eta^2=0.445.

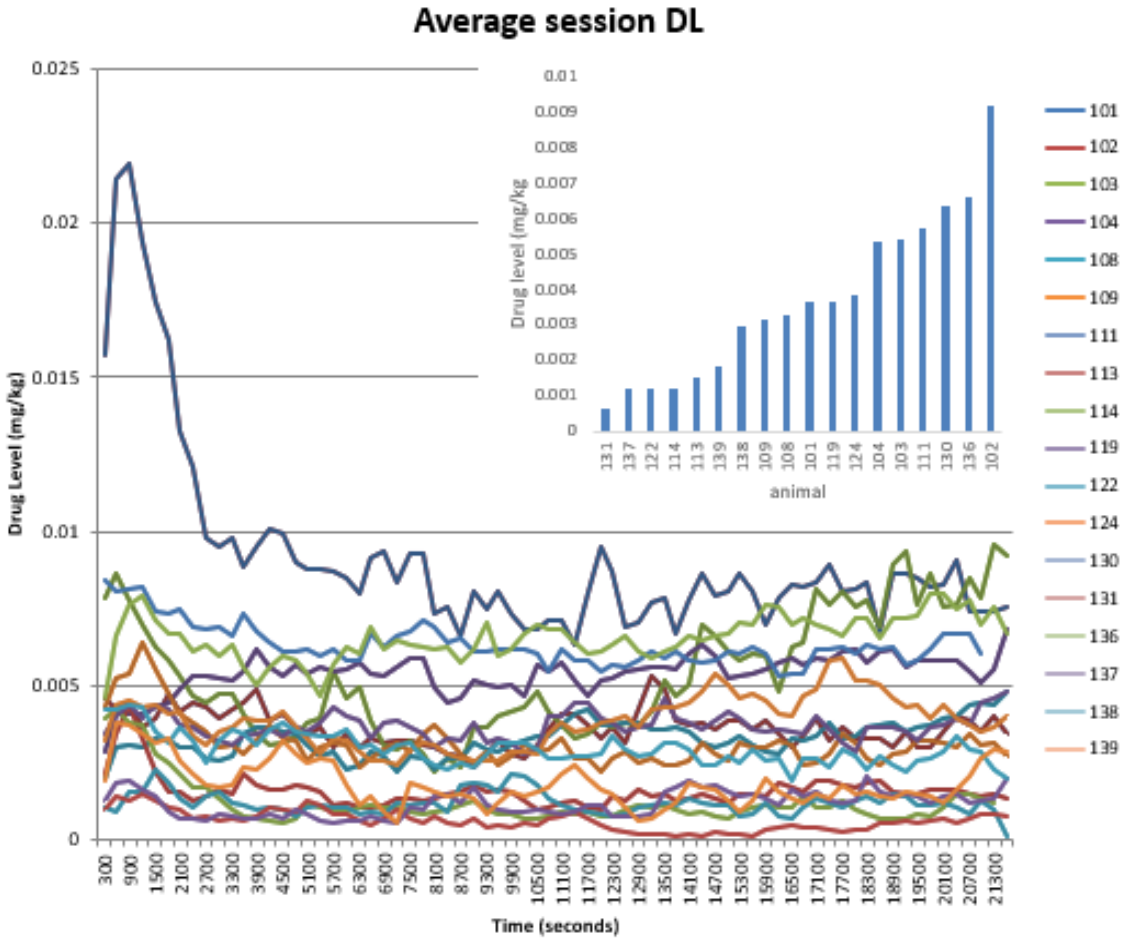

Figure 4: Average session DL for each individual animal was calculated by averaging second by second DL over the course of each session for all 30 sessions. *Inset:* Average amount of fentanyl consumed (mg/kg) in a single session, averaged over all 30 sessions for each animal.

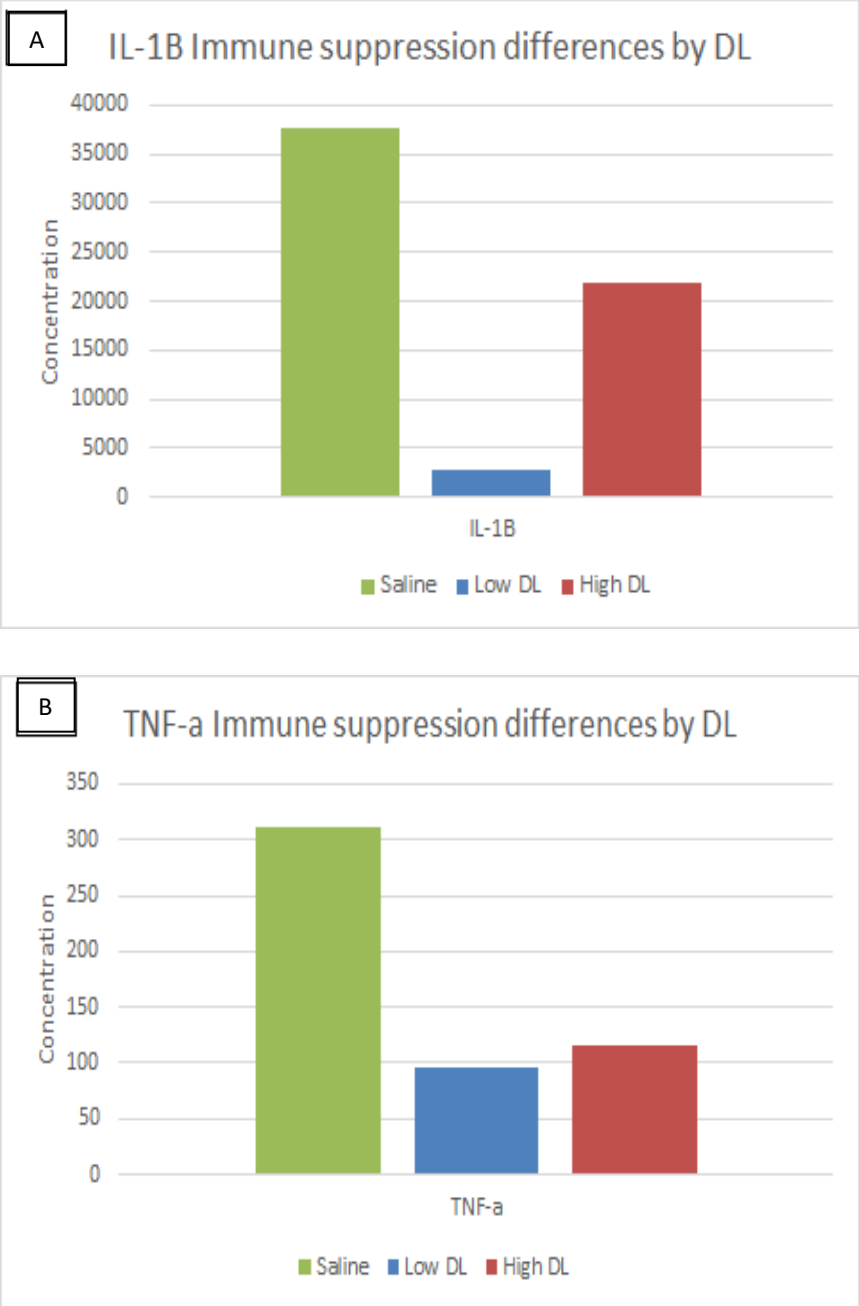

Figure 5: Drug level differences in immune suppression. *A)* Low DL correlates to lower IL-1B concentrations than high DL. *B)* No dose-dependent effects were found regarding TNF-α concentrations.