Treatment of SIV-infected sooty mangabeys with a type-I IFN agonist results in decreased virus replication without inducing hyper immune activation

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Short Title: IFN-alpha treatment of SIV-infected sooty mangabeys.

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Abstract

A key feature differentiating non-pathogenic SIV infection of sooty mangabeys (SMs) from pathogenic HIV/SIV infections is the rapid resolution of type I interferon (IFN-I) responses and interferon stimulated gene (ISG) expression during the acute-to-chronic phase transition, and establishment of an immune quiescent state that persists throughout the chronic infection. We hypothesized that low levels of IFN-I signaling may help prevent chronic immune activation and disease progression in SIV-infected SMs. To assess the effects of IFN-I signaling in this setting, we administered recombinant rhesus macaque IFNα2-IgFc (rmIFNα2-IgFc) to eight naturally SIV-infected SMs weekly for 16 weeks. Gene expression profiling revealed a strong up-regulation of ISGs in the blood of treated animals confirming the reagent’s bioactivity. Interestingly, we observed a ~1-log decrease in viral load that persisted through day 35 of treatment. Flow cytometric analysis of lymphocytes in the blood, lymph nodes, and rectal biopsies did not reveal a significant decline of CD4+ T cells, a robust increase in lymphocyte activation, or change in the level of SIV-specific CD8+ T-cell. Taken together these results indicate that administration of type I IFNs in SIV-infected SMs induces a significant antiviral effect that is not associated with a detectable increase of chronic immune activation.
Introduction

In stark contrast to HIV infection in humans and experimental SIV infection of macaques, that both lead to AIDS, SIV infection of natural host sooty mangabeys (SMs) is typically non-progressive despite similarly high levels of virus replication\(^1,2\). The reasons why SIV-infected SMs are resistant to AIDS remain incompletely understood, and it is hoped that their elucidation will help define the mechanisms responsible for the development of AIDS in HIV-infected individuals\(^3\). Several studies have shown that two consistent features of naturally SIV-infected SMs are the absence of generalized immune activation during the chronic phase of the infection, and a pattern of in vivo infected cells that results in a preferential preservation of central memory CD4+ T cells from SIV infection\(^4,5\).

The low immune activation observed in chronically SIV-infected SMs represents a key phenotypic difference with pathogenic HIV/SIV infection of humans and macaques, in which chronic immune activation is a major marker and predictor of disease progression, both in the natural history and in the setting of antiretroviral treatment\(^6-11\). In SIV-infected SMs, the low immune activation that is observed during the chronic phase of infection is established as the result of the relatively rapid resolution of a strong innate and adaptive immune response to the virus that occurs during the acute phase of infection and lasts approximately 4-6 weeks after the initial inoculation\(^4,12\). Of note, similar kinetics of the immune responses to SIV has been reported in another natural host, the African green monkeys (AGMs)\(^13,14\). The mechanisms by which SIV-infected SMs are able to tune down their immune activation remain unclear but may involve specific virus properties, better ability to activate immune regulatory pathways, decreased sensing of viral antigens, preservation of mucosal immunity with consequent absence of microbial translocation, and differences in the pattern and anatomic location of infected cells\(^3\).

Regardless of the mechanisms involved, the low immune activation observed during the chronic phase of SIV infection in SMs is associated with the absence of up-regulation of type I interferon (IFN-I) stimulated genes (ISGs) which is a consistent feature of the transcriptional profile of pathogenic HIV/SIV infections\(^4,13\). At this time, however, it remains unknown whether and to what extent this lack of IFN-I gene expression signature in chronically SIV-infected natural hosts represents a cause or a consequence of the low immune activation. To address this issue, we treated eight naturally SIV-infected SMs for 16 weeks with a recombinant IFN-I agonist (a recombinant rhesus macaque IFNa2-Ig fusion protein, rmIFN\(\alpha\)2) that induces a strong ISG upregulation both in vitro and in vivo, thus demonstrating that SMs are not intrinsically resistant to IFN-I signaling. The main result of this treatment was a significant ~1 Log
decline in viral load that persisted for ~6 weeks, coincident with the presence of a IFN-I transcripational signature in the blood. No major immunological effects were observed and in particular we observed that rmIFNα2 treatment failed to induce a significant increase in immune activation and did not augment the level of SIV-specific CD8+ T-cells. This study emphasizes the important antiviral role of IFN-I and ISG upregulation during SIV infection of SMs, and suggests that the mechanisms involved in the ability of these animals to maintain low immune activation are likely multi-factorial and not entirely dependent on type I interferon and ISG expression.

Methods

Ethics Statement. These studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Emory University (AWA# A3180-01) and University of Pennsylvania (AWA# A3079-01) Institutional Animal Care and Use Committees. All animals were anesthetized prior to the performance of any procedure, and proper steps were taken to ensure the welfare and to minimize the suffering of all animals in these studies.

Animals and study design. Eight naturally (i.e. not experimentally inoculated) SIV-infected sooty mangabeys were selected for this study based on availability. Baseline viral quantification and immunophenotyping was performed 3 weeks before the beginning of treatment. On the first day of treatment and every week subsequently for a total of 16 weeks, each SM was given a sub-cutaneous dose of 500,000 units of recombinant rhesus macaque interferon-α2-Ig fusion protein (median dose: 46,098 units/kg). Throughout the treatment period and afterward, peripheral blood mononuclear cells (PBMC), lymph node biopsies (LN) and biopsies of the rectal mucosa (RB) were collected for virological and immunological analysis.

Recombinant rhesus macaque interferon-α2-Ig fusion protein. The coding sequence of interferon-α was cloned and sequenced from several non-human primate species including sooty mangabeys and macaques as previously described 15 (http://pathology.emory.edu/Villinger/index.htm). The fusion of the interferonα to IgG, was done as described previously for PD-1-IgG and IL15RA-IgG2 16,17. Briefly, the Fc portion of a macaque IgG2 was mutated at two positions (L235A and P331S) to inactivate potential binding of the fusion protein to complement and to Fc receptors respectively, to avoid the potential for
complement and cell-mediated cytotoxicity of the cytokine targets. The mature interferon-α2 (165 aa) was amplified with primers IFNa12pMT (CC GGATCC TGT GAT CTA CCT CAA ACC) and IFNa13 Ig (GGT GGC GCA GTG AGA TCT ACC TTC ACT TCT TAA ACT TTC TTG C) and the IgG2 amplified with primers PAmigg2b (GGTAGATCTACGTGCCCACCGTGCCCAGCTGAA) and IgG6ae (TATGACGTCGAATTCTCTTTACCCGGAGACACGGAGA). These two fragments were then concatenated by another round of PCR using the primers IFNa12pMT and IgG6ae and the overlap created in the previous amplifications. The gene coding for this fusion construct was subcloned using BamHI and EcoRI into the pMT-BIP vector designed to produce soluble proteins from Schneider-2 (S-2) insect cells in spinner cultures (Invitrogen). All constructs were verified by sequencing. The protein released in the supernatant of these cultures at a concentration of 7-10 mg/Lt was then purified by passage over a Protein-G-sepharose capture column and eluted with diluted acetic acid (pH 2.8). The ~50 kDa purified protein was then dialyzed extensively against PBS and tested for purity, presence of endotoxin, protein content and biological activity. The protein appeared >90% pure and devoid of any significant endotoxin activity. Testing for rmIFNα2 bioactivity was performed using an EMCV neutralization bioassay on vero cells as previously described.

Detection of antibodies against rmIFNα2 by western blot. To verify that the sooty mangabeys treated with rmIFNα2 did not generate an anti-cytokine neutralizing response, we tested sequential plasma samples from all treated monkeys including pre-treatment to several weeks after the last administration by Western blots against the protein. RmIFNα2 was separated through 12.5% SDS-PAGE gels transferred to a PVDF membrane. This membrane was then blocked with a 5% nonfat dry milk solution in PBS overnight before assembling into Mini-PROTEAN II multiscreen apparatus (Bio-Rad). The plasma samples were tested at a 1:200 dilution. Bound antibodies were then detected with a secondary goat anti-human kappa/alkaline phosphatase conjugate cross-reactive with monkey Ig and developed with a BioRad substrate at room temperature.

Plasma RNA and cell-associated DNA viral quantification. Plasma viral quantification was performed as previously described. For the quantification of cell-associated viral DNA in CD4+ peripheral blood cells, frozen PBMCs were thawed in a 37°C water bath and immediately washed in DMEM supplemented with 10% fetal bovine serum, L-glutamine, and pen/strep. CD4+ peripheral blood cells were then separated by positive selection using the Miltenyi Biotec (Boston, MA) CD4 microbeads for non-human primates on an LD column per the manufacturer’s specifications. CD4+ peripheral blood cells were counted and resuspended in buffer RLT+ and extracted using Qiagen’s (Valencia, CA) Blood DNA Mini Kit.
Quantiative real-time PCR was then performed on the extracted cell-associated DNA, similar to above. Viral genome copies were normalized by the number of cells.

**Immunophenotyping by flow cytometry.** Peripheral blood lymphocytes were separated by centrifugation in sodium citrate CPT tubes. Lymph node and rectal mucosa samples were processed as previously described using standardized procedures. Multi-color flow cytometric analysis was performed using pre-determined optimal concentrations of the following fluorescently conjugated monoclonal antibodies: anti-HLA-DR-PerCP-Cy5.5 (G46-6), anti-Ki67-FITC (B56), anti-CD69-APC-Cy7 (FN50), anti-CD25-APC-Cy7 (M-A251), anti-CD20-APC-H7 (L27), anti-CD3-Alexa700 (SP34-2), anti-HLA-DR-APC (G46-6), anti-CCR5-APC (3A9), anti-CD14-PE-Cy7 (M5E2), anti-CD95-PE-Cy5 (DX2), and anti-CD62L-PE (SK11) from BD Biosciences; anti-CD28-PECy7 (CD28.2) from Beckman Coulter; anti-CD8-PE-Texas Red (3B5) and the Aqua Blue Live/Dead Discriminator from Invitrogen; anti-CD8-PerCP-Cy5.5 (RPA-T8), anti-CD28-PE-Cy7 (CD28.2), anti-CD4-Pacific Blue (OKT4) from eBiosciences. Flow cytometric acquisition and analysis of samples was performed on at least 100,000 events on a LSRII flow cytometer driven by the DiVa software package (Becton Dickinson). Analysis of the acquired data was performed using FlowJo software (Tree Star, Inc., Ashland, OR).

**SIVmac239 gag and env peptide stimulations.** Peptide stimulations were performed as previously described. The function of SIV-specific CD8+ T-cells was assessed by flow cytometry after stimulation with peptide pools of 15-mers (overlapping by 11 amino acids) spanning the SIVmac239 Gag and Env proteins. Peptides were prepared from peptide stocks obtained from the NIH AIDS Research and Reference Reagent Program, reconstituted in DMSO and pooled. All peptides were used at a final concentration of 2 µg of each peptide per milliliter. Purified PBMCs were thawed, resuspended, and stimulated as previously described. Anti-CD107a FITC (BD Biosciences, San Jose, California) was added at the start of all stimulation periods, as described previously. The cocktail of antibodies for surface staining included anti-CD8-APC-Cy7 (SK1), anti-CD3-Alexa700 (SP34-2), anti-CD95-PE-Cy5 (DX2), from BD Biosciences; anti-CD28-ECD (CD28.2) from Beckman Coulter; anti-CD4-Pacific Blue (OKT4) from eBiosciences; Aqua Blue Live/Dead Discriminator from Invitrogen. The cocktail for intracellular staining included anti-CD107a-FITC (H4A3), anti-IL-2-APC (MQ1-17H12), anti-TNF-α-PE-Cy7 (Mab11), and anti-IFN-γ-PE (B27) from BD Biosciences.

**RNA purification, array hybridization and hemoglobin blocking.** Methodology for purifying total RNA and microarray hybridization were described previously. 2.5 ml of venous blood was collected into PAXgene blood RNA tubes (Becton Dickinson, San Diego, CA) and stored at –80°C. Total RNA was
purified with PAXgene Blood RNA kits (QIAGEN) according to manufacturer’s protocol with on-column DNAse digestion. RNA quality was quantitated by Nanodrop analysis Agilent Bioanalyzer capillary electrophoresis was used for quality assessment; all samples had a RIN > 8.0. RNA was hybridized to Affymetrix GeneChip Rhesus Macaque Genome Arrays (Affymetrix, Santa Clara, CA). 0.5 ug of total RNA was amplified using the Affymetrix 3’ IVT Express Kit using techniques previously described in 21, including the inclusion of a set of 5 peptide nucleic acid (PNA) oligonucleotides (Bio-Synthesis Inc.) specific for regions of hemoglobin α and β mRNA into the reverse transcription cocktail as described previously 4 to inhibit nonspecific binding.

Microarray data analysis. Background adjustment, normalization and median polish summarization of .CEL files was performed using the robust multi-chip average (RMA) algorithm 22. RMA was performed using Bioconductor and downstream analyses were performed using Partek Genomics Suite software v6.4 (Partek Inc). One array with poor hybridization was excluded based on NUSE and RLE centroids outside of expected boundaries. To determine genes statistically changed after rmIFNα treatment we performed 2-way ANOVA (individual animal, time after treatment). Significantly expressed genes were defined by exhibiting an FDR-corrected p-values < 0.00062. Differentially expressed genes with similar expression patterns were organized using agglomerative clustering with Pearson dissimilarity distance metric and average linkage.

Annotation of the Rhesus Genome array with human Gene Symbol identifiers. Affymetrix annotation of the Rhesus GeneChip maps to the rhesus genome, and > 11,000 probesets have provisional Gene Symbols in NCBI Gene (ie. “LOC” symbols). To increase the mapping of the array, probesets lacking definitive annotation were cross-referenced to potential human orthologs identified by the online resources provided by the laboratory of Robert Norgren (version 3, June 2010) (http://www.unmc.edu/rhesusgenechip/), the InParanoid ortholog database (release 7.0, June 2009) (http://inparanoid.sbc.su.se) 23, using probeset IDs and Ensembl Gene/Protein IDs, respectively.

GEO Accession numbers. The microarray dataset was submitted to the GEO online repository according to MIAME (Minimum Information About a Microarray Experiment) standards (GEO accession number GSE35460.)
Results

Treatment of chronically SIV-infected SMs with rmIFNα2 results in a strong increase in ISG expression in peripheral blood.

In contrast to pathogenic HIV and SIV infections of humans and macaques, chronic SIV infection of SMs is associated with low levels of immune activation and absence of a IFN-I transcriptional signature. In order to test the hypothesis that low levels of IFN-I production and ISG expression contribute to the low levels of immune activation, eight naturally SIV-infected SMs were treated with 500,000 units of a recombinant rhesus macaque IFNα2-Ig Fc fusion protein (rmIFNα2) sub-cutaneously once a week for 16 weeks. The fusion of the interferon-α2 to IgG, was done as described previously for PD-1-IgG and IL15RA-IgG2 with the Fc portion of a macaque IgG2 mutated at two positions (L235A and P331S) to inactivate binding to complement and to Fc receptors, respectively. Of note, previous work from our laboratory showed that in vitro stimulation of SM PBMCs with rmIFNα2 resulted in a significant upregulation of several ISGs as measured by real time PCR. As expected based on these in vitro results, in vivo administration of rmIFNα2 induced a strong and specific upregulation of numerous ISGs (e.g. antiviral genes: OAS1, OAS2, MX1) at days 7 and 21 of treatment relative to baseline (FDR < 0.0006) as measured by gene array analysis using the Affymetrix rhesus macaque gene chip that has been previously validated for use in SMs (Figure 1). The observed upregulation of ISGs was transient with expression levels returning to baseline by day 84 of treatment and at two post treatment time points (days 118 and 180; Figure 1). Although the reasons why the rmIFNα2-induced ISG upregulation lasted less than 12 weeks remain unclear, it is unlikely that antibodies against the reagent were responsible for the decline in IFN-I sensitivity as western blots of serial serum samples (days -21, 7, 21, 42, 84, and 180) from the treated animals were inconclusive (data not shown). Ultimately, these observations, taken together, indicate that rmIFNα2 was bioactive in vivo in SMs and capable of inducing significant ISG expression, thus demonstrating that chronically SIV-infected SMs are not intrinsically resistant to IFN-I stimulation.

Treatment of chronically SIV-infected SMs with rmIFNα2 results in a ~1 log decline in plasma viremia without changes in cell-associated viral load.

While a series of recent studies have suggested that IFN-I is a potential cause for the aberrant chronic immune activation associated with pathogenic HIV/SIV infections, the canonical role of IFN-I is to induce a cellular antiviral state in response to viral infections by upregulating the expression of a
number of antiviral genes. In the context of HIV and SIV infection, IFN-I can suppress virus replication by upregulating antiretroviral genes such as APOBECs, TRIMs, SAMHD1 and BST-2/tetherin. In order to determine the effect of rmIFNα2 on viral replication in the eight treated SIV-infected SMs, viral RNA genome copy number was measured in plasma (Figure 2A) by real-time PCR. By day 14 of treatment, mean viral load had significantly declined by ~1 log (Friedman’s test with Dunn’s multiple comparisons, p < 0.01), remained lower than baseline through day 35, and returned to pre-treatment levels by 42 of treatment (Figure 2A). Interestingly, the effect of rmIFNα2 on plasma viral load was consistent with the observation that the used reagent induced a significant upregulation of ISGs, with elevated levels at day 7 and day 21 but not at day 84 of treatment as compared to baseline (see Figure 1). To better define the mechanism by which rmIFNα2 reduced plasma viral load, we also measured cell-associated SIV-DNA by real-time PCR in column-separated, CD4+ peripheral blood cells. In contrast to the significant decrease in plasma viral load observed at day 14 of treatment, the mean levels of cell-associated viral DNA did not change relative to pre-treatment time points (Figure 2B). Overall, these data indicate that rmIFNα2 induced a significant but transient decline in virus replication in SIV-infected SMs.

**Administration of rmIFNα2 to chronically SIV-infected SMs does not result in CD4+ T cell depletion.**

IFN-I treatment of HIV-HCV co-infected humans is widely used, and has been found to be associated with a modest decrease in HIV viral load and a coincident development of mild lymphopenia. In order to determine the effects of IFN-I signaling augmentation as induced by rmIFNα2 on CD4+ T cell homeostasis in chronically SIV-infected SMs, peripheral blood mononuclear cells (PBMC) as well as mononuclear cells derived from lymph nodes and rectal biopsies were analyzed by multiparametric flow cytometry. This analysis revealed that rmIFNα2 did not induce any significant change in the absolute number or percentage of CD4+ T cells in any of the examined anatomic compartments (Figure 3A-D), aside from an isolated finding of lower CD4+ T cell percentage and count at day 84 in peripheral blood and rectal biopsies (Friedman’s test with Dunn’s multiple comparisons, p < 0.05) which return to baseline levels by the last time point during treatment. A similar analysis conducted on CD8+ T cells revealed no significant changes of their absolute numbers or percentage in peripheral blood and lymph nodes relative to baseline, aside from the reciprocal changes expected in the percentage of CD8+ T cells in peripheral blood and RB (Figure 3E-G). Finally, no changes were observed in the absolute numbers of circulating NK and B cells (Figure 3I-J), nor in the levels of plasmacytoid dendritic cells (data not shown). Taken together these immunophenotypic data indicate that treatment with rmIFNα2 was not associated
with any major change in the levels of the main lymphoid subpopulations and, in particular, with no significant negative effect on CD4+ T cell homeostasis.

**Treatment of chronically SIV-infected SMs with rmIFNα2 does not result in increased levels of lymphocyte proliferation and activation.**

In chronically HIV-infected humans and SIV-infected rhesus macaques high levels of immune activation are accompanied by strong ISG expression, while in chronically SIV-infected SMs low levels of lymphocyte activation are associated with ISG expression similar to that observed in uninfected animals. To determine whether rmIFNα2 treatment and associated ISG up-regulation in chronically SIV-infected SMs results in increased levels of immune activation, we longitudinally measured the percentages of CD8+ (Figure 4A-C) and CD4+ (Figure 4D-F) T cells expressing several markers of lymphocyte proliferation and activation in peripheral blood, rectal mucosa, and lymph nodes. We first examined the expression levels of the proliferation marker Ki67. This analysis revealed a significant but transient increase in the percentage of circulating CD8+ Ki67+ T cells at day 21 of treatment relative to baseline (Friedman’s test with Dunn’s multiple comparisons, p < 0.05), and no significant changes in the fraction of circulating CD4+Ki67+ T cells throughout treatment (Figure 4A,D). No significant changes in the fraction of CD8+Ki67+ or CD4+Ki67+ T cells were observed in lymph nodes (Figure 4B,E) or rectal biopsies (Figure 4C,F). In addition, rmIFNα2 treatment was not associated with significant changes in the level of circulating or tissue-resident CD4+ or CD8+ T cells expressing the activation markers CD69, HLA-DR, and CD25 (data not shown). Collectively, these data indicate that treatment with a bioactive IFN-I agonist did not induce a robust increase in the prevailing level of immune activation in chronically SIV-infected SMs.

**Treatment with rmIFNα2 is not associated with increased SIV-specific CD8+ T cell responses.**

In several experimental systems IFN-I provides an important third signal for the induction of robust antiviral CD8+ T cell responses. Therefore, it is conceivable that in our study the administration of rmIFNα2 may have resulted in an increased level of SIV-specific CD8+ T cells, which in turn could have contributed to the decrease in SIV viral load observed during treatment. To determine whether rmIFNα2 treatment resulted in an augmentation of SIV-specific CD8+ T cell responses in our group of SIV-infected SMs, PBMCs collected at various time points before and after rmIFNα2 administration were stimulated in vitro with SIVmac239 gag and env peptide pools, and the levels of IFN-γ, TNF-α, IL-2, and CD107a were measured by intracellular staining and flow cytometric analysis. As shown in Figure 5, this analysis...
did not reveal any significant increase in the level of SIV-specific CD8+ T cell responses in the tested animals, with only relatively minor fluctuations in the magnitude and functionality of the response observed between different time-points. As such, these data do not support the hypothesis that the decline of viral load observed during rmIFNα2 treatment of SIV-infected SMs is mediated by the induction of stronger antiviral cellular immune responses.

Discussion

HIV and SIV infection of humans and rhesus macaques, respectively, results in a progressive immunodeficiency characterized by depletion of CD4+ T cells, destruction of lymphoid architecture, and chronic immune activation, which all lead to an increased susceptibility to opportunistic infections. In striking contrast to the pathogenic infection of humans and macaques, SIV-infected sooty mangabeys avoid AIDS despite high levels of virus replication\(^1^,\(^2\). While the mechanisms responsible for the lack of AIDS in these animals are incompletely understood, one recent study has shown that, in contrast to pathogenic SIVmac239 infection of RMs, SIV infection of SMs results in a rapid resolution of innate and adaptive antiviral immune responses coincident with the transition from the acute to chronic phase of infection\(^4\). This lower level of immune activation exhibited during the chronic phase of infection is associated with a dramatic downmodulation of interferon stimulated genes, thus suggesting that resolution of the innate antiviral immune responses may be required to reach a state of non-pathogenicity in SIV-infected SMs\(^4\). Consistent with this possibility is the growing body of experimental evidence suggesting that despite the canonical antiviral mechanisms induced by IFN-I, long-term production of IFN-I and ISGs during a chronic viral infection can have detrimental effects on disease progression by fueling aberrant CD8+ T cell responses\(^42^,\(^43\), increasing apoptosis of uninfected CD4+ T cells\(^44^,\(^45\), and indirectly inducing higher viral loads\(^25\). While it is clear that IFN-I is an important contributor to the dynamics of virus-host interactions, additional studies are required to elucidate the direct and indirect influences of this pleiotropic immune effector molecule during pathogenic and nonpathogenic lentiviral infection, and how the balance of these effects may be beneficial or detrimental to the host immune function.

In this study we administered rmIFNα2, a IFN-I agonist, to eight chronically SIV-infected SMs in an effort to determine whether the addition of exogenous IFN-I could recapitulate some aspects of the pathogenic HIV and SIV infections of humans and macaques, such as induction of higher levels of
immune activation, decline of CD4 T cell count, and ultimately progression to AIDS. We did not use a
group of control SIV-infected SMs treated with an ‘irrelevant’ construct containing the IgG2 Fc portion
coupled with an irrelevant protein. This decision was made due to the limited availability of SMs for this
type of longitudinal in vivo studies that require extensive tissue collections. While this design is
admittedly not ideal, it should be noted that the used fusion protein contains an IgG2-Fc that was
mutated at two amino acid positions (L235A and P331S) in order to inactivate potential binding to
complement and to Fc receptors respectively \textsuperscript{16,17}. As such, the possibility that any biological effect of
rmIFNα2 is simply a consequence of an IgG2-Fc administration is extremely unlikely. Strikingly, we
observed that four months of administration of IFN-I to chronically infected SMs did not result in any
significant increase in immune activation, nor in any signs of immune deficiency. Interestingly, IFN-I
treated SMs did exhibit a \textasciitilde 1-log decline in plasma viral load during the first two months of treatment,
therefore confirming the well-known antiviral effect of these molecules. This virological effect of IFN-I
was transient, with levels of SIV viral load returning to baseline coincident with the loss of a IFN-I
transcriptional signature in the blood. The observed suppression of virus load in SIV-infected SMs is
similar in magnitude to that seen in HIV-Hepatitis C virus (HCV) coinfected patients who were treated for
their HCV infection with pegylated-IFNα (PEG-IFN) \textsuperscript{34}. Of note, the viral decline described in this study of
HIV/HCV coinfection was more durable than what we observed in our cohort of IFN-I-treated, SIV-
infected SMs \textsuperscript{34}. This discrepancy could be due to differences in the structures and bioavailabilities of
rmIFNα2 and PEG-IFN or the lack of chronic viral hepatitis in our SIV-infected SMs. Nevertheless, the
predominant effect of IFN-I administration to SIV-infected SMs was to induce a heightened, but
transient antiviral state without resulting in chronic lymphocyte activation. The reason(s) why the
effects of rmIFNα2 were transient and lost by day 84 of treatment in SIV-infected SMs may be complex
and include possible generation of anti- rmIFNα2 antibodies and down-modulation of the expression of
IFN-I receptors.

While these results may downplay the pathogenic role of chronic IFN-I stimulation in immune activation
and AIDS pathogenesis, it is important to note that four months of treatment (or, in fact, two months, if
the rebound in viral load at day 42 is an indicator of the longevity of rmIFNα2 pharmacokinetics and
bioavailability) may not have been sufficient to induce an immunopathological state. However, while we
acknowledge this limitation of the current study, we would have predicted that some effect on immune
activation be observed within a few weeks of treatment if the used IFN-I agonist were to have a major
impact on the phenotype of SIV infection in SMs. Particularly, this effect would have been even more
pronounced in the setting of a chronic viral infection in which low basal levels of IFN-I and ISG
expression allow for a strong and immediate increase in ISG expression. One possible explanation for the lack of an activating effect of rmIFNα2 treatment is that infection of SMs with SIV triggers a species-specific gene expression program that actively inhibits the activating effects of IFN-I stimulation. This phenomenon would be reminiscent of the rapid resolution of IFN-I and ISG responses observed during the transition from acute to chronic phase of SIV infection in SMs\(^4\). An additional, non-mutually exclusive possibility is that the immunological effect of “natural”, i.e., virus-induced IFN-I production is different from the effect of a rapid infusion of a single, massive dose of IFN-I. Indeed, upon sensing the virus, plasmacytoid dendritic cells (pDCs), the primary IFN-I producing cell during viral infections, traffic to lymph nodes where they secrete high levels IFN-I that appears to act mostly on cells located in close proximity\(^{46,47}\). Conceivably, the dose (500ku) and/or route of administration (sub-cutaneous) of rmIFNα2 in our system, while similar to that used to treat HCV infection in humans, may not fully mimic the high levels of endogenous IFN-I observed in lymphoid tissues during pathogenic HIV and SIV. In this experiment, no effect of rmIFNα2 on the levels of circulating pDCs was observed, and whether SIV pathogenesis is at least in part dependent on the presence of pDCs remains to be determined. Hopefully the generation of reagents specific for this cell population may enable us to answer this important question.

The main goal of this experiment was to provide insights on the mechanisms responsible for the striking differences in chronic immune activation observed between pathogenic and non-pathogenic primate lentivirus infections. In this regard, the presented data do not unequivocally support the hypothesis that lack of chronic IFN-I signaling is a key requirement to induce a state of non-pathogenicity in SIV-infected SMs. Instead, the observation that exogenous IFN-I treatment in SMs resulted in a significant, albeit transient, decline in SIV viral loads, suggests that its antiviral effect is at least initially predominant among the many \textit{in vivo} functions of this highly pleiotropic system of cytokines. In this context, the current experiment is also consistent with a pathogenic model in which the chronic immune activation observed in HIV-infected humans or SIV-infected macaques is a complex and multi-factorial phenomenon in which chronic IFN-I stimulation may simply be one of many players.

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Authorship

THV designed and performed the study, collected and analyzed the data, and wrote the manuscript; CS, BOL, and RO collected and analyzed the data; KAR and FV designed and produced the rmIFNα2 reagent and contributed to the study design; JE coordinated veterinary needs for the animals in the study; SEB collected and analyzed the microarray data; GS designed and secured funding for the study, and wrote the manuscript.

The authors declare no competing financial interests.
References


Figure Legends

Figure 1: Recombinant rhesus macaque IFNα induces a transient increase of ISGs in vivo. A heat map of fold change in gene expression relative to day -21 of treatment as measured from mRNA extracted from PBMCs of SIV-infected SMs (n = 8) treated longitudinally with recombinant rmIFNα2 and hybridized to Affymetrix Rhesus Arrays shows a significant upregulation of ISGs during treatment. Genes with differential expression over time (ANOVA, p < 0.0006) and documented function as ISGs are shown at left, p-values are denoted on the right. Individual colored panels represent the average fold-change. The color scale is indicated at bottom.

Figure 2: Viral load decreased by approximately 1-log during the first two weeks of recombinant IFN-α treatment, irrespective of the levels of infection of CD4+ cells. (A) Viral load remained low for 5 weeks, but returned to pre-treatment levels by day 42 of treatment. The nadir of viral load at day 14 was significantly lower than day -21 and day 0 (Friedman test with Dunn’s multiple comparisons). **: p < 0.01; *** p <0.001. (B) CD4+ peripheral blood cells were sorted from PBMCs using CD4-Ig labeled magnetic beads. Viral load was determined by real-time PCR.

Figure 3: The percentage of major lymphocyte populations do not change significantly during the course of recombinant IFN-α treatment of SIVsmm infected sooty mangabeys, however the numbers of CD8 T cells do decrease significantly between days 7 and 21 of treatment. Counts and percentages of the major lymphocyte populations, (A) CD4+ T cell counts, %CD4+ T cells in (B) blood, (C) lymph nodes, and (D) rectal biopsies, (E) CD8+ T cell counts, %CD8+ T cells in (F) blood, (G) lymph nodes, and (H) rectal biopsies, (I) CD16+CD8+ NK cell counts, and (J) CD20+ B lymphocyte counts were determined by flow cytometry. Differences were determined using the Friedman test with Dunn’s multiple comparisons. *: p < 0.05; **: p < 0.01; *** p <0.001.

Figure 4: T cell activation, measured by the proliferation antigen Ki67, changed significantly relative to baseline (day -21 and day 0) only in CD8+ T cells sampled from peripheral blood. The levels of Ki67 on CD8+ (A, B, and C) and CD4+ (D, E, and F) T subsets in PBMC (A, D), RB (B, E), and LN (C, F) were determined by flow cytometry and analyzed using Friedman's test with Dunn's multiple comparisons.

Figure 5: CD8 T cells stimulated did not produce more IFN-γ or TNF-α in response to stimulation with gag and env peptide pools during rmIFNα2 treatment. PBMCs were stimulated with gag and env
peptides, separately, and analyzed by fluorescent antibody mediated flow cytometry for CD8 T cell IFN-γ and TNF-α production.
### Figure 1

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+2-fold: Red
-2-fold: Blue
No change: Gray
Figure 2

A

Log_{10} Viral RNA copies per mL plasma

Treatment Day

B

Log_{10} SIVsmm DNA copies per million CD4+ cells

Day -21  Day 21  Day 42
Figure 3
Figure 4
Figure 5

% Gag+Env responding memory CD8+ T cells

Day 0  Day 14  Day 35  Day 56

IFN-\(\gamma\)  TNF-\(\alpha\)
Treatment of SIV-infected sooty mangabeys with a type-I IFN agonist results in decreased virus replication without inducing hyper immune activation

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