## Title

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# An oral form of methylglyoxal-bis-guanylhydrazone reduces monocyte activation and traffic to the dorsal root ganglia in a primate model of HIV-peripheral neuropathy

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## Abstract

Peripheral neuropathy (PN) is a major comorbidity of HIV infection that is caused in part by chronic immune activation. HIV-PN is associated with infiltration of monocytes/macrophages to the dorsal root ganglia (DRG) causing neuronal loss and formation of Nageotte nodules. Here, we used an oral form of methylglyoxal-bis-guanylhydrazone (MGBG), a polyamine biosynthesis inhibitor, to specifically reduce activation of myeloid cells. MGBG is selectively taken up by monocyte/macrophages *in vitro* and inhibits HIV p24 expression and DNA viral integration in macrophages. Here, MGBG was administered to nine SIV-infected, CD8-depleted rhesus macaques at 21 days post-infection (dpi). An additional nine SIV-infected, CD8-depleted rhesus macaques were used as untreated controls. Cell traffic to tissues was measured by *in vivo* BrdU-pulse labeling. MGBG treatment significantly diminished DRG histopathology and reduced the number of CD68+ and CD163+ macrophages in DRG tissue. The number of recently trafficked BrdU+ cells in the DRG was significantly reduced with MGBG treatment. Despite diminished DRG pathology, intraepidermal nerve fiber density (IENFD) did not recover after treatment with MGBG. These data suggest that MGBG alleviated DRG pathology and inflammation.

### Keywords

HIV; rhesus; peripheral neuropathy; dorsal root ganglia; monocyte; polyamine biosynthesis inhibitor

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Conflicts of Interest

Michael McGrath is a shareholder and consultant in Pathologica, LLC. For the remaining authors, there are no conflicts of interests.

## INTRODUCTION

HIV-associated peripheral neuropathy (PN) affects more than half of HIV-infected individuals on durable antiretroviral therapy (ART) [1]. The most common type of HIV-PN is distal sensory polyneuropathy (DSP) that results in chronic, drug resistant bilateral pain, tingling, and numbness in the extremities[2,3]. We and others have shown that HIV-DSP is associated with chronic monocyte activation, which results in increased traffic of monocytes to the dorsal root ganglia (DRG). Monocyte activation and cell traffic to the DRG is associated with a reduction of neuronal cell density in the DRG and a “dying back” of small, unmyelinated nerve fibers in distal skin regions [4-7]. Importantly, HIV-DSP occurs despite effective ART [1,8].

ART drugs suppress viral RNA replication efficiently in T cells; however, function less efficiently in macrophages due in part to lower intracellular drug concentrations in these cells [9]. Additionally, many of ART drugs fail to eliminate integrated proviral DNA, particularly in macrophages [10,11]. Macrophages are long-lived and not susceptible to viral-induced cell lysis, making them a potent viral reservoir [12]. Increased numbers of HIV DNA+ macrophages in the central nervous system (CNS) are associated with HIV-associated neurocognitive disorders (HAND), demonstrating the negative effect of virally infected cells even in the absence of active viral replication [13]. Additionally, even when viral replication is suppressed with ART, monocytes and macrophages remain chronically activated and are thought to contribute to co-morbidities including HAND, cardiovascular disease, and neuropathic pain [14]. Targeting persistently infected and/or activated macrophages is a major hurdle in combatting HIV-associated diseases. Viral replication in macrophages in DRG is present in some animals, but not all. Additionally, the number of virally infected cells in the DRG does not correlate with pathology, suggesting that viral proteins are not the main source of neuronal death [4]. Identifying drugs that will target viral replication and macrophage activation is likely to help treat and prevent HIV-associated co-morbidities.

Methyl-bis-guanylhydrazone (MGBG) is a polyamine analog that interferes with polyamine biosynthesis by inhibiting S-adenosine-methionine decarboxylase (SAMDC) [15]. This process is required for cell differentiation and proliferation and thus, MGBG has been studied as an anti-tumor cell proliferation drug [16]. Virally infected macrophages treated with MGBG *in vitro* displayed reduced integration of proviral DNA compared to control macrophages, although the levels of total intracellular DNA were unchanged [17]. MGBG is selectively taken up by monocyte/macrophages *in vitro* and inhibits activation by depleting the intracellular pool of spermine and spermidine[18,19]. Depletion of these intracellular polyamines results in prevention of alternatively-activated M2 macrophage induction and also inhibits LPS induced cytokine production from M1 macrophages [20]. Thus, MGBG could function to reduce viral replication in monocytes/macrophages through blocking viral integration and will reduce alternative activation of these cells through polyamine depletion *in vivo*. We have recently shown that MGBG can reduce cardiovascular inflammation, CIMT, and fibrosis that correlated with a decrease in number of macrophage in SIV-infected macaques [21].

Here, we aimed to specifically target monocytes and macrophages with MGBG in a primate model of HIV-PN in an attempt to reduce DRG pathology associated with macrophage differentiation and trafficking [4]. Eighteen rhesus macaques were infected with SIVmac251 and CD8 depleted to allow for rapid disease progression. Nine animals received an oral form of MGBG and the remaining nine were untreated controls. Here, we show that MGBG treatment results in less severe DRG pathology by reducing numbers of macrophages in tissue.

## METHODS

### Ethical Statement

All animals used in this study were handled in strict accordance with American Association for Accreditation of Laboratory Animal Care with the approval of the Institutional Animal Care and Use Committee of Harvard University (protocol number 04420) and Tulane University (protocol numbers P0066 and P0263). Animals were housed at the New England Primate Research Center (NERPC, Southborough, MA) or Tulane National Primate Research Center (TNPRC, Covington, LA).

### Animals, viral infection, and CD8 lymphocyte depletion

Eighteen male rhesus macaques (*Macaca mulatta*) were utilized in this study. All animals were inoculated intravenously with SIVmac251 (a generous gift from Dr. Ronald Desrosiers, University of Miami) and administered 10 mg/kg of anti-CD8 antibody subcutaneously at day 6 after infection, and 5 mg/kg intravenously at days 8 and 12 after infection in order to achieve rapid AIDS. The human anti-CD8 antibody was provided by the NIH Non-human Primate Reagent Resource (RR016001, AI040101). Animals were sacrificed between 55 and 89 dpi based on progression of disease or termination of the study. They were anesthetized with ketamine-HCl and euthanized by an intravenous pentobarbital overdose and exsanguinated. The diagnosis of simian AIDS was determined post-mortem by the presence of opportunistic infections, including SIV giant cell pneumonia, CMV pneumonia, giant cell encephalitis, pneumocystis carinii and lymphoma.

### MGBG administration

Nine animals received 30mg/kg of MGBG (provided by Pathologica, LLC; formulated as flavored syrup by Wedgewood Pharmacy, Swedesboro, NJ) daily beginning at 21 dpi. Previous experiments demonstrated that a daily dose of 30mg/kg of MGBG was able to achieve an effective concentration of 0.7μM in tissue and plasma (data not shown).

### BrdU administration

BrdU was administered as a slow bolus intravenous injection at a dose of 60mg/kg body weight as previously described [22]. BrdU was administered on 7, 19, 56 dpi, and 24 hours prior to necropsy.

### Plasma viral load quantification

Plasma SIV-RNA was quantified using real-time PCR for all animals used in this study, as previously described [21]. EDTA plasma (500μL) was collected and SIV virions were pelleted by centrifugation at 20,000 g for 1 hour. The threshold sensitivity was 100 copy Eq/mL, with an average interassay coefficient variation of less than 25%.

### Intraepidermal nerve fiber density measurements

Skin biopsies (3mm) were taken in all SIV+ animals at 20 dpi (pre-drug) and at necropsy (post-drug). Biopsies were taken near the sural innervation site just distal to the lateral malleolous and were fixed in Zamboni's fixative. 50μm thick sections were stained with a pan-axonal marker (anti-PGP 9.5 at 1:10,000 dilution; ABD Serotec). Nerve fiber length (mm) per volume of epidermis ($mm^3$) (IENFD) was quantified using computer software (Space balls program; Microbrightfield Bioscience) as previously described [4,5].

### Necropsy and Histopathology

Animals were necropsied immediately following death and representative sections of all major organs were collected, fixed in 10% neutral buffered formalin (NBF), embedded in paraffin, and sectioned at 5μm. Hematoxylin and eosin stained sections of DRG were evaluated blindly for histopathologic lesions by a board-certified veterinary anatomic pathologist (ADM) and scored based on the presence and severity of infiltrating mononuclear cells, neuronophagia, and Nageotte nodules as previously described [4,23]. Overall pathology was scored on a scale of 0 to 3 at increments of 0.5 via the following criteria: (0) No significant findings; (1) Mild: scattered infiltrating mononuclear cells with rare evidence of neuronophagia and/or neuronal loss; (2) Moderate: increased numbers of infiltrating mononuclear cells with occasional neuronophagia and/or neuronal loss; and (3) Severe: abundant infiltrating mononuclear cells, frequent neuronophagia, and neuronal loss were all present.

### Immunohistochemistry and quantification satellite cells in DRGs

DRG sections were stained with either the pan-macrophage marker anti-CD68 (Clone KP1, Dako, Carpinteria, CA), the scavenger receptor anti-CD163 (Clone MCA1853, Serotec, Raleigh, NC), the early inflammatory marker anti-MAC387 (Clone M0747, Dako, Carpinteria, CA), anti-BrdU (Clone M0744, Dako, Carpinteria, CA), or the T lymphocyte marker anti-CD3 (Clone A0452, Dako, Carpinteria, CA) as previously described [4,23]. For quantitation of monocyte/macrophage and T cell populations by immunohistochemical analyses, at least eight non-overlapping fields at 200x magnification were quantitated per DRG tissue as previously described [4,23]. Data were expressed as mean ± standard error of the mean (SEM).

### Statistical methods

Prism version 5.0f (GraphPad Software, Inc., San Diego, CA) software was used for statistical analyses. Mann-Whitney t tests were used to detect variation in cell numbers between untreated and MGBG treated rhesus macaques. Wilcoxon matched-pairs signed

rank test was used to detect a change of IENFD within an individual over time. A P value of <0.05 was considered significant for all analysis performed.

## RESULTS

### MGBG treatment diminishes overall DRG pathology

Eighteen SIV-infected, CD8-depleted rhesus macaques were used in this study (Table 1). Nine of the animals received MGBG starting at 21 days post-infection (dpi). The remaining nine animals were untreated SIV-infected controls. All animals used in these studies were male and the age of the placebo (5.6yrs ± 0.7) and the MGBG treated (4.6yrs ± 0.1) group was not statistically different (P= 0.2). All animals were sacrificed between 55 and 89 dpi. The viral load at necropsy in the MGBG treated group was 7.38 ± 0.25 copies/ml and 7.58 ± 0.24 copies/ml in the control group (p=0.61; Table 1).

Histopathology of lumbar, sacral, and thoracic dorsal root ganglia was assessed for the presence and degree of satellitosis, neuronophagia, and Nageotte nodules and are reported in Table 1. Overall DRG pathology was ranked on a scale of zero to three as previously described [4,5] with a score of zero indicating no significant findings and a score of three indicating severe pathology. Treatment with MGBG significantly reduced the overall DRG pathology from an average score of 1.4 ± 0.2 in control animals to 0.9 ± 0.1 ($p<0.05$; Figure 1). Notably, none of the MGBG treated animals developed Nageotte nodules. Additionally, the degree of satellitosis and neuronophagia never reached a pathology score above 1.5 (mild-moderate) in MGBG treated animals compared to untreated which ranged from a score of 1 (mild) to 2.5 (moderate-severe) (Table 1).

### MGBG treatment decreases the number of macrophages in the DRG

We sought to determine if MGBG affected immune activation in the DRG and the composition of macrophages that surround the DRG. We have previously shown that the numbers of CD68+, CD163+, and MAC387+ macrophages are increased in DRG tissues of SIV-infected animals compared to uninfected animals. Additionally, elevated numbers of CD68+ and MAC387+ macrophages in DRG tissue were associated with severe pathology [4]. Here, we sought to determine if MGBG treatment would affect resident and recently trafficked macrophages. We investigated the number of CD68+, CD163+, and MAC387+ macrophages in the DRG. CD163 is a hemoglobin-haptoglobin scavenger receptor that has been associated with an M2 (alternative-activation) phenotype and can also overlap with the CD68+ macrophage population, which is considered to be a marker of mature tissue macrophages [24]. MAC387 is expressed by recently infiltrated, inflammatory M1-like (classically-activated) monocytes/macrophages and does not co-localize with CD68 or CD163 [25]. Here, we found that the number of CD68+ macrophages in the DRG decreased significantly with MGBG treatment (784.3±86.8 versus 473.9±44.2 cells/mm$^2$; $p<0.01$; Figure 2A-C). Additionally, MGBG reduced the number of CD163+ macrophages in DRG (789±107.5 versus 369.5±45.7 cells/mm$^2$; $p<0.01$; Figure 2D-F). The number of MAC387+ macrophages, while not statistically significant, did trend towards a decrease with MGBG treatment (133.0±15.6 versus 91.9±11.4 cells/mm$^2$; p=0.06; Figure 2G-I).

Next, we assessed cell traffic to the DRG using BrdU labeling [26]. We have previously shown that increased monocyte traffic to the DRG was associated with severe pathology and a loss of IENFD. The majority (78.1%) of BrdU+ cells in the DRGs were found to be MAC387+ macrophages [4]. Here, we found that MGBG significantly reduced traffic of BrdU+ cells (78.3±6.3 versus 41.9±4.3 cells/mm$^2$; $p<0.0001$; Figure 2J-L).

Finally, we examined the number of CD3+ T lymphocytes in the DRG in both MGBG-treated and untreated groups. We have previously shown that the number of T cells in the DRG did not increase with SIV infection (compared to uninfected animals) [4,23]. We found that there was no change in the number of CD3+ T cells with MGBG treatment (168.1±18.1 versus 122.9±11.1 cells/mm$^2$; $p=0.08$; Figure 2M-O), suggesting that MGBG had no effect on T cells.

### There is no regeneration of intraepidermal nerve fiber density (IENFD) with MGBG treatment

All animals received skin biopsy punches before MGBG treatment (20 dpi) and at necropsy to assess if MGBG treatment would allow for or prevent regeneration of IENFD. We have previously reported an early decline in IENFD following SIV infection that never recovers to baseline levels [4]. As expected, IENFD in SIV-infected untreated animals did not change between 20 dpi and necropsy (298.8±50.9 IENFD at 20 dpi versus 304.8±47.1 IENFD at necropsy; $p=0.84$; Figure 3A). Interestingly, MGBG treated animals had a significant additional loss of IENFD after drug treatment was initiated at 21 dpi. IENFD in the MGBG treated group decreased from 551.8±73.3 IENFD at 20 dpi to 387.8±53.0 IENFD at necropsy ($p<0.05$; Figure 3B). We suspect that this is because macrophages are required for peripheral nerve regeneration and MGBG targets these M2 repair macrophages [27,28]. MGBG treatment in the absence of SIV infection did not significantly affect the IENFD (369.3±108.0 IENFD at pre-drug versus 291.7±113.3 IENFD at necropsy; $p=0.75$; data not shown). This demonstrates that MGBG is not neurotoxic and that the drug's effect on macrophages combined with SIV infection is the cause of IENFD reduction.

## DISCUSSION

In a recent study, we showed that MGBG treatment in SIV-infected macaques reduced cardiovascular inflammation, CIMT, and fibrosis that correlated with a decrease in number of macrophages [21]. Here, we found that MGBG administration in a rhesus model of HIV-PN diminished DRG pathology with reduced numbers of CD68+ and CD163+ macrophages. We have previously shown that the number of CD68+ and CD163+ macrophages in DRG tissue increased after SIV infection. Additionally, we have shown that macrophage cell traffic to the DRG is associated with severe DRG pathology and a greater loss of IENFD [4]. Monocyte traffic to the DRG appears to be a driving force of formation of Nageotte nodules, neuronophagia and DRG pathology. When cell traffic was blocked with an anti-VLA-4 antibody, natalizumab, on the day of infection there was a reduction in these pathological processes. However, blocking cell traffic during late infection did not completely prevent formation of Nageotte nodules suggesting that Nageotte nodules and neuronophagia form

during early infection [29]. Here, we found that MGBG treatment reduced BrdU+ cell traffic to the DRG and reduced tissue pathology.

Despite the reduction of cell traffic and pathology in the DRGs, we did not observe regeneration of IENFD in the footpad. It has been previously shown that IENFD does regenerate in the setting of HIV infection, but was slowed [30]. Loss of IENFD in the SIV-infected CD8 depleted macaque model occurs early after infection (dpi 8-21) and never recovers to the baseline level [4]. Because MGBG was not administered until 21 dpi, where significant loss of IENFD has already occurred, it may not be reversible. Early MBGB treatment may be warranted to maintain IENFD in addition to protecting DRGs. Macrophages play a role in tissue remodeling and peripheral nerve regeneration [31]. We suspect that the diminished numbers of alternatively activated macrophages in MGBG-treated animals prevented PNS recovery. Polyamines, which are depleted with MGBG treatment, aid in sciatic nerve regeneration *in vivo* and promote axonal regeneration *in vitro* [32,33]. Additionally, mac

It is thought that HIV-PN may be caused by direct neurotoxicity of viral proteins and by indirect damage of cytokines and chemokines that are secreted by activated myeloid cells [3]. HIV- and SIV-PN occurs even when viral replication is suppressed and non-neurotoxic ART drugs are used [1,8]. Therefore, it is possible that immune activation that occurs with or without active viral replication is a main cause of persistent PNS damage. We have previously shown that elevated plasma sCD163 and RANTES (both of which are associated with myeloid cell activation and chemotaxis) correlates with reduced IENFD in SIV+ macaques [5]. HIV-PN does not typically respond to pain medicine [35,36]. Thus, identifying treatment options that target the cause of damage to the nerves or DRG neurons has great clinical implications.

Few drugs specifically target monocyte and macrophage activation as therapy for HIV-PN. One drug that may improve PNS pathology in HIV+ patients is maraviroc. Maraviroc is a CCR5 inhibitor that blocks viral entry into CCR5+ cells [37]. RANTES (the ligand for CCR5) and CCR5+ cells in the DRG are elevated after SIV infection and are associated with SIV-PN pathologies [5]. Additionally, *in vitro* maraviroc treatment to DRG neurons inhibited gp120-induced tumor necrosis factor-alpha expression and thus reduced neurotoxicity [38]. Cenicriviroc (CVC), which is currently in clinical trials for HIV treatment, targets both CCR5 and CCR2 [39,40]. The ligand for CCR2 is CCL2 or monocyte chemoattractant protein-1 (MCP-1) is elevated in SIV+ DRG [5] and most likely plays a role in recruiting inflammatory CCR2+ monocytes to the tissue. Additionally, CCR2-CCL2 interaction has been implicated in models of neuropathic pain [41,42]. It is unclear if CVC treatment will have a beneficial effect on PNS nerve pathologies.

MGBG is specifically taken up by monocytes and macrophages inhibiting HIV integration *in vitro*, although the exact mechanism of this phenomenon is not clear [17]. There was no difference in plasma viral load between the placebo and MGBG-treated animals, which was expected since most plasma virus is determined from T cells and not monocytes and MGBG only effects HIV replication in myeloid cells. MGBG has been well studied as a potent inhibitor of SAMDC and therefore depletes the intracellular polyamine pool [15,43]. Polyamines are regulators of macrophage activation, are needed for alternative macrophage

(M2) polarization and can inhibit M1-cytokine production [20]. In this study, we found that MGBG significantly reduced the number of CD163+ (M2) macrophages, but not the number of MAC387+ (M1-like) macrophages in the DRG, supporting a mechanism of MGBG inhibition of M2-activation.

Identifying drugs that target myeloid cells to reduce their traffic and activation during HIV and SIV disease pathogenesis is important to prevent HIV comorbidities. However, one must be careful to not completely block macrophage function, as they can play essential roles in innate immunity and tissue remodeling, as demonstrated by the lack of IENFD regeneration in this study. Future studies should seek to understand the diverse role of different macrophage phenotypes and identify drugs that specifically target destructive and inflammatory macrophages. Treatment with MGBG before PNS damage begins would likely have a greater preventative impact on the development of PN. Additionally, using MGBG in conjunction with ART to reduce monocyte/macrophage activation and viral replication may also be beneficial. In this study, we did not see an effect on plasma viral load as expected since MGBG only targets HIV integration in myeloid cells and not T cells. Suppressing viral replication with ART and reducing monocyte activation with MGBG may be a successful combination to control disease progression and prevent inflammation-induced comorbidities and these studies are currently underway.

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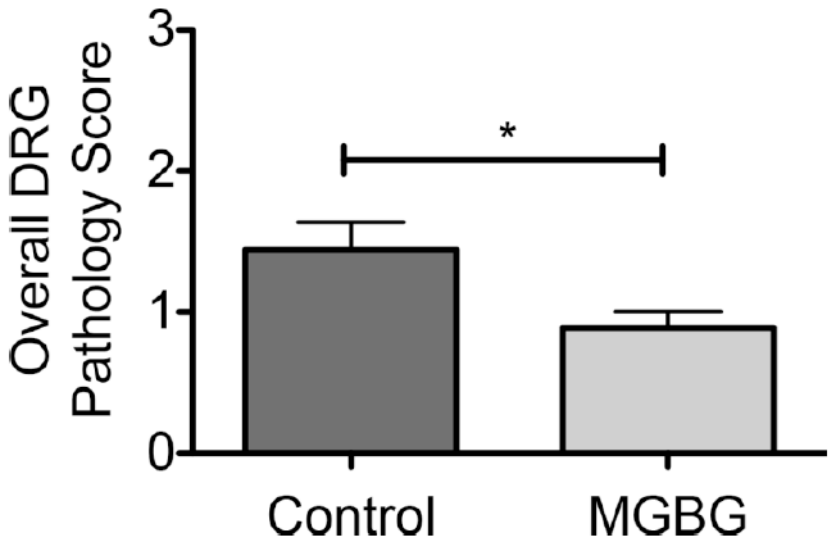


**Figure 1. Overall DRG pathology is reduced in MGBG-treated animals compared to controls**

Overall DRG pathology was scored on a scale of zero (no significant findings) to three (severe pathology) at increments of 0.5 in lumbar, sacral, and thoracic regions and were averaged in each animal. Pathology was scored based on the degree and presence of satellitosis, neuronophagia, and Nageotte nodules. Bars represent the average overall DRG pathology mean ± SEM. Groups were compared with a Mann-Whitney T-test. *P<0.05.

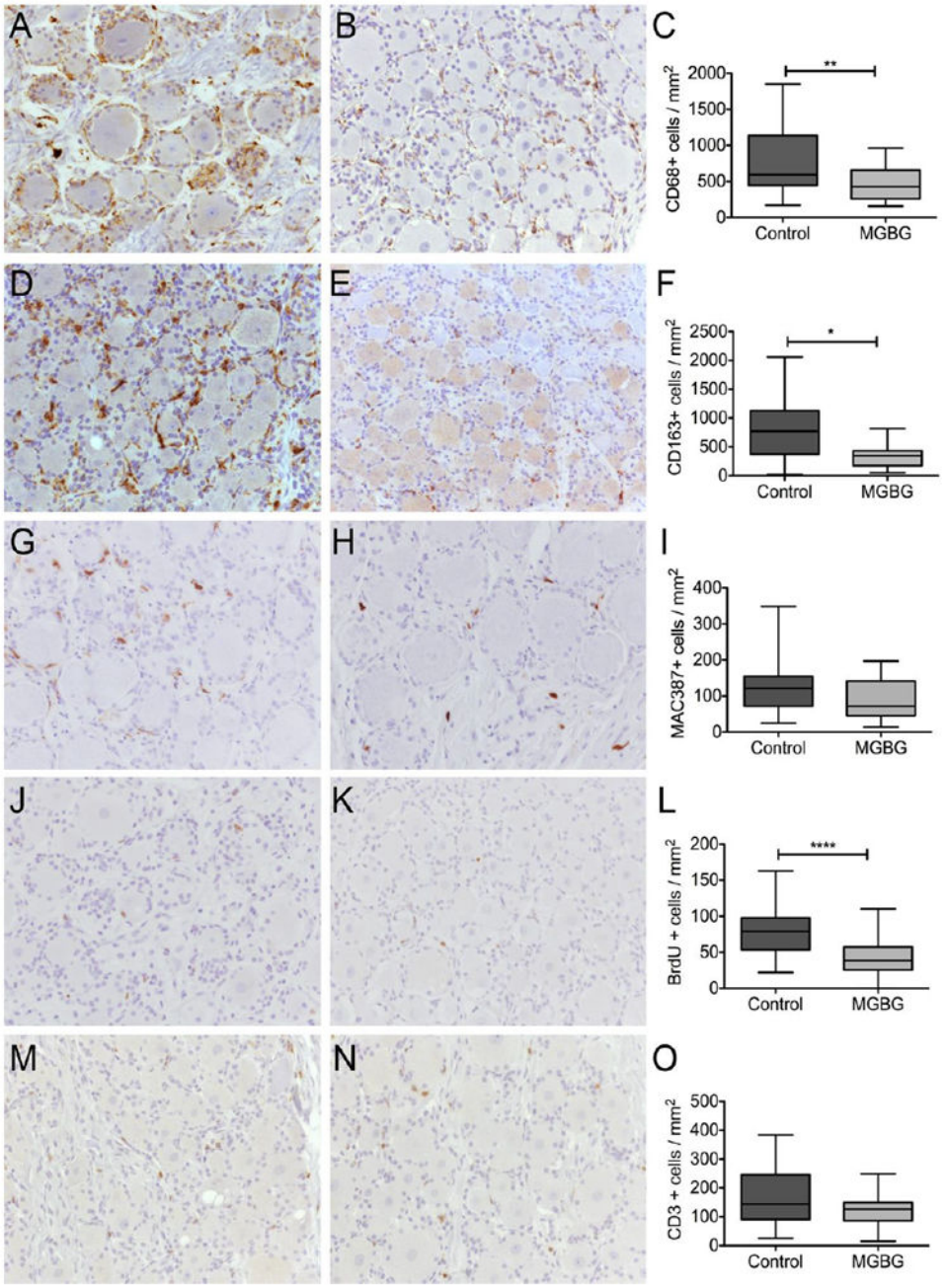


**Figure 2. MGBG treatment reduces the number of macrophages in and cell traffic to the DRG**

The numbers of CD68+ (A-C), CD163+ (D-F), MAC387+ (G-I), BrdU+ (J-L), and CD3+ (M-O) cells per $mm^2$ of tissue were counted in DRG from control animals and MGBG-treated animals. Representative images of DRG from control animals (A, D, G, J, M) and MGBG-treated animals (B, E, H, K, N) are shown. Data are shown as mean ± SEM (C, F, I, L, O). Groups were compared with a Mann-Whitney T-test. *P<0.05; **P<0.01; ****P<0.0001.

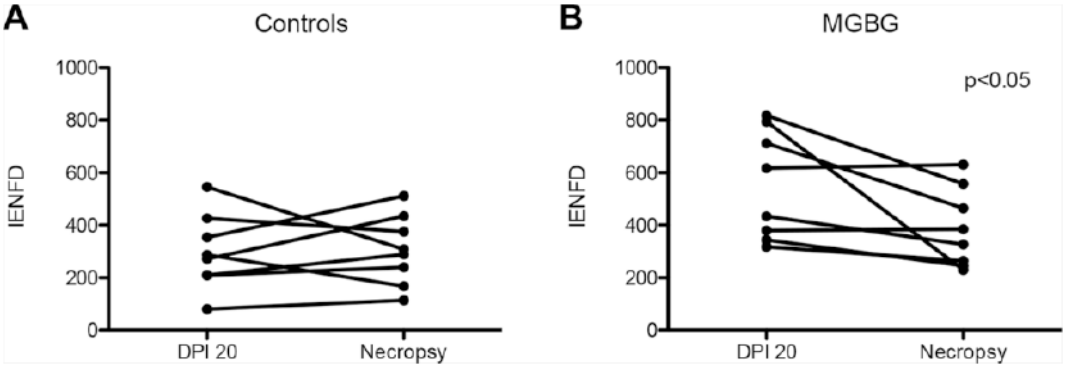


**Figure 3. MGBG treatment does not allow for regeneration of peripheral nerves**

IENFD was measured at 20 dpi and at necropsy in control (A) and in MGBG-treated (B) groups. Changes in IENFD between time points assessed with a Wilcoxon matched-pairs signed rank test. A p value less than 0.05 was considered significant.

**Table 1**

SIV+ rhesus macaques used in this study and DRG pathology.

| Animal group | ID | Survival (days) | Age at necropsy (years) | Sex of animal | Viral load at necropsy (log10) | Overall DRG pathology | Satellitosis | Neuronophagia | Nageotte nodules |
|---|---|---|---|---|---|---|---|---|---|
| Control (n=9) | C1 | 63 | 4.4 | Male | 5.89 | Mild | Moderate | Rare | None |
| | C2 | 70 | 4.4 | Male | 7.74 | Mild | Mild | Mild | None |
| | C3 | 77 | 5.4 | Male | 7.58 | Mild | Mild-Mod | Mild | None |
| | C4 | 83 * | 4.3 | Male | 7.63 | Mild | Mild | Mild | None |
| | C5 | 84 * | 4.4 | Male | 8.08 | Mild | Mild | Mild | Rare |
| | C6 | 77 | 3.8 | Male | 7.23 | Mod-Severe | Severe | Mod-Severe | Severe |
| | C7 | 77 | 5.8 | Male | 8.54 | Moderate | Moderate | Moderate | Rare |
| | C8 | 89 | 10.4 | Male | 7.71 | Moderate | Moderate | Moderate | Rare |
| | C9 | 55 | 7.3 | Male | 7.83 | Mild-mod | Moderate | Mild | Rare |
| MGBG (n=9) | M1 | 63 | 4.5 | Male | 8.11 | Mild | Mild | None | None |
| | M2 | 70 * | 4.4 | Male | 5.89 | NSF | Rare | Rare | None |
| | M3 | 70 | 4.4 | Male | 7.85 | Mild | Mild-Mod | Mild | None |
| | M4 | 77 * | 4.4 | Male | 6.66 | Mild | Mild | Mild | None |
| | M5 | 83 * | 4.5 | Male | 7.32 | Mild | Mild-Mod | Mild | None |
| | M6 | 84 * | 5.4 | Male | 7.11 | Mild | Mild-Mod | Mild | None |
| | M7 | 63 | 4.4 | Male | 8.04 | Mild | Mild | None | None |
| | M8 | 77 | 4.4 | Male | 8.18 | Mild | Mild-Mod | Mild | None |
| | M9 | 83 * | 4.5 | Male | 7.23 | Mild | Mild | Mild | None |

* = timed or paired sacrificed;

SIVE = SIV encephalitis; Mod = Moderate.