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Methamphetamine Increases Brain Viral Load and Activates Natural Killer Cells in Simian Immunodeficiency Virus-Infected Monkeys

Maria Cecilia Garibaldi Marcondes,* Claudia Flynn,* Debbie D. Watry,* Michelle Zandonatti,* and Howard S. Fox*†

From the Molecular and Integrative Neurosciences Department, The Scripps Research Institute, La Jolla, California; and the Department of Pharmacology and Experimental Neuroscience,† University of Nebraska Medical Center, Omaha, Nebraska*

Methamphetamine (Meth) abuse increases risky behaviors that contribute to the spread of HIV infection. In addition, because HIV and Meth independently affect physiological systems including the central nervous system, HIV-induced disease may be more severe in drug users. We investigated changes in blood and brain viral load as well as differences in immune cells in chronically simian immunodeficiency virus-infected rhesus macaques that were either administered Meth or used as controls. Although Meth administration did not alter levels of virus in the plasma, viral load in the brain was significantly increased in Meth-treated animals compared with control animals. Meth treatment also resulted in an activation of natural killer cells. Given the prevalence of Meth use in HIV-infected and HIV at-risk populations, these findings reveal the likely untoward effects of Meth abuse in such individuals. ***(Am J Pathol 2010, 177:355–361; DOI: 10.2353/ajpath.2010.090953)***

The infection of rhesus macaques with simian immunodeficiency virus (SIV) results in disease signs and symptoms that are similar to the ones produced by the infection of humans with HIV and is the best animal model for AIDS study. In both SIV and HIV, infection of CD4 T cells and macrophages causes progressive loss or dysfunction of important components of the immune system, resulting in immune deficiency in nearly all untreated infected subjects. In addition, the central nervous system (CNS) is infected, which results in cognitive and motor impairment in susceptible individuals. The symptoms of CNS disease vary in severity, ranging from minor cognitive disorders to severe dementia.[1] The exact etiology of CNS alterations remains unknown, and the virus does not infect neurons. However, indirect effects that follow infection of brain macrophages and microglia, such as production of inflammatory molecules, and the innate and adaptive response to infection in the CNS can all contribute to brain dysfunction.[2–4]

D-Methamphetamine hydrochloride (Methamphetamine, Meth) is a psychostimulant used illegally for diverse reasons, including weight loss, maintenance of alertness, and enhanced sexual pleasure. Meth is a drug that is increasing in popularity among the drug-abuser population because of its price, availability, and effects. However Meth is highly addictive, and chronic Meth use leads to untoward CNS effects including depression and psychosis.[5] The popularity of Meth in particular populations such as gay men and sex workers and the increasing intravenous administration of Meth are leading to a significant overlap between those who use Meth and those at risk for HIV infection.[6–10]

The action of Meth in the brain involves its ability to increase dopamine levels. Meth is a substrate for the dopamine transporter, which allows its access to dopamine neurons, where it inhibits the vesicular monoamine transporter, leading to increased dopamine in the synaptic cleft. However, damage to the nerve terminals can result, as well as frank neurotoxicity. Although the exact mechanisms are not known, monoamine metabolites, excitotoxic neurotoxicity, oxidative free-radical chemistry, and metabolic stress are potential candidates.[11,12] Interestingly a temporal study revealed that microglia activation, induced by Meth administration to rats, preceded

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Address reprint requests to Howard Fox, M.D. Ph.D., Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, 985800 Nebraska Medical Center, Omaha, NE 68198-5800. E-mail: hfox@unmc.edu.

pathological changes in the striatal dopaminergic fibers, indicating that the activated microglia may contribute to, rather than react to, the Meth-induced damage.[13] This same study also demonstrated microglia activation outside of the striatum, revealing additional targets of Meth in the brain.

Little is known about the effects of chronic Meth use on the immune system, much less its effect on the pathogenesis of a chronic viral infection such as HIV. Given the increasing overlap between Meth abusers and HIV-infected individuals and potential neuropathogenic commonalities such as affects on microglia, understanding the effects of Meth on parameters of HIV-induced disease become urgent. Intriguingly HIV-infected individuals who are Meth users have a higher rate of neuropsychological impairment than those that do not use Meth, as well as Meth users that are not HIV-infected.[14] In addition, HIV-infected Meth users taking antiretroviral agents exhibit a higher plasma viral load than infected individuals who are not using Meth.[15] Interestingly, expression profiling studies revealed that among those with HIV encephalitis, Meth users had significantly increased expression of a group of interferon-inducible genes in their brains.[16]

In the present study, we evaluated the hypothesis that in HIV infection (modeled in SIV-infected monkeys) Meth increases viral load and CNS damage through effects on the immune system and brain. We observed a marked influence of Meth on natural killer (NK) cells, both in the brain and in peripheral sites. In addition, we observed an increase in viral load and $CD14^+CD16^+$ macrophages in the brain. Together, the results suggest that Meth has significant consequences on SIV and probably on HIV infection, with untoward effects, especially in the brain.

Materials and Methods

Monkeys and SIV Infection

SIV-, simian retrovirus type D-, and herpes B virus-free rhesus macaques of Chinese origin purchased from Valley Biosystems (West Sacramento, CA) were infected with a cell-free SIV stock derived from SIVmac251.[17,18] All animal experiments were performed with approval from the Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines. Animals kept in containment were anesthetized with 10 to 15 mg/kg of ketamine intramuscularly before experimental procedures. Blood was serially drawn from the femoral vein, and plasma was obtained from EDTA-treated blood. At necropsy, performed after terminal anesthesia, animals were intracardially perfused with sterile PBS containing 1 U/ml heparin. Tissue samples were taken for cell isolation, virus quantification, and formalin fixation for histology.

Meth Treatment

Meth was administered by intramuscular injection as described in the Results.

Viral Quantitation

Plasma and brain SIV RNA was calculated by using a quantitative branched DNA signal amplification assay, performed by Siemens Clinical Laboratory (Emeryville, CA).

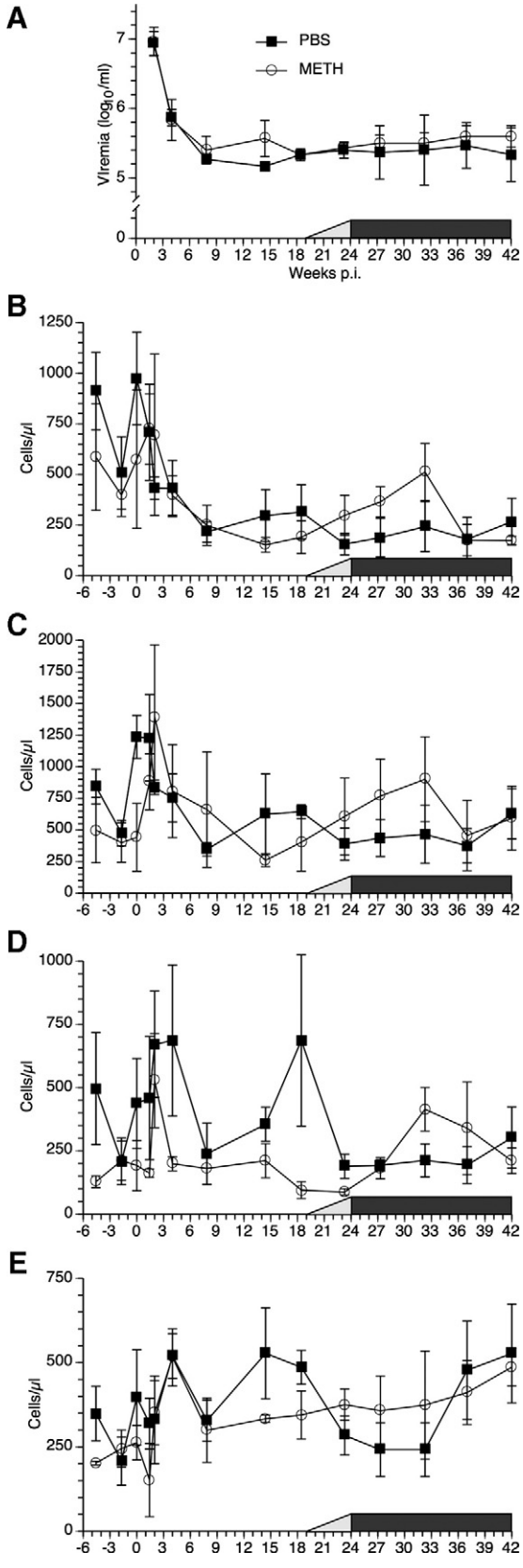
Cells

Cells from centrifuged EDTA-treated blood (buffy coat) or 70-μm nylon-mesh sieved cells from spleen and lymph nodes were subjected to Histopaque (Sigma-Aldrich, St. Louis, MO) gradient for isolation of the mononuclear fraction. For the organs, tissues were removed at necropsy after intravascular perfusion. For isolation of cells from the brain, the brain was carefully freed of meninges. Brain immune cells were isolated by enzymatic digestion of minced tissue, followed by Percoll (Sigma-Aldrich) gradient, as described previously.[19] The absolute numbers of peripheral blood monocytes and lymphocytes were determined by a complete blood count (Antech Diagnostics, Irvine, CA). Cells from tissues were quantified in a Z2 Coulter Counter (Beckman Coulter, Brea, CA). For quantifying blood and tissue lymphocyte and brain macrophage subsets, the absolute numbers were multiplied by the fraction in the specific subset as determined by flow cytometric analysis described below.

Flow Cytometry

Isolated cells were stained with labeled antibodies in PBS containing 2% fetal calf serum and 0.01% NaN_3. For cell surface phenotyping, the antibodies used were anti-CD14-PE (clone M5E2, BD Pharmingen, San Diego, CA), anti-CD16-FITC (clone 3G8, BD Pharmingen), anti-monkey CD3-biotin (clone FN-18, Invitrogen Biosource, Carlsbad, CA) followed by streptavidin-PerCP or -APC (BD Pharmingen), anti-human CD8-PE, -FITC, or -PeCy5 (clone DK25, Dako, Carpinteria, CA), or isotype controls (BD Pharmingen). Stained cells were acquired by a FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer, and analyzed in FlowJo 6.2.1 software (Tree Star Inc., Ashland, OR).

For functional (CD107a/degranulation or cytokine) assessment samples were treated with protein transport blockers. Cells were treated with 10 mg/ml brefeldin-A (Sigma-Aldrich) for cytokine detection or with 1 μmol/L of monensin (Sigma-Aldrich) for CD107a detection, both for 6 hours. Cells were washed and cell surface-stained with anti-CD3 and anti-CD8 as above. They were then surface-stained with anti-CD107a (BD Pharmingen) or treated with Cytofix/Cyoperm solution (BD Pharmingen), washed in Perm/Wash Buffer (BD Pharmingen), and incubated with anti-interferon-γ (BD Pharmingen) or anti-tumor necrosis factor-α (BD Pharmingen) in Perm/Wash buffer for 30 minutes. Cells were kept in 3% paraformaldehyde and acquired in a FACSCalibur instrument using CellQuest software. Analysis was performed using FlowJo 6.2.1 software.



Histopathology

Tissue was fixed in 10% formalin and paraffin-embedded. Five-micrometer sections were stained with H&E and examined microscopically. Indirect immunohistochemical staining for CD163 and Glut5 for macrophages and microglia (Vector Laboratories, Burlingame, CA and AbD Serotec, Raleigh, NC, respectively), CD45 leukocyte common antigen for lymphocytes (Dako), glial fibrillary acidic protein for astrocytes (Abcam, Cambridge, MA), and HLA-DR for activated microglia, endothelia, and immune cells (Invitrogen) was performed as described previously.[20] Colorimetric development was performed with NovaRed Chromagen (Vector) followed by a hematoxylin counterstain (Sigma-Aldrich).

Statistics

Group comparisons were performed using the tests described in the text and figure legends. The difference between the means was considered significant at $P < 0.05$. Tests were performed using Excel (Microsoft Corporation, Redmond, WA) and Prism software (GraphPad Software Inc., San Diego, CA) for Macintosh.

Results

Study Design

Studies on the pathogenesis of SIV infection are often complicated by the inclusion of animals with an aberrant, rapidly progressive disease course that does not reflect human HIV infection, and such animals lack the development of the initial immune response to the infection. Therefore, to effectively assess the effects of Meth on SIV infection, nine rhesus monkeys were infected with SIV and assessed for the first 19 weeks for viral and immune parameters. Three animals did not show control of viral load and were sacrificed because of early development of simian AIDS. The other six animals developed an acute viremia with viral load reaching an average of 7.0 (log_{10}) at 2 weeks postinoculation (p.i.), which then dropped and began at a steady-state level at 8 weeks p.i. (Figure 1A).

At 19 weeks p.i. plasma viral load averaged 5.3 (log_{10}), and three animals were begun on a escalating protocol of Meth administration. Importantly, we mimicked the amounts of Meth used by chronic abusers with our drug administration schedule. Human chronic Meth abusers, who generally increase dose with use, reach up to 1 g/day. In a recent high-resolution magnetic resonance imaging study demonstrating Meth-induced defects in human brains, the subjects averaged 3.4 g of Meth/

Figure 1. Plasma viral load and blood immune cell numbers in PBS- and Meth-treated animals. **A:** Viral load was longitudinally measured by the branched DNA assay, before and after introduction of treatment in both PBS-treated (closed square) and Meth-treated (open circle) animals. **B–E:** The numbers of CD4 (**B**), CD8 (**C**), NK (**D**), and monocytes (**E**) in the blood before and during the course of infection. All figures show the average ± SEM. The Meth initiation and ramp-up period are indicated by the **lightly shaded triangle**, and the Meth maintenance period is indicated by the **darkly shaded rectangle**.

week.[21] Assuming an average of 70 kg body weight and 50% purity of the Meth (the average according to US Department of Justice statistics), this would be 24 to 25 mg/kg/week. Because a typical abuser takes Meth 1 to 3 times a day on >20 days a month,[22] we used a modification of our escalating dose regimen, giving drug 5 days a week, twice a day, and further increasing the dose to reach a final dose of 2.5 mg/kg twice daily, after a 5 week ramp-up, for a total of 25 mg/kg/week, approximating the amounts used by chronic Meth abusers. This level was then maintained for an additional 18 weeks. The other three animals received PBS injections on the same schedule. All animals were sacrificed at 42 weeks p.i.

Longitudinal Analysis

Meth treatment did not alter the plasma viral load over the course of the experiment (Figure 1A). Although cerebrospinal fluid was sampled less frequently, no difference was found in the viral load in the cerebrospinal fluid (data not shown). In addition, although infection induced a drop in CD4 cells and an acute rise and then recovery to baseline in CD8 cells, there were no differences between the groups for CD4, CD8, NK, or monocytic cells (Figure 1, B–E) during treatment.

CNS and Immune System Analysis at Necropsy

Histopathological examination of the brain did not reveal SIV-specific lesions. Immunohistochemical examination of the brain for lymphocytes and macrophages showed occasional perivascular lymphocytes and macrophages (Figure 2, A–D), with no differences between the groups. Although there was no appreciable microglial activation, astrocyte activation was present and was more pronounced in Meth-treated animals than in PBS-treated controls (Figure 2, E–H).

Immune cells were isolated from the brain and assessed by fluorescence-activated cell sorter analysis, revealing that the proportion of $CD4^+$, $CD8^+$, and NK cells in the brain did not differ between groups (CD4 T cells: 1.65 ± 0.75% in PBS group and 1.28 ± 0.47% in Meth group; CD8 T cells: 3.57 ± 1.02% in PBS group and 2.10 ± 0.85% in Meth group; NK cells: 2.40 ± 1.62% in PBS group and 2.33 ± 0.3% in Meth group). Meth treatment did not alter the overall percentage of $CD11b^+$ cells (microglia and macrophages) in the brain; in both groups these cells comprised more than 85% of immune cells isolated from the brain. Interestingly, significantly more virus was found in the brains of the Meth-treated animals than in the brains of the control animals (Figure 3).

Although their proportions did not differ, to examine potential activation/activity of the brain-infiltrating cells as well as lymphocytes, we assessed their release of granule contents through analysis of cell surface expression of CD107a (lysosomal-associated membrane protein 1), a marker of secretory lysosomes present on the cell surface, after degranulation, representing the functional ability to secrete cytokines and/or cytotoxic molecules.[23] In addition, we assessed the percentage of cells producing cytokines, on a single-cell basis by intracellular staining. Increased amounts of CD107a were present on the cell surface of $CD8^+$ T cells and NK cells in the Meth-treated group in the brain, blood, and lymphoid organs; increased CD107a was also present in the $CD4^+$ T cells in the brain (Figure 4A), indicating a higher degree of active cell degranulation. Intracellular cytokine staining indicated that in the Meth-treated animals, NK cells were markedly affected, exhibiting higher levels of tumor necrosis factor-α and interferon-γ production in the brain and other sites (Figure 4, B and C).

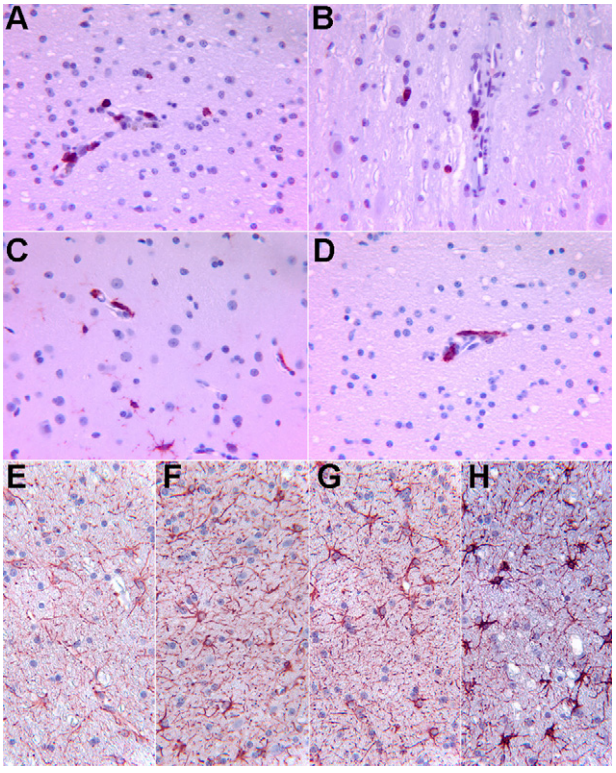


Figure 2. Photomicrographs of CNS immunohistochemical analyses. **Left** (**A, C, E,** and **F**): PBS-treated animals. **Right** (**B, D, G,** and **H**): Meth-treated animals. Staining for leukocyte common antigen (**A** and **B**) and CD163 (**C** and **D**) revealed no differences in perivascular lymphocytes and macrophages, respectively, and occasional $CD163^+$ microglia. Staining for glial fibrillary acidic protein (**E–H**) revealed astrocytes in both the PBS-treated (**E** and **F**) and Meth-treated (**G** and **H**) animals, with increased reactivity in the Meth-treated group (photomicrographs from subcortical white matter). Original magnification, ×500.

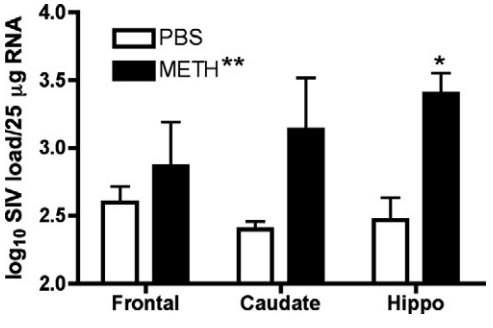


Figure 3. Viral load in the brain. Viral load was measured in different areas of the brain by the branched DNA assay in PBS-treated (white bar) and Meth-treated animals (black bar). $^{**}P < 0.01$ in two-way analysis of variance. $^{**}P < 0.01$ in hippocampus in a Bonferroni post hoc test. Figure shows the average ± SEM.

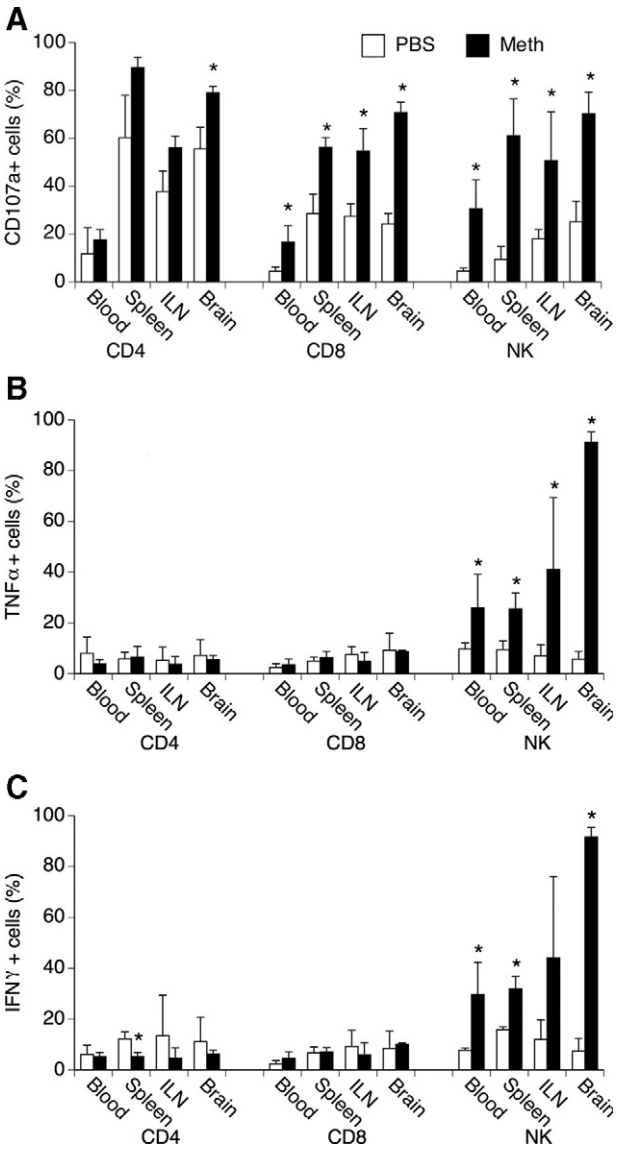


Figure 4. Lymphoid activation/activity markers (**A**) CD107a/degranulation, (**B**) intracellular tumor necrosis factor-α (TNFα) and (**C**) interferon-γ (IFNγ) in lymphoid cells from various sites of SIV-infected animals receiving PBS or Meth treatment, measured from necropsy samples. Cells were gated on markers for CD4, CD8, and NK cells. $^*P < 0.05$, Student's *t*-test between groups. ILN, inguinal lymph node.

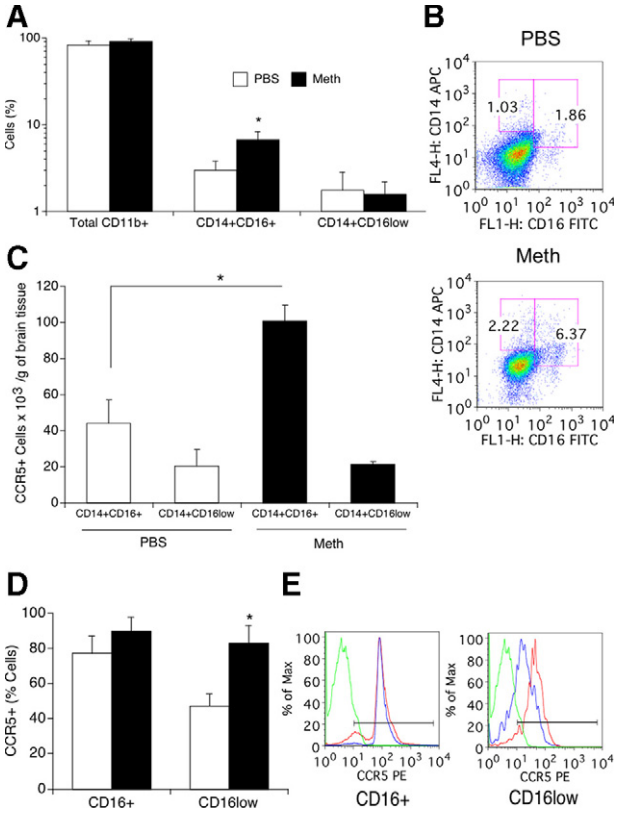


Figure 5. Subpopulations of macrophages in the brains of PBS- or Meth-treated SIV-infected monkeys. **A:** Total CD11b, and percentage of $CD14^+CD16^+$ and $CD14^+CD16^{low}$ within $CD11b^+$-gated cells in PBS-treated (white bar) or Meth-treated (black bar) SIV-infected animals. **B:** Fluorescence-activated cell sorter plots illustrating CD11b-gated cells in brain cell suspensions from a representative SIV-infected animal treated with PBS (**top:** animal 518) or Meth (**bottom:** animal 554). **C:** Average absolute numbers of the two macrophage subsets in the brains of PBS- or Meth-treated SIV-infected animals. **D:** Percentage of CCR5-expressing cells within CD11b-gated $CD14^+CD16^+$ and $CD14^+CD16^{low}$ cells in PBS-treated (white bar) or Meth-treated (black bar) SIV-infected animals. **E:** CCR5 expression histograms on CD11b-gated $CD14^+CD16^+$ (**left**) and $CD14^+CD16^{low}$ cells (**right**) in the brain of a representative animals in each group (animal 514: blue line, PBS-treated; animal 555: red line, Meth-treated). $^*P < 0.05$; Bonferroni post hoc test after two-way analysis of variance with $P < 0.05$.

Phenotypic changes were also observed in the brain macrophages, which were identified by the presence of CD14, and further analyzed for the activation marker CD16. The percentage of $CD14^+CD16^+$ cells within the brain-derived $CD11b^+$ population was significantly increased in the Meth-treated animals compared with controls receiving PBS (Figure 5A). Brain cell suspension scatter plots of one representative control animal and one Meth-treated animal (Figure 5B) show the increase of $CD14^+CD16^+$ macrophages within the $CD11b^+$ gate. On average there were almost double the number of $CD14^+CD16^+$ macrophages in the brains of Meth-treated animals compared with the number in the PBS group (Figure 5C). Interestingly, although the majority of these $CD14^+CD16^+$ cells in the brain expressed high levels of CCR5 in both SIV-infected groups regardless of the treatment, in the Meth-treated group the $CD14^+CD16^{low}$ macrophages had a higher percentage of CCR5-highly expressing cells (Figure 5D). Representative CCR5 expression histograms illustrate the high levels of CCR5 on $CD14^+CD16^+$ cells (left panel) in both groups and the up-regulation of CCR5 within the $CD14^+CD16^{low}$ compartment (right panel) in Meth-treated animals (red lines) compared with PBS-treated animals (blue lines) (Figure 5E). Overall the mean fluorescence intensity of CCR5 in the $CD14^+CD16^{low}$ brain macrophages more than doubled, increasing from 23.3 ± 11.9 (mean ± SEM) in PBS-treated animals to 56.4 ± 15.4 in the Meth-treated animals.

Discussion

Here we report changes in the virus-host interaction in SIV-infected macaques as a result of Meth administration. Although no change in plasma viral load was found

after Meth administration, Meth led to a significantly increased viral load in the brain and increased numbers of activated brain macrophages. Interestingly, effects on peripheral as well as brain lymphoid cells were found, with markedly increased NK cell activation.

We performed this study because of the overlap in individuals who use Meth and who are HIV-infected and because of the potential interacting effects of these agents on the immune system as well as the brain. SIV/HIV enters the brain early after infection and remains in the brain throughout the course of infection. Increased virus was present in the brain of Meth-treated animals as well as an increase in $CD14^+CD16^+$ inflammatory macrophages, known targets for SIV/HIV infection in the brain.[24,25] These cells express high levels of CCR5, the SIV coreceptor; in addition there is also increased expression of CCR5 on $CD14^+CD16^{low}$ macrophages in Meth-treated animals. This result suggests that the increase in brain viral load is correlated to an enrichment of target cells. At this stage of infection, we did not find significant inflammatory foci, either in the presence or absence of Meth treatment. The increased macrophages in Meth-treated animals are probably diffuse within the brain, although histopathological studies are limited to a small proportion of the brain volume.

Recent *in vitro* studies support an effect of Meth on monocytic lineage cells. Meth has been shown to increase macrophage and dendritic cell CCR5 expression and HIV infection.[26,27] In addition, dopamine itself, which is increased by Meth, leads to increased HIV infection of macrophages.[28] Because the plasma viral load is largely linked to T-cell infection and brain viral load is due to macrophage infection, a Meth-induced increase in CCR5 and macrophage infectivity is consistent with our data, in which plasma virus (resulting largely from T cells) is unchanged, but brain virus (derived from macrophages) is increased along with an increase in brain $CCR5^+$ macrophages. Our data on the unchanged plasma viral load are also consistent with findings in HIV-infected people, as the increase in viremia in Meth users is attributed to lack of effectiveness or compliance with antiviral medications.[15,29]

In the periphery as well as the brain, distinct changes due to Meth treatment were found in lymphocytes. The expression of CD107a showed a significant increase in CD8 and NK cells in Meth-treated animals in comparison with PBS controls. The increased expression of CD107a correlated with an increased capacity to secrete tumor necrosis factor-α and interferon-γ in NK cells but not in the CD8 compartment; however we did not examine other important molecules produced by CD8 T cells in SIV/HIV infection, such as perforin and granzymes.[19] These markers suggest that in Meth-treated animals there is more NK cell activation and possibly $CD8^+$ T-cell activation than in PBS-treated animals. Studies in HIV and SIV infection reveal that NK cells do not seem to play a major role in viral control.[30,31] Given the nature of NK cells and the broad range of activation, we speculate that Meth may be causing a relatively nonspecific activation of NK cells, but further work is required to assess the true basis of this effect.

Neuropathologically the only difference noted between the groups was increased astrocyte activation in the monkeys administered Meth. Reactive astrocytes represent a fairly nonspecific finding and are seen in response to SIV/HIV infection as well as to other neuropathological insults. Meth use may lead to increased astrocyte activation[21]; in addition, the increased brain virus and activated NK cells may also have contributed to this finding in the Meth-treated animals.

Whether the impact of Meth on immune cells is direct is not known. Meth raises concentrations of dopamine, which can directly affect immune cells.[32,33] Regarding NK cells, both stimulatory and inhibitory effects of Meth have been found in mice and monkeys.[34–36] However, the markedly different Meth pharmacokinetics in rodents as opposed to primates (approximately a 10-fold shorter half-life in rodents) complicates interpretation of rodent studies.[37] Furthermore, these are largely acute studies, and chronic administration studies such as ours have not been performed to examine the effects of Meth on immune parameters.

In addition to the activation of NK cells, the introduction of a Meth administration regimen during stable SIV infection led to an increase in activated macrophages and virus in the brain, which are probably connected events and both linked to the potential development of CNS disease. Meth may act on immune cells both directly and indirectly, affecting different functional levels in distinct ways. Given the prevalence of Meth use in HIV-infected and HIV at-risk populations, Meth represents a significant comorbid factor in HIV-induced disease, particularly in the brain.

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