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A Single Amino Acid Mutation in the Envelope Cytoplasmic Tail Restores the Ability of an Attenuated Simian Immunodeficiency Virus Mutant To Deplete Mucosal CD4$^+$ T Cells

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Disruption of the conserved motif GYxxØ in the simian immunodeficiency virus (SIV) SIVmac239 envelope (Env) cytoplasmic tail resulted in a virus (ΔGY) that exhibited a high plasma peak but uniquely failed to acutely deplete mucosal CD4$^+$ T cells. Here, we show that ΔGY containing a flanking S727P mutation that was acquired in ΔGY-infected macaques reacquired the ability to rapidly deplete CD4$^+$ T cells in lamina propria. This suggests that the GYxxØ motif and S727P each contribute to SIV's targeting to mucosal tissues.

Pathogenic human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections uniformly cause rapid and profound acute depletion of mucosal CD4$^+$ T cells (1–5). Recently we described a SIVmac239 variant, known as ΔGY (6), containing a Gly-Tyr deletion of amino acids (aa) 720 to 721 in a highly conserved GYxxØ trafficking motif (where G = Gly, Y = Tyr, x = any amino acid, and Ø = an amino acid with a bulky hydrophobic side chain) in the envelope (Env) glycoprotein cytoplasmic tail (i.e., GYRPV for SIVmac239). This model was unique in demonstrating that the high acute peak viral load and acute loss of mucosal CD4$^+$ T cells could be disassociated (6). In rhesus macaques, ΔGY exhibited an acute plasma viral RNA peak that was comparable to that of highly pathogenic parental SIVmac239 and exhibited robust replication in peripheral lymphoid tissues (e.g., tonsil, spleen, and mesenteric lymph nodes) but displayed an attenuated phenotype within the intestinal mucosa, with only patchy and transient infection of CD4$^+$ T cells in the lamina propria (6). Consistent with this lack of gut pathology, these animals showed no evidence of microbial translocation, which is associated with a loss of epithelial barrier function and has been implicated as a driver of immune activation and disease progression (7–16). Nonetheless, even in the absence of microbial translocation, ΔGY-infected animals progressed to disease with immune activation and a gradual depletion in mucosal CD4$^+$ T cells. Although the ΔGY mutation was maintained, disease progression was associated with several mutations in the cytoplasmic tail that flanked the ΔGY deletion, including R722G, S727P, and a 9-nucleotide deletion resulting in loss of QTH at aa 735 to 737 (6, 17). We proposed that one or more of these mutations could restore pathogenicity and/or the ability of ΔGY to target and deplete mucosal CD4$^+$ T cells.

We examined the effects of one of these mutations, S727P, on the ability of ΔGY to target and deplete mucosal CD4$^+$ T cells and determined its impact on disease progression. We focused on this mutation because it had previously been described in a ΔGY-infected rhesus macaque that rapidly progressed to AIDS with a high viral load (17). SIVmac239 containing both the ΔGY and the S727P mutations, designated ΔGY+S/P, was produced from transfected 293T cells. Four male Indian-origin rhesus macaques (Table 1) were inoculated intravenously with ΔGY+S/P (100 50% tissue culture infective doses [$TCID_{50}$]) and compared to macaques inoculated with ΔGY or either SIVmac239 or the closely related SIVmac251, as previously described (6). Two animals were euthanized at week 4 to provide a comprehensive assessment of ΔGY+S/P infection in gut and other tissues, and two were followed through chronic infection. All animals were maintained at the Tulane National Primate Research Center in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Research Council (28). The Tulane Institutional Animal Care and Use Committee approved all studies.

Acute plasma viral RNA for the 4 ΔGY+S/P-inoculated macaques was compared to data from 8 ΔGY- and 8 SIVmac239-infected macaques (Fig. 1A). The acute peak of infection for ΔGY+S/P occurred more rapidly (day 17) than that for ΔGY (day 21), although both were slower than SIVmac239 (day 14). Interestingly, the mean viral peak for ΔGY+S/P was higher than that for ΔGY (4.4×10^7 copies/ml versus 1.0×10^7 copies/ml, respectively; $P < 0.05$) but comparable to that for SIVmac239 (1.3×10^7 copies/ml; $P > 0.05$) (Fig. 1B).

We next compared the impact of ΔGY+S/P on mucosal CD4$^+$ T cells during acute and chronic infection to those of ΔGY- and SIVmac251-infected controls. Flow cytometry was performed on serial jejunal biopsy specimens to measure CD4$^+$ T-cell populations over time in lamina propria (Fig. 1C and D). In marked contrast to ΔGY-infected animals, ΔGY+S/P-infected animals exhibited a rapid and profound depletion of mucosal CD4$^+$ T cells

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TABLE 1 Results from individual rhesus macaques infected with ΔGY+S/P[a]

Stage of infection	Animal	Duration of infection (wk)[b]	*Mamu* type(s)[c]
Acute	GM76	4	*DRBw201*, *A*11*, *B*01*
	GT39	4	*DRBw201*
Chronic	GK58	**40**	*A*02*, *B*01*
	GM20	>100	*DRBw201*, *A*02*, *A*08*

[a] Results from four rhesus macaques infected with ΔGY+S/P are shown. (Two were euthanized at week 4 to assess pathological features during acute infection, and 2 were followed during chronic infection.) The control animals for this study have been reported previously (6) and included 8 animals infected with ΔGY and 15 animals infected with either SIVmac239 or SIVmac251.

[b] Animals were euthanized at the indicated week postinfection. Boldface text indicates euthanasia due to progressive disease. Regular text indicates euthanasia at the end of the predetermined period during acute infection. GM20 controlled infection and remained alive for >100 weeks.

[c] *Mamu* alleles of infected animals are shown.

by day 21 (7.9% for ΔGY+S/P versus 36.5% for ΔGY; $P < 0.05$) (Fig. 1C). For the two ΔGY+S/P-infected macaques followed through chronic infection, this difference was sustained for up to 34 weeks, at which time these animals showed 6 and 10% $CD4^+$ T cells, in contrast to a mean of 32.7% for 4 chronic ΔGY-infected animals (Fig. 1D). Depletion of $CD4^+$ T cells was clearly evident in animals infected with ΔGY+S/P compared to naive animals ($P < 0.01$) and animals infected with ΔGY at a similar time postinfection ($P < 0.05$) (Fig. 1E); however, this reduction was less than for SIVmac251 (i.e., 3.2% at week 4 and 1.3% at week 36; $P < 0.05$) (Fig. 1D and E). These findings indicate that the S727P mutation significantly restores, at least in part, the ability of ΔGY to deplete mucosal $CD4^+$ T cells.

To further examine the ability of ΔGY+S/P to infect mucosal $CD4^+$ T cells, we performed SIV RNA *in situ* hybridization and multilabel confocal microscopy on intestinal tissues, as described previously (18, 19). The intestinal tissues examined included serial jejunal biopsy specimens collected during acute infection from animals inoculated with ΔGY ($n = 6$), ΔGY+S/P ($n = 4$), and SIVmac239 ($n = 6$).

SIV-infected cells were quantified in lamina propria at the peak of mucosal infection for ΔGY+S/P, ΔGY (week 2), and SIVmac239 (week 1) (Fig. 1F). SIVmac239-infected animals showed a high level of infection (mean ± standard error of the mean [SEM], 450 ± 10 cells/mm^2) at week 1, which declined to 10 ± 4 cells/mm^2 at week 2 due to their rapid depletion (Fig. 1C). While no infected cells were seen for ΔGY+S/P or ΔGY at week 1, by week 2, ΔGY+S/P had infected a significantly greater number of cells than ΔGY (295.5 ± 80.4 versus 57.9 ± 16.8, respectively; $P < 0.01$), which was similar to SIVmac239-infected animals at week 1 ($P > 0.05$) (Fig. 1F). Thus, although delayed, the frequency of lamina propria $CD4^+$ T-cell infection was similar for ΔGY+S/P and SIVmac239. This observation appears consistent with the plasma viral RNA for ΔGY+S/P, which showed delayed kinetics (Fig. 1A) but a comparable peak viremia (Fig. 1B). In addition, SIV-infected cells were more diffusely distributed throughout the jejunum in SIVmac239- and ΔGY+S/P-infected animals (Fig. 2A and B) than in ΔGY-infected animals, where infection was sparse and more focally distributed (Fig. 2C) (6).

We then characterized the immunophenotype of ΔGY+S/P-infected cells in jejunal biopsy specimens using multilabel confocal microscopy, as described previously (18, 19). Similar to ΔGY infection, during the acute infection ΔGY+S/P-infected cells were predominantly $CD3^+$ and $CD68^-$, indicating that they were T cells and not macrophages (data not shown). We then used immunohistochemistry and a specific memory T-cell marker, OPD4 (anti-CD45RO) that was shown to label only $CD4^+$ T cells (20). For both ΔGY and ΔGY+S/P, the majority of infected cells were

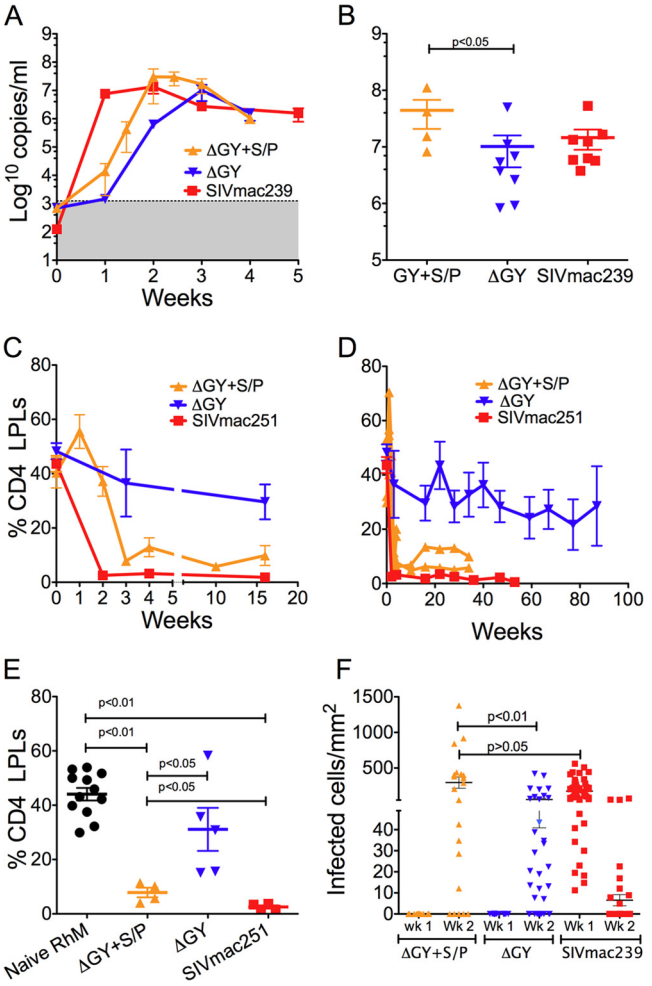

FIG 1 Plasma viral load and gut $CD4^+$ T-cell infection in rhesus macaques infected with ΔGY+S/P, ΔGY, or parental SIVmac (SIVmac239 or SIVmac251). (A) Plasma viral loads (mean ± SEM) for ΔGY+S/P-, ΔGY-, and SIVmac239-infected animals are shown during acute infection. The shaded area in panel A indicates the limits of sensitivity of the viral RNA assay; (B) peak viral RNA levels are shown for individual animals (mean ± SEM). ΔGY+S/P had an earlier (A) and a higher (B) peak viremia than ΔGY. (C and D) The percentages of $CD4^+$ T cells in the intestinal lamina propria (lamina propria lymphocytes [LPLs]), determined by flow cytometry, are shown (mean ± SEM) during acute (C) and chronic (D) infection. Values for the 2 ΔGY+S/P-infected animals followed during chronic infection are plotted individually (D). $CD4^+$ T-cell depletion was greater and occurred more rapidly for ΔGY+S/P than for ΔGY. (E) Individual percentages of $CD4^+$ T cells in lamina propria are shown in naive rhesus macaques ($n = 12$) and at the nadir of mucosal CD4 depletion for ΔGY+S/P- and ΔGY-infected animals (week 3) and for SIVmac251-infected animals (week 2; mean ± SEM). (F) The numbers of infected cells detected by *in situ* hybridization per mm^2 of tissue surface area are shown. ΔGY+S/P infected a greater number of cells per mm^2 of lamina propria at week 2 than did ΔGY, but the level of infection was similar to that of SIVmac239 at week 1 (F). The number of cells infected by SIVmac239 is reduced at week 2 due to depletion of these cells (C).

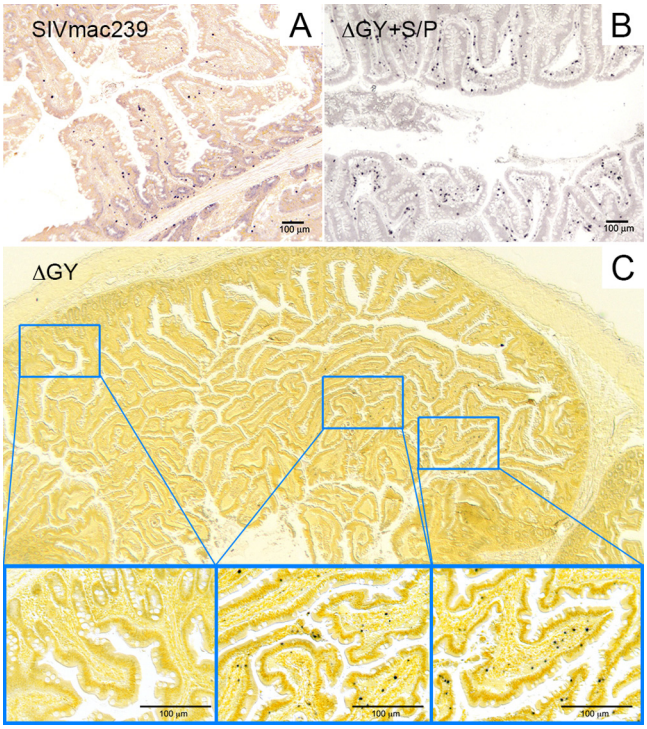

FIG 2 SIV RNA *in situ* hybridization of jejunal tissues at the peak of mucosal infection. *In situ* hybridization of SIV RNA is shown for SIVmac239 infection at week 1 (A) and for ΔGY+S/P infection at week 2 (B). Infection for both viruses is diffuse and distributed throughout lamina propria. (C) Jejunum from a ΔGY-infected animal at week 2 with magnified insets demonstrating the sparse multifocal pattern of infection with focal areas of intense infection and large areas of no infection.

CD45RO$^+$, indicating that memory CD4$^+$ T cells were the predominant target of infection (Fig. 3). However, as shown in Fig. 3, a strikingly higher density and more diffuse distribution of infected cells were found with ΔGY+S/P than with ΔGY. These findings are consistent with flow cytometry and morphometric quantification of infected cells in the gastrointestinal mucosa (Fig. 1C to F), indicating that acquisition of the S727P mutation resulted in a marked gain of function in the extent of CD4$^+$ T-cell infection and depletion in the intestine by ΔGY (1, 2).

Given the marked acute depletion of mucosal CD4$^+$ T cells in ΔGY+S/P infection, which was similar to pathogenic SIVmac239 and SIVmac251, the long-term follow-up of these animals to assess disease outcome was of particular interest. One animal (GK58) developed a viral set point above 5×10^5 RNA copies/ml, similar to SIVmac239-infected animals, with declining peripheral CD4$^+$ T cells, increased monocyte turnover, thrombocytopenia, and progression to clinical AIDS within 1 year (see Fig. S1A to C in the supplemental material). However, the other (GM20) developed a viral set point of <1,000 RNA copies/ml, retained normal numbers of CD4$^+$ T cells, and maintained monocyte turnover at preinfection levels (see Fig. S1A to C). The survival of the two ΔGY+S/P-infected rhesus macaques fell between those observed for ΔGY and SIVmac239 (see Fig. S1D) (6). Although the numbers of animals are small, these findings suggest that while the ΔGY+S/P mutation is able to restore, at least in part, the ability of the ΔGY mutant to cause depletion of mucosal CD4$^+$ T cells, it may not be sufficient to restore virulence for all infected animals.

Single genome amplification (SGA) analysis, as described previously (6), was performed to assess the stability of the S727P mutation and possible evolution of the ΔGY+S/P Env. Plasma samples were collected for SGA analysis at weeks 8 and 34 from GK58, although only at week 8 for GM20, due to its low viral set point during chronic infection. The ΔGY and S727P mutations were maintained in all amplicons (see Fig. S1E in the supplemental material). An R751G consensus mutation was also observed in both animals by week 8, as has been reported for SIVmac239 (21) and ΔGY (6) in rhesus macaques, reflecting an apparent optimizing effect of this mutation on SIVmac239-based viruses *in vivo* (6, 21). For GK58, additional mutations were also acquired by week 34 in the context of a high viral set point and progression to AIDS. Interestingly, one of these changes was a L786Y at the transmembrane C terminus in 7/9 amplicons, which resulted in a new YxxØ consensus sequence (YTLL) (see Fig. S1E). Possible functional effects of this new motif are under investigation.

The S727P mutation was first described in a ΔGY-infected rhesus macaque that developed a high viral load and rapidly progressed to AIDS, in contrast to 2 other ΔGY-infected animals that controlled viral replication (17). Furthermore, recently we described the appearance of the S727P mutation associated with disease progression in 3 chronically ΔGY-infected rhesus macaques (6). Notably, the acquisition of a proline at this position does not create a recognizable trafficking signal. Indeed, preliminary findings have confirmed that neither an endocytosis nor a

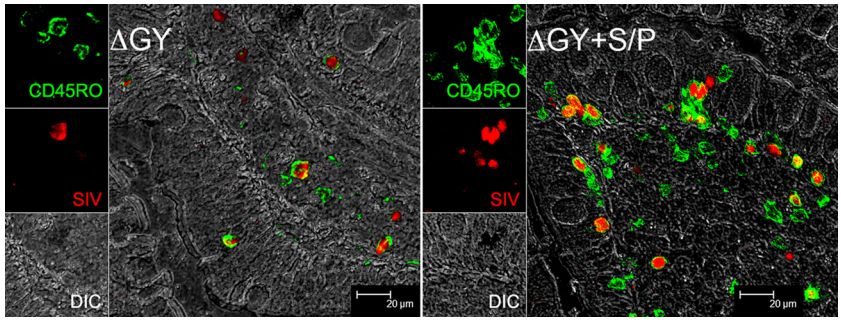

FIG 3 Analysis of SIV-infected cells in jejunal biopsy specimens from ΔGY- and ΔGY+S/P-infected animals at week 2 postinfection by double-label confocal microscopy for CD45RO/OPD4 (green) and SIV *in situ* hybridization (red). Differential interference contrast images (DIC) of the tissue are shown in gray. Merged images show that infected cells for both viruses are memory CD4 cells expressing CD45RO, although their frequency is greater for ΔGY+S/P infection. The individual channels are shown on the left.

basolateral sorting signal is conferred by this change (M. Marsh and Scott Lawrence, University College London, personal communication). These results suggest that S727P could be compensating for additional functions of the GYxxØ trafficking signal. Given that ΔGY infection *in vivo* is remarkable for its selectively attenuated replication in mucosal but not peripheral $CD4^+$ T cells (6) and the results of the present study that S727P partially restores this effect, taken together, these data indicate that there are strong selection pressures *in vivo* to maintain the ability to target and deplete mucosal $CD4^+$ T cells. Of interest, an S727P mutation was also reported in a pathological variant of the attenuated SIVmac239 derivative, ΔNef, that emerged *in vivo* (21). This change was not required for an interaction of Env with rhesus tetherin, a function that was gained through more distal mutations in the Env cytoplasmic tail (22). The role of the S727P mutation in pathogenicity of this virus is unknown.

While the ability of SIV (1, 23) and HIV-1 (4, 24) to selectively target and deplete gut $CD4^+$ T cells is well recognized, the mechanism that underlies this effect is unknown. For HIV-1, the binding of gp120 to the integrin alpha-4/beta-7, expressed on gut $CD4^+$ T cells, has been proposed as one explanation (25, 26), although this finding is controversial based on findings for transmitted/founder HIV-1 Env proteins (27). Our findings for SIVmac indicate that the GYxxØ trafficking signal (aa 720 to 723), which is highly conserved in HIV-1 (aa 711 to 714; HXB numbering) and all nonhuman primate lentiviruses, contributes to mucosal $CD4^+$ T-cell infection and depletion during acute infection. Further studies of the role of S727P in reestablishing this function, as well as quantitative studies of cell types infected and viral loads in multiple tissues when this trafficking signal is disrupted, could provide insights as to the underlying mechanism.

In conclusion, we found that a single amino acid mutation (S727P), which developed in ΔGY *in vivo* in two independent experiments (6, 17), largely restored the ability of this virus to infect and rapidly deplete mucosal $CD4^+$ T cells. In contrast to animals infected with ΔGY, animals infected with ΔGY+S/P had a higher and earlier acute peak of plasma viremia and exhibited a more rapid and sustained depletion of gut mucosal $CD4^+$ T cells than ΔGY. Furthermore, using *in situ* hybridization, we found greater numbers of ΔGY+S/P-infected cells in intestinal tissues and showed that these cells were more diffusely distributed in the lamina propria than ΔGY. Collectively, these findings (i.e., the attenuated infection and depletion of mucosal $CD4^+$ T cells in ΔGY-infected animals being compensated for by S727P) strongly implicate the importance of a domain in the proximal Env cytoplasmic tail that modulates SIV mucosal tropism and pathogenesis. Further *in vivo* and *in vitro* studies of ΔGY and related mutants will help to identify the basis for this effect.

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