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3,4-Methylenedioxymethamphetamine (MDMA) alters acute gammaherpesvirus burden and limits Interleukin 27 responses in a mouse model of viral infection

Daniel A. Nelson^a, Sam J. Singh^a, Amy B. Young^a, Melanie D. Tolbert^a, and Kenneth L. Bost^{a,*}

^a Department of Biology, University of North Carolina at Charlotte, 9201 University City Blvd., Charlotte, NC 28223, USA

Abstract

Aims—To test whether 3,4-methylenedioxymethamphetamine (MDMA, “Ecstasy”) abuse might increase the susceptibility, or alter the immune response, to murine gammaherpesvirus 68 (HV-68) and/or bacterial lipopolysaccharide.

Methods—Groups of experimental and control mice were subjected to three day binges of MDMA, and the effect of this drug abuse on acute and latent HV-68 viral burden were assessed. In vitro and in vivo studies were also performed to assess the MDMA effect on IL-27 expression in virally infected or LPS-exposed macrophages and dendritic cells, and latently infected animals, exposed to this drug of abuse.

Results—Acute viral burden was significantly increased in MDMA-treated mice when compared to controls. However the latent viral burden, and physiological and behavioral responses were not altered in infected mice despite repeated bingeing with MDMA. MDMA could limit the IL-27 response of HV-68 infected or LPS-exposed macrophages and dendritic cells in vitro and in vivo, demonstrating the ability of this drug to alter normal cytokine responses in the context of a viral infection and/or a TLR4 agonist.

Conclusion—MDMA bingeing could alter the host’s immune response resulting in greater acute viral replication and reductions in the production of the cytokine, IL-27 during immune responses.

Keywords

3, 4-methylenedioxymethamphetamine; Ecstasy; gammaherpesvirus; IL-27

*Corresponding author at: Department of Biology, University of North Carolina at Charlotte, 9201 University City Blvd., Charlotte, NC 28223, USA. Tel: +1 704 687 8677; fax +1 704 687 3128 klbost@uncc.edu.

Contributors

D. A. Nelson, S. J. Singh, A. B. Young, M. D. Tolbert and K. L. Bost each participated in performing selected assays for this publication and organizing the data into a form suitable for publication. All authors contributed to the experimental design, data interpretation, and participated to some extent in writing the manuscript. All authors approved the final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest.

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1. Introduction

Ecstasy, or 3,4 methylenedioxymethamphetamine (MDMA), is currently one of the most popular club drugs of abuse, with abuse being especially prevalent in young adults (Wu et al., 2010). Our understanding of the dangers of MDMA abuse has focused primarily on the acute physiological effects (Badon et al., 2002; Green et al., 2003) and on the short term and long term neurological consequences, of abusing this drug (Capela et al., 2009; Lyles and Cadet, 2003). There is a solid rationale for such studies, since the acute physiological effects can be life threatening, and since there are clearly deleterious effects of MDMA abuse on central nervous system function.

However, less consideration has been given to the consequences that MDMA abuse might have on the immune response against infectious diseases (Connor, 2004). In general, drug abusers have long been known to be more susceptible to infectious diseases (Friedman et al., 2003; Matta et al., 1995), however the exact mechanisms for such increased susceptibility as a result of such drug abuse are not always clear. While we suspect that there is a general link between infection and club drug abuse, there have been few studies to specifically investigate whether Ecstasy might predispose young adults to microbial infections. One survey of MDMA abusers suggested that there was a perceived correlation between MDMA and increased numbers of infections (Parrott et al., 2002). Despite such suspicions, few studies have been carried out to establish a deleterious cause and effect relationship between this drug of abuse and microbial infections.

The lack of such studies is somewhat surprising since Ecstasy is one of the most commonly abused drugs by young adults, and since club drug abuse within this population contributes to the very social behaviors that would increase the likelihood of exposure to a variety of transmissible infectious diseases. Teenagers and young adults are the age group most likely to abuse MDMA (Wu et al., 2010), and use of this drug leads to elevated moods, extroversion, and enhancement of sensual experiences (Freese et al., 2002). The use of MDMA at parties and at “raves” appears to promote close-contact behaviors between individuals (Freese et al., 2002; Roper-Miller and Goldberger, 1998) that would favor transmission of infectious diseases.

Mononucleosis (Cohen, 2000) is a disease often seen in young adults, and this incidence correlates with an increase in socialization (i.e. dating, parties, etc.) in this age group. One behavioral effect of Ecstasy abuse is to stimulate the very social interactions (Freese et al., 2002; Roper-Miller and Goldberger, 1998) which might facilitate the spread of such pathogens. Mononucleosis is caused by the gammaherpesvirus, Epstein Barr virus (EBV), and has been called the “kissing disease” since this viral infection can be transmitted following close contact between individuals. While this common viral infection is seldom life threatening, the immune status of the individual influences the severity and length of the acute illness. Furthermore, upon recovering from mononucleosis, EBV becomes latent and persists for the life of the individual. During later life, EBV can contribute to the development of malignancies (Thompson and Kurzrock, 2004), can be life threatening for immunosuppressed patients (Holmes and Sokol, 2002; McClain, 2001), and may contribute to the severity and development of periodontal diseases (Contreras and Slots, 2000) or autoimmune diseases (Mockridge et al., 2004). Whether the use of MDMA can contribute to the severity of EBV infections in young adults has not been addressed, and would be extremely difficult to investigate due to concern over the ethical nature of such studies (O’Leary et al., 2001; Vollenweider et al., 1999).

Fortunately, there are some excellent animal models, which can be used to address MDMA-altered immune responses during infection. We have used a mouse model of

gammaherpesvirus infection to test whether Ecstasy abuse might increase the susceptibility, or alter the immune response, to this viral infection. Murine gammaherpesvirus 68 (HV-68) has been used as a mouse model for EBV and human herpesvirus 8 infection (Doherty et al., 1997; Simas and Efstathiou, 1998), and is the only well-characterized rodent model for studying gammaherpesviruses. Upon intranasal or oral inoculation in mice (Elsawa and Bost, 2004; Peacock and Bost, 2000), there is a productive infection of epithelial cells, followed by infection of B lymphocytes, and also macrophages and dendritic cells. A marked leukocytosis (i.e. mononucleosis) and splenomegaly occurs, which peaks around days 15 post-infection. Given an appropriate stimulus (Moser et al., 2005), gammaherpesviruses can emerge from latency, resulting in a productive infection and re-establishment of latency. This virus cycle ensures that the host remains infected for life. The pathophysiology of murine gammaherpesvirus 68 closely mimics that observed for EBV infections (Doherty et al., 1997; Simas and Efstathiou, 1998), making it an excellent model to investigate such viral infections.

In the present study, we demonstrate that exposure of mice to MDMA can affect the acute, infectious viral burden, however no significant alterations were observed in levels of latent virus. MDMA-induced alterations in the cytokine response of HV-68 infected macrophages and dendritic cells were also observed, and this study focused on defining alterations of one member of the IL-12 family of cytokines, IL-27.

2. Methods

2.1 Acute and chronic MDMA exposures

Racemic 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) was provided by the National Institute of Drug Abuse (NIDA; Research Technology Branch, Research Triangle Park, NC). A stock solution of MDMA was prepared at 1.6 mg/ml in sterile PBS and administered intragastrically at 20 mg/kg.

Mice were exposed to acute and chronic MDMA bingeing protocols to assess drug effects on viral burden and cytokine responses. The rationale for selecting such exposure regimens came from a review of the literature demonstrating the common practice of bingeing by MDMA drug abusers (Hammersley et al., 1999; Pacifici et al., 2002), and from recent reports in the literature demonstrating effects of similar drug exposure regimens on acute alpha herpesvirus infections (Pennock et al., 2009; Zwick et al., 2005). Our goal in designing these experiments was to mimic situations that might actually occur for young adults who abuse this drug while engaging in behaviors that would expose them to viruses like EBV. Specifically, the short-term or “acute” protocol exposes mice to three consecutive days of MDMA with HV-68 infection, which might reflect a long weekend of Ecstasy abuse (e.g. Thursday, Friday, and Saturday nights). The extended or “chronic” protocol has multiple three day exposures to MDMA separated by one week prior to HV-68 infection, which might represent several consecutive weekends of drug abuse by young adults. Obviously, there are enumerable protocols which might be proposed for exposing mice to MDMA, but we designed two likely scenarios which reflect a very plausible series of events which might occur.

The concentration of MDMA used in these *in vivo* studies (i.e. 20 mg/kg) was selected based on its ability to induce clear physiological and behavioral responses in mice, and previous studies by other investigators who have used similar concentrations of drug in their rodent studies (Baumann et al., 2007; Baumann et al., 2009; Green et al., 2009).

2.2 LPS

Phenol-water purified *Salmonella typhimurium* lipopolysaccharide (LPS) was purchased from Sigma-Aldrich and contained less than 1% protein as reported by the manufacturer. Aliquots of LPS at 2 mg/ml in sterile PBS were stored at -80°C and diluted in sterile PBS for both in vitro and in vivo experiments. For in vivo experiments, LPS was administered intraperitoneally at 50 $\mu\text{g}/\text{mouse}$.

2.3 Animals

Six to eight week old female C57BL/6 mice (18–22 g) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the vivarium in filter top cages containing sterile bedding. After arrival, mice were quarantined for at least five days, and fed chow and water *ad libitum*. All studies were performed according to the Guide for Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Experimental protocols were approved by the Animal Care and Use Committee at the University of North Carolina at Charlotte.

2.4 HV-68 propagation, isolation, infection and assay for reactivation

Murine gammaherpesvirus-68 (HV-68; American Type Culture Collection # VR-1465) stocks were prepared by infecting baby hamster kidney cells (BHK-21; American Type Culture Collection # CCL-10) at a low multiplicity of infection, followed by preparation of cellular lysates, as described previously (Elsawa and Bost, 2004; Nelson et al., 2010; Peacock and Bost, 2000). Replicating HV-68 in viral stocks was quantified using 1:3 serial dilution of the cell lysates on BALB/3T12-3 cell (American Type Culture Collection # CCL-164) monolayers, as described previously (Elsawa and Bost, 2004; Nelson et al., 2010; Peacock and Bost, 2000).

For viral infection, mice were anesthetized with 2–3 % isoflurane in oxygen and infected intranasally with 6×10^4 plaque forming units (pfu) of HV-68 in 20 μl sterile PBS.

The presence of lytic virus was quantified by plaque assay (Elsawa and Bost, 2004; Nelson et al., 2010; Peacock and Bost, 2000). Approximately 25 mg of spleen was weighed, homogenized in 0.5 ml PBS and pulse-sonicated for 15 seconds. Samples were serially diluted 1:3 and absorbed onto 3T12-3 cell monolayers for 1–2 hour at 37°C . Wells were overlaid with 0.5% Plaque Assay Agarose (BD Biosciences, San Jose, CA), and incubated for 5–7 days. Cells were fixed with formol saline (4 % formaldehyde, 1 % NaCl) and stained with crystal violet (0.1 % crystal violet, 2 % EtOH) to improve the ability to observe plaques.

Latent virus was quantified using an infectious centers assay (Elsawa and Bost, 2004; Nelson et al., 2010; Peacock and Bost, 2000). For this assay, approximately 25 mg of spleen was passed through a 30-gauge wire mesh to generate single cells. Cells were washed one time with RPMI and red blood cells lysed by resuspension in Red Blood Cell Lysing Buffer (Sigma-Aldrich, St. Louis, MO). Splenocytes were pelleted at $1000 \times g$, washed two times with RPMI and resuspended at 10^7 cells per ml in RPMI plus 10% FBS. Samples were serially diluted 1:3 and plated onto 3T12-3 cell monolayers for 24 hour at 37°C . Wells were overlaid with 0.5% Plaque Assay Agarose (BD Biosciences, San Jose, CA), and incubated for another 5–7 days. Plates were processed and plaques counted as described for the acute viral burden assay.

2.5 Core temperature and locomotor activity

Two weeks after HV-68 infection, BMDS temperature transponders were implanted subcutaneously into mice (Bio Medic Data Systems, Seaford, DE). Baseline temperature

measurements were obtained prior to MDMA bingeing. Thirty days after viral infection, animals were administered MDMA and temperatures measured every thirty minutes for six hours.

One week prior to MDMA bingeing, mice were handled for three days and habituated to a radial six-arm maze for at least five minutes each day to allow animals to become comfortable with the apparatus and to become familiar with extra-maze cues (Wenk, 2004). During the days of MDMA administration, animals were placed in the maze for eight minutes and the number of arm visits determined during this time.

2.6 Isolation of bone marrow cells

Mice were anesthetized with isoflurane and euthanized. Bone marrow cells were isolated by flushing femurs with RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated and filtered fetal bovine serum (FBS), 25 mM HEPES, 4.5 g/L glucose, 1 mM Na pyruvate, 2 g/L NaHCO₃, 50 μ M 2-mercaptoethanol and 25 μ g/ml gentamicin, with the addition of either M-CSF or GM-CSF. After suspension of bone marrow cells by pipetting, bone matrix was allowed to settle to the bottom of a 50 ml conical tube and the pellet discarded. 10 ml aliquots of bone marrow cells were plated into T-25 T-flasks or 6-well plates and incubated in a 5% CO₂ incubator at 37° C.

2.7 Generation of bone marrow-derived macrophages

Bone marrow-derived macrophages in 6-well plates were matured in M-CSF containing medium as previously described (Elsawa and Bost, 2004; Nelson et al., 2008). Cell culture medium was supplemented with 20 % LADMAC cell-conditioned medium as a source of M-CSF (Sklar et al., 1985). After 2 days in culture additional supplemented medium was added and differentiation and proliferation continued for 3 more days. After 5 days, medium and non-adherent cells were discarded and fresh, supplemented medium added to the adherent macrophages. Virtually all cells were CD11b positive (Nelson et al., 2004) and ready for experiments after culturing for 6–7 days.

2.8 Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells were generated as previously described (Nelson et al., 2004). Briefly, medium was supplemented with 5 ng/ml murine rGM-CSF (BD Pharmingen, San Diego, CA) and after 3 days incubation in T-25 T-flasks, an additional 10 ml of medium was added per T-25 flask and the incubation continued for 3 more days. After 6 days, nonadherent GM-CSF-stimulated cells were pelleted at 1000 $\times$ g for 5 min and resuspended in fresh medium containing GM-CSF. Cells were plated into 6-well plates at a density of $\sim 1 \times 10^6$ cells per well, and experiments initiated after 24 hr. As described previously, the bulk of the cells at day 7 appear phenotypically as dendritic cells, and ~ 75 % were CD11c positive.

2.9 Macrophage and dendritic cell activation

Cells in 6-well plates were incubated for 24 hr in the presence or absence of 500 μ M MDMA, and the incubation continued with either *Salmonella typhimurium* LPS, or with mouse gammaherpesvirus-68 (HV-68) as described in the figure legends and previous publications (Bowman and Bost, 2004; Elsawa and Bost, 2004; Gasper-Smith et al., 2006a; Gasper-Smith et al., 2006b). It should be noted that 500 μ M MDMA was selected for use in these studies since this concentration has previously been shown not to affect HV-68 infection in cultured macrophages or dendritic cells and is not toxic to these cells (Nelson et al., 2008).

2.10 Nucleic acid analyses

2.10.1 Isolation of DNA—Five to twenty-five milligrams of tissue or virally-infected cells were suspended in 250 µl of ice-cold phosphate-buffered saline (PBS) and homogenized briefly in 1.5 ml microfuge tubes. An equal volume of PBS containing 0.4% SDS, 10 mM EDTA and 50 µg/ml Proteinase K (Sigma Chemical Co.) was added and homogenates incubated overnight at 37° C. Nucleic acid was extracted 2X with saturated phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 2 volumes of EtOH, and resuspended in PBS with 25 µg/ml RNase A (Sigma-Aldrich). After incubation for 30 min at 37° C, DNA was extracted 1X with phenol:chloroform:isoamyl alcohol, precipitated with 2 volumes EtOH, microfuged for 10 min at 13,000 rpm and washed with 75% EtOH. The DNA pellet was air dried and resuspended in 10 mM Tris, pH 8.0. DNA concentration was determined by absorbance at 260 nm.

2.10.2 Total RNA isolation and synthesis of cDNA—Total RNA was isolated using Trizol (Invitrogen; Carlsbad, CA), as described by the manufacturer. For the analysis of HV-68 viral gene expression, RNA samples were treated with DNase (Promega; Madison, WI) prior to precipitation with EtOH and resuspension in 50 µl of nuclease-free H₂O. RNA concentrations were determined with a Gene Spec III spectrophotometer (Naka Instruments, Japan) using a 10 µl cuvette. For cDNA synthesis, one µg of RNA was reverse-transcribed in the presence of random hexamers (50 ng/µl), 10 mM dNTPs, 2.5 mM MgCl₂ using ImProm-II reverse transcriptase (Promega) in the buffer supplied by the manufacturer. cDNA was precipitated with one-tenth volume of 3 M sodium acetate (pH 5.2) and 3 volumes of EtOH, and resuspended in 50 µl of nuclease-free H₂O.

2.10.3 Real time polymerase chain reaction (RT-PCR) to quantify mRNA expression—p28 and Ebi3 mRNA expression was quantified relative to GAPDH expression using an Applied Biosystems 7500 Fast Real-time PCR System (Foster City, CA). PCR amplifications were performed in a total volume of 10 µl containing QuantiTect SYBR Green PCR Master Mix (Qiagen), primer pairs (0.5 µM), template cDNA (20 ng) and nuclease-free water. Samples were cycled 45 times, starting with an initial activation step of 15 min at 95°C, followed by denaturation at 95°C, annealing at 60°C and extension at 72°C. Real-time PCR primer sets were designed with SciTools from IDT (Integrated DNA Technologies, Coralville, IA). Primer sets used for amplification are as follows:

HV-68 **ORF50** (murid herpesvirus 4; accession no. NC 001826; 355 bp):

Forward: 5' – ATG GCA CAT TTG CTG CAG AAC – 3'

Reverse: 5' – ACG GCG CCT GTG TAC TCA A – 3'

HV-68 **M3** (murid herpesvirus 4; accession no. NC 001826; 275 bp):

Forward: 5' - TCT CTT GAC CCA GCT CTT CCA CAG - 3'

Reverse: 5' - AGG AGG ATT GGC TGA GAG CCT TAC - 3'

p28 (73 bp – spans exons 4 to 5):

Forward: 5' – GGC CAT GAG GCT GGA TCT C – 3'

Reverse: 5' – AAC ATT TGA ATC CTG CAG CCA – 3'

Ebi3 (126 bp – spans exons 3 to 4):

Forward: 5' – TTG TGG CTG AGC GAA TCA TCA AGC – 3'

Reverse: 5' – TTG AGA GAG AAG ATG TCC GGG AAG – 3'

GAPDH (133 bp – spans exons 5 to 6):

Forward: 5' - CAA TGT GTC CGT CGT GGA TCT GAC – 3'

Reverse: 5' - AGA CAA CCT GGT CCT CAG TGT AGC – 3'

Changes in mRNA expression are presented as mRNA fold-induction, where Fold-induction = $2^{(C_T(\text{Cytokine}) \text{ Control} - C_T(\text{Cytokine}) \text{ Sample})} / 2^{(C_T(\text{GAPDH}) \text{ Control} - C_T(\text{GAPDH}) \text{ Sample})}$ and C_T = the threshold cycle.

2.11 Enzyme-linked immunosorbant Assay (ELISA) to quantify p28 and IL-27

The p28 subunit of IL-27 was quantified using an ELISA (R&D Systems, Minneapolis, MN). The heterodimeric IL-27 (p28 + Ebi3) was quantified by ELISA (eBioscience, San Diego, CA).

2.12 Data Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc. San Diego, CA). Statistical analyses were performed using one-way analysis of variance (ANOVA) and analyzed post-test using Tukey's Multiple Comparison Test. Figures show mean values $\pm$ the Standard Error of the Mean (SEM). Results labeled with an (*) were determined to be statistically significantly different at $P < 0.05$.

3. Results

3.1 MDMA exposure increases acute viral burden in the lungs following HV-68 infection

Groups of mice were given an acute viral infection intranasally, while also receiving MDMA intragastrically. At day 3 post infection, mice binged with MDMA had a significantly higher viral burden in the lungs (Fig. 1A). However this increase was somewhat transient since by day 6 post infection there were no significant differences in infectious virus found in drug-treated mice (Fig. 1B). HV-68 *ORF50* is a viral mRNA species expressed only during acute infection, and has been used a marker to assess viral replication (Wu et al., 2001). Supporting the notion of increased virus replication in the lungs following MDMA exposure were complementary studies demonstrating significant increases in HV-68 *ORF50* mRNA expression (Fig 1C and 1D). We concluded from these studies that even a limited 3 day exposure to MDMA was sufficient to increase the susceptibility of animals to acute HV-68 infection.

3.2 MDMA-induced alterations in physiological and behavioral responses in mice latently infected with HV-68

Gammaherpesvirus replication in epithelial cells is a necessary preamble for the ultimate goal of establishing latency (Doherty et al., 1997; Simas and Efstathiou, 1998). The fact that MDMA exposure increased acute HV-68 replication suggested that mice exposed to this drug might also have alterations in latent viral burden and/or the host response following infection. To address this possibility, groups of mice were acutely infected with HV-68 and 30 days was allowed for infectious virus to abate, and viral latency to become established (Elsawa and Bost, 2004; Peacock and Bost, 2000). Mice were then subjected to two different 3 day MDMA exposure paradigms, including a single three day binge and four weekly three day binges. Splenomegaly is a common physiological response to the host's inflammatory response to viral infection. Though splenomegaly was observed in all HV-68 infected mice (Fig. 2A and 2B), there were no significant differences in those mice that received MDMA. It should be noted that there was a general trend for infected animals exposed to MDMA to have less splenomegaly than mice that were infected, but not exposed to the drug. However such differences never reached a p value < 0.05 .

Similarly, while MDMA exposure resulted in the expected 6 hour weight loss (Fig. 3A) and hyperthermia (Fig. 3B), mice latently infected with HV-68 and exposed to MDMA had similar responses as the animals receiving only the drug (Fig. 3A and 3B). In addition, locomotor activity measured using a six arm maze was not different in infected versus non-infected mice exposed to MDMA (Fig. 3C). Therefore, we found no physiological or behavioral differences in mice latently infected with HV-68 that were drug treated when compared to those receiving MDMA alone.

3.3 Exposure to MDMA does not increase latent viral burden in the spleen

Despite the lack of any demonstrable effect of MDMA on latently infected mice, we considered it likely that latent viral burden would be altered in drug-treated mice based on the results obtained when measuring increases in acute viral burden (Fig. 1). Again, latently infected mice were subjected to two different 3 day MDMA exposure paradigms, including a single three day binge and four weekly three day binges. Surprisingly, no differences in latent viral burden were detected following a single three day binge (data not shown). We reasoned that this limited exposure might not have been sufficient to observe quantifiable differences. However as shown in Figure 4, when latently infected mice were exposed to four weekly three day binges of MDMA, there were no significant differences in latent viral burden when compared to mice infected with HV-68 alone (Fig 4A).

HV-68 *M3* is a viral mRNA species expressed only during latency, and has been used a marker to detect latent virus (Simas et al., 1999). Supporting the notion of similar levels of latent virus in the spleen of MDMA treated mice were complementary studies demonstrating comparable HV-68 *M3* mRNA expression in this peripheral tissue (Fig 4B). We concluded from these studies that even an extensive exposure to MDMA during a 4 week period was insufficient to affect the latent viral burden in mice with an already established gammaherpesvirus infection.

It should also be noted that we found no detectable increase in infectious virus following one week or four week bingeing of latently infected mice (data not shown). This result strongly suggests that exposure to MDMA is not a stimulus which results in a significant reactivation of latent virus to its infectious form.

3.4 Exposure to MDMA reduces HV-68-induced macrophage and dendritic cell p28 mRNA expression, ebi3 mRNA expression, p28 protein secretion, and IL-27 protein secretion

IL-27 is a member of the IL-12 family of heterodimeric cytokines, being composed of the proteins, p28 and Epstein Barr virus gene 3 (Ebi3) protein subunits (Pflanz et al., 2002). Following HV-68 infection, cultured macrophages (Fig. 5) and dendritic cells (Fig. 6) respond by upregulating p28 mRNA expression and by increasing secretion of p28 and IL-27 proteins. MDMA was a potent suppressor of p28 mRNA expression in both cultured macrophages (Fig. 5A) and dendritic cells (Fig. 6A), which translated into significant decreases in the ability of these cells to secrete p28 (Fig. 5C and Fig. 6C) and IL-27 (Fig. 5D and Fig. 6D) proteins.

Several monoamines were tested for their affects on p28 mRNA expression by cultured macrophages (Fig. 7A) and dendritic cells (Fig. 7B). Upon comparison, only MDMA, and not the other trace amines investigated, were able to significantly suppress expression of this mRNA species. These results support the notion that the effects of MDMA are not mediated by trace amine receptors on these cell populations.

3.5 Exposure to MDMA reduces LPS-induced macrophage and dendritic cell p28 mRNA expression and IL-27 protein secretion

The ability of MDMA to decrease p28 mRNA expression and IL-27 protein secretion was not restricted to HV-68 infection. Cultured macrophages (Fig. 8) and dendritic cells (Fig. 9) stimulated with the TLR4 agonist, salmonella LPS, also responded to the presence of MDMA with decreased p28 mRNA expression (Fig. 8A and Fig. 9A) and decreased IL-27 secretion (Fig. 8B and Fig. 9B). These results demonstrate the ability of MDMA to reduce cytokine expression to two very different microbial challenges (e.g. HV-68 infection and bacterial LPS).

3.6 Exposure to MDMA reduces serum p28 and IL-27 levels in HV-68-infected mice after injection of LPS

MDMA-induced alterations in p28 and IL-27 protein production were investigated in mice latently infected with HV-68. When mice were binged for three consecutive days for four weeks, no significant alterations in p28 (Fig. 10A) or IL-27 (Fig. 10B) protein production in sera were noted in control or HV-68 infected animals. Conversely, when HV-68 infected mice were challenged with the TLR4 agonist, LPS, exposure of MDMA could significantly reduce p28 (Fig. 10A) and IL-27 (Fig. 10B) protein production when compared to mice treated similarly, but not infected with HV-68. These results demonstrated that MDMA could alter an *in vivo* immune response by limiting production of this cytokine.

4. Discussion

Early studies did not provide much insight regarding the ability of MDMA abuse to alter the protective immune response against microbial pathogens. Several previous studies assessed the direct effects of MDMA exposure on leukocytes by adding drug to normal cell cultures (Connor, 2004; House et al., 1995). There were surprisingly few effects of this drug on isolated or mixed cells, leading to the suggestion that MDMA did not exert any significant effects by directly interacting with leukocytes in culture (Connor, 2004).

Ex-vivo studies were also performed. Typically, animals (Connor, 2004) or human subjects (Pacifici et al., 2001b) were exposed to MDMA, and then their leukocytes were isolated to evaluate responses to mitogenic stimulation *in vitro*. In drug-treated rats, altered cytokine secretion and altered T cell proliferative responses to mitogens were observed in these cultured cells (Connor, 2004; Connor et al., 2005). Human blood leukocytes isolated from exposed volunteers showed decreased IL-1, IL-6, IL-10, and interferon gamma secretion, and decreased T cell proliferation in response to the mitogen, phytohemagglutinin (Pacifici et al., 2001b).

Finally, *in vivo* studies have been conducted in animals and human subjects. Rat serum levels of IL-1, TNF- α , and IL-10 were altered, as well as early decreases in blood lymphocyte numbers, following *in vivo* exposure to MDMA (Connor, 2004; Connor et al., 2005). Human subjects exposed to MDMA also showed early decreases in blood lymphocyte numbers (Pacifici et al., 2004; Pacifici et al., 2001a; Pacifici et al., 2001b), as well as alterations in natural killer cell numbers following acute (Pacifici et al., 2004; Pacifici et al., 2001a; Pacifici et al., 2001b) and chronic (Pacifici et al., 2002) drug regimens.

Despite the differences observed in these *ex vivo* and *in vivo* studies, no clear mechanism of MDMA-mediated immune modulation has emerged. A general consensus that MDMA exposure was immunosuppressive has been proposed (Connor, 2004). This conclusion is supported by more recent studies using an LPS challenge model (Boyle and Connor, 2007). Using this TLR4 agonist, MDMA could suppress interferon gamma production by increasing IL-10 secretion, possibly via beta-adrenoreceptors (Boyle and Connor, 2007).

While these previous studies seek to define MDMA-mediated immune dysfunction, they do not provide much insight regarding how this drug of abuse might alter protective immune responses against particular pathogens that young adults might encounter.

In the present study, we used a mouse model of gammaherpesvirus infection to directly address the consequences of MDMA exposure on an acute and latent viral infection. HV-68 provides an excellent model to investigate events during acute infection, as well as the establishment of latency (Doherty et al., 1997; Simas and Efstathiou, 1998). We found that even a limited 3 day exposure to MDMA was sufficient to increase the susceptibility of animals to acute HV-68 infection (Fig 1). This result is consistent with a recent report where MDMA increased susceptibility to herpes simplex virus type 2 infection using a mouse model of genital herpes (Pennock et al., 2009). Here the authors demonstrated increased viral burden, as well as an earlier onset of disease in animals exposed to this drug of abuse. These previous studies (Pennock et al., 2009), together with the present work, are significant for at least two reasons. First while HSV-2 and HV-68 are both herpesviruses, HSV-2 is an alphaherpesvirus known for its ability to infect genitalia and establishes latency in neurons (Gupta et al., 2007). HV-68 is a gammaherpesvirus that can induce a systemic mononucleosis-like disease and establishes latency in leukocytes (Doherty et al., 1997; Simas and Efstathiou, 1998). Despite these differences in the initial site of infection, disease progression, and cellular tropism, exposure to MDMA resulted in an increased susceptibility to each of these viruses. These results suggest that some fundamental alterations in the initial anti-viral response could explain increased viral susceptibility. Second, future attempts to define potential mechanisms to explain MDMA-mediated modulation of the host's antiviral response should focus on innate immunity. Mouse models of herpesvirus infection employ naïve mice for infection that have no existing immunological memory against these viruses. Since alterations in susceptibility to acute viral infection were noted within days following inoculation (Fig. 1 and (Pennock et al., 2009)), it is likely that MDMA can alter early events important for defense against herpesviruses.

In an effort to dissect the early, innate immune responses which MDMA might alter, our laboratory has focused on macrophages and dendritic cells as possible targets. Previously, we discovered that cultured mouse macrophages exposed to MDMA and infected with HV-68 had reduced expression of a variety of cytokines and chemokines, while continuing to allow for normal viral gene expression (Nelson et al., 2008). In the present study, we extend these findings to demonstrate the ability to MDMA to limit macrophage (Fig. 5 and Fig. 8) and dendritic cell (Fig. 6 and Fig. 9) IL-27 expression following exposure to HV-68 or LPS. This drug-induced reduction in cytokine expression was not mediated by trace amine-associated receptors in cultured cells (Fig. 7).

IL-27 is a member of the IL-12 family of heterodimeric cytokines, being composed of the proteins, p28 and ebi3 (Pflanz et al., 2002). This cytokine is produced predominantly by macrophages and dendritic cells (Collison and Vignali, 2008) and its production can have profound effects on T helper type 1 responses (Kastelein et al., 2007; Yoshida et al., 2009) which contribute to the control of HV-68 infection and pathophysiology (Elsawa and Bost, 2004). Transcription of both the Ebi3 (Wirtz et al., 2005) and p28 (Liu et al., 2007; Molle et al., 2007) genes are induced by ligand binding to a number of toll-like receptors (TLRs) on antigen-presenting cells, including LPS activation of TLR4 (Nelson et al., 2010). IL-27 can have pro- or anti-inflammatory effects (Hunter, 2005; Kastelein et al., 2007; Yoshida et al., 2009), via the heterodimeric receptor complex of WSX-1 and gp130. When IL-27 functions as a proinflammatory cytokine, it does so by augmenting IL-12-mediated responses via induction of IL-12 receptor expression. Previous work in our laboratory has demonstrated that endogenous IL-12 production during HV-68 infection participates in the protective

immune response (Elsawa and Bost, 2004). Therefore, we have focused studies on these IL-12 family members produced by macrophages and dendritic cells as potential mediators of anti-gammaparvovirus responses during acute infection. Previous work in our laboratory demonstrated that HV-68 induced expression of IL-27 mRNA and protein in cultured macrophages and dendritic cells (Nelson et al., 2010). Furthermore, mice infected with this gammaparvovirus were primed for IL-27 production in vivo when challenged with a TLR4 agonist (Nelson et al., 2010). In the present study, we extend these findings to demonstrate MDMA suppression of the LPS-induced IL-27 response. Ongoing studies are aimed at defining the importance of early IL-27 production in the protective host response against acute gammaparvovirus infection, and at determining the mechanisms of MDMA-induced suppression.

We were somewhat surprised to find that bingeing mice with MDMA had little effect on latent viral burden (Fig. 4) and on physiological and behavioral parameters measured in latently infected mice (Fig. 3) following drug exposure. The explanation for this result may lie in the fact that we could detect no reactivation of HV-68 from latency to its infectious form following exposure to MDMA (data not shown). A variety of physiological and emotional “stressors” can induce herpesviruses to emerge from latency (Cohen, 2000), and exposure to MDMA causes a variety of physiological changes (e.g. hyperthermia). The fact that these changes did not reactivate HV-68 is consistent with our findings that latently infected mice responded to MDMA exposure in a manner similar to uninfected controls (Fig. 3). Exposure to LPS is one “stressor” that can induce the reactivation of HV-68 from latency to its infectious form (Moser et al., 2005). As shown in Figure 10, latently infected mice exposed to LPS had an exaggerated IL-27 response. It will be important to determine whether this exacerbated IL-27 response facilitates the protective host response against viral reactivation, and whether MDMA-induced suppression of this IL-27 response (Fig. 10) is immunosuppressive.

4.1 Limitations

At present we do not understand the exact mechanism by which exposure to MDMA can increase susceptibility to acute gammaparvovirus infection. As noted above, one possibility is that MDMA can alter the nature of the innate immune response, resulting in viral replication and dissemination. Additional studies to dissect such events will be required to make definitive statements about such mechanisms, however it is clear from our work (Nelson et al., 2008) and from others (Pennock et al., 2009) that MDMA exposure can alter the host response during herpesvirus infections.

While it is likely that early IL-27 production is an important cytokine in the protective host response against herpesvirus infections due to its ability to influence the IL-12 response and T cell mediated immunity (Hunter, 2005; Kastelein et al., 2007; Yoshida et al., 2009), the extent of this cytokine’s contribution needs additional study. With the availability of WSX-1 (IL-27 receptor) deficient mice (Yoshida et al., 2001), such future studies are now possible.

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Figure 1. MDMA exposure increases acute viral burden in the lungs following HV-68 infection
Groups of mice were exposed to vehicle (control) or intranasally infected with HV-68 (HV-68) and given vehicle (control) or 20 mg/kg MDMA (MDMA) intragastrically daily for three consecutive days. At days 3 (**Panel A**) and 6 (**Panel B**) post-infection, animals were euthanized and acute viral burden in lung tissue quantified. Results are presented as mean plaque forming units per mg of lung tissue of triplicate determinations $\pm$ SEM. In addition, levels of HV-68 mRNA encoding ORF-50 (ORF-50) were quantified using real time PCR at days 3 (**Panel C**) and 6 (**Panel D**) post-infection. Results are presented as mean levels of ORF-50 mRNA expression normalized to GAPDH mRNA expression of triplicate determinations for four individual mice per group $\pm$ SEM. Asterisks denote a statistically significant difference ($p < 0.05$) when compared to mice infected with HV-68 alone. This entire experiment was performed twice with similar results.

Figure 2. Splenomegaly following MDMA bingeing in mice latently infected with HV-68
Groups of mice were exposed to vehicle (control) or intranasally infected with HV-68 (HV-68) followed by a 30 day incubation period to allow viral latency to be established. At 30 days post-infection groups of mice were then given vehicle (control) or 20 mg/kg MDMA (MDMA) intragastrically daily for three consecutive days (**Panel A**) or given vehicle or MDMA for three consecutive days at weekly intervals for a total of four weeks (**Panel B**). One day after MDMA treatment, mice were euthanized and spleens were excised and weighed. Asterisks denote a statistically significant difference ($p < 0.05$) when compared to control mice or mice treated with MDMA alone. This entire experiment was performed twice with similar results.

Figure 3. Physiological and behavioral effects of MDMA in C57BL/6 mice latently infected with HV-68

Groups of mice were exposed to vehicle (control) or intranasally infected with HV-68 (HV-68) followed by a 30 day incubation period to allow viral latency to be established. At 30 days post-infection groups of mice were then given vehicle (control) or 20 mg/kg MDMA (MDMA) intragastrically and weight loss at 6 hours post-exposure (**Panel A**), body temperature during the first 6 hours post-exposure (**Panel B**), and maze activity 1.5 hours post-exposure (**Panel C**) were determined. Asterisks denote a statistically significant difference ($p < 0.05$) when compared to control mice. This entire experiment was performed twice with similar results.

Figure 4. Exposure to MDMA does not increase latent viral burden in the spleen

Groups of mice were exposed to vehicle (control) or intranasally infected with HV-68 (HV-68) followed by a 30 day incubation period to allow viral latency to be established. At 30 days post-infection groups of mice were then given vehicle (control) or 20 mg/kg MDMA (MDMA) intragastrically daily for three consecutive days at weekly intervals for a total of four weeks. Mice were euthanized and latent viral burden quantified using an infectious centers assay (**Panel A**). Results are presented as mean infectious centers per total spleen of triplicate determinations for 4 mice per group $\pm$ SEM. In addition, the latent viral mRNA transcript, *M3*, was also quantified using real time PCR (**Panel B**). Results are

normalized to the expression of GAPDH and presented as mean values of triplicate determinations for four mice per group $\pm$ SEM.

Figure 5. Exposure to MDMA reduces HV-68-induced macrophage p28 mRNA expression, ebi3 mRNA expression, p28 protein secretion, and IL-27 protein secretion

Cultured mouse bone marrow-derived macrophages were pre-incubated for 24 hr with 500 μ M MDMA and then infected for 0 to 48 hr with HV-68 at a multiplicity of infection of 1:1 (virus to cell). Cells and medium were harvested at the indicated times and assayed for p28 mRNA expression (**Panel A**), ebi3 mRNA expression (**Panel B**), p28 protein secretion (**Panel C**) and IL-27 protein secretion (**Panel D**) protein levels, respectively. Real time PCR results for mRNA expression are normalized to the expression of GAPDH and presented as mean values of triplicate determinations for four mice per group $\pm$ SEM. ELISA results for protein secretion are presented as mean values of triplicate determinations for four mice per group $\pm$ SEM. Asterisks denote a statistically significant reduction ($p < 0.05$) in mRNA expression or protein secretion for MDMA treated macrophages when compared to

the corresponding time post infection without MDMA treatment. This entire experiment was performed twice with similar results.

Figure 6. Exposure to MDMA reduces HV-68-induced dendritic cell p28 mRNA expression, ebi3 mRNA expression, p28 protein secretion, and IL-27 protein secretion

Cultured mouse bone marrow-derived dendritic cells were pre-incubated for 24 hr with 500 μ M MDMA and then infected for 0 to 48 hr with HV-68 at a multiplicity of infection of 1:1 (virus to cell). Cells and medium were harvested at the indicated times and assayed for p28 mRNA expression (**Panel A**), ebi3 mRNA expression (**Panel B**), p28 protein secretion (**Panel C**) and IL-27 protein secretion (**Panel D**) protein levels, respectively. Real time PCR results for mRNA expression are normalized to the expression of GAPDH and presented as mean values of triplicate determinations for four mice per group $\pm$ SEM. ELISA results for protein secretion are presented as mean values of triplicate determinations for four mice per group $\pm$ SEM. Asterisks denote a statistically significant reduction ($p < 0.05$) in mRNA expression or protein secretion for MDMA treated dendritic cells when compared to

the corresponding time post infection without MDMA treatment. This entire experiment was performed twice with similar results.

Figure 7. Exposure to MDMA, but not other monoamines, reduces HV-68-induced macrophage and dendritic cell p28 mRNA expression

Bone marrow-derived macrophages (**Panel A**) and dendritic cells (**Panel B**) were incubated for 24 hr with 500 μ M NaCl, glycine, monoamines, or MDMA, and then further incubated for 6 hr with or without HV-68 (MOI of 1:1). p28 mRNA induction was assayed by real time PCR. Results are presented as mean levels of p28 mRNA expression normalized to GAPDH mRNA expression of triplicate determinations $\pm$ SEM. Asterisks denote a statistically significant reduction ($p < 0.05$) in p28 mRNA expression when compared to all other monoamine treatments. This entire experiment was performed twice with similar results.

Figure 8. Exposure to MDMA reduces LPS-induced macrophage p28 mRNA expression and IL-27 protein secretion

Cultured bone marrow-derived macrophages were pre-incubated for 24 hr with 500 μ M MDMA and then stimulated with increasing concentrations of LPS for 6 hr. p28 mRNA induction (**Panel A**) and IL-27 cytokine production (**Panel B**) were quantified using real time PCR and ELISA, respectively. Real time PCR results for mRNA expression are normalized to the expression of GAPDH and presented as mean values of triplicate determinations $\pm$ SEM. ELISA results for protein secretion are presented as mean values of triplicate determinations $\pm$ SEM. Asterisks denote a statistically significant reduction ($p < 0.05$) in mRNA expression or protein secretion for MDMA treated macrophages when compared to the corresponding dose of LPS without MDMA treatment. This entire experiment was performed twice with similar results.

Figure 9. Exposure to MDMA reduces LPS-induced dendritic cell p28 mRNA expression and IL-27 protein secretion

Cultured bone marrow-derived dendritic cells were pre-incubated for 24 hr with 500 μ M MDMA and then stimulated with increasing concentrations of LPS for 6 hr. p28 mRNA induction (**Panel A**) and IL-27 cytokine production (**Panel B**) were quantified using real time PCR and ELISA, respectively. Real time PCR results for mRNA expression are normalized to the expression of GAPDH and presented as mean values of triplicate determinations $\pm$ SEM. ELISA results for protein secretion are presented as mean values of triplicate determinations $\pm$ SEM. Asterisks denote a statistically significant reduction ($p < 0.05$) in mRNA expression or protein secretion for MDMA treated dendritic cells when compared to the corresponding dose of LPS without MDMA treatment. This entire experiment was performed twice with similar results.

Figure 10. Exposure to MDMA reduces serum p28 and IL-27 levels in HV-68-infected mice after i.p. injection of LPS

30 days after intranasal infection with HV-68, groups of mice were binged for three consecutive days each week for four weeks with 20 mg/kg MDMA. Three days after the final binge, animals were injected with LPS (50 µg/mouse). Six hours following LPS injection, mice were euthanized and serum was assayed by ELISA for p28 (**Panel A**) and IL-27 (**Panel B**) protein secretion. ELISA results are presented as mean values of triplicate determinations for four mice per group $\pm$ SEM. Asterisks denote a statistically significant decrease ($p < 0.05$) in serum protein secretion for HV-68-infected, MDMA-treated, LPS-exposed mice when compared to infected mice receiving LPS alone. This entire experiment was performed twice with similar results.