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Short Communication
HIV$^+$ Viremic Slow Progressors Maintain Low Regulatory T Cell Numbers in Rectal Mucosa but Exhibit High T Cell Activation

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Abstract

Viremic slow progressors (VSP) are a rare subset of HIV-infected persons who exhibit slow immunologic progression despite high viremia. The mechanisms associated with this slow progression remain to be defined. Clinical characteristics of VSP are similar to those of natural hosts for simian immunodeficiency virus (SIV), such as sooty mangabeys (SM) and African green monkeys (AGM), who maintain near-normal CD4 counts despite high-level viremia but maintain low immune activation. Immune activation is a powerful predictor of disease progression, and we hypothesized that low immune activation might also explain the VSP phenotype. Using multiparameter flow cytometry, we assessed levels of T cell activation and regulatory T cells (Treg) in blood and rectal mucosa of VSP, typical progressors, virologic controllers, and seronegative controls. We also assessed Treg function and CD4 T cell proliferative capacity in VSP. Contrary to expectations, we found that VSP subjects have high levels of T cell activation in the gastrointestinal mucosa. The ratio of Treg to CD3$^+$ T cells in the mucosa of VSP was relatively low, potentially contributing to increased immune activation. Nonetheless, CD4$^+$CD25$^-$ T cells isolated from these individuals displayed a comparatively weak proliferative response to anti-CD3 stimulation. These data reveal that the VSP phenotype is associated with elevated markers of mucosal immune activation and low numbers of mucosal Treg, suggesting that factors other than immune activation account for this phenotype.

Although most untreated HIV-infected individuals display high levels of viremia and progressive CD4 T cell loss, the rate of CD4 decline varies significantly. In the absence of treatment, time to AIDS is predicted by both CD4 count and viral load (VL).[1,2] However, unusual phenotypes have been described in which the typical association between viral load and outcome is not evident. For example, despite undetectable HIV RNA and high CD4$^+$ T cell counts, HIV "elite" controllers experience higher than expected levels of atherosclerosis.[3] Recently, several groups have begun to study rare individuals who fail to exhibit disease progression despite years of high-level viremia.[4,5] These "viremic slow progressors" (VSP) represent a unique cohort of HIV$^+$ subjects for whom mechanisms associated with slow progression have yet to be defined.

The clinical characteristics of VSP are similar to those of natural hosts for simian immunodeficiency virus (SIV), sooty mangabeys (SM), and African green monkeys (AGM).[6] Chronically infected SM and AGM maintain near-normal CD4 counts despite high-level viremia, but also maintain low peripheral immune activation.[7] Immune activation is a powerful predictor of disease progression,[8–11] and multiple groups have suggested low levels of immune activation may similarly explain the VSP phenotype.[4,5]

HIV and SIV are known to target the gastrointestinal mucosa, an area rich in CD4$^+$CCR5$^+$ T-lymphocytes.[12–15] A damaged mucosal barrier during acute pathogenic infection may contribute to chronic immune activation fueled by microbial translocation.[16] SM and AGM experience increased immune activation and mucosal CD4 T cell depletion during acute infection[17]; however, rapid induction of immunosuppressive responses prevents the generalized T cell activation characteristic of progressive infection.[18,19] Such attenuated

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activation may be mediated, in part, by regulatory T cells (Treg).[20–22] Our group and others have found a significantly higher frequency of Treg and greater T cell activation in the gut of noncontrollers compared to controllers or seronegatives,[6,23,24] suggesting Treg from HIV progressors are responsive to, yet unable to limit, immune activation.

To determine whether VSP recapitulate the SM/AGM profile, we compared T cell activation in blood and rectal mucosa of VSP to typical progressors, controllers, and seronegatives. We also examined Treg frequency, number, and function and $CD4^+$ non-Treg proliferative capacity. Surprisingly, our results indicated that VSP are characterized by increased mucosal T cell activation, low Treg numbers, and decreased $CD4^+$ T cell proliferation e*x vivo*. Thus, in contrast to SM and AGM, protection in VSP appears to be mediated by a mechanism that is not reliant upon maintenance of low T cell activation.

We defined VSP as individuals with VL >10,000 copies RNA/ml, infected >7 years, with $CD4^+$ T cell counts maintained above 500 cells/μl. These individuals were compared to progressors (VL >10,000, CD4 decline below 500 cells/μl) and controllers (VL <1,000, infected >5 years). A previous study from our group reported on mucosal Treg and immune activation in HIV controllers and progressor subjects, who were also used as comparison groups for this study[25]; however, data and analysis of VSP subjects were not presented in the earlier report. Subjects were recruited at the UC Davis Medical Center, Sacramento, CA or San Francisco General Hospital, UC San Francisco (UCSF). Informed consent was obtained under protocols approved by the UCSF Committee on Human Subjects Research and the UC Davis Institutional Review Board.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Hypaque (GE Healthcare, UK). Rectal biopsies were obtained by flexible sigmoidoscopy[26] and mucosal leukocytes isolated using collagenase and mechanical disruption.[25] PBMCs and rectal cells were stained with anti-CD3 (clone UCHT1; BD Pharmingen), anti-CD4 (SFCI12T4D11, Beckman Coulter, Brea, CA, or L200, BD Pharmingen), anti-CD8 (SK1 or 3B5, Invitrogen), anti-CD25 (M-A251, BD Pharmingen), anti-CD127 (hIL-7R-M21, BD Pharmingen), anti-CD38 (HIT2, BD Pharmingen), anti-programmed-death-receptor 1 (PD-1) (eBioJ105, eBioscience), and Live/Dead Fixable Aqua dead cell stain (Invitrogen, Carlsbad, CA), followed by intracellular staining with anti-FOXP3 (PCH101, eBioscience) using the FOXP3 Staining Buffer Set (eBioscience, San Diego, CA). After acquisition on an LSRII flow cytometer (BD Pharmingen), data were analyzed using FlowJo software (Tree-Star Inc., Ashland, OR).

Treg suppression assays were performed using bead-isolated $CD4^+CD25^-$ non-Treg and $CD4^+CD25^+$ Treg as described previously.[25] Briefly, CFSE-stained non-Treg and irradiated, autologous PBMCs were left unstimulated or stimulated with plate-bound anti-CD3 and cultured alone or in the presence of increasing numbers of Treg for 4 days. Cultures were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD25, and anti-FOXP3 and analyzed by flow cytometry. GraphPad Prism (GraphPad Software, La Jolla, CA) was used to graph and analyze data. Intergroup comparisons were analyzed using a two-tailed Mann–Whitney test. The Wilcoxon matched pairs test was used for intragroup comparisons between blood and rectal samples. p values ≤ 0.05 were considered significant.

We studied six VSP, eight progressors, 11 controllers, and 12 uninfected controls. The median CD4 counts were 645, 418, 728, and 793 cells/μl for VSP, progressors, controllers, and controls, respectively. The median viral loads were 28,647, 24,650, and 50 copies/ml plasma; the median numbers of years infected were 17, 12, and 19 for these same groups, respectively (Fig. 1). Three consecutive time points where the CD4 count was <500 cells/μl indicated disease progression with the exception of two progressors who had only two CD4 counts available for analysis (all of which were <400 cells/μl). Clinical data for controllers and seronegatives have been reported elsewhere.[25] All patient groups were assayed during the same time frame to eliminate potential bias due to reagent or protocol variability.

Two approaches were used to assess T cell activation: augmented CD38/PD-1 coexpression and decreased expression of CD127.[25, 27–29] Although the combination of HLA-DR and CD38 is most commonly used to assess T cell activation in HIV disease,[10] both increased CD38/PD-1 coexpression and reduced CD127 expression have been linked to disease progression.[25,27–29] $CD4^+$ Treg are typically $CD127^-$, therefore only non-Treg, defined as $FOXP3^-CD127^-$ cells, were considered when analyzing the $CD4^+$ subset for CD127 expression. In blood, progressors displayed significantly higher percentages of $CD38^+PD\text{-}1^+$ $CD4^+$ and $CD8^+$ T cells compared to controllers or seronegatives and a strong trend toward increased activation compared to VSP (Fig. 2A). Although VSP had lower frequencies of peripheral $CD38^+/PD\text{-}1^+$ T cells than progressors, their T cell activation was significantly elevated compared to controllers ($CD8^+$ cells only) or seronegatives ($CD4^+$ and $CD8^+$ cells) (Fig. 2A). Interestingly, while no significant differences in peripheral $CD4^+CD127^-$ percentages were observed, all HIV^+ subjects displayed significant expansion of $CD8^+CD127^-$ T cells (Fig. 2B).

Strikingly, in rectal mucosa, CD38/PD-1 coexpression was high in progressors and VSP, with both groups demonstrating increased $CD4^+$ and $CD8^+$ T cell activation compared to controllers or seronegatives (Fig. 2A). Loss of CD127 expression by $CD4^+$ T cells mirrored what was observed for CD38/PD-1 coexpression, with progressors and VSP displaying decreased CD127 compared to controllers or seronegatives. However, as in blood, the $CD8^+$ subset again exhibited an expansion of $CD127^-$ cells within all HIV-infected groups compared to seronegatives (Fig. 2B).

In chronically infected SM, $CD4^+$ T cell preservation closely correlates with a low frequency of circulating $CD127^-$ effector T cells and high frequency of Treg.[30] Because Treg can suppress immune activation, we hypothesized that high Treg levels might compensate for augmented T cell activation in VSP. As previously reported,[25] the frequency of Treg as a percentage of $CD4^+$ T cells was significantly higher in mucosa of progressors compared to controllers or seronegatives; surprisingly, however, VSP did not exhibit increased mucosal Treg frequencies relative to seronegatives or HIV controllers (Fig. 2C). Indeed, VSP had the lowest mucosal ratio of Treg to $CD3^+$ T cells of any subject group (number of $CD4^+$ $FOXP3^+CD25^+$ cells/10,000 $CD3^+$ cells) (Fig. 2C).

To examine Treg functionality, suppression assays were performed using cells isolated from four progressors, seven controllers, four seronegatives, and four VSP; data on the first three groups were reported previously.[25] Peripheral and

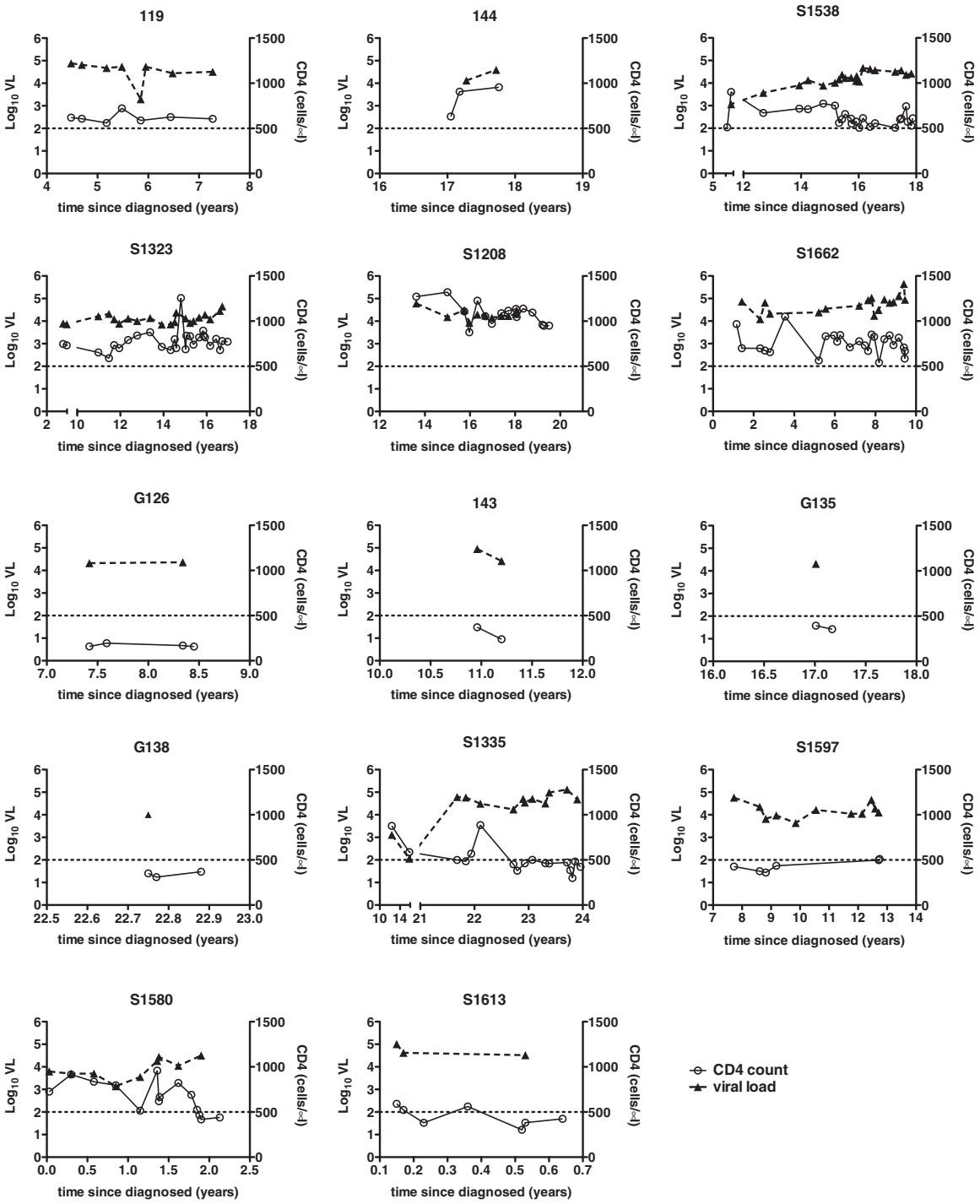

FIG. 1. Longitudinal viral load (VL) and $CD4^+$ T cell counts for viremic slow progressors (VSP) and progressors. Viral load (left *y*-axes, filled triangles, dashed lines) and $CD4^+$ T cell counts (right *y*-axes, open circles, solid lines) are plotted against time since HIV^+ diagnosis (*x*-axis) in years for VSP (upper six graphs) and progressors (lower eight graphs). The horizontal dashed line at 500 CD4 cells/μl serves as a reference point. Numbers above each graph indicate patient code numbers.

rectal Treg displayed similar suppressive capacity in all groups, indicating that although few in number, Treg of VSP are functionally suppressive (data not shown). Interestingly, in control cultures containing peripheral blood non-Treg alone, both VSP and controllers demonstrated reduced T cell proliferation, measured by CFSE dilution, compared to progressors or seronegatives (Fig. 2D). In rectal cell cultures, only VSP T cells were distinguished by weak proliferative responses; this effect was statistically significant compared to seronegatives (Fig. 2D).

Multiple studies have suggested that low immune activation contributes to slow HIV progression; however, our results indicate that although VSP have low levels of activated T cells in blood as compared to typical progressors, this unusual

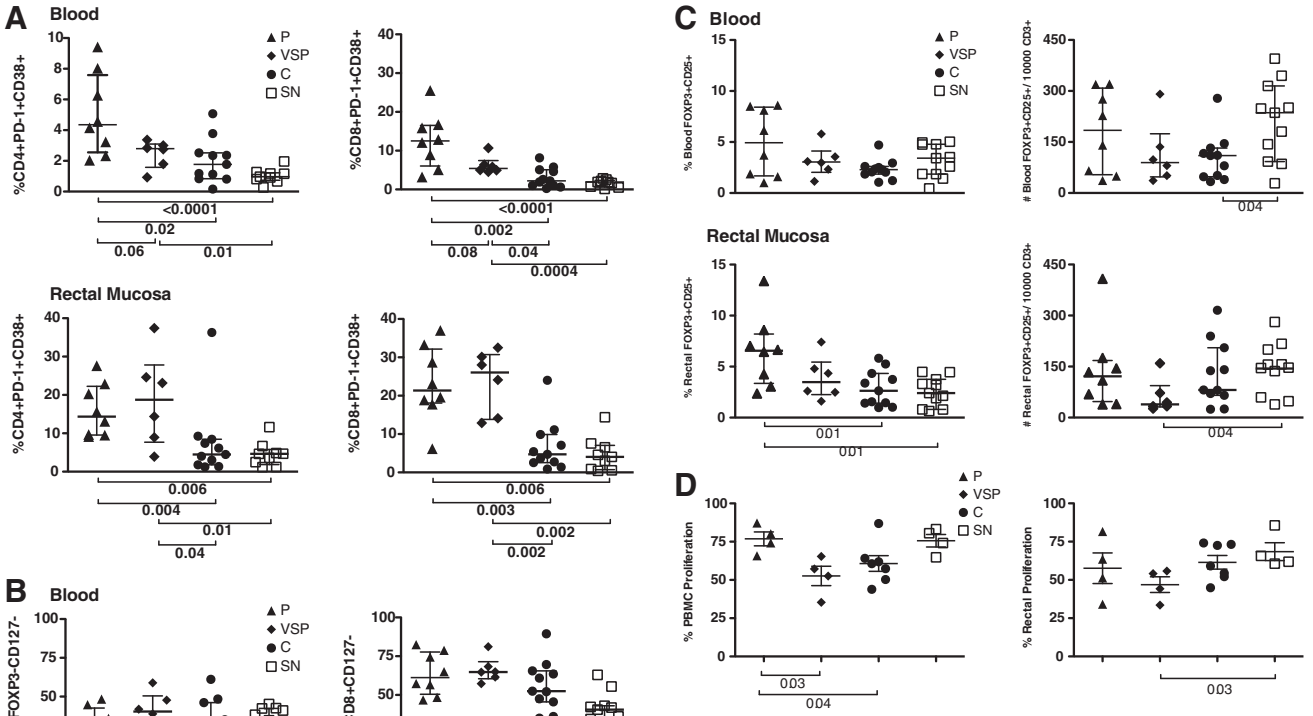

FIG. 2. **(A)** CD38/PD-1 coexpression in peripheral and mucosal T cells. Peripheral and mucosal $CD4^+$ (left panels) and $CD8^+$ (right panels) T cells from progressors (P), viremic slow progressors (VSP), controllers (C), and seronegative subjects (SN) were analyzed for expression of CD38 and PD-1 by flow cytometry. Vertical lines and whiskers indicate 25th–75th percentiles with median values indicated by a horizontal bar. p-values ≤ 0.05 were considered significant. **(B)** CD127 expression in peripheral and mucosal T cells. $CD4^+Foxp3^{neg}CD127^{neg}$ (left panels) and $CD8^+CD127^{neg}$ T cells (right panels) from progressors (P), viremic slow progressors (VSP), controllers (C), and seronegative subjects (SN) were analyzed by flow cytometry. Vertical lines and whiskers indicate 25th–75th percentiles with median values indicated by a horizontal bar. p-values ≤ 0.05 were considered significant. **(C)** $CD4^+$ Treg frequency and number. Left panels: Peripheral and rectal mucosal Treg frequencies are expressed as percentages of $CD4^+$ T cells coexpressing Foxp3 and CD25. Right panels: Peripheral and rectal mucosal Treg numbers are expressed as number of $Foxp3^+CD25^+CD4^+$ T cells per 10,000 $CD3^+$ T cells. These numbers were determined from flow cytometry data using the following formula: [total $CD4^+FOXP3^+CD25^+$ cells/total $CD3^+$ cells] $\times$ 10,000. **(D)** T cell proliferation. $CD4^+CD25^-$ non-Treg were stimulated with 0.2 μg/ml plate-bound anti-CD3 for 4 days and proliferation analyzed by CFSE-dye dilution for T cells isolated from peripheral blood (left panel) and rectal (right panel) cell populations of progressors (P), VSP, controllers (C), and seronegatives (SN). Vertical lines and whiskers represent the 25th–75th percentiles with median values indicated by a horizontal bar. p-values ≤ 0.05 were considered significant.

group of patients also has high levels of mucosal T cell activation. This "disconnect" between blood and mucosa in VSP was unexpected. Additionally, we observed low Treg numbers in the rectal mucosa of VSP, which may contribute to immune activation, but may also support stronger anti-HIV immune responses[31] and a preserved Treg:Th17 cell ratio.[32,33] Alternatively, it is possible that a blunted mucosal Treg response allows immune activation to persist without inhibiting normal T cell homeostasis, allowing the ongoing proliferation of $CD4^+$ T cells despite high-level viremia.

Despite increased frequencies of $CD38^+PD\text{-}1^+$ and $CD127^-$ T cells, VSP CD4s displayed reduced $CD4^+$ T cell proliferation when stimulated *ex vivo*. In agreement with this finding, decreased Ki67 expression has been described in T cells of viremic patients with long-term asymptomatic infection.[4] Additionally, low percentages of $CD4^+Ki67^+$ cells have been linked to CD4 preservation in SM.[34] While activated and effector memory cells support high levels of virus replication, limited proliferation and CCR5 expression in central memory $CD4^+$ T cells (T_{CM}) of SIV-infected SM may protect these cells from depletion.[7] Additional studies will be required to determine whether VSP T_{CM} have a similar selective advantage.

The clinical significance of our findings will need to be explored in longitudinal studies. Notably, after this study was completed VSP patient S1538 began showing signs of progression, declining from 611 to 390 $CD4^+$ cells/μl in 4 months with a concomitant increase in viral load from 26,323 to 67,431

copies/ml. This observation indicates susceptibility of some VSP to eventual disease progression.

These data present a complex picture of VSPs' ability to resist $CD4^+$ T cell depletion despite high-level viremia. The apparent dichotomy between activation and CD4 proliferation in VSP highlights the need to better define how immune activation influences HIV progression. Further studies of VSP are warranted to elucidate how Treg, immune activation, and adaptive immune responses are related to $CD4^+$ T cell preservation in the context of high plasma viremia. The analysis of virus isolated from VSP subjects was beyond the scope of the present study; however, it will also be critical to determine whether virologic features contribute to the VSP phenotype.

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S.G.D., P.W.H., J.M.S., and B.L.S. conceived the study. J.M.S. performed experiments and analyzed data. J.W.C. assisted with assay development. S.G.D., D.H.M., J.C.G., P.W.H., R.B.P., and M.S. provided patient samples. J.M.S. and B.L.S. wrote the manuscript. All authors reviewed and edited the manuscript.

Author Disclosure Statement

No competing financial interests exist.

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