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Experimentally-induced immune activation in natural hosts of SIV induces significant increases in viral replication and $CD4^+$ T cell depletion

Running title: Experimental immune activation increases SIV replication

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Key words: AIDS, cell activation, T cells, Lipopolysaccharide, cell proliferation, Tregs

Abstract

Chronically SIVagm-infected African green monkeys (AGMs) have a remarkably stable non-pathogenic disease course, with levels of immune activation in chronic SIVagm infection similar to those observed in uninfected monkeys and stable viral loads (VLs) for long periods of time. *In vivo* administration of lipopolysaccharide (LPS) or an IL-2/diphtheria toxin fusion protein (Ontak) to chronically SIVagm-infected AGMs triggered increases in immune activation and subsequently of viral replication and depletion of intestinal $CD4^+$ T cells. Our study indicates that circulating microbial products can increase viral replication by inducing immune activation and increasing the number of viral target cells, thus demonstrating that immune activation and T cell proliferation are key factors in AIDS pathogenesis.

Introduction

Comparative studies of pathogenic HIV/SIV infection in humans and rhesus macaques (Rh) and SIV infection in African nonhuman primate (NHP) natural hosts that generally do not progress to AIDS have resulted in the emergence of a new paradigm of AIDS pathogenesis in which immune activation is a central factor underlying disease progression. According to this model, viral replication during acute infection results in rapid, massive mucosal $CD4^+$ Tcell depletion in both progressive and non-progressive SIV infections (1-3). During pathogenic HIV/SIV infection, immunologic and structural damage to the mucosal barrier results in microbial translocation from the gut lumen into the systemic circulation, which contributes to chronic immune activation and progression to AIDS (4). It was reported that the degree of immune activation in HIV-infected patients is a better predictor of disease progression rate than plasma viral load (VL) (5). Natural hosts (such as African green monkeys-AGMs, mandrills, and sooty mangabeys) generally do not progress to AIDS when infected with SIV and are able to maintain their intestinal barrier integrity (1, 3), perhaps through the suppression of inflammation (6) and lack of enteropathy, despite significant mucosal $CD4^+$ T cell depletion (1, 3). Consequently, natural hosts maintain normal levels of T cell activation, proliferation and apoptosis and show significant recovery of mucosal $CD4^+$ T cells during chronic infection despite high levels of viral replication (1, 3, 7). A direct causal relationship between immune activation, viral replication and $CD4^+$ T cell depletion has not yet been established in natural hosts and no successful attempt to experimentally induce immune activation was reported thus far. We addressed the question of whether immune activation can be experimentally-induced in AGMs and if it results in increased VL and $CD4^+$ T cell depletion.

Materials and Methods

Animals, infection, treatments and samples

Ten Caribbean AGMs (*Chlorocebus sabaeus*) were included. They received plasma equivalent to 300 tissue culture-infective dose (TCID) 50 of SIVagm.sab92018 (7). Animals were housed and handled at the Tulane National Primate Research Center in accordance with American Association for Accreditation of Laboratory Animal Care, *Guide for the Care and Use of Laboratory Animals* (U.S. Public Health Service) and the Animal Welfare Act. Tulane University Institutional Animal Care and Use Committee approved all protocols and procedures.

Acute infection follow-up was done on blood samples collected as described (3, 8). During chronic infection, the AGMs were split in 2 groups: monkeys in the first group (n=6) received intravenously 20 U/kg of LPS (*Escherichia coli*, lot G; U.S. Pharmacopeia, Rockville, MD). After treatment, blood samples were collected every 2 hours for 6 hours and then daily for the first 4 days. Additional samples were collected at days 7 and 14 post-LPS treatment. To evaluate if the observed effect was not due to repeated sampling and anesthesia episodes, 4 of the 6 AGMs received a control inoculation consisting of saline in the same volume as the LPS administration 1 month after the last blood draw. The same sampling schedule as for the LPS study was employed during saline administration.

Monkeys in the second group (n=4) were treated with Ontak® (Ligand Pharmaceutical Incorporated, San Diego, CA). Three Ontak treatments consisting of daily administration (15 mg/kg) for five consecutive days every 2 months were administered to each monkey in this study. Blood samples were collected prior to Ontak administration and then at days 4, 8, 12, 15 and 18 post-treatment initiation.

78 ***Tissue sampling***

Blood samples were collected as reported (3, 7) and plasma and mononuclear cells were isolated
80 as described (3, 7) for VL quantification, cytokine determination and flow-cytometry.

Viral quantification

82 Plasma VLs were quantified as described (6, 9). Assay sensitivity was 100 copies/ml.

Abs and flow cytometry

84 Whole blood was stained for flow cytometry as described (3, 47). Monoclonal Abs used were: CD3- FITC or CD3-PerCP; CD20-PE; CD8-PerCP or CD8-PE; CD4-allophycocyanin or CD4-
86 PerCP; HLA-DR-PerCP; CD95-FITC or CD95-allophycocyanin; CD28-allophycocyanin or CD28-PE; and Ki-67-FITC (BD Biosciences). All Abs were validated and titrated using AGM
88 and Rh PBMC. Data were acquired with a FACSCalibur flow cytometer (BD Immunocytometry Systems) and analyzed with CellQuest software (BD Biosciences). $CD4^+$ and $CD8^+$ T cell
90 percentages were obtained by first gating on lymphocytes, then on $CD3^+$ T cells. Memory, activation, and proliferation markers were determined by gating on lymphocytes, then on $CD3^+$
92 T cells and finally on $CD4^+CD3^+$ or $CD8^+CD3^+$ T cells.

Cytokine determination

94 Cytokine testing in plasma was done using a sandwich immunoassay-based protein array system, the Human Cytokine 25-Plex (Biosource International, Camarillo, CA, USA) as
96 instructed by the manufacturer and read by the Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA, USA) which uses Luminex fluorescent-bead-based technology (Luminex
98 Corporation Austin, TX, USA).

sCD14 measurement

Plasma sCD14 levels were measured using a commercially available ELISA (R&D systems). Plasma was diluted 1:300 and assay was performed in duplicate according to the manufacturer's protocol.

Statistics

Paired t-test was used to analyze data when we only had one series of measurements (LPS experiments and RT-PCR data). Linear-mixed effects models were used to analyze the Ontak data with three series of measurements, using the data of the first 10 days after administration. Monkeys were considered random effects; time and Ontak treatment were used as additive fixed covariates. Calculations were done in Excel (Microsoft, WA) and the software package R.

Results and Discussion

We previously reported that, in chronically SIVagm-infected AGMs, normal levels of immune activation are associated with normal levels of T cell apoptosis and proliferation, as well as a lack of enteropathy and plasma lipopolysaccharide (LPS) levels which are similar to those in uninfected monkeys (3). To provide proof-of-concept data that release of bacterial components into the systemic circulation results in increased immune activation and viral replication, we initially administered a single dose of LPS to six chronically SIVagm-infected AGMs. This resulted in a transient increase in the frequency of activated $CD4^+$ T-cells (Figure 1a) ($p=0.02$) and was accompanied by a transient but very rapid increase in VL (Figure 1b). One of the animals had VL comparable to those observed at the peak of viral replication during acute infection (Figure 1b). Moreover, the LPS was bioactive *in vivo* as indicated by increases in plasma levels of sCD14 (Figure 1c). To confirm that these increases in immune activation and viral replication were specifically induced by the LPS administration, we then treated 4 of the animals with saline alone and observed no effect (Figure 1a and b).

A second mechanism which may explain the lack of immune activation in natural SIV infections involves regulatory T cells (Tregs) (6). Previously we determined that the ratio between the total number of Tregs and conventional $CD4^+$ T cells is higher in uninfected AGMs compared to uninfected Rh in both the inductive and effector lymphoid sites of the intestine (10). Furthermore, the level of FoxP3 in $CD4^+$ T cells sorted from intestine was higher in AGMs compared with Rh (10). Additionally, we reported differences in the dynamics of Tregs in SIVagm-infected AGMs compared to SIVmac-infected Rh, such that FoxP3 expression was increased at day 1 post-SIVagm infection in AGMs but at a later time point in SIVmac-infected Rh (6). The same applies to cytokines associated with Tregs in that TGFβ and IL-10 are

increased very early in SIVagm infection but only late in SIVmac infection (6). Thus, the
134 mobilization of Tregs may be of insufficient rapidity and quantity to be effective in controlling immune activation in SIVmac-infected Rh.

136 To examine Treg involvement in the suppression of immune activation in AGMs, we conducted an *in vivo* Treg-depletion study in SIVagm-infected AGMs. We used Ontak (Denileukin
138 diftitox), an FDA-approved drug which depletes $CD25^+$ cells in humans. Ontak is a fusion protein which combines a recombinant IL-2 and a cytocidal diphtheria toxin moiety. Three
140 Ontak treatments (of 5 daily injections) were administered at 2-month intervals to chronically SIV-infected AGMs. Ontak did not change the total numbers of $CD4^+$ $CD25^+$ T-cells (p=0.79)
142 (Figure 2a) but it reduced the percentage of these cells (p=0.0001) (Supplementary Figure 1 and Supplementary Table 1). The absolute numbers (p=0.28) and percentages (p=0.48) of Tregs
144 defined by FoxP3 expression were also not decreased, however a transient increase in FoxP3 expression was observed by quantitative PCR (p=0.006 at day 4) during Ontak administration
146 (Supplementary Figure 2). Ontak also failed to induce a decrease in Treg function, as sorted Tregs from treated AGMs were as capable of inhibiting the cell proliferation of conventional
148 $CD4^+CD25^{neg}$ T cells as Tregs purified from non-treated AGMs (Supplementary Figure 3).

However, T-cell functional measurements revealed increased proliferation of conventional $CD4^+$
150 T cells in the Ontak-treated AGMs, which was confirmed by significant increases in the frequency of T-cells expressing the proliferation marker Ki-67 (p=0.003 for $CD4^+$ and p=0.0002
152 for $CD8^+$, Figure 2c, d). Over the 5 days of Ontak administration, we also found significant increases of both percentages (p<0.00005) and absolute numbers (p=0.02) of peripheral $CD4^+$ T
154 cells (Supplementary Figure 4), as well of effector memory $CD4^+$ T cells (p=0.006), which are preferentially depleted in SIV/HIV infections (Supplementary Figure 5). It is therefore possible

156 that this increase in overall T cell proliferation masked any partial depletion of both $CD4^+$ $CD25^+$ T cells and Tregs. Importantly, Ontak administration induced increases in T-cell immune
158 activation, as measured by HLA-DR expression on both $CD4^+$ ($p=0.07$) and $CD8^+$ T-cells ($p=0.002$) (Figure 2e, f), as well as by the dynamics of pro-inflammatory cytokines IL-1
160 ($p=0.004$), IL-8 ($p=0.02$) and IL-12 ($p=0.01$) (Figure 2g-i). As can be seen in the figures these had different dynamics, IL-1 and IL-12 showed an early increase (less than 5 days), whereas DR
162 expression and IL-8 had a slower increase (over the first 8-11 days). The levels of CCR5 by RT-PCR were also increased ($p=0.005$ at day 4), similar to primary infection, confirming an increase
164 of immune activation after the Ontak treatment (Supplementary Figure 6). Ontak administration resulted in significant upregulation of genes associated with immune activation, such as CD80,
166 HLA-DR, IL-3, IL-4, Toll-like receptors 4 and 6, IL12 receptor and IL-17 receptor as well downregulation of TGF-β1, CD3z, CD3d, CD3g and T-box21 genes (data not shown). Finally,
168 this marked increase in immune activation resulted in a significant increase in viral replication in all treated chronically SIVagm-infected AGMs ($p=0.0001$, Figure 2j) These increases in viral
170 replication were up to the levels of the peak VLs. Moreover, in two animals, increased viral replication was maintained between the first two treatments. Consequently, at the end of the
172 Ontak treatment, $CD4^+$ T cell were significantly depleted in the intestine ($p=0.0001$, Figure 2b).

Taken together, our data provide proof-of-concept that experimental induction of immune
174 activation in natural hosts of SIV infection drives increases in viral replication and mucosal $CD4^+$ T cell depletion. In the first set of the experiments we observed that a single low-dose LPS
176 administration results in increased immune activation and a consequent increase in viral replication, thus adding weight to the concept that microbial translocation contributes to systemic
178 immune activation and disease progression. In the second set of experiments, a more persistent

and significant increase in immune activation, and consequently a more robust and sustained increase in VL and significant depletion of mucosal $CD4^+$ T cells, was obtained after Ontak administration in chronically SIVagm-infected AGMs. Although Ontak did not induce significant Treg depletion, the drug has potential to induce immune activation as it contains an IL-2 moiety combined with a TLR ligand (the diphteria toxin) that interacts with the innate immune system. The increases in immune activation in both experiments through TLR ligand stimulation demonstrate that interaction between innate and adaptative immune systems are functional in the natural hosts and can induce significant increase of viral replication and $CD4^+$ T cell depletion in this animal model that otherwise is characterized by very stable chronic lentiviral infection. Our results are in agreement with a recent study showing augmentation of viral replication at mucosal sites during early SIVmac infection of Rh due to increased immune activation driven by CTLA-4 blockade (11) and with data reporting that the levels of VLs in sooty mangabeys depends on the availability of activated $CD4^+$ T cells (12).

Therefore, our results demonstrate that immune activation and proliferation of the target cells for the virus are key factors in AIDS pathogenesis. These data suggest that therapeutic strategies to reduce immune activation should be explored, in addition to the classic antiretroviral therapies, in preventing progression to AIDS in chronically HIV-infected individuals.

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Disclosures

The authors have no financial conflict of interest.

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Footnotes

1. **Grant support**

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2. **Abbreviations**

AGM, African green monkey; LPS, Lipopolysaccharide; Rh, rhesus macaque; VL, viral load; PCR, polymerase chain reaction; LN, lymph node; Tregs, T regulatory cells.

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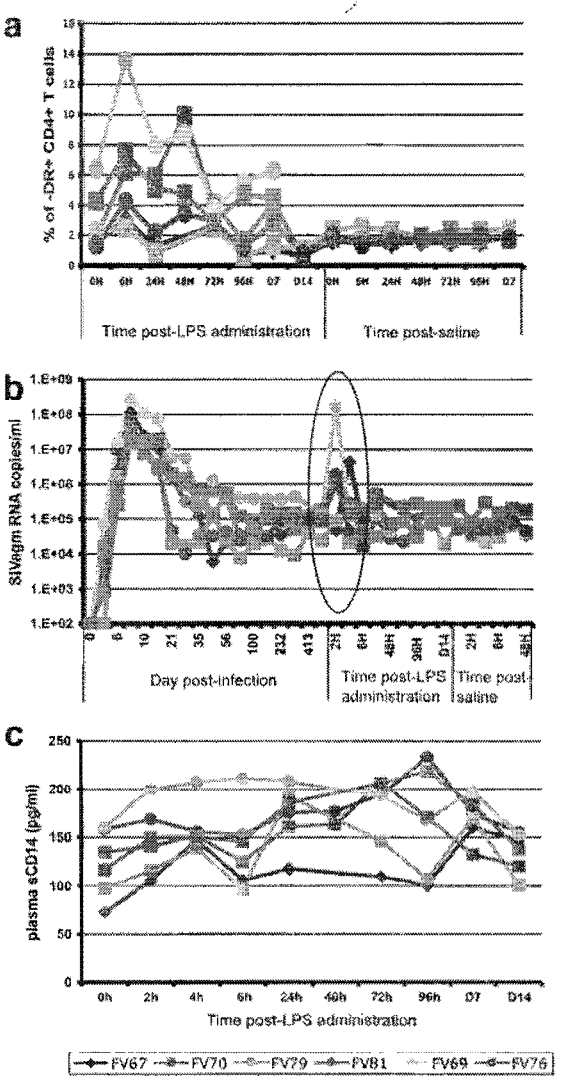
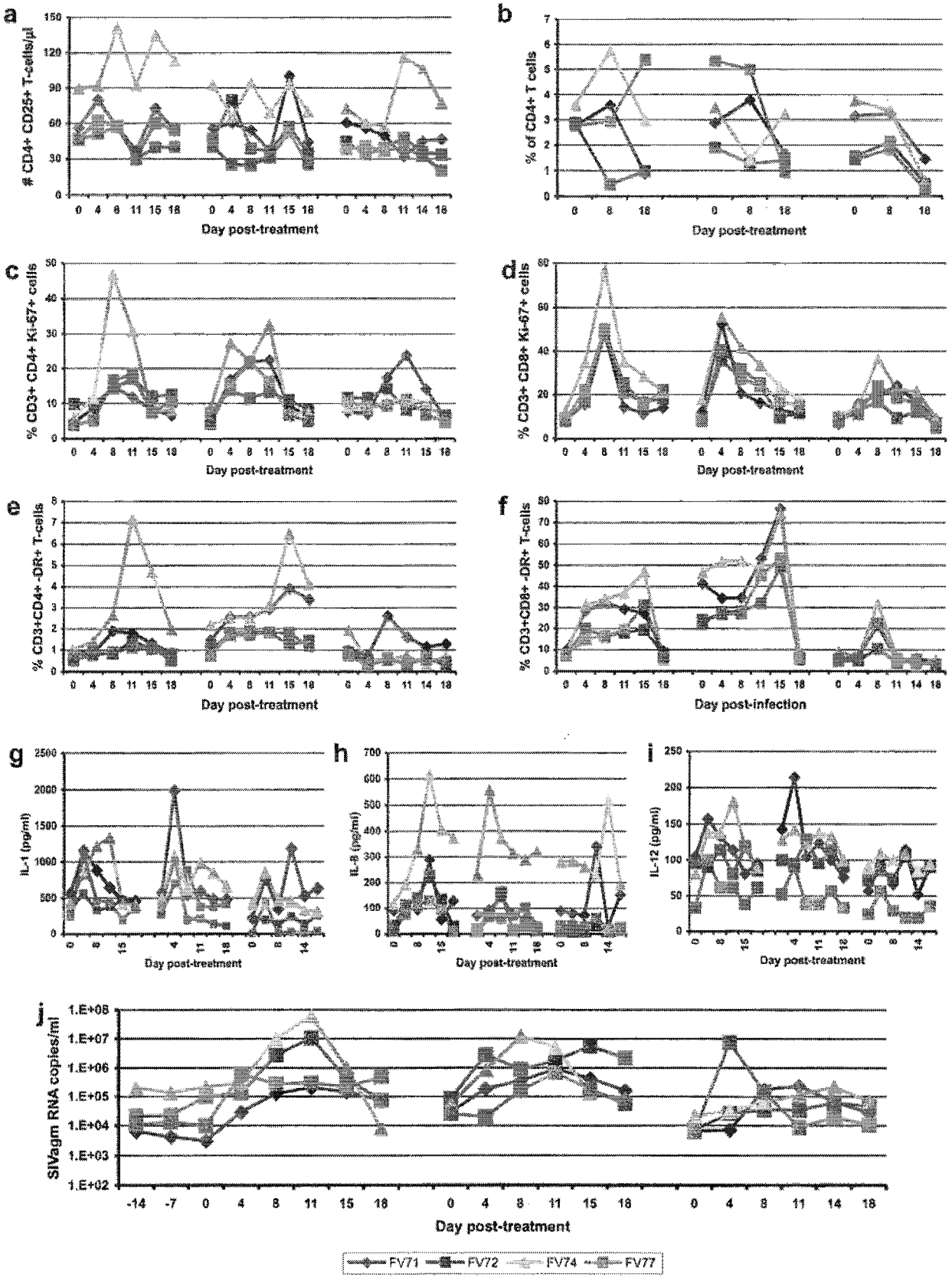
Figure legends

Figure 1. *In vivo* LPS and saline administration in chronically SIVagm-infected AGMs. A single dose of LPS induces a transient but significant increase in activation of $CD4^+$ T cells (a), a transient increase in the VL (b) and an increase in plasma sCD14 (c). In FV75, VL reaches the levels of virus replication from the primary infection. Saline administration in the same AGMs did not modify the steady-state of the chronic SIVagm infection (a, b).

Figure 2. Impact of Ontak administration in chronically SIVagm-infected AGMs. Although Ontak did not induce significant depletion of $CD25^+$ $CD4^+$ T-cells (Tregs) (a), at the end of treatment, mucosal $CD4^+$ T cells were significantly depleted (b). Significant increases in the cell proliferation of $CD4^+$ (c), $CD8^+$ T cells (d), as well as immune activation of $CD4^+$ (e) and $CD8^+$ T cells (f) were observed after Ontak administration. This increase in immune activation was confirmed by the dynamics of pro-inflammatory cytokines: IL-1 (g), IL-8 (h) and IL-12 (i) and resulted in a significant increase in VLs (j).



Pandrea et al., Figure 1



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