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Effect of Chronic Morphine Administration on Circulating Dendritic Cells in SIV-Infected Rhesus Macaques

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Abstract

We studied the effect of chronic morphine administration on the circulating dendritic cell population dynamics associated with SIV infection using rhesus macaques. Animals were either first infected with SIV and then given chronic morphine, or visa versa. SIV infection increased the numbers of myeloid DCs (mDCs), but morphine treatment attenuated this mDC expansion. In contrast, morphine increased the numbers of plasmacytoid DCs (pDCs) in SIV-infected animals. Finally, chronic morphine administration (no SIV) transiently increased the numbers of circulating pDCs. These results show that chronic morphine induces a significant alteration in the available circulating levels of critical antigen-presenting cells.

Graphical abstract

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Conflict of Interest

The authors have no known conflicts of interest concerning the results reported in this manuscript.

Keywords

Dendritic cells; myeloid; plasmacytoid; SIV; CD83; morphine

1. Introduction

Dendritic cells (DCs) are a critical component of the early response to infectious agents, and play an important role in both innate and adaptive immune responses. These cells can play

both positive (protective) and negative roles in the host response to HIV (or SIV) infection. It is well established that DCs are capable of expressing CD4 and most of the chemokine co-receptors, including both CCR5 and CXCR4, which are the major co-receptors involved in HIV attachment (Granelli-Piperno et al., 1996; Rubbert et al., 1998; Ignatius et al., 2000; Turville et al., 2001). While DCs are susceptible to infection, it appears that the level of viral replication in these cells is relatively low (Cameron et al., 1992; McIlroy et al., 1995; Pope et al., 1995), due in part to the low level of CD4 and coreceptor expression (Wu and KewalRamani, 2006; Liu et al., 2009a). Because of their vigorous migratory activity, DCs spread the virus to lymph node compartments where T cells can eventually become infected. It is apparent that DCs transmit the virus to T cells during the process of cognate antigen-presentation resulting in preferential infection of HIV-specific CD4-positive T cells (Douek et al., 2002; Lore et al., 2005; Moris et al., 2006). There is evidence that HIV/SIV infection results in reduced DC function, and this appears to be due to a period of reduced absolute numbers of circulating DCs, as well as reduced functional activity of these cells (Pacanowski et al., 2001; Donaghy et al., 2003; Chehimi et al., 2007). For example, peripheral blood DCs from HIV-infected patients exhibit less efficient stimulation of T cells, suggesting that these cells fail to carry out antigen-presentation at a normal level (Macatonia et al., 1990; Knight et al., 1991). Moreover, recent studies show that HIV-infected DCs induce elevated levels of the immunosuppressive cytokine IL-10 (Granelli-Piperno et al., 2004), which would be expected to attenuate antigen-driven T cell activation induced through the DCs.

There are two major subset of DCs: CD11c-positive myeloid DCs (mDCs), and CD11c-negative plasmacytoid DCs (pDCs) (O'Doherty et al., 1994; Robinson et al., 1999; Shortman and Liu, 2002). These cell populations express distinct collections of TLRs and exhibit divergent cytokine expression profiles in response to activation (Liu et al., 2009a). Both populations of DCs express high levels of MHC class II proteins, costimulatory molecules, and are efficient antigen-presenting cells. The pDCs express TLR7 and TLR9, and respond to bacterial and viral RNA and DNA by producing very high levels of type I interferon (IFN) (Asselin-Paturel et al., 2001; Kadowaki et al., 2001; Liu, 2005). These cells are capable of strong antiviral activity by virtue of the IFN production, but they are typically less efficient antigen-presenting cells than mDCs (Asselin-Paturel et al., 2001). In general, the mDCs express TLR3 and respond to microbial patterns by producing IL-12p70, which promotes Th1 development (Cella et al., 1997; Banchereau and Steinman, 1998). While it is apparent that the mDCs are somewhat heterogeneous, the majority of these cells produce high levels of IL-12 and promote Th1 immunity (Chang et al., 2000; Johnson et al., 2011), and exhibit potent antigen-processing and presenting activity (Chang et al., 2000).

Recent data shows that chronic intravenous opioid abusers make up approximately 33% of HIV infections in the United States, and the development of neurodegeneration is more rapid and more severe in this population (Bell et al., 1998; Donahoe and Vlahov, 1998; Shor-Posner, 2000; Nath, 2002; Royal et al., 2003; Compton and Volkow, 2006; Mathers et al., 2010; Vlahov et al., 2010). Opioid abuse is associated with reduced resistance to a number of opportunistic infections, and work reported by a number of investigators, based on both clinical and laboratory research, have documented the capacity of heroin (or morphine) to inhibit adaptive and innate immune responses (Novick et al., 1989; Kreek et al., 1990;

McCarthy et al., 2001; Finley et al., 2008; Madera-Salcedo et al., 2011; Dutta and Roy, 2012). Experimental animal studies have shown that morphine administration modulates monocyte/macrophage, neutrophil, T and B lymphocyte, and NK cell function (Reviewed in (McCarthy et al., 2001; Finley et al., 2008; Dutta and Roy, 2012). However, very little is known about the effects of morphine on DC populations, especially in view of the significance of these cells in the host-parasite interaction with HIV. There is evidence that both human and murine DCs express μ -, κ -, and δ -opioid receptors, and in vitro administration of μ -opioid receptor (MOR) agonists (including morphine) has been shown to inhibit IL-23 expression by DCs (Makarenkova et al., 2001; Messmer et al., 2006; Li et al., 2009; Ma et al., 2010; Wang et al., 2011). However, to our knowledge, there are no studies which address the effects of chronic morphine administration on circulating DCs in either HIV/SIV-infected or non-infected primates. Using an SIV model for infection of rhesus macaques, the present studies show that chronic morphine administration reduces circulating levels of mDCs in SIV-infected animals. However, the levels of circulating pDCs is elevated in both SIV-infected and non-infected animals.

2. Materials and Methods

2.1. Animals

A total of 44 Indian Rhesus macaques were used in these studies. In study A, there were 32 animals (20 female and 12 male), and study B in which there were 12 female animals, with an average age of 4.4 ± 1.7 years at study initiation. All animals were housed at BIOQUAL, Inc., Rockville, MD, according to standards and guidelines as set forth in the Animal Welfare Act and The Guide for the Care and Use of Laboratory Animals, as well as according to animal care standards deemed acceptable by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All experiments were performed with the approvals of both the Temple University and Bioqual Institutional Animal Care and Use Committees (IACUC). Animals were housed on site for more than three months to allow for stabilization of the immune system prior to initiation of the studies described herein. In addition, the animals were tested for retroviral pathogens and herpes B virus (Virus Reference Laboratories (VRL), Gaithersburg, MD) and were negative.

2.2. Study Designs

In Study A (Fig. 1), 27 animals were infected intravenously with SIVmac251 at a dose of 30 ID₅₀. Blood was collected at baseline (1 day prior to infection), and at 4 week intervals. At 20 weeks, morphine administration was initiated in 14 infected animals, and in 5 non-infected animals. Animals were allowed to proceed for an additional 12 weeks for the completion of this study (see Figure 1). In Study B, following a baseline blood collection, 7 animals were treated with morphine, and after 8 weeks, 5 of the morphine-treated animals and 5 non-morphine treated animals were infected with SIVmac251 at a dose of 30 ID₅₀. Blood was taken from these animals at 4 week intervals until the study was terminated at 24 weeks (Figure 1).

2.3. Quantitative assay for SIVmac251 viral RNA levels

For measurement of plasma SIVmac251 RNA levels, quantitative RT-PCR analysis was carried out which measures a conserved region of SIV gag mRNA (Lewis et al., 1994). The sensitivity of this method is two copies per analysis, and this results in a detection limit of approximately 40 RNA copies/mL. Briefly, RNA was isolated from plasma using RNA-STAT 60, and RT-PCR was carried out using One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA), with the PCR run for 40 cycles at 95°C for 15 sec. and 60°C for 1 min. The following PCR primer/probes were used: SIV2-U 5' AGTATGGGCAGCAAATGAAT 3' (forward primer), SIV2-D 5' GGCACCTATTGGAGCTAAGAC 3' (reverse primer), SIV-P 6FAMA-GATTTGGATTAGCAGAAAGCCTGTTGGA-TAMRA (probe). The signal was finally analyzed using a standard curve of known concentrations from 10⁷ to 1 copy (the linear range of concentration/signal relation spans eight orders of magnitude).

2.4. Morphine treatment

In both studies, morphine was administered in saline by intramuscular injection at 5 mg/kg, 3 times daily beginning 24 hours following the baseline bleed. This dose was introduced stepwise from 3mg/kg to 5mg/kg over the initial 2 week period. Blood was drawn at 4 week intervals following the initiation of the morphine treatment. Control animals received injections of saline on the same schedule.

2.5. Peripheral blood mononuclear cell (PBMC) isolation

Blood was layered onto 15ml of Ficoll-Paque (GE Healthcare Bio-Science AB, Uppsala, Sweden) and centrifuged at 250g for 40 minutes at RT. The PBMC layer was collected and diluted 1:1 with HBSS. Cells were pelleted at 250g for 10 min at 4C, and the cells were resuspended, and any remaining erythrocytes were lysed with ACK solution (Invitrogen) for 10 minutes at 4C. Cells were washed and suspended in RPMI1640 containing 10% FCS.

2.6. Analysis of PBMCs by flow cytometry

The PBMCs were blocked with human IgG for 30 min at 4C, and then stained for 30 min at 4C in a FACS buffer (composed of PBS containing 0.2% BSA and 0.09% sodium azide) with a combination of antibodies which included CD3-PacBlue (BD Bioscience; clone SP34-2), CD123-PerCP-Cy5.5 (BD Bioscience; clone 7G3), CD11c-Alexa700 (eBioscience; clone 3.9), CD14-Qdot605 (Life Technologies; clone Tuk4), CCR7-PE-Cy7 (BD Bioscience; clone 3D12), CD83-APC (Biolegend; clone HB15e), CD8-Qdot655 (Life Technologies; clone 3B5) or CD8-APC-H7 (BD Bioscience; clone SK1), CD1c-FITC (Miltenyi; clone AD5-8E7), CD16-V500 (BD Bioscience; clone 3G8), and CD20-V450 (BD Bioscience; clone L27). The stained cells were washed twice with FACS buffer, fixed with 2% paraformaldehyde for 10 min at 4C prior to analysis using an LSRII instrument (BD Bioscience). The LSRII cytometer was equipped with 355 nm, 405 nm, 488 nm, and 640 nm lasers, and a potential for up to 13 channels of fluorescence. The instrument was calibrated daily with BD Cytometer Setup & Tracking beads. In addition, BD Compbeads were stained individually and used as single color controls to determine the compensation adjustment within each channel. Data were obtained from at least 200,000 cellular events, and analysis

was conducted using BD FACSDIVA v6.1.3 acquisition software and exported for compensation and analysis in FlowJo v7.6.5 software. Debris, dead cells, and doublets were gated as previously described (Autissier et al., 2010). Lineage markers CD3, CD20, CD8, and CD14 were used to gate out T cells, B cells, and monocytes, respectively, prior to analysis of the individual DC populations (Fig. 2). The remaining cells were gated on CD123 and CD11c. pDCs were defined as the CD123+CD11c⁻ cells and the mDCs were defined for the purposes of this report as the CD123⁻CD11c+CD16⁺ cells. We obtained data for both CD16⁺ mDCs and CD1c⁺ mDCs, but the data presented for mDCs in our figures is limited to the CD16⁺ cells since the numbers of CD1c⁺ cells were small, and the results for the CD16⁺ and CD1c⁺ mDCs were essentially identical. Non-specific binding of antibodies was determined using isotype-matched control antibodies. Absolute numbers of the DCs were determined using a clinical automated hematology analyzer to determine the total leukocyte count for each blood sample, and calculated absolute numbers by multiplying this number times the percentages of the DC samples.

2.7. Statistical Analysis

All of the data obtained from these studies were subjected to statistical analysis by the Temple University Biostatistics Core Facility. The numbers of circulating mDC and pDC data, by treatment, were analyzed using two-way repeated measures ANOVA. Percent of control data were analyzed using one-way repeated measures ANOVA. To avoid multiple comparisons, post-hoc comparisons were pre-planned and limited to a single comparison of the last observations for the two-way ANOVA analyses and to a single comparison of the first and last observations for the one-way ANOVA analyses. Changes in baseline to last mDC or pDC levels by treatment were assessed using the two-sample t-test.

3. Results

3.1. Morphine administration does not alter circulating levels of SIV or circulating levels of CD4⁺ T cells

We carried out two studies to assess the effects of morphine on the levels of circulating DCs in rhesus macaques (Fig. 1). In Study A, animals were first infected with SIV, and allowed to stabilize for 20 weeks prior to initiation of chronic morphine administration. In addition, noninfected animals were also subjected to morphine administration at the same time. Blood was obtained from these animals at 4 week intervals until the termination of the study at 32 weeks. In Study B, chronic morphine administration (or placebo treatment) was initiated at the start of the experiment, and then most of these animals were infected with SIV after 8 weeks. Blood was obtained from these animals at 4 week intervals until the termination of the study at 24 weeks.

In Study A, we examined the SIV titer in the blood over the course of the infection in animals before and after the initiation of daily morphine administration. The results show that the SIV level in the blood reaches a stable plateau by the end of 4 weeks of infection (Fig. 3A). The results also show that the administration of morphine after 20 weeks of infection, does not induce a detectable change in the circulating level of SIV. Furthermore, we also divided the SIV-infected animals into arbitrary high titer (titer above the median)

and low titer (titer below the median) groups (Fig. 3A), and independently analyzed the viral titers for the “high” and “low” titer animals. When the animals are divided into “high” and “low” titer groups, the circulating levels of SIV are still not significantly different for the morphine-treated animals for the duration of the morphine treatment. We conducted a similar analysis of the animals in Study B (Fig. 3B), except that we focused the determination of SIV levels during the initial 4 weeks of infection. The data show that while the pre-administration of morphine resulted in somewhat higher SIV levels among the high titer animals during the first 4 weeks, there was no statistically significant difference between morphine-treated and non-treated animals.

We also assessed the levels of circulating CD4+ and CD8+ T cells in both of the studies. The results from this analysis (Fig. 4) show that the numbers of circulating CD4+ T cells declines rapidly following SIV infection ($p < 0.007$), and we observed this expected result in both Study A and B (Fig. 4A and 4D). In contrast, SIV infection had no statistically significant effect on circulating CD8+ T cell numbers (Fig. 4B and 4E), but there was the expected decline in the CD4:CD8 ratio ($p < 0.0001$) following SIV infection in both Study A and B (Fig. 4C and 4F). There were no statistically significant differences in CD4 numbers or the CD4:CD8 ratio following morphine administration (without SIV infection) in either Study A or B (Fig. 4C and 4F), and there was no statistically significant difference comparing morphine-treated and non-treated SIV infected animals (Fig. 4C and 4F). It should be pointed out that the data suggest (Fig. 4F) that the decline in CD4:CD8 ratio following SIV infection in Study B may be less substantial with morphine administration. However, this difference in CD4:CD8 ratio between morphine-treated and non-treated animals following SIV infection was not statistically significant ($p = 0.0511$).

3.2. Chronic morphine administration reduces circulating mDC levels in SIV-infected macaques

In Study A, the levels of circulating mDCs was assessed in SIV-infected animals following initiation of morphine or placebo administration. The results show (Fig. 5) a gradual increase in the levels of mDCs in the SIV-infected animals, which reached approximately a 3-fold increase in circulating total mDCs by the end of 12 weeks. In contrast, morphine administration resulted in a significant attenuation of the levels of these cells over the 12 week course of the study. Additional analysis to determine whether the impact of morphine on mDC levels might correlate with plasma SIV mRNA levels, and we determined that there was no correlation between the SIV mRNA and mDC levels in the morphine treated animals (data not shown). However, we also divided the SIV-infected animals into arbitrary high titer and low titer groups (Fig. 5A), and independently analyzed the mDC numbers for the high and low titer animals. The data show that the effect of morphine was essentially the same both high and low titer animals (Fig. 5B and 5C). We conducted a similar analysis in Study B, and the results show (Fig. 5D) that the mDC levels gradually began to increase at about 16 weeks (8 weeks following SIV infection), and reached a level of about a 2.5-fold increase in total mDCs. However, we found that the increase in total mDCs was significantly attenuated in the morphine treatment group during this late stage of the study.

We wished to extend our analysis of the mDC populations, and determine the effect of morphine on the expression of cells which express the activation marker CD83. The results from Study A show (Fig. 5E) that the percentage of CD83-positive mDCs increased significantly in the placebo-treated SIV-infected animals, while the percentage of mDCs expressing the CD83 activation marker was significantly attenuated in the morphine-treated SIV infected animals. We also analyzed the expression of CD83 expression in mDCs in Study B, and we found that the morphine administration reduced the circulating numbers of CD83+ cells at the later time points (Fig. 5F).

3.3. Chronic morphine administration modulates the numbers of circulating pDCs in SIV-infected macaques

In Study A, the numbers of circulating pDCs was determined in SIV-infected animals following initiation of morphine or placebo administration as described above. The results show (Fig. 6A) that in contrast to the numbers of circulating mDCs, SIV infection did not induce a significant change in the numbers of pDCs. However, the data show that morphine induced a statistically significant increase in the level of circulating pDCs relative to the placebo-treated SIV-infected animals. This increase in the pDC level was transitory, with a 2-fold increase at 8 weeks following the initiation of morphine treatment. We analyzed the results further by grouping the animals based on plasma SIV mRNA levels measured just prior to the initiation of the morphine administration (as described above in Fig. 5). Animals were arbitrarily segmented into the half with higher SIV levels (high SIV) and the lower SIV level (low SIV). These individual groups were then analyzed for the levels of circulating pDCs, and the results show that the numbers of circulating pDCs increased in the SIV-infected animals beginning at 8 weeks. Moreover, in the high titer animals (Fig. 6B), morphine administration depressed pDC levels, in contrast to the low titer group (Fig. 6C) or the combined high and low animals (Fig. 6A).

We conducted a similar analysis of animals in Study B, and the results (Fig. 6D) showed that the administration of morphine 8 weeks prior to SIV infection had little detectable impact on circulating pDC levels in this experimental paradigm. In contrast to the results obtained from Study A where animals were first infected with SIV 20 weeks prior to morphine administration, in Study B there was no change in circulating levels of pDCs in either high SIV or low SIV groups (data not shown).

We also wished to determine the levels of circulating activated pDCs in both of the study groups. The results from Study A show (Fig. 7A) that circulating CD83+ pDCs were increased with morphine administration to SIV-infected animals. The results reflect the effect of morphine on the total numbers of pDCs shown in Fig. 6, and these data suggest that while morphine induces a transient increase in the levels of CD83+ pDCs in SIV-infected animals, morphine does not selectively alter the activation state of these cells in animals that were previously infected. Additional analysis of the CD83+ pDCs in high and low titer subsets (Fig. 7B and 7C) shows essentially the same results as reported above in Fig. 6 for the total pDCs. We conducted a similar analysis of the animals in Study B, and our results show (Fig. 7D) that a transitory increase in the numbers of CD83+ pDCs is apparent at 4

weeks post infection for both the morphine-treated and placebo-treated SIV-infected animals.

3.4. Selective induction of circulating levels of DCs following chronic morphine administration

We wanted to examine the effect of chronic morphine administration on circulating levels of mDCs and pDCs in the absence of SIV infection. In Study A we initiated morphine administration and then assessed circulating DC levels over a period of 12 weeks. The results show (Fig. 8A) that circulating mDC levels are not significantly altered following morphine administration. Similarly, in Study B we administered morphine to noninfected animals and determined the levels of mDCs over a period of 24 weeks. The results show that there was no detectable significant change in mDC levels with morphine administration in Study B.

We also analyzed the effect of chronic morphine administration on circulating levels of pDCs. The results show (Fig. 8B) that in both Study A and B the morphine treatment resulted in a significant 2–3 fold increase in circulating pDCs by 12 weeks. Further analysis shows that the levels of activated CD83+ pDCs were increased at 12 weeks by about 5-fold, with a similar increase in both study A and B (Fig. 8D). We observed similar results for CCR7+ pDCs (Fig. 8C), in which we observed a 3–4 fold increase in circulating CCR7+ pDCs. It is apparent from the results that the morphine-driven increase in circulating pDC populations is transient, and after 12 weeks there is a decline in both studies.

4. Discussion

In the present studies we have utilized two experimental paradigms to test the effect of chronic morphine administration on the DC population dynamics in SIV-infected rhesus macaques. In Study A, animals were first infected with SIV, and after 20 weeks to allow for the establishment of the viral set point, the infected animals were randomly assigned into morphine or control groups. In this study we were able to assure that the infected animals with high and low viral titers were equally represented in the morphine and control groups. In Study B, animals were divided into control or morphine-treated groups, and after 8 weeks, these animals were infected. The advantage of this paradigm is that the chronic opioid administration precedes the infection, which more closely reflects the conditions in which an opiate abuser becomes infected with HIV. In both studies, we included groups in which the animals received chronic morphine treatment without infection. We were unable to detect an effect of morphine administration on the level of SIV in the blood in either of these studies. The absence of an effect in the first study may have been due to the fact that a viral set point had been established several months prior to the initiation of the morphine administration, and the level of viral replication may have been insensitive to the effects of the opiate at this point. However, pre-administration of morphine in the second study also had little effect on circulating viral titers. It should be pointed out that we were unable to assess tissue reservoirs of the virus, and it is entirely possible that morphine did manifest an effect at the level of the reservoir. In any case, we suspect that the effects of morphine are more apparent at the level of infection-associated neurodegeneration and

neuroinflammation, parameters which were not assessed in these studies. This would be consistent with epidemiological evidence from analysis of intravenous opiate abusers (Bell et al., 1998).

Several published reports have shown that the absolute numbers of circulating DCs are rapidly reduced within the first 1–2 weeks of either HIV or SIV infection (Grassi et al., 1999; Donaghy et al., 2001; Chehimi et al., 2002; Brown et al., 2007; Reeves and Fultz, 2007; Malleret et al., 2008b; Barratt-Boyes and Wijewardana, 2011). The decline in circulating numbers of both mDCs and pDCs occurs within the first 1–2 weeks of infection, but a rebound in the numbers of these cells begins to become apparent by 4–5 weeks following SIV infection. It should be noted that the timing of the circulating DC decline and rebound, and the degree of the reduced DC numbers, varies depending on the SIV strain (Reeves and Fultz, 2007; Diop et al., 2008). In the present studies, we did not examine the numbers of DCs until 4 weeks post infection (in Study B), and as expected, the numbers of both mDCs and pDCs were not substantially reduced relative to baseline at this time point.

Our results show a gradual increase in the numbers of circulating mDCs beginning at 20 weeks post-infection (Fig. 5; Study B). We observed the same gradual increase in mDC numbers in the second study, with a statistically significant two-fold increase by 16 weeks. Previous studies have documented a similar increase in the circulating numbers of mDCs in SIV-infected rhesus or cynomolgus macaques (Malleret et al., 2008a; Wijewardana et al., 2010). Using the same SIVmac251 strain, Barratt-Boyes and Wijewardana (Barratt-Boyes and Wijewardana, 2011) have reported that in non-progressor SIV-infected macaques, after a transient mDC loss, the circulating mDC numbers steadily increase to reach 200% of the pre-infection levels. However, in the present study, the increased numbers of mDCs are apparent in both the animals with a “high” and “low” SIV titer, suggesting that the macaques exhibiting a high titer in our experiments behave more like the non-progressor animals in the Barratt-Boyes and Wijewardana report. Our data are somewhat surprising given the documented inverse correlation between circulating mDCs and viral load in studies of both HIV and SIV infection (Feldman et al., 1995; Feldman et al., 2001; Pacanowski et al., 2001; Brown et al., 2007). It should be pointed out that the SIV “high” or “low” titers in our studies is an arbitrary designation (titers above/below the median, $10^{5.1}$) and are not necessarily comparable to these titer designations that may be reported in the literature for other SIV studies.

The numbers of circulating mDCs represent the balance between the mobilization of precursor cells from the bone marrow, and the exodus of cells which are recruited to extravascular tissues. The Common DC Precursors (CDPs) develop in the bone marrow, and mDC development is controlled by a number of factors, including M-CSF, GM-CSF, the FMS-like tyrosine kinase-3 ligand (Flt-3L), and the influence of T regulatory cells (Waskow et al., 2008; Liu et al., 2009b; Geissmann et al., 2010; Collin et al., 2013). The capacity of HIV to induce the expression of M-CSF and GM-CSF in both the blood and CNS is very well documented (Gruber et al., 1995; Haine et al., 2006; Kogan et al., 2012). It is possible that an up-regulation in one or more of these cytokines may be responsible for the gradual increase in mDC numbers we observed in the blood at the later time points.

Our data show that this steady increase in circulating numbers of mDCs following SIV infection was attenuated following morphine treatment in both studies. It is possible that the failure of mDCs to expand in the morphine-treated animals was due to reduced expression of the cytokines reviewed above, and an examination of the impact of chronic morphine administration on the production of these cytokines is warranted. A number of studies using either in vitro cell culture methods, or in vivo studies using experimental animal models, have shown a reduction in the expression of a several pro-inflammatory cytokines following acute administration of morphine (reviewed in (Rogers and Peterson, 2003)). However, it is also possible that depressed levels of mDCs in the chronic morphine-treated group may be due to increased exodus of these cells from the circulation. A number of reports have shown that mDCs are recruited from the bloodstream to extravascular tissues following SIV infection, particularly to the lymph nodes (Brown et al., 2007; Brown et al., 2009; Wijewardana et al., 2010). A recent report shows that SIV infection leads to recruitment of DCs to the brain, and the numbers of DCs in brain tissue were slightly greater with the combination of SIV infection and morphine administration (Hollenbach et al., 2014).

In contrast to our results with mDCs, in both of our studies we observed little long-term effects of SIV infection on the circulating numbers of pDCs. However, in the first study we did find an unexpected significant elevation in the numbers of blood pDCs in those animals with a high SIV titer that was absent from the animals with a low titer. Published literature has shown that the circulating levels of pDCs is typically inversely correlated with HIV titer, and the numbers of pDCs in the blood decline in the first 2 weeks following infection (Donaghy et al., 2001; Soumelis et al., 2001). Our data also show that the pDCs in the animals with a high SIV titer express the activation marker CD83 (Fig. 7), and it is possible that the more highly activated pDCs may have directly or indirectly promoted expansion of this DC population. Nevertheless, an explanation for the expansion of pDCs in the context of elevated SIV replication will require further analysis.

A number of recent studies have shown a relationship between pDC function, and the generation of T regulatory (Treg) cells. It is apparent that pDCs induce FoxP3+ Treg cell activity (Moseman et al., 2004; Ito et al., 2008), and DCs isolated from the lymph nodes of HIV-infected patients are able to induce CD4+ T cells to express FoxP3 (Krathwohl et al., 2006). In addition, the circulating Treg cells numbers are reduced early after SIV infection (Karlsson et al., 2007; Qin et al., 2008), and this is in parallel with the numbers of circulating pDCs. Moreover, we have recently reported results which show that chronic morphine administration in the absence of SIV infection induces an increase in circulating Treg cells which become significant at 12 weeks (Cornwell et al., 2013). It is tempting to associate this increase Treg cell numbers at 12 weeks, with our data herein showing an increase in circulating pDCs at the same time.

Our results show that chronic morphine administration induced a small but significant increase in the numbers of circulating pDCs in our first study, and this was particularly apparent in animals with a low SIV titer. Our overall results, in which chronic morphine administration attenuated expansion of mDCs, and increased the numbers of pDCs, suggests that the effect(s) of morphine are highly selective in the context of SIV infection. Moreover, morphine administration in the absence of SIV infection (Fig. 8), did not alter numbers of

circulating mDCs, but induced a significant but transient increase in numbers of pDCs. These results strongly suggest that the combination of SIV infection and chronic morphine administration alters DC population dynamics in a manner which is distinct from the effects induced by either the drug or the infection alone. The analysis of the expansion of pDC numbers in response to morphine alone shows that these cells are activated (CD83-positive) and express the homing receptor CCR7. It is particularly remarkable that with the induction of CCR7 expression, these cells apparently fail to relocate to lymph nodes or the gut mucosa (the typical sites for CCR7– driven recruitment) (Forster et al., 2008). The increase of pDCs in the bloodstream was consistent in both studies, and reached a peak at 12 weeks post-morphine treatment initiation. The mechanism for this increase will require more extensive experimental analysis.

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Abbreviations

mDC	myeloid dendritic cell
MOR	μ -opioid receptor
pDC	plasmacytoid dendritic cell

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Highlights

- SIV infection alters the number of circulating mDCs and pDCs in rhesus macaques.
- Chronic morphine administration attenuates the SIV-induced increase in the number of circulating mDCs.
- Chronic morphine administration up-regulates the number of circulating pDCs following SIV infection.
- Chronic morphine transiently increases the number of circulating pDCs in the absence of SIV infection.

Fig. 1.

Diagrammatic summary of experimental paradigms. Study A, 5 animals were treated with morphine beginning at 20 weeks, 27 animals were infected on day 1, and 18 of these infected animals were treated with morphine beginning at 20 weeks post infection. In Study B, 7 animals were treated with morphine, and then all 10 of the animals were infected with SIV at 8 weeks. The diagram shows the organization of groups: yellow: no morphine/no SIV; red: morphine treated/no SIV; black: no morphine/SIV infected; and green: morphine treated/SIV infected.

Fig. 2.

Flow cytometric gating strategy to define DC populations. **a.** Forward and side scatter was used to identify lymphocyte and monocytes regions and a gate was drawn (red oval). **b.** Events in this gate were further selected to exclude doublets using the area and height of the forward scatter. **c.** Lineage markers, CD3, CD20, and CD14 were used to exclude T cells, B cells, and monocytes. **d.** CD8+ cells were excluded in panel D and the CD8– cells contain both CD16+ and CD16– populations (red box). **e–g.** The red box in panel D which contains lin– cells, are further characterized and subdivided by CD11c expression and CD16+CD11c+

mDCs (**e**) (red circle), CD1c+ mDCs (**f**) (red square), and pDC defined as CD11c–CD123+ cells (**g**) are shown.

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Fig. 3.

Analysis of SIV titers in morphine-treated and non-treated macaques. (a). In study A, animals were infected with SIV and viral titers were measured at 4 week intervals. Animals with viral titers greater than the median (log 4.76) were designated “high”, and the remaining animals were designated “low” titer animals. Morphine was administered to an equal number of high and low titer animals at week 20 post infection. (b) In study B, animals were infected with SIV following 8 weeks of morphine or placebo administration, viral titers were measured weekly for 4 weeks post infection. Based on the week 4 viral

titers, animals with viral titers greater than the median (log 5.06) titer for all animals were designated high titer animals while the remaining were designated as low titer animals.

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Fig. 4.

Chronic morphine administration does not alter circulating CD4 or CD8 cell numbers. **a-c.** In Study A, animals were infected with SIV 20 weeks prior to receiving morphine (●) or saline (■), as well as animals which were not infected with SIV but did receive morphine immediately following the zero time point (▼). The numbers of circulating CD4 and CD8 cells/ μ l, as well as the CD4:CD8 ratio, were determined every 4 weeks. **d-f.** In Study B, animals received either saline (◆) or morphine (▼) for 2 months prior to receiving SIV. The figure shows saline-treated animals which were then SIV infected (black ■), morphine-

treated animals which receive SIV, (green ●), and morphine treated animals which do not receive SIV (red ▼). Data are presented as the mean $\pm$ SEM. Analysis by two-way repeated measures ANOVA show that both the SIV only, and SIV + morphine groups were significantly reduced after SIV infection ($p < 0.007$) (panels **a** and **d**). The analysis also shows that SIV infection (with or without morphine) significantly reduced CD4:CD8 ratio ($p < 0.0001$) (panels **c** and **f**).

Fig. 5.

Chronic morphine administration attenuates the SIV-induced expansion of circulating mDC numbers. Results are presented for Study A (a–c, e) and Study B (d, f). **a.** In Study A, animals were first infected with SIV and at 20 weeks a portion of these animals began treatment with morphine. The number of mDC increases with SIV infection (■), but this is attenuated with morphine treatment (●). **b–c.** Results from animals with a high SIV titer and low SIV titer demonstrate the same effects. **d.** In Study B, animals were first treated with morphine (or control), and at 8 weeks were infected with SIV. Animals treated with

morphine and then infected with SIV show an attenuation of the increase in circulating mDC numbers. **e.** CD83+ mDCs were obtained as described in panel (a), and show the same pattern as the total mDCs in Study A. **f.** CD83+ mDCs were obtained as described in panel (d), and show the same pattern as the total mDCs in study B. Data are presented as the mean $\pm$ SEM. Repeated measures ANOVA analysis shows a significant effect of morphine vs saline over time ($p < 0.05$) (panels **a–e**). Analysis of individual data points vs baseline by t-test shows: * = $p < 0.05$ as compared with the 20 week time point (Study A, panels **a–c, e**), or the 8 week time point (Study B, panel **d, f**). S = SIV infected; S+M = SIV infected followed by morphine-treated; C = control (no morphine/no SIV); M+S = Morphine-treated followed by SIV infected.

Fig. 6.

Chronic morphine exposure modulates the number of circulating pDCs. **a.** In Study A the number of pDCs is modestly increased with morphine treatment of SIV-infected animals. **b.** In animals with a high SIV titer, there is a significant increase in circulating pDC numbers, which is absent in the morphine-treated SIV-infected animals. **c.** In animals with a low SIV titer, there is a transient increase in the numbers of pDCs in morphine-treated SIV-infected animals. **d.** In study B, there is no significant change detected in pDC numbers in any group. Data are presented as the mean $\pm$ SEM. Repeated measures ANOVA analysis shows a

significant effect of morphine vs saline over time ($p < 0.05$) (panels **a**, **b**). Analysis of individual data points vs baseline by t-test shows: * $p < 0.05$ as comparing morphine vs saline at a given time point (Study A).

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Fig. 7.

Chronic morphine exposure modulates the number of circulating activated (CD83+) pDCs.

a. In Study A the number of CD83+ pDCs is modestly increased with morphine treatment of SIV-infected animals relative to the non-morphine-treated SIV infected animals. **b.** In animals with a high SIV titer, there is a significant increase in circulating CD83+ pDC numbers, which is absent in the morphine-treated SIV-infected animals. **c.** In animals with a low SIV titer, there is a transient increase in the numbers of CD83+ pDCs in morphine-treated SIV-infected animals relative to the non-morphine-treated SIV infected animals. **d.** In

study B, there is a transient increase in the numbers of CD83+ pDCs 4 weeks after SIV infection with or without morphine treatment. Data are presented as the mean $\pm$ SEM. Repeated measures ANOVA analysis shows a significant effect of morphine vs saline over time ($p < 0.05$) (panels **a**, **c**). Analysis of individual data points vs baseline by t-test shows: * $p < 0.05$ as comparing morphine vs saline at a given time point (Study A).

Fig. 8.

Chronic morphine exposure without SIV infection modulates the number of circulating pDCs. **a.** The number of circulating mDCs in Study A (▲) and Study B (▼) are not altered in animals treated with morphine (without SIV infection). **b.** The number of circulating pDCs exhibits a transient increase at 12 weeks in animals from both Study A and Study B. **c** – **d.** The numbers of either activated CCR7+ pDCs (**c**), or CD83+ pDCs (**d**), show a transient increase in both Study A and Study B. Data are presented as the mean $\pm$ SEM. Repeated measures ANOVA analysis shows a significant effect of morphine over time ($p < 0.05$) (panels **b–d**). Analysis by t-test shows: * $p < 0.05$ as compared with animals prior to initiation of morphine treatment for both Study A and B.