Short-Term Pegylated Interferon α 2a Treatment Does Not Significantly Reduce the Viral Reservoir of Simian Immunodeficiency Virus-Infected, Antiretroviral Therapy-Treated Rhesus Macaques

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ABSTRACT The major obstacle to human immunodeficiency type 1 (HIV-1) eradication is a reservoir of latently infected cells that persists despite long-term antiretroviral therapy (ART) and causes rapid viral rebound if treatment is interrupted. Type I interferons are immunomodulatory cytokines that induce antiviral factors and have been evaluated for the treatment of HIV-infected individuals, resulting in moderate reduction of viremia and inconclusive data about their effect on reservoir size. Here, we assessed the potential of pegylated IFN- α 2a (pIFN- α 2a) to reduce the viral reservoir in simian immunodeficiency virus (SIV)-infected, ART-treated rhesus macaques (RMs). We found that pIFN- α 2a treatment of animals in which virus replication is effectively suppressed with ART is safe and well tolerated, as no major clinical side effects were observed. By monitoring the cellular immune response during this intervention, we established that pIFN- α 2a administration is not associated with either CD4⁺ T cell depletion or increased immune activation. Importantly, we found that interferon-stimulated genes (ISGs) were significantly upregulated in IFN-treated RMs compared to control animals, confirming that pIFN- α 2a is bioactive *in vivo*. To evaluate the effect of pIFN- α 2a administration on the viral reservoir in CD4⁺ T cells, we performed cell-associated proviral SIV DNA measurements in multiple tissues and assessed levels of replication-competent virus by a quantitative viral outgrowth assay (QVOA). These analyses failed to reveal any significant difference in reservoir size between IFN-treated and control animals. In summary, our data suggest that short-term type I interferon treatment in combination with suppressive ART is not sufficient to induce a significant reduction of the viral reservoir in SIV-infected RMs.

IMPORTANCE The potential of type I interferons to reduce the viral reservoir has been recently studied in clinical trials in HIV-infected humans. However, given the lack of mechanistic data and the potential for safety concerns, a more comprehensive testing of IFN treatment *in vivo* in SIV-infected RMs is critical to provide rationale for further development of this intervention in humans. Utilizing the SIV/RM model in which virus replication is suppressed with ART, we addressed experimental limitations of previous human studies, in particular the lack of a control group and specimen sampling limited to blood. Here, we show by rigorous testing of blood and lymphoid tissues that virus replication and reservoir size were not significantly affected by pIFN- α 2a treatment in SIV-infected, ART-treated RMs. This suggests that intensified and/or prolonged IFN treatment regimens, possibly in combination with

Received 16 February 2018 **Accepted** 26 April 2018

Accepted manuscript posted online 2 May 2018

Citation Palesch D, Bosinger SE, Mavigner M, Billingsley JM, Mattingly C, Carnathan DG, Paiardini M, Chahroudi A, Vanderford TH, Silvestri G. 2018. Short-term pegylated interferon α 2a treatment does not significantly reduce the viral reservoir of simian immunodeficiency virus-infected, antiretroviral therapy-treated rhesus macaques. *J Virol* 92:e00279-18. <https://doi.org/10.1128/JVI.00279-18>.

Editor Viviana Simon, Icahn School of Medicine at Mount Sinai

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other antilateness agents, are necessary to effectively purge the HIV/SIV reservoir under ART.

KEYWORDS AIDS, HIV, SIV, antiretroviral therapy, interferons, latency, rhesus macaque, viral reservoir

Despite the tremendous success of antiretroviral therapy (ART) in prolonging the survival and improving the quality of life of human immunodeficiency virus (HIV)-infected individuals, a treatment that can eradicate or functionally cure the infection is not available. In addition, long-term ART is complicated by drug toxicities, incomplete immune reconstitution, and residual immune activation/inflammation which increases the risk of various end-organ diseases, including cardiovascular disease, kidney disease, bone disease, cancer, and neurocognitive decline (1–4). The main obstacle to HIV eradication or functional cure is the presence of a persistent reservoir of latently infected cells that is not affected by ART. This viral reservoir predominantly comprises a population of resting memory CD4⁺ T cells which harbor integrated, replication-competent proviruses (5, 6).

In this context, the potential of type I interferons (IFN-I) as an anti-reservoir therapeutic approach has been the subject of significant interest. Interferons are major effector cytokines that have antiviral, antiproliferative, and immunomodulatory effects. Interferons are classified into three groups: type I (IFN- α , IFN- β , IFN- ω , IFN- ϵ , and IFN- κ), type II (IFN- γ), and type III (IFN- λ 1-3) IFN (7). IFN-I, and particularly IFN- α 2 and IFN- β , have been used for many years in humans as antiviral and immunomodulatory agents for viral infections and autoimmune diseases, respectively (8). The role of IFN in HIV/simian immunodeficiency virus (SIV) infection and whether it is beneficial or harmful has been intensely investigated over the past decade. IFN-I production is induced and IFN-stimulated genes (ISGs) are upregulated during acute SIV infection (9, 10). In addition, it has also been shown that treatment with IFN-I antagonists is followed by increased virus replication and accelerated disease progression in SIV-infected rhesus macaques (11). Importantly, IFN-I and ISGs normalize in nonpathogenic SIV infections of sooty mangabeys and African green monkeys during the transition from acute to chronic infection, while they remain upregulated throughout the chronic phase of pathogenic HIV and SIV infections (9, 10, 12, 13). However, the antiviral activity of this endogenously produced IFN-I is not sufficient to suppress virus replication in the absence of ART, and in fact persistently elevated circulating IFN- α levels are associated with higher levels of virus replication and predict disease progression (14). On the other hand, IFN-I induces the expression of numerous host factors that can potentially restrict lentiviral replication, including TRIM5 α , APOBEC-3G, Tetherin, SAMHD1, and others (reviewed in reference 15). This potent antiviral activity has prompted the investigation of the effects of exogenous administration of IFN-I during untreated HIV infection, and these studies revealed inconsistent effects on viremia and CD4⁺ T cell counts with no change in disease outcomes (16–20). Intriguingly, two more recent studies showed a modest, though significant, reduction of the virus reservoir in a small cohort of ART-treated, HIV-infected individuals (21, 22). However, the lack of an appropriate control group has complicated the interpretation of this result. In addition, these studies in humans were limited to the analysis of peripheral blood, omitting secondary lymphoid organs which harbor a substantial portion of the latent reservoir.

To address these potential limitations of previous studies in humans, we used the model of SIV infection of rhesus macaques (RMs), which is very well established and robust and features a number of important similarities with HIV infection in terms of early acute infection events, viral and CD4⁺ T cell dynamics, establishment of reservoirs, and events associated with late disease progression (23, 24). Importantly, the RM model of SIV infection has recently been sufficiently validated for studies of virus eradication and/or functional cure by developing an optimized ART regimen that fully and consistently suppresses virus replication in SIV-infected RMs (25–27). The current study included 12 SIV-infected RMs in which virus replication was effectively suppressed with

FIG 1 Experimental design. Twelve RMs were infected intrarectally (I.R.) with 10,000 TCID₅₀ of SIVmac239. At week 6 postinfection (w.p.i.), all RMs started a four-drug antiretroviral therapy (ART) regimen consisting of PMPA (tenofovir) at 20 mg/kg/day, FTC (emtricitabine) at 40 mg/kg/day, dolutegravir at 2.5 mg/kg/day, and darunavir at 375 mg b.i.d. Six animals were initiated on pIFN-α2a in addition to ART at 32 w.p.i., and 6 animals were continued on ART alone as controls.

a potent, four-drug ART regimen to investigate the effect of additional pIFN-α2a administration for a 4-week period. We found that in this *in vivo* experimental setting, pIFN-α2a (i) is clinically safe, (ii) does not deplete CD4⁺ T cells, (iii) does not induce excessive immune activation and exhaustion associated with disease progression, and (iv) induces marked ISG upregulation. However, we also found that pIFN-α2a intervention fails to significantly deplete the viral reservoir of latently infected cells, suggesting that intensified and/or prolonged IFN treatment regimens, possibly in combination with other antiretroviral agents, will be required to effectively purge the HIV/SIV reservoir under ART.

RESULTS

Experimental design, SIV infection, and ART treatment. In this study, whose overall experimental design is shown in Fig. 1, we performed a short-term (i.e., 4 weeks) treatment with pegylated IFN-α2a (pIFN-α2a) in SIV-infected RMs in which virus replication is suppressed by a potent ART regimen. The main goal of this study was to test whether a signal of reservoir reduction could be detected in pIFN-α2a-treated animals compared to untreated controls. To this end, we longitudinally collected blood, lymph node, and rectal biopsy specimens throughout the course of the study and monitored a number of virological and immunological parameters during ART, as well as prior to and during pIFN-α2a treatment (Fig. 1). We infected 12 RMs intrarectally with 10,000 50% tissue culture infective doses (TCID₅₀) of SIVmac239, which resulted in a robust infection with peak viral loads of 10⁶ to 10⁸ viral RNA copies/ml (Fig. 2A). After 6 weeks of infection, all RMs started a three-class, four-drug ART regimen consisting of two nucleoside reverse transcriptase inhibitors (PMPA [tenofovir], 20 mg/kg of body weight/day; FTC [emtricitabine], 40 mg/kg/day), one integrase inhibitor (dolutegravir, 2.5 mg/kg/day), and one protease inhibitor (darunavir, 375 mg twice a day [b.i.d.]). Once viral loads were consistently undetectable, six RMs were administered 1 dose of pIFN-α2a per week for 4 weeks with each weekly intramuscular application at 6 μg/kg, as previously described (11). Six animals did not receive IFN treatment but were kept on ART and served as controls. All SIV-infected RMs in this study were continued on ART until necropsy. As shown in Fig. 2A, all animals receiving ART experienced a rapid and highly significant decline in plasma viremia, and by week 30 postinfection all animals showed plasma viremia below the limit of detection of our standard assay (i.e., <60 SIV RNA copies/ml of plasma). This result is in line with previous studies from us and others, which showed that this recently optimized ART regimen is (i) safe and well-tolerated and (ii) fully and consistently suppresses virus replication in SIV- and SHIV-infected RMs (25, 27–29). As shown in Fig. 2B and in accordance with many previous studies, we observed in all animals the well-characterized progressive depletion of circulating CD4⁺ T cells, measured as the fraction of CD3⁺ T lymphocytes, during acute SIV

FIG 2 Longitudinal assessment of virological and immunological parameters. (A) Levels of plasma viral load using a standard SIV RT-PCR assay. Viremia is shown as RNA copies per ml of plasma. The dotted line indicates the limit of detection (i.e., <60 SIV-RNA copies/ml of plasma). Gray shading depicts the period of antiretroviral therapy (ART). The arrow indicates pIFN- α 2a treatment initiation. (B) CD4⁺ T cell frequency is depicted as a percentage of CD3⁺ T cells in blood, as monitored using flow cytometry. Means $\pm$ standard deviations are shown.

infection. As expected, this was followed by a partial reconstitution of CD4⁺ T cell levels during ART. Importantly, pIFN- α 2a treatment was not associated with a decline of CD4⁺ T cell levels.

Safety profile of pIFN- α 2a treatment in SIV-infected ART-treated rhesus macaques. Having established that, in our cohort of SIV-infected RMs, virus replication was suppressed by ART to an extent that closely resembled the level of virus suppression observed in HIV infection of humans, we next examined several clinical and laboratory parameters to assess the safety of exogenous pIFN- α 2a administration in this preclinical setting. We also monitored the animals daily for clinical signs of discomfort (i.e., weight loss, lethargy, loss of appetite, weakness, diarrhea, etc.) and for hematological parameters, such as white blood cell (WBC) count, lymphocyte count, platelet count, and hemoglobin levels, as well as concentrations of creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) (Fig. 3). Aside from a very modest increase in platelets upon IFN treatment, we observed no signs of liver dysfunction, chemical or hematologic abnormalities, or any significant deviations relative to pretreatment values or corresponding time points in the control animals.

pIFN- α 2a administration induces the expression of ISGs. The establishment of an ART-suppressed model system in which we were able to safely administer pIFN- α 2a

FIG 3 pIFN- $\alpha 2a$ treatment is safe. (A to C) Cell counts per μL of blood for white blood cells (A), lymphocytes (B), and platelets (C), as determined by complete blood count (CBC). (D to G) Hemoglobin (D), aspartate aminotransferase (AST) (E), alanine aminotransferase (ALT) (F), and creatinine (G) were measured by blood chemistry analysis. Week 32, pre-IFN treatment; week 36, post-IFN treatment; gray shading depicts the period of IFN treatment. Unpaired Mann-Whitney test was used for analysis of week 36 post-IFN versus week 36 postsham treatment; paired Wilcoxon matched-pairs signed-rank test was used for analysis of week 32 pre-IFN versus week 36 post-IFN; P values are indicated. Means $\pm$ standard deviations are shown.

to RMs allowed us to assess the effect of short-term pIFN- $\alpha 2a$ administration on the reservoir of latently infected cells. To this end, we first performed a comprehensive analysis of the specific bioactivity of pIFN- $\alpha 2a$ by measuring the induction of interferon-stimulated genes (ISGs) (Fig. 4). Human IFN- $\alpha 2$ and the homologous rhesus macaque protein have an amino acid sequence identity of more than 92%. Hence, several studies have previously shown that recombinant human pIFN- $\alpha 2a$ induces a strong IFN response in RMs. IFN- α induces the production of hundreds of ISGs, most of which exhibit antiviral or immunomodulatory activity. Due to the broadness of the type I IFN response, we developed a protocol and bioinformatics pipeline that uses high-throughput RNA deep-sequencing (RNA-seq) technology to quantify ISG expression *en masse*. This assay uses a novel bioinformatics resource, namely, a new reference genome for RM, MacaM, and a custom gene-filtering list of ISGs (30). We have employed this strategy in several studies, including a recent publication in which we validated the bioactivity of an IFN-antagonist treatment and of pIFN- $\alpha 2a$ treatment in ART-naïve, SIV-infected RMs (11). The *in vivo* bioactivity of pIFN- $\alpha 2a$ treatment was

A

B

assessed by RNA-seq analyses of sorted CD4⁺ T cells prior to and at 1 week after IFN treatment. As shown in Fig. 4, we observed a clear upregulation of multiple ISGs of various signaling pathways of the innate immune response to lentiviral infection. This includes the antiviral factor tripartite motif-containing protein 5 (TRIM5), myxovirus resistance 2 (MX2), a GTPase that restricts HIV replication, and pattern recognition receptors of viral RNA retinoic acid-inducible gene I (RIG-I/DDX58) and melanoma differentiation-associated protein 5 (MDA5/IFIH1) (31–34). Figure 4 summarizes the expression distribution in 23 prototypical ISGs. On a technical level, RNA-seq offers an advantage over microarrays in that it provides an absolute estimate of expression and not simply fold change/relative expression. Interestingly, we observed induction of interferon regulatory factors (IRFs), such as IRF7, which is considered to be the master regulator transcription factor controlling type I interferon responses (35). Collectively, these data unambiguously demonstrate that the pIFN- α 2a treatment achieved a high level of *in vivo* biological efficacy in ART-treated SIV-infected RMs.

pIFN- α 2a does not induce T cell immune activation. We next evaluated the immunological impact of pIFN- α 2a treatment during ART by determining the relative frequencies of a comprehensive number of cell populations representing key players of the innate as well as the adaptive immune response. As shown in Table 1, IFN treatment did not induce any change in CD4⁺ T cell frequencies in either the peripheral blood or the lymph node (IFN treated, 58.08 ± 1.99 ; controls, 59.58 ± 1.85 ; IFN treated, 60.85 ± 2.67 ; controls, 57.95 ± 2.56 ; each as a percentage of CD3⁺ T cells). In contrast, we observed a nonsignificant trend toward lower frequencies of CD8⁺ T cells (IFN treated, 24.05 ± 3.94 ; controls, 32.83 ± 1.50 ; each as a percentage of CD3⁺ T cells) and higher frequencies of B cells (IFN treated, 13.72 ± 2.64 ; controls, 8.37 ± 2.33 ; each as a percentage of peripheral blood mononuclear cell [PBMC]) in blood. CD4⁺ and CD8⁺ T cell frequencies in the rectal mucosa were also not significantly affected by IFN administration (data not shown). Moreover, NK cell numbers were not significantly affected; however, CD16 is downregulated in NK cells of IFN-treated animals. Frequencies of myeloid cells, including mDC, pDC, and monocytes, remained largely unchanged, with the exception of a significant decrease of CD16⁺ nonclassical monocytes in IFN-treated animals. However, this cell population represents only a very small fraction of the total monocyte population, which overall has not been significantly affected by IFN treatment.

Lymphocyte immune activation is a classic marker of disease progression in HIV and SIV infection (9–11). Hence, we assessed the expression of key markers of lymphocyte activation (HLA-DR) and proliferation (Ki-67) on cells from peripheral blood and lymph node specimens. Although HLA-DR and Ki67 levels were slightly reduced in CD4⁺ T cells and slightly increased in CD8⁺ T cells, no significant changes were found in the level of either HLA-DR or Ki-67 T cells (either CD4 or CD8) in peripheral blood or lymph node of IFN-treated SIV-infected RMs on ART (Table 1). Of note, we found that, somewhat unexpectedly, activation marker HLA-DR was significantly, but transiently, decreased at 7 days after IFN treatment compared to pretreatment values in CD4⁺ and CD8⁺ T cells from PBMCs and lymph nodes (data not shown). In contrast, we observed a significant upregulation of proliferation marker Ki67 in B cells (IFN treated, 8.37 ± 0.56 ; controls, 5.40 ± 0.78 ; each as a percentage of Ki67⁺ cells), which is reflected by higher numbers of B cells in blood (Table 1). Overall, these data demonstrate that pIFN- α 2a administration does not induce lymphocyte activation, which has been associated with disease progression; however, it is followed by a small increase in

FIG 4 pIFN- α 2a treatment effectively induced ISG expression in ART-suppressed RMs. (A) Median read counts for 23 ISGs before and after IFN and sham treatment are shown. A total of 500,000 CD4⁺ T cells were purified from cryopreserved PBMCs taken immediately prior to and at 1 week after IFN administration. The level of induction of ISGs was assessed using RNA sequencing. Line plots represent the median read count of week 32 (pre-IFN, presham) and week 33 (post-IFN, postsham) time points. (B) Differential ISG expression between week 32 (pre-IFN, presham) and week 33 (post-IFN, postsham) for individual animals, as represented by heatmap analysis. Red indicates upregulation, white indicates no change, and blue indicates downregulation relative to pretreatment. Data for five out of six IFN-treated animals is shown due to insufficient RNA yield. Expression values (baseline-normalized log₂ read counts) of each transcript represent the week 33 (post-IFN, postsham) time point. Expression values are baseline normalized by subtraction of each animal's week 32 time point value.

TABLE 1 Levels, proliferation, and activation of leukocyte populations by flow cytometry

Cell type and subset	Tissue	Treatment	Cell frequency ^a (%)	P value	Significance ^b
T cells					
CD4 ⁺ cells (% of T cells)	Blood	IFN	58.08 ± 1.99	0.56	ns
		Controls	59.58 ± 1.85		
KI67 ⁺ CD4 ⁺ cells	Blood	IFN	14.15 ± 2.06	0.47	ns
		Controls	12.61 ± 2.01		
HLADR ⁺ CD4 ⁺ cells	Blood	IFN	3.07 ± 0.65	0.18	ns
		Controls	1.78 ± 0.29		
CD4 ⁺ cells (% of T cells)	Lymph node	IFN	60.85 ± 2.67	0.47	ns
		Controls	57.95 ± 2.56		
KI67 ⁺ CD4 ⁺ cells	Lymph node	IFN	4.88 ± 0.93	0.24	ns
		Controls	3.56 ± 1.04		
HLADR ⁺ CD4 ⁺ cells	Lymph node	IFN	4.95 ± 1.13	0.14	ns
		Controls	6.89 ± 1.07		
CD8 ⁺ cells (% of T cells)	Blood	IFN	24.05 ± 3.94	0.10	ns
		Controls	32.83 ± 1.50		
KI67 ⁺ CD8 ⁺ cells	Blood	IFN	10.23 ± 0.72	0.68	ns
		Controls	10.46 ± 1.10		
HLADR ⁺ CD8 ⁺ cells	Blood	IFN	3.73 ± 1.07	0.51	ns
		Controls	4.94 ± 0.76		
CD8 ⁺ cells (% of T cells)	Lymph node	IFN	30.68 ± 2.48	0.42	ns
		Controls	33.53 ± 2.34		
KI67 ⁺ CD8 ⁺ cells	Lymph node	IFN	8.36 ± 1.96	0.09	ns
		Controls	24.65 ± 8.35		
HLADR ⁺ CD8 ⁺ cells	Lymph node	IFN	3.41 ± 0.29	0.07	ns
		Controls	4.56 ± 0.55		
B cells					
B cells (% of PBMC)	Blood	IFN	13.72 ± 2.64	0.24	ns
		Controls	8.37 ± 2.33		
KI67 ⁺ B cells (%)	Blood	IFN	8.37 ± 0.56	0.02	*
		Controls	5.40 ± 0.78		
B cells (% of LN cells)	Lymph node	IFN	21.38 ± 2.13	0.99	ns
		Controls	19.25 ± 2.10		
KI67 ⁺ B cells (%)	Lymph node	IFN	10.00 ± 1.60	0.09	ns
		Controls	6.010 ± 1.180		
NK cells					
NK cells (% of PBMC)	Blood	IFN	0.72 ± 0.20	0.62	ns
		Controls	1.20 ± 0.47		
CD16 ⁺ NK cells (%)	Blood	IFN	82.03 ± 1.86	0.03	*
		Controls	91.85 ± 2.57		
CD56 ⁺ NK cells (%)	Blood	IFN	4.11 ± 1.49	0.79	ns
		Controls	3.03 ± 1.11		
KI67 ⁺ NK cells (%)	Blood	IFN	36.92 ± 7.78	0.42	ns
		Controls	28.43 ± 7.41		
NK cells (% of LN cells)	Lymph node	IFN	0.09 ± 0.02	0.90	ns
		Controls	0.12 ± 0.04		
CD16 ⁺ NK cells (%)	Lymph node	IFN	14.60 ± 2.33	0.24	ns
		Controls	18.66 ± 3.88		
CD56 ⁺ NK cells (%)	Lymph node	IFN	34.98 ± 4.69	0.79	ns
		Controls	35.17 ± 7.78		
KI67 ⁺ NK cells (%)	Lymph node	IFN	20.88 ± 2.33	0.06	ns
		Controls	14.78 ± 1.52		
DC					
mDC (% of PBMC)	Blood	IFN	0.31 ± 0.10	0.97	ns
		Controls	0.32 ± 0.09		
KI67 ⁺ mDC (%)	Blood	IFN	2.51 ± 0.67	0.18	ns
		Controls	1.19 ± 0.77		
pDC (% of PBMC)	Blood	IFN	0.03 ± 0.01	0.85	ns
		Controls	0.03 ± 0.01		
KI67 ⁺ pDC (%)	Blood	IFN	60.28 ± 7.80	0.47	ns

(Continued on next page)

TABLE 1 (Continued)

Cell type and subset	Tissue	Treatment	Cell frequency ^a (%)	P value	Significance ^b
mDC (% of LN cells)	Lymph node	Controls	51.87 ± 3.76	0.06	ns
		IFN	20.88 ± 2.33		
KI67 ⁺ mDC (%)	Lymph node	Controls	14.78 ± 1.52	0.55	ns
		IFN	10.72 ± 5.21		
pDC (% of LN cells)	Lymph node	Controls	5.55 ± 5.55	0.02	*
		IFN	0.006 ± 0.001		
KI67 ⁺ pDC (%)	Lymph node	Controls	0.02 ± 0.007	0.24	ns
		IFN	53.28 ± 13.06		
		Controls	21.99 ± 9.50		
Monocytes					
Classical monocytes (% of PBMC)	Blood	IFN	2.31 ± 0.66	0.57	ns
		Controls	2.06 ± 0.39		
Proinflammatory monocytes (% of PBMC)	Blood	IFN	0.07 ± 0.04	0.31	ns
		Controls	0.18 ± 0.09		
Nonclassical monocytes (% of PBMC)	Blood	IFN	0.008 ± 0.002	0.18	ns
		Controls	0.02 ± 0.005		
Classical monocytes (% of LN cells)	Lymph node	IFN	0.04 ± 0.01	0.43	ns
		Controls	0.03 ± 0.003		
Proinflammatory monocytes (% of LN cells)	Lymph node	IFN	0.014 ± 0.004	0.29	ns
		Controls	0.023 ± 0.006		
Nonclassical monocytes (% of LN cells)	Lymph node	IFN	0.003 ± 0.0007	0.04	*
		Controls	0.01 ± 0.003		

^aData are means and standard errors.^bAnalyses were by unpaired Mann-Whitney test. ns, not significant; *, $P < 0.05$.

frequency and activation status of B cells and a slight reduction of CD16 on NK cells in peripheral blood.

Virus replication is not affected by pIFN- α 2a administration. Having confirmed that the pIFN- α 2a treatment regimen was able to establish a broad and potent induction of antiviral effector molecules, we next tested if short-term pIFN- α 2a treatment would affect virus replication and/or the size or composition of the SIV reservoir under ART. We first sought to assess whether the level of residual viremia was affected by IFN treatment. Thus, we repeated the viral load measurement by using an ultrasensitive viral load assay with a detection limit of 1 copy/ml (25, 36). Consistent with previous studies in ART-treated, SIV-infected RMs and clinical observations in ART-treated, HIV-infected humans, low levels of plasma SIV RNA were detected in all animals at 32 weeks postinfection (w.p.i.) and 36 w.p.i. (necropsy) (Fig. 5A, B, and C) (25, 37, 38). Plasma viremia continued to decrease in all animals and at the time of necropsy, resulting in 2 out of 12 RMs in which plasma SIV-RNA was undetectable even when using the highly sensitive assay. However, no significant differences in plasma viral load were noted between IFN-treated animals and controls.

We then evaluated whether IFN affects SIV transcriptional activity by quantification of cell-associated SIV RNA, since it has been shown that lentiviral RNA remains detectable in most chronically infected individuals despite suppressive ART (39). Furthermore, it has been recently reported that cell-associated HIV RNA was significantly decreased after short-term IFN treatment in HIV-infected individuals on ART (40). Nonetheless, we did not observe any effect of IFN treatment on SIV RNA levels in CD4⁺ T cells from PBMCs (Fig. 5D, E, and F). To characterize low-level virus replication even more rigorously in multiple anatomic sites, we also determined cell-associated SIV RNA levels in lymphatic tissue, a major contributor to the viral reservoir, which has been reported to feature a lower ART drug penetration than blood (41, 42). We found that levels of cell-associated SIV RNA were not significantly different when CD4⁺ T cells isolated from lymph node-derived lymphocytes of IFN-treated and control RMs were compared (Fig. 5G, H, and I). Similar results were obtained for CD4⁺ T cells from the spleen (Fig. 5J). Taken together, our results demonstrate that levels of SIV RNA in plasma and within CD4⁺ T cells from all tissues tested were similar between IFN-treated and control

FIG 5 Assessment of viral replication in IFN-treated, ART-suppressed RMs. (A to C) Ultrasensitive plasma viral load levels of IFN-treated (A) and control (B) animals, represented as a ratio of pre- to posttreatment values (C). (E to F) Cell-associated viral RNA in CD4⁺ T cells from PBMCs of IFN-treated (D) and control animals (E), represented as ratio of pre- to posttreatment values (F). (G and H) Cell-associated viral RNA in CD4⁺ T cells from lymph nodes of IFN-treated (G) and control (H) animals, represented as ratio of pre- to posttreatment values (I). (J) Cell-associated viral RNA in CD4⁺ T cells from spleen at necropsy. *P* values were calculated using paired Wilcoxon matched-pairs signed-rank test comparing CD4-normalized SIV *gag* copy numbers at the pre- and posttreatment time points separately for the IFN treatment and control groups. Ratios of pre- to posttreatment values were statistically compared using an unpaired Mann-Whitney test. Pre-IFN/sham time point, 32 w.p.i.; post-IFN/sham time point, 36 w.p.i. (necropsy). Pre-/posttreatment ratios were calculated as 32 w.p.i./36 w.p.i., and means are denoted.

animals, suggesting that IFN treatment does not significantly affect virus production in ART-treated RMs.

pIFN- α 2a treatment is not associated with a decrease in the viral reservoir of latently infected cells. To elucidate whether IFN treatment had an impact on the overall viral reservoir in ART-treated SIV-infected RMs, we determined the amount of total, proviral SIV DNA in CD4⁺ T cells from blood and lymphatic tissues. To this end, total circulating CD4⁺ T cells were isolated by flow cytometry and tested for the relative quantity of cell-associated SIV DNA using PCR specific for SIV Gag. As shown in Fig. 6A and B, prior to pIFN- α 2a treatment the levels of cell-associated SIV DNA were similar (median, 1,275 copies/million CD4⁺ T cells in controls compared to 825 copies/million in the IFN treatment group). However, after 4 weeks of pIFN- α 2a treatment, levels of SIV-DNA were significantly (*P* value of 0.0313) reduced to a mean of 704 copies/million

FIG 6 Quantification of the viral reservoir in IFN-treated, ART-suppressed RMs. (A to C) Cell-associated SIV DNA levels in CD4⁺ T cells from PBMCs of IFN-treated (A) and control animals (B), represented as a ratio of pre- to posttreatment values (C). (D to F) Cell-associated SIV DNA levels in CD4⁺ T cells from lymph nodes of IFN-treated (D) and control (E) animals, represented as ratio of pre- to posttreatment values (F). (G) SIV DNA levels in CD4⁺ T cells from spleen at necropsy. (H) Quantitative viral outgrowth assay (QVOA) using CD4⁺ T cells from mesenteric lymph nodes at necropsy. Cell-associated SIV-DNA was assessed by RT-PCR of the SIV *gag* gene. A total of 500,000 CD4⁺ T cells were used, and samples were normalized for cell input. P values were calculated using a paired Wilcoxon matched-pairs signed-rank test comparing SIV *gag* copy number at the pre- and posttreatment time points separately for the IFN treatment and control groups. Ratios of pre- to posttreatment values were statistically compared using an unpaired Mann-Whitney test. Pre-IFN/sham time point, 32 w.p.i.; post-IFN/sham time point, 36 w.p.i. (necropsy). Pre-/posttreatment ratios were calculated as 32 w.p.i./36 w.p.i., and means are denoted.

CD4⁺ T cells. In comparison, the postsham treatment levels of cell-associated DNA were decreased after the 4-week period as well but to a slightly reduced degree (1,216 versus 895 copies/million CD4⁺ T cells; P value of 0.0625). To estimate the magnitude of the reduction in SIV DNA levels, we then calculated the ratio of SIV DNA values between w.p.i. 32 and 36 (necropsy) separately for IFN-treated as well as control animals (Fig. 6C). We found that there is no significant difference in the decline of the SIV DNA between these two groups. Hence, these data suggest that the drop in the amount of SIV DNA, observed in IFN-treated as well as control RMs, is defined by the half-life of the CD4⁺ T cell population under ART. This model is strongly supported by many studies that demonstrated that the size of the reservoir of latently infected cells declines continuously but slowly during ART (estimated mean half-life of 44 months under long-term ART treatment and even 9 months or less at earlier time points after ART initiation) (6, 43–45). We next sought to determine whether IFN treatment has an effect on the reservoir of latently infected CD4⁺ T cells in multiple lymphoid tissues, as quantified by PCR (DNA quantification) for total cell-associated SIV DNA. As shown in Fig. 6D to G, pIFN- α 2a administration did not induce any significant change in the number of SIV DNA-positive CD4⁺ T cells from either lymph nodes (LNs) or spleen. Finally, we determined the levels of resting CD4⁺ T cells harboring inducible, latent virus using a

quantitative viral outgrowth assay (QVOA), which is considered the gold standard for measuring replication-competent virus in HIV-infected humans and SIV-infected RMs (26, 46). CD4⁺ T cells were enriched from mesenteric lymph node cells and were cocultured with CEMx174 T cells in a limiting dilution assay. Frequencies of infected cells were determined by SIV *gag*-specific quantitative reverse transcription-PCR (qRT-PCR) and were expressed as infectious units per million (IUPM) CD4⁺ T cells. The results of the QVOA clearly showed that the numbers of CD4⁺ T cells harboring replication-competent virus were overall very similar between IFN-treated and control animals, with a frequency of 0.44 and 0.48 per 1 million CD4⁺ T cells, respectively. In summary, our data indicate that the reservoir of latently infected cells in SIV-infected RMs on ART is not significantly reduced by the short-term administration of pIFN- α 2a.

DISCUSSION

The key obstacle to curing HIV/SIV infection is the presence of a latent reservoir of virus-infected cells that persists for very long periods in the setting of ART-mediated reduction of HIV viremia below detectable limits. Among the multiple therapeutic concepts that are currently being explored in clinical trials in HIV-infected humans is type I interferon. IFN-I plays a complex role during lentiviral infection, as it may exert strong antiviral activity, while at the same time the presence of persistent IFN-I production and ISG upregulation has been associated with disease progression and poor immunological response to ART (12, 47, 48). Multiple lines of evidence suggest that IFN is generally of benefit to the host in acute HIV infection, as it exerts a strong antiviral activity by inducing antiviral restriction factors, activates NK cells, and facilitates CD8⁺ T cell responses (15). Furthermore, we have previously shown that IFNAR blockade in RMs during acute infection leads to accelerated mortality (11). In contrast to the acute phase, IFN-I signaling in chronic HIV infection is thought to be detrimental, since it has been demonstrated that sooty mangabeys and other natural host primate species that avoid AIDS despite high viremia shut off systemic IFN signaling during the transition from acute to chronic infection, and a failure to recover CD4⁺ T cells after a virological response to ART is associated high expression of ISGs (9, 10, 47, 49). In order to harness the benefits of IFN for HIV therapy and affect the latent reservoir, several clinical trials administering IFN to HIV-infected patients have been performed and have reported modest reductions in plasma viremia and/or CD4⁺ T cell-associated HIV DNA (21, 22, 40).

Here, we used the well-established nonhuman primate model of SIV infection of RMs to evaluate the potential impact of pIFN- α 2a on the overall size and anatomic location of the virus reservoirs in ART-treated, SIV-infected animals. The use of the SIV macaque model provides a number of important advantages when performing studies aimed at testing the impact of novel therapeutic regimens, such as type I IFN, on the viral reservoir, since (i) the identity, dose, and route of the virus challenge were known in all animals; (ii) we controlled for various clinical parameters that are virtually impossible to control in humans (time of infection, duration of ART, and adherence to ART); and (iii) exogenous pIFN- α 2a administration was conducted in a timely and controlled fashion using a standardized weight-adjusted dose. Furthermore, previous studies that tested IFN administration in HIV-infected humans on antiretroviral therapy commonly lacked a control group (21, 22). In contrast, our study was adequately controlled by the presence of a group of SIV-infected RMs receiving ART alone.

In the present study, we elucidated directly *in vivo* and in multiple tissues whether and to what extent pIFN- α 2a administration results in a reduction of the virus reservoir compared to those of animals treated with ART alone. To this end, we have infected 12 RMs with pathogenic SIVmac239 and then, at week 6 postinfection, initiated a potent, four-drug ART regimen which was maintained for 6 months. Administration of pIFN- α 2a was conducted in six RMs for 4 weeks, with regular monitoring of the main virological, immunological, and clinical parameters of the infection; at the end of this cycle all animals underwent elective necropsy. Our data demonstrate that IFN administration is safe and well tolerated, as we observed no discernible clinical or laboratory side effects

due to exogenous pIFN- α 2a treatment and peripheral blood CD4⁺ T cell counts remained stable. Hence, all animals were included in the analysis of the present study.

We and others have previously shown that unabated type I IFN signaling is linked to chronic immune activation in chronic ART-naïve HIV/SIV infection (9–11). As such, we explored the possibility that pIFN- α 2a treatment increases immune activation, thereby potentially exacerbating disease progression in ART-treated SIV-infected RMs. Importantly, we observed that short-term exogenous pIFN- α 2a treatment was not associated with an increase in markers of immune activation. Although our data show that pIFN- α 2a administration does not result in excessive immune activation and exhaustion with a potential detrimental effect on disease progression, this observed lack of a CD8⁺ T cell response might contribute to the poor anti-reservoir effect, as it has been shown that these cells are critical for controlling the viral reservoir in SIV-infected RMs on ART (11). Having demonstrated the safety of IFN intervention, we show that the administered dose of pIFN- α 2a was indeed effective *in vivo* as measured by ISG upregulation, as has been shown previously in RMs (11). ISGs that are viral restriction factors have been described to have potent antiviral effects, interfering with multiple steps of the virus life cycle, including virion uncoating, reverse transcription, and virion budding (15). It remains unclear, however, whether ISG antiviral factors also have an effect against integrated provirus, which does not actively replicate and does not produce infectious virus progeny.

The level of proviral DNA closely reflects the size of the viral reservoir of latently infected cells, as more than 90% of cell-associated HIV DNA is constituted by integrated DNA (50, 51). However, PCR-based methods cannot distinguish defective proviruses from intact replication-competent ones. Accordingly, the latent reservoir as defined by total HIV DNA can be overestimated up to 300-fold compared to the inducible viral reservoir as measured by QVOA (52). Hence, we used the QVOA for reservoir quantification, in addition to the cell-associated SIV DNA assay. Consistent with similar levels of SIV DNA upon pIFN- α 2a administration, we did not find any difference in the replication-competent reservoir by QVOA between IFN-treated and control RMs. The pIFN- α 2a treatment in the present study was rather short in duration (four weeks) compared to the typical course of pIFN- α 2a treatment used for hepatitis C virus infection in humans (48 weeks). As such, the observed minor reduction in cell-associated SIV DNA in both IFN-treated and untreated controls is likely due to the relatively short period of ART suppression in the study, in which the reservoir might not have achieved a stable equilibrium. However, the possibility that pIFN- α 2a reduced SIV production and/or replication through the activity of interferon-induced lentiviral restriction factors cannot be ruled out. This finding suggests that further testing of pIFN- α 2a administration, in which the initial ART suppression period is prolonged to the standard of 1 year to ensure full suppression and stable size of the viral reservoir and the therapeutic window of IFN administration is extended, might be more effective. In addition, an intensified pIFN- α 2a treatment schedule might translate into a delayed rebound or even a lack of rebound of virus replication after a structured interruption of ART. However, studies of intensified pIFN- α 2a administration must be closely monitored, as it has been reported that the likelihood of serious adverse effects increases with the duration of IFN treatment (18, 21, 53).

In conclusion, we showed that short-term pIFN- α 2a treatment, although safe and bioactive, does not significantly reduce the viral reservoir of latently infected CD4⁺ T cells from blood and lymphoid tissue of ART-treated SIV-infected RMs. Nonetheless, intensified therapeutic interventions with more potent agonists of the IFN-I pathway or IFN-I as part of a combination therapy with other immunomodulatory agents (e.g., TLR7 agonists, HDAC inhibitors, or CD8⁺ T cell depletion) might represent new avenues to effectively harness the antiviral potential of IFN-I for viral reservoir reduction and, ultimately, for a functional cure of HIV/SIV infection (11, 54).

MATERIALS AND METHODS

Animals. Twelve male rhesus macaques (*Macaca mulatta*) of Indian origin were included in this study (ranging in age from 2.3 to 3.5 years). Mamu-A*01, Mamu-B*08, and Mamu-B*17 are protective alleles in the rhesus macaque model of SIV/AIDS, as RMs possessing these alleles tend to show slower disease progression. To have comparable disease progression in all animals, the RMs used in the present study were negative for known protective alleles, or the IFN-treated group and control group were balanced for a certain allele. All RMs were Mamu-B*08 and -B*17 negative. Nine of 12 animals were Mamu-A*01 positive; four Mamu-A*01-positive RMs were assigned to the IFN-treated group, and 5 Mamu-A*01 RMs were assigned to the control group. This study was conducted in strict accordance with USDA regulations and the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (55), and it was approved by the Emory University Institutional Animal Care and Use Committee (YER-2002891-ENTRPR-A; Targeting SIV Reservoirs with Type 1 Interferons, NIH). Commercial dry food supplemented with fruit was provided by the veterinary personnel, and water was available *ad libitum*. SIV-infected RMs are singly caged but have visual, auditory, and olfactory contact with at least one social partner, permitting the expression of noncontact social behavior. Cages also contained additional sources of animal enrichment, including objects such as perching and other manipulanda. Animal welfare was monitored daily. Appropriate procedures were performed to ensure that potential distress, pain, or discomfort was alleviated. The sedatives ketamine (10 mg/kg) and telazol (4 to 5 mg/kg) were used for blood draws and biopsy specimens. Euthanasia of RMs, using pentobarbital (100 mg/kg) under anesthesia, was performed only when deemed clinically necessary by veterinary medical staff and according to IACUC endpoint guidelines.

SIV infection and antiretroviral therapy. All animals were intrarectally infected with 10,000 50% tissue culture infective doses (TCID₅₀) of SIV_{mac239}. All 12 RMs were put on a four-drug ART regimen at week 6 postinfection. ART included 20 mg/kg tenofovir (PMPA), 40 mg/kg emtricitabine (FTC), and 2.5 mg/kg dolutegravir administered once a day by subcutaneous (s.c.) injection, along with 400 mg darunavir orally twice daily. ART was continuously administered until necropsy.

IFN treatment. In addition to ART, six SIV-infected, ART-treated RMs received 6 μ g/kg pegylated IFN- α 2a (Pegasys, Genentech) intramuscularly weekly for 4 weeks. As controls, six SIV-infected, ART-treated RMs did not receive pIFN- α 2a and were continued on ART alone.

Standard plasma viral load. SIV_{mac239} plasma viral load was quantified in the Core Virology Laboratory of the Emory Center for AIDS Research using a quantitative real-time PCR (qPCR) assay as described previously (56).

US viral load assay. Ultrasensitive (US) quantification of plasma viral RNA was performed in the laboratory of Jeff Lifson from Leidos, Inc., National Cancer Institute, Frederick, MD, as described previously (36). Please note that while the viral load results obtained with the standard and US assay are highly correlated, occasional discrepancies may occur due to minor differences in the protocol for these two assays.

Sample collection and processing. Peripheral blood (PB), lymph node (LN), and colorectal mucosa biopsy (RB) specimens were collected longitudinally at various experimental points throughout the study, as described previously (Fig. 1) (25, 57, 58). Briefly, EDTA-anticoagulated blood samples were collected regularly and used for a complete blood count, routine chemical analysis, and immunostaining. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation. Lymphocytes were isolated from freshly obtained lymph node and spleen by passing homogenized tissue through a 70- μ m cell strainer and lysing red blood cells with ACK lysis buffer.

Flow cytometry and intracellular cytokine staining. Multiparametric flow cytometry was performed on whole blood, LN mononuclear cells, and cells isolated from RBs with fluorescently labeled monoclonal antibodies cross-reactive in RMs. The Aqua Live/Dead amine dye-AmCyan (ThermoScientific) and the following antibodies were used as previously described (59): allophycocyanin (APC)-Cy7 mouse anti-human CD3 (SP34-2), phycoerythrin (PE)-Cy7 mouse anti-human CD14 (M5E2), PE-Cy5 mouse anti-human CD20 (2H7), peridinin chlorophyll protein (PerCP)-Cy5.5 mouse anti-human HLA-DR (G46-6), APC mouse anti-human CCR5 (3A9), APC mouse anti-human CD11c (S-HCL-3), Alexa Fluor 700 mouse anti-Ki-67 (B56), PE mouse anti-human CD80 (L307.4), BV711 mouse anti-human CD86 (2331), fluorescein isothiocyanate (FITC) mouse anti-human CD56 (NCAM16.2), FITC mouse anti-human CD197/CCR7 (150503), PE mouse anti-human anti-T-bet (4B10), PE mouse anti-human CD62L (SK11), and PE-Cy7 mouse anti-human CD45RA (5H9), from BD Biosciences; BV650 mouse anti-human CD4 (OKT4), BV711 mouse anti-human CD8 (RPA-T8), BV421 mouse anti-human CD16 (3G8), Brilliant Violet 421 anti-human CD279/PD-1 (EH12.2H7), BV605 mouse anti-human CD95/FAS (DX2), and PE mouse anti-human CD123 (6H6), from BioLegend; APC mouse anti-human CD159/NKG2a (Z199) and ECD mouse anti-human CD28 (CD28.2), from BeckmanCoulter; and PE-Texas red mouse anti-human granzyme B, from ThermoFisher Scientific. All samples were acquired on an LSR II (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star). Cell populations were defined as CD4⁺ T helper cells (singlets, lymphocytes, live, CD3⁺, CD4⁺, and CD8⁻), CD8⁺ cytotoxic T lymphocytes (singlets, lymphocytes, live, CD3⁺, CD4⁻, and CD8⁺), B cells (singlets, lymphocytes, live, CD3⁻, HLADR⁺, and CD20⁺), NK cells (singlets, lymphocytes, live, CD3⁺, CD8⁺, HLADR⁻, CD14⁻, and CD20⁻, and NKG2a⁺), mDC (singlets, lymphocytes, live, CD3⁻, CD14⁻, CD20⁻, HLADR⁺, CD11c⁺, and CD123⁻), pDC (singlets, lymphocytes, live, CD3⁻, CD14⁻, CD20⁻, HLADR⁺, CD11c⁻, and CD123⁺), classical monocytes (singlets, live, CD3⁻, CD20⁻, HLADR⁺, CD11c⁻, CD123⁻, CD14⁺, and CD16⁻), proinflammatory monocytes (singlets, live, CD3⁻, CD20⁻, HLADR⁺, CD11c⁻, CD123⁻, CD14⁺, and CD16⁺), and nonclassical monocytes (singlets, live, CD3⁻, CD20⁻, HLADR⁺, CD11c⁻, CD123⁻, CD14⁻, and CD16⁺).

Cell sorting. CD4⁺ T cells (CD3⁺ CD4⁺ CD8[−] CD14[−] CD16[−] CD20[−]) were sorted from freshly isolated PBMC and LN cells as well as frozen splenocytes on a FACS Aria II flow cytometer (BD Biosciences). The following reagents were used according to standard protocols: Aqua Live/Dead amine dye-AmCyan, from ThermoScientific; AF700 mouse anti-human CD3 (SP34-2) and APC-Cy7 mouse anti-human CD8 (SK1), from BD Biosciences; and BV650 mouse anti-human CD4 (OKT4), Pacific Blue mouse anti-human CD14 (M5E2), BV421 mouse anti-human CD16 (3G8), and Pacific Blue mouse anti-human CD20 (2H7), from BioLegend.

Cell-associated SIV DNA assay. Quantitative PCR for SIV *gag* DNA from 500,000 sorted CD4⁺ T cells that were stored as a frozen cell pellet at −80°C was performed as described previously (60, 61).

Cell-associated SIV RNA assay. Cell-associated viral RNA was quantified in 450,000 sorted CD4⁺ T cells, stored in 350 μ l of RLT buffer at −80°C, using RNA extracted with the Qiagen RNeasy kit, reverse transcribed using random hexamers, and expressed as copies of SIV *gag* RNA per copy of rhesus macaque CD4. Primer and probe sequences for CD4 TaqMan qPCR are based on the rhesus macaque CD4 sequence: CD4-F, 5'-ACATCGTGGTCTAGCTTCCAGA-3'; CD4-R, 5'-AAGTGTAAGGCGAGTGGGAAGGA-3'; and CD4-Pr, 5'-AGGCCTCCAGCACAGTCTATAAGAAAGAGG-3'.

RNA-seq library preparation. RNA-seq analysis was conducted at the Yerkes Nonhuman Primate Genomics Core Laboratory (http://www.yerkes.emory.edu/nhp_genomics_core/). RNA was purified from 50,000 sorted CD4⁺ T cells, which were stored in 350 μ l of RLT buffer at −80°C, using Qiagen Micro RNeasy columns, and RNA quality was assessed using an Agilent Bioanalyzer. Two nanograms of total RNA was used as input for mRNA amplification using 5' template switch PCR with the Clontech SMART-Seq v4 ultra-low-input RNA kit according to the manufacturer's instructions. Amplified mRNA was fragmented and appended with dual-indexed bar codes using Illumina NexteraXT DNA library preparation kits. Libraries were validated by capillary electrophoresis on an Agilent 4200 TapeStation, pooled, and sequenced on an Illumina HiSeq 3000 using 100-bp SR at an average read depth of 28 million (M). Reads were aligned to the rhesus macaque Refseq (v7.8.2) using STAR software (v2.5.2b) (<http://code.google.com/p/rna-star/>) (62). Transcripts were annotated using the UNMC rhesus annotation v. 7.6.8. (<https://www.unmc.edu/rhesusgenomechip/index.htm#NewRhesusGenome>) (30). Estimates of gene-wise and isoform-wise expression levels for individual genes were performed using DESeq2, which normalizes gene expression level estimates across samples and also corrects for nonuniformity in read distributions across each gene (63).

Analysis of ISG expression before and after interferon treatment. A list of 169 previously identified ISGs that have been described to be involved in the host response to HIV infection was compiled from recent publications (9, 10, 64, 65). A subset of 28 of the 169 ISGs were selected as being significantly differentially expressed (*P* value of ≤ 0.02 by negative binomial generalized linear models) between the treated group pre- and posttreatment time points and not significantly differentially expressed between control group pre- and posttreatment time points (63). Genes were additionally filtered using the interferome.org database (<http://interferome.org/interferome/home.jsp>), requiring that they have greater than 10 experimental references supporting their classification as interferon-stimulated genes (66).

SIV QVOA. Latently infected cells were quantified by using a limiting-dilution culture assay in which CD4⁺ T cells, enriched from mesenteric LN cells using magnetic beads and column purification (Miltenyi Biotec), were cocultured with CEMx174 cells in duplicate 5-fold serial dilutions ranging from as many as 5×10^6 cells per well to as few as 320 cells per well. The cells were cultured in RPMI containing 10% fetal bovine serum and 100 U/ml interleukin-2 (Sigma). The ratio of target cells added was 4:1 for the 2 highest dilutions. A constant number of 50,000 CEMx174 cells was added to all other wells. The cultures were split every 2 to 3 days, and fresh medium was added. After 21 days, growth of virus was detected by qRT-PCR. SIV RNA was isolated from 1,250 μ l of culture supernatant using the Zymo viral RNA isolation kit (Zymo Research). The cell culture supernatant was centrifuged for 90 min at $21,000 \times g$ before extraction by following the manufacturer's instructions. DNase treatment was performed using an RQ1 RNase-free DNase kit (Promega). A one-step qRT-PCR targeting SIV *gag* was performed using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems) and the Veriquest probe one-step qRT-PCR master mix (Affymetrix) with the following primers and probe: SIVgagFwd, 5'-GCAGAGGAGGAAATTAC CAGTAC-3'; SIVgagRev, 5'-CAATTTTAC CCAGGCATTTAATGTT-3'; SIVgag-probe, 5'-6FAM-TGTCCACCTGCCATTAAGCCCGA-3IBFQ-3'. The frequencies of infected cells were determined by the maximum-likelihood method and were expressed as infectious units per million (IUPM) CD4⁺ T cells (67).

Statistical analysis. Comparisons between pre-IFN and post-IFN treatment time points as well as presham and postsham treatment time points were determined by paired Wilcoxon matched-pairs signed-rank test. Ratios of pre- to posttreatment data were compared using unpaired Mann-Whitney test. Significance was attributed at *P* values of < 0.05 . Analysis was done using GraphPad Prism 6.0. RNA-seq differential expression analysis and statistical analyses were performed using the DESeq2 package (63).

Accession number(s). RNA-seq data were submitted to the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information NCBI database (accession number GSE114561).

ACKNOWLEDGMENTS

We thank M. Zanon, E. Cartwright, C. S. McGary, and L. Micci for helpful discussions. We also thank Sherrie Jean, Stephanie Ehner, Christopher Souder, and all of the animal care and veterinary staff at the Yerkes National Primate Research Center; Barbara

Cervasi and Kiran Gill at the Emory University Flow Cytometry Core; R. Dawoud, P. Dhadvai, E. Gebru, C.A. Enemu, and F. Viviano for technical support; the Emory CFAR Virology Core for viral loads; Nirav Patel at the Emory Nonhuman Primate Genomics Core for RNA sequencing and analysis; and Jeff Lifson from Leidos, Inc., National Cancer Institute, Frederick, MD. We gratefully acknowledge Gilead (I.MTA.15.225), Janssen Sciences Ireland (I.MTA.15.225), and Viiv Healthcare (I.MTA.15.446) for providing anti-retroviral drugs.

This work was funded by NIH grant R21AI116200. Research reported in this publication was also supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number P51 OD011132 (to the YNPRC).

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