Elite Control, Gut CD4 T Cell Sparing, and Enhanced Mucosal T Cell Responses in *Macaca nemestrina* Infected by a Simian Immunodeficiency Virus Lacking a gp41 Trafficking Motif


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ABSTRACT

Deletion of Gly-720 and Tyr-721 from a highly conserved GYxxØ trafficking signal in the SIVmac239 envelope glycoprotein cytoplasmic domain, producing a virus termed ΔGY, leads to a striking perturbation in pathogenesis in rhesus macaques (*Macaca mulatta*). Infected macaques develop immune activation and progress to AIDS, but with only limited and transient infection of intestinal CD4+ T cells and an absence of microbial translocation. Here we evaluated ΔGY in pig-tailed macaques (*Macaca nemestrina*), a species in which SIVmac239 infection typically leads to increased immune activation and more rapid progression to AIDS than in rhesus macaques. In pig-tailed macaques, ΔGY also replicated acutely to high peak plasma RNA levels identical to those for SIVmac239 and caused only transient infection of CD4+ T cells in the gut lamina propria and no microbial translocation. However, in marked contrast to rhesus macaques, 19 of 21 pig-tailed macaques controlled ΔGY replication with plasma viral loads of <15 to 50 RNA copies/ml. CD4+ T cells were preserved in blood and gut for up to 100 weeks with no immune activation or disease progression. Robust antiviral CD4+ T cell responses were seen, particularly in the gut. Anti-CD8 antibody depletion demonstrated CD8+ cellular control of viral replication. Two pig-tailed macaques progressed to disease with persisting viremia and possible compensatory mutations in the cytoplasmic tail. These studies demonstrate a marked perturbation in pathogenesis caused by ΔGY’s ablation of the GYxxØ trafficking motif and reveal, paradoxically, that viral control is enhanced in a macaque species typically predisposed to more pathogenic manifestations of simian immunodeficiency virus (SIV) infection.

IMPORTANCE

The pathogenesis of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) reflects a balance between viral replication, host innate and adaptive antiviral immune responses, and sustained immune activation that in humans and Asian macaques is associated with persistent viremia, immune escape, and AIDS. Among nonhuman primates, pig-tailed macaques following SIV infection are predisposed to more rapid disease progression than are rhesus macaques. Here, we show that disruption of a conserved tyrosine-based cellular trafficking motif in the viral transmembrane envelope glycoprotein cytoplasmic tail leads in pig-tailed macaques to a unique phenotype in which high levels of acute viral replication are followed by elite control, robust cellular responses in mucosal tissues, and no disease. Paradoxically, control of this virus in rhesus macaques is only partial, and progression to AIDS occurs. This novel model should provide a powerful tool to help identify host-specific determinants for viral control with potential relevance for vaccine development.

Hallmarks of human immunodeficiency virus type 1 (HIV-1) infection and pathogenic models of simian immunodeficiency virus (SIV) infection in Asian macaques include early targeting and rapid depletion of CD4+ effector memory T cells that express the chemokine receptor CCR5, particularly in gut lymphoid tissue where they are abundant (1). A subsequent loss of epithelial barrier function and translocation of microbial products to the systemic circulation contributes to generalized immune activation, which likely generates new CD4+ target T cells that serve to sustain ongoing viral replication (2). With associated contributions from macrophage and dendritic cell dysfunction (3–6), a state of persistent immune activation and inflammation is established. These processes, coupled with viral escape from adaptive immune responses, lead to persistent and progressive infection and depletion of central memory CD4+ T cells and an eventual loss of CD4+ T cell regenerative capacity (2) that underlies progression to AIDS.

Among nonhuman primate models, marked differences in the outcome of SIV infection have been observed, reflecting a complex interplay of viral and host factors (1, 7, 8). SIV infection of several African monkey species that are natural hosts of different SIV strains results in only a transient depletion of mucosal CD4+ T cells, with limited immune activation and little or no disease despite ongoing viral replication (9–11). However, even among Asian macaque species that succumb to pathogenic SIV infection, differences in the natural history of infection have been observed.
In Chinese rhesus macaques, SIVmac infection leads to disease in approximately two-thirds of animals, with the other one-third controlling plasma viral RNA to low or undetectable levels (12–14), while in Indian rhesus macaques, viral replication levels are on average significantly higher and progression to disease is more commonly seen and occurs more rapidly, although the rate of progression can be modulated in the setting of protective major histocompatibility complex (MHC) class I alleles (15, 16). Pig-tailed macaques (*Macaca nemestrina*) have been shown to be particularly sensitive to pathogenic effects of SIVmac and SIVagm infection and typically exhibit a more uniform and rapid progression to disease and death than do rhesus macaques (8, 17–19). The enhanced pathogenicity of SIV infection seen in this species has been attributed to an increased basal level of immune activation caused by a “leaky” gut epithelial barrier and elevated baseline microbial translocation in animals prior to infection (20, 21). This increased basal immune activation likely enhances viral replication and accelerates disease progression (17, 20). Transcriptional profiling of lymphoid tissues during acute SIVagm infection of pig-tailed macaques, which leads rapidly to AIDS, has revealed a markedly robust and sustained expression of mRNAs associated with cellular stress pathways, type I and II interferon (IFN) responses, and T and B cell activation (18). Pig-tailed macaques also exhibit a mutated Trim5-alpha gene due to retrotranspositioning of the cyclophilin A gene between exons 7 and 8, which ablates the function and accelerates disease progression (17, 20). These findings indicate that while epithelial damage and systemic translocation of microbial products have been associated with chronic immune activation and disease progression, these processes are not absolutely required for immunopathogenesis and additional factors can contribute. The work also demonstrated a remarkable alteration of cellular and tissue tropism of infection caused by the ΔGY mutation within the GYxO trafficking motif, leading to sparing of mucosal CD4+ T cells and no detectable macrophage infection (24).

In the current study, we evaluated the effects of ΔGY infection in pig-tailed macaques, in which SIV infection is typically more pathogenic than in rhesus macaques. We found that infection in pig-tailed macaques was similar to infection in rhesus macaques: this virus established a high acute peak of viremia, largely spared CD4+ T cells in intestinal lamina propria, and failed to cause detectable infection of tissue macrophages. However, ΔGY viremia in pig-tailed macaques contrasted markedly with that in rhesus macaques. In pig-tailed macaques, ΔGY viremia was rapidly suppressed in the majority of animals to levels of <15 to 50 copies/ml, with preservation of CD4+ T cells in blood and gut for >100 weeks. Anti-CD8 cell depletion studies suggested that host control of ΔGY was, at least in part, mediated by CD8+ cells. However, this control was also strongly associated with the appearance of robust, SIV-specific CD4+ T cell responses, particularly in intestinal lamina propria, which was spared during acute ΔGY infection. These findings extend the novel in vivo effects of the ΔGY mutation in the SIV Env cytoplasmic domain and reveal a paradoxical species-specific difference in rhesus compared to pig-tailed macaques, with superior control occurring in pig-tailed macaques, a species that typically exhibits more rapid disease progression following wild-type SIV infection.

**MATERIALS AND METHODS**

**Ethics statement.** The Tulane and University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committees approved all experiments using rhesus and pig-tailed macaques (protocols P0088R and P0147 at Tulane and 041205386 at UAB). The Tulane National Primate Research Center (TNPRC) and UAB facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and closely follow the recommendations made in the Guide for the Care and Use of Laboratory Animals (29). The NIH Office of Laboratory Animal Welfare assurance number for TNPRC is A4499-01, and that for UAB is A3255-01. All clinical procedures, including administration of anesthesia and analgesics, were carried out under the direction of a laboratory animal veterinarian. Animals were anesthetized with 10 mg/kg ketamine hydrochloride for blood collection procedures. Laboratory animal veterinarians performed intestinal resections and lymph node biopsies. Animals were preanesthetized with acepromazine and glycopyrrolate, anesthesia was induced with either 10 mg/kg ketamine hydrochloride or 8 mg/kg tiletamine-zolazepam, and animals were then intubated and main-
TABLE 1 Summary of animals, virus stock, outcome, and experimental group for ΔGY- and control SIVmac239-infected pig-tailed macaques

<table>
<thead>
<tr>
<th>Group</th>
<th>Animala</th>
<th>Sexb</th>
<th>Status</th>
<th>Euthanasia (wk)</th>
<th>Mane-A</th>
<th>Mane-B</th>
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<td>0.028, B123</td>
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<tr>
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<td>0.016b, B044</td>
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<tr>
<td></td>
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<td>HJ98</td>
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<td>0.043, B104</td>
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<td></td>
<td>CT2B</td>
<td>M</td>
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<td>0.056, B104</td>
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<td>0.017b, B052</td>
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<tr>
<td></td>
<td>CT2J</td>
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<td>Controller</td>
<td>A006, A082</td>
<td>0.016a, B052</td>
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</tbody>
</table>

| SIVmac239    | GR06    | M    | AIDS     | 51              | 0.052, A061 |
|              | GR08    | F    | AIDS     | 51              | 0.007, A061 |
|              | IK37    | M    | AIDS     | 25              | 0.019, A061 |
|              | IP82    | F    | AIDS     | 27              | 0.010, A084 |
|              | AD7C    | M    | AIDS     | 76              | 0.082, A082 |
|              | AK93    | M    | AIDS     | 77              | 0.082, A084 |
|              | 99P040  | M    | AIDS     | 112             | Not determined |
|              | 99P041  | M    | AIDS     | 93              | Not determined |
|              | 99P051  | M    | AIDS     | 89              | Not determined |

<table>
<thead>
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<th>Haplotypesa</th>
<th>Mane-A</th>
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<td>A084</td>
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<td>B017</td>
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<td>AT4D</td>
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<tr>
<td>CT2J</td>
<td>0.016a, B052</td>
<td></td>
</tr>
</tbody>
</table>

a Bold text indicates pig-tailed macaques that progressed to disease.
b M, male; F, female.
c The A084 and B017 MHC haplotypes have been associated with exceptional control of chronic-phase SIV replication in a subset of macaques (41–43).

tained on a mixture of isoflurane and oxygen. Buprenorphine was given intraoperatively and postoperatively for analgesia. All possible measures are taken to minimize discomfort of all the animals used in this study. Animals were closely monitored daily following surgery for any signs of illness, and appropriate medical care was provided as needed. Euthanasia was performed in accordance with the recommendations of the panel on Euthanasia of the American Veterinary Medical Association. Tulane University and UAB comply with NIH policy on animal welfare, the Animal Welfare Act, and all other applicable federal, state, and local laws.

Animals, viral inoculations, and sample collection. A total of 30 pig-tailed macaques were used in this study and were inoculated intravenously (i.v.) with 100 TCID50 of tail macaques were used in this study and were inoculated intravenously (i.v.) with 100 TCID50 of tail macaques were used in this study and were inoculated intravenously (i.v.) with 100 TCID50 of tail macaques were used in this study and were inoculated intravenously (i.v.). Prior to use, all animals tested negative for antibodies to SIV, simian T cell leukemia virus (STLV), and type D retrovirus and by PCR for type D retrovirus. Multiple blood samples and small intestinal biopsy samples (endoscopic duodenal pinch biopsy samples or jejunal resection biopsy samples) were collected under anesthesia (ketamine hydrochloride or isoflurane) at various times from each animal (see the figures and figure legends). Animals were euthanized if they exhibited a loss of more than 25% of maximum body weight, anorexia for more than 4 days, or major organ failure or medical conditions unresponsive to treatment (e.g., severe pneumonia or diarrhea).

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Lymphocyte isolation from intestinal tissues. Intestinal cells were collected either by endoscopic pin biopsy of the small intestine or by 2-cm surgical resections of the jejunum from animals at various times. Intestinal biopsy procedures and isolation of cells from intestinal tissues were described previously (12, 31–33). Intestinal cells were isolated using EDTA-collagenase digestion and Percoll density gradient centrifugation.

Immunophenotyping and analysis of cell turnover with BrdU. Immunophenotyping of cells was performed on isolated lamina propria lymphocytes (LPLs) and antigen-coated whole blood using antibodies reactive with CD3 (SP34), CD8 (SK1 or SK2), CD4 (L200), CD14 (M5E2), CD45 (MB4-6D6), CD20 (B9E9), 5-bromo-2′-deoxyuridine (BrdU) (3D4), and HLA-DR (L234), all from BD Biosciences, San Jose, CA. Additional antibodies included anti-CD8 (3B5) (Invitrogen, Carlsbad, CA), -CD20 (Beckman Coulter, Indianapolis, IN), and -CD45 (Miltenyi Biotech, San Diego, CA). Viability was determined using LIVE/DEAD stain (L34957) (Life Technologies, Grand Island, NY). Protocols for performing immunophenotyping have been described previously (24) CD4+ and CD8+ lymphocytes and monocytes were further analyzed for turnover and immune activation. For turnover experiments, BrdU (Sigma) was administered to animals, as described previously (34, 35). Pig-tailed macaques, infected with AGY (n = 8) or SIVmac239 (n = 4), had BrdU administered before infection and at multiple times postinfection; at least one month was allowed between BrdU inoculations. Control groups for the BrdU labeling experiments included naive, AGY-infected, or SIVmac-infected rhesus macaques (24, 34). Flow cytometry data were analyzed using FlowJo software version 9.1 (TreeStar Inc., Ashland, OR).

Intracellular cytokine analysis. Intracellular cytokine analysis of SIV-specific CD4 and CD8 peripheral blood and lamina propria lymphocytes was performed in a subset of the 19 AGY-controlling pig-tailed macaques, at week 66 for animals GC07, GC72, GG10, GR29, HC67, and HJ98 and before and after weeks 8 after CD8+ depletion for HC67 and HJ98, as described previously (36, 37). An additional four pig-tailed macaques infected with SIVmac239 (GR06, GR08, IK37, and IP82) were similarly assessed for SIV-specific CD4 and CD8 responses in peripheral blood. Briefly, cells were stimulated with SIV peptides (Env, Gag, or Pol; NIH AIDS Research & Reference Reagent Program), and then brefeldin A (Sigma) was added. Cytokine production was determined immunocytochemically using a protocol wherein intracellular cytokine expression was assessed using polyclonal antibodies. Intracellular cytokine expression at the single-cell level was determined using flow cytometry.

The gating strategy was similar to the immunophenotyping described above. In brief, cells were gated first on singlets, lymphocytes, followed by live cells, and then on CD3+ T cells and subsequently on CD3+ CD4+ and CD3+ CD8+ T cell subsets. The percentages of IFN-γ, TNF-α, and IL-2-positive responses in each subset against each antigen as well as negative or positive controls were assessed using FlowJo software, version 9.1 (TreeStar). The analysis of absolute cytokine expression was performed with PESTLE and SPICE software version 5.3 (J. Nozzi and M. Roederer, VRC, NIAID, Bioinformatics and Computational Biosciences Branch). The analysis of absolute cytokine expression was performed with PESTLE and SPICE software version 5.3 (J. Nozzi and M. Roederer, VRC, NIAID, Bioinformatics and Computational Biosciences Branch). The analysis of absolute cytokine expression was performed with PESTLE and SPICE software version 5.3 (J. Nozzi and M. Roederer, VRC, NIAID, Bioinformatics and Computational Biosciences Branch). The analysis of absolute cytokine expression was performed with PESTLE and SPICE software version 5.3 (J. Nozzi and M. Roederer, VRC, NIAID, Bioinformatics and Computational Biosciences Branch). The analysis of absolute cytokine expression was performed with PESTLE and SPICE software version 5.3 (J. Nozzi and M. Roederer, VRC, NIAID, Bioinformatics and Computational Biosciences Branch). The analysis of absolute cytokine expression was performed with PESTLE and SPICE software version 5.3 (J. Nozzi and M. Roederer, VRC, NIAID, Bioinformatics and Computational Biosciences Branch).

Analysis of plasma LPS and sCD14 levels. To measure lipopolysaccharide (LPS), plasma samples (collected in EDTA) were diluted to 10% in endotoxin-free water, heated to 80°C for 10 min, and then quantified in duplicate with a commercially available Limulus amoebocyte lysate assay (Lonza, Basel, Switzerland) (38, 39). Plasma soluble CD14 (sCD14) was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Samples were diluted to 0.5% and tested in duplicate, as described previously (40).

MHC typing of pig-tailed macaques. MHC class I genotyping was carried out as described previously (41–43). Total cellular RNA was isolated from whole blood or PBMCs, and first-strand cDNA was synthesized by reverse transcription with SuperScript III (Life Technologies, USA). This cDNA served as a template for PCR with SBT568 primers that bind conserved sequences flanking the highly polymorphic peptide-binding domain and universally amplify MHC class I transcripts. After pyrosequencing of these amplicons on a GS Junior instrument (Roche/454, USA), a custom genotyping workflow was used to map the resulting sequences against a curated database of known Macaca nemestrina (Mance) class I sequences. Ancestral Mance-A and Mance-B haplotypes were inferred on the basis of the combinations of class I alleles that were identified for each animal as described previously (Table 1) (41–43).

Assays for neutralizing antibodies. Neutralizing antibody responses were determined on viruses produced in 293T cells using TZM-bl cells expressing CD4 and CCR5. All assays were conducted in triplicate as described previously (44). The 50% inhibitory dilution (ID50) was calculated as the reciprocal serum dilution causing 50% reduction of relative light units compared with the virus alone (without test sample). Viruses used in the neutralization panel included SIVmac239 and SIVmac251.6 (45) and variants of SIVsmE660 that are neutralization sensitive (SIVsmE660-VTRN) or neutralization resistant (SIVsmE660-2A5) (46). Neutralization-sensitive SIVsmE660-2A5.VTRN was generated by introducing 4 amino acid changes into neutralization-resistant SIVsmE660-2A5 that have been shown previously to confer this phenotype (46).

ISH. Tissue samples collected at biopsy or necropsy were fixed in Z-Fix (Anatech, Battle Creek, MI) and embedded in paraffin, and 5-μm-thick sections were cut from select tissues and adhered to charged glass slides. SIV RNA in situ hybridization (ISH) was then performed, as described previously (24, 28, 47, 48), using SIV RNA sense/antisense probes tagged with digoxigenin (DIG) and obtained from Lofstrand Laboratories (Gaithersburg, MD) or provided by Jake Estes (AIDS and Cancer Virus Program at the Frederick National Laboratory of Cancer Research) (49). The chromogens used were nitroblue tetrazolium (NBT)–5–bromo-4-chloro-3-indolyl phosphate (BCIP) for light microscopy or liquid permanent red for fluorescence microscopy. Results were assessed by bright-field optical microscopy (DM LB microscope [Leica, Buffalo Grove, IL] and spot imaging [camera and software from Insight, Sterling Heights, MO]) or confocal microscopy (Leica TCS SP2 confocal microscope equipped with three lasers [Leica Microsystems, Buffalo Grove, IL]).

Confocal microscopy. Double- and triple-label confocal microscopy was performed to colocalize SIV RNA with cell type-specific markers to determine the immunophenotype of infected cells, as described previously (47). Immunofluorescent labeling of T cells (rabbit polyclonal to CD3 [Biocare Medical, Concord, CA]), CD4 memory T cells (mouse IgG1 monoclonal to OPD4/CD45RO [Dako]), macrophages (mouse IgG1 monoclonal to CD68 [Dako] or CD163 [Serotec]), and BrdU (rat monoclonal BU1/75 [Novus Biologicals]) was performed after ISH, as previously described (24, 28, 34, 47, 50, 51). After incubation with the primary antibodies (anti-CD3, anti-OPD4/CD45RO [which only detects CD45RO on CD4+ T cells [34]], anti-CD68, anti-CD163, or anti-BrdU) and subsequent washes, the appropriate species-specific secondary antibodies were applied (Alexa Fluor 488- or 633-conjugated goat anti-rabbit or goat anti-mouse IgG1 or goat anti-rat [Invitrogen, Carlsbad, CA]). Confocal microscopy was performed using the sequential mode to capture separately the fluorescence from the different fluorochromes (Leica Microsystems, Exton, PA). NIH Image v1.62 and Adobe Photoshop v7 software were used to correct the colors collected in the different channels: Alexa 488 (green), Alexa 568 (red), Alexa 633 (blue), and differential
interference contrast (DIC) (gray scale). In selected animals and tissues, the numbers of infected cells by phenotype were quantitated as described previously (24).

**SGA analysis.** Single genome amplification (SGA) analyses were performed on plasma samples from two /H9004 GY-infected pig-tailed macaques that progressed to disease (GR26 and HA94): GR26 at weeks 2, 6, 22, and 58 after infection and from HA94 at week 76 at necropsy. For two animals that were controlling infection (HC67 and HJ98), SGA analyses were performed at the plasma viral RNA peak at 2 weeks after CD8/H11001 cell depletion. The entire env gene was sequenced using a limiting-dilution PCR to ensure that only one amplifiable molecule was present in each reaction mixture, as previously described (24, 52). Sequence alignments were generated with Clustal W and presented as highlighter plots (www.hiv.lanl.gov). Unrooted neighbor-joining trees were constructed in Clustal W, excluding gaps and correcting for multiple substitutions. APOBEC signature mutations were identified with HyperMut (www.hiv.lanl.gov), and diversity/divergence measurements were determined using pairwise comparisons implemented in DIVEIN (53).

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism v5.0c (GraphPad Software, Inc., La Jolla, CA). The non-parametric Mann-Whitney test was used to determine  P  values when comparing two groups that were not normally distributed.

**Nucleotide sequence accession numbers.** All sequences in this study were deposited in GenBank (accession numbers KP313060 to KP313239).

**RESULTS**

**Viral dynamics of /G infection in pig-tailed macaques.** Pig-tailed macaques were inoculated intravenously (i.v.) with SIVmac239 or /G, and analyses were performed on blood, small intestinal biopsy specimens (excisional or endoscopic), and tissues collected at necropsy. Plasma viral loads for /G-infected macaques were divided into controllers (black) or progressors (blue); SIVmac239-infected animals are shown (red). The threshold of sensitivity for the viral RNA assay varied from 15 to 50 RNA copies/ml and is represented by the shaded area. RNA values for individual animals are shown in Fig. S1 in the supplemental material. P[r] values were determined using the Mann-Whitney nonparametric test. NS, not significant.

**FIG 1** Plasma viral loads in pig-tailed and rhesus macaques infected with /G or SIVmac239. Plasma viral loads are shown for pigtailed macaques (A and B) (diamonds) and previously reported rhesus macaques (C and D) (triangles), (24). †, time points at which individual animals were euthanized with AIDS. (A and C) Plasma RNA levels postinfection. One /G-infected pig-tailed macaque (JV15) died of causes unrelated to SIV infection at week 16 with a plasma viral RNA level of 200 copies/ml and is not included in the viral set point analysis (see Fig. S1A in the supplemental material). (B and D) Peak viremia and viral set points for individual animals (mean ± SEM). /G-infected macaques were divided into controllers (black) or progressors (blue); SIVmac239-infected animals are shown (red). The threshold of sensitivity for the viral RNA assay varied from 15 to 50 RNA copies/ml and is represented by the shaded area. RNA values for individual animals are shown in Fig. S1 in the supplemental material. P[r] values were determined using the Mann-Whitney nonparametric test. NS, not significant.
HA94) (Table 1) exhibited higher set-point viremia (4.1 viremia with intermittent RNA increases of 10^2 to 10^3 copies/ml, assay), with the other 4 animals showing persisting but low level (Fig. 1A; see Fig. S1B in the supplemental material). At necropsy, AIDS-associated disease by approximately 70 weeks postinfection pig-tailed previously described tent with cytomegalovirus infection. esophageal candidiasis, and intranuclear inclusion bodies consisted following: meningoencephalitis with multinucleated giant cells, inhibited findings typical of simian AIDS, with one or more of the propria (see below). In contrast, SIVmac239-infected animals exhibited numbers and percentages of CD4^+ T cells in blood and gut lamina propria (see below). In contrast, SIVmac239-infected animals exhibited findings typical of simian AIDS, with one or more of the following: meningoencephalitis with multinucleated giant cells, severe anemia and thrombocytopenia, giant cell pneumonia, esophageal candidiasis, and intranuclear inclusion bodies consistent with cytomegalovirus infection.

Two ΔGY-infected pig-tailed macaques that controlled infection (JV13 and JV15) were euthanized due to complications unrelated to SIV infection. JV13 had recurrent rectal prolapse and was euthanized at week 38 with a viral load of <15 copies/ml; JV15 had a testicular torsion at week 15 and was euthanized with a viral load of 200 copies/ml that was rapidly declining (see Fig. S1 in the supplemental material).

Deep-sequencing analysis showed that animals in both the ΔGY and SIVmac239 challenge groups carried a diverse array of MHC haplotypes (Table 1), indicating that differences in control of chronic-phase viral replication were unlikely to be associated with MHC class I genetics, including the Mane-A allele (formerly Mane-A^*10), which has been implicated as an SIVmac-controlling allele in pig-tailed macaques in early (54) although not more recent (41, 55) studies. Viral set points for ΔGY-controlling pig-tailed macaques were also reduced compared to those in 4 previously described ΔGY-infected rhesus macaques (P < 0.01), which had RNA levels of 3.8 × 10^4 ± 1.4 × 10^4 copies/ml and progressed to disease (Fig. 1C and D) (24).

Two of 21 ΔGY-infected pig-tailed macaques (GR26 and HA94) (Table 1) exhibited higher set-point viremia (4.1 × 10^5 and 1.3 × 10^5 RNA copies/ml, respectively) and progressed to simian AIDS-associated disease by approximately 70 weeks postinfection (Fig. 1A; see Fig. S1B in the supplemental material). At necropsy, both had evidence of SIV infection, including generalized lymphoid hyperplasia and thrombocytopenia, pulmonary arteriopathy, and thrombosis (56–58), despite only slight reductions in numbers and percentages of CD4^+ T cells in blood and gut lamina propria (see below). In contrast, SIVmac239-infected animals exhibited findings typical of simian AIDS, with one or more of the following: meningoencephalitis with multinucleated giant cells, severe anemia and thrombocytopenia, giant cell pneumonia, esophageal candidiasis, and intranuclear inclusion bodies consistent with cytomegalovirus infection.

Characterization of ΔGY-infected cells in gastrointestinal lymphoid tissue during acute infection. Small intestinal biopsy specimens were obtained during acute infection (up to day 28
postinfection), and in situ hybridization (ISH) for SIV RNA and immunohistochemistry were performed to evaluate the distribution and immunophenotype of ΔGY-infected cells. At day 7, infected cells were not identified despite detectable, albeit moderate, levels of RNA in plasma (Fig. 3A) (110, 1.6 × 10^5, and 3 × 10^4 copies/ml for animals GR29, GC07, and GG10, respectively [Fig. 1A; see Fig. S1 in the supplemental material]). By day 15, infected cells were present but were sparsely distributed in a patchy, multifocal pattern throughout immune inductive sites (organized lymphoid nodules and Peyer’s patches) and effector sites (lamina propria and epithelium) (Fig. 3B). By day 21 (not shown) and day 28 (Fig. 3C), infected cells were detectable only at low levels in immune inductive sites (Peyer’s patches) but were absent throughout the diffuse lamina propria. This finding was remarkable but agrees with the observation that CD4+ T cells in gut lamina propria were only mildly depleted from preinfection levels (Fig. 2C). These results were in contrast to those for small intestinal biopsy specimens from SIVmac239-infected pig-tailed macaques, where extensive and diffuse infection throughout the lamina propria occurs during acute infection (references 17 and 20 and data not shown), corresponding to the massive and rapid depletion of CD4+ T cells from this site (Fig. 2C).

To identify the immunophenotype of ΔGY-infected cells in gastrointestinal tissues, we performed multilabel confocal microscopy on jejunum at day 15 using antibodies to CD3 (T cells), CD68 and CD163 (macrophages), CD45RO (memory CD4+ T cells) (50), and BrdU (proliferating cells) and digoxigenin-labeled probes to detect SIV RNA (Fig. 3D and E). As expected, T cells, including memory (CD45RO+) and proliferating (BrdU+) cells (Fig. 3E), were infected. In addition, although macrophages that had phagocytosed SIV-infected T cells were observed (Fig. 3D), SIV-infected macrophages were rare in ΔGY-infected pig-tailed macaques. By day 163, for SIVmac239, 14.9% of CD68+ cells were positive for SIV RNA, whereas for ΔGY, only 0.42% were positive (Fig. 3F and data not shown).

Thus, while ΔGY infection of intestinal lamina propria CD4+ T cells occurred during acute infection, similar to ΔGY infection in rhesus macaques and in marked contrast to SIVmac239 infection in both macaque species, infection was transient and sparsely distributed in this compartment, with no detectable infection beyond day 28 and no macrophage infection (24, 28).

Progressive infection for ΔGY is altered compared to that for SIVmac239. As noted above, although the majority of ΔGY-infected pig-tailed macaques controlled ΔGY infection and retained normal numbers of gut and peripheral CD4+ T cells, two animals (GR26 and HA94) progressed to disease. Tissues at necropsy were analyzed by ISH for SIV RNA and compared to necropsy samples from 4 SIVmac239-infected pig-tailed macaques that progressed to AIDS (Fig. 4). All SIVmac239-infected animals exhibited high levels of plasma RNA at necropsy (2.1 × 10^8 ± 1.8 × 10^8 copies/ml [mean ± SEM]), and viral RNA in tissues was abundant throughout the small and large intestines, lymph nodes, spleen, and brain (selected images are shown in Fig. 4). In contrast, both ΔGY-infected animals that progressed to disease (referred to as ΔGY-progressing animals below) exhibited more moderate levels of plasma RNA at necropsy (3.6 × 10^5 and 6.4 × 10^5 copies/ml for GR26 and HA94, respectively) (see Fig. S1B in the supplemental material), and viral infection was difficult to detect, with only rare infected cells detected in mesenteric lymph nodes and none detected in intestine or brain (Fig. 4).

Evaluation of immune activation and microbial translocation in ΔGY-controlling pig-tailed macaques. Increased monocyte turnover (34, 35), chronic immune activation (5, 38, 63), and microbial translocation (5, 38) are correlates of disease progression in virulent HIV and SIV infection. Given the transient nature of ΔGY’s infection in lamina propria and early sparing of CD4+ T cells from this site, parameters of cellular turnover, immune activation, and microbial translocation were evaluated in ΔGY-controlling, ΔGY-progressing, and SIVmac239-infected pig-tailed macaques.

BrdU incorporation in peripheral blood CD4+ and CD8+ T cells and in CD14+ monocytes was assessed as an indicator of cell turnover (34, 51). SIVmac239-infected pig-tailed macaques exhibited an early and sustained elevation in BrdU+ cells, particularly CD8+ T cells and monocytes, with a more variable incorporation in CD4+ T cells. In contrast, ΔGY-progressing macaques exhibited little to no increase in BrdU incorporation in any of these cell types (Fig. 5A to C). Both ΔGY-progressing macaques displayed late increases in CD8+ and CD4+ T cell turnover, although the small numbers precluded statistical comparisons (Fig. 5A and B). With the exception of a single point at week 34 for animal GR26, no increase in monocyte turnover was seen for ΔGY-progressing pig-tailed macaques (Fig. 5C).

HLA-DR expression on CD8+ and CD4+ T cells was also followed as a marker of T cell activation (Fig. 5D and E) and was increased on CD8+ T cells of all SIVmac239-infected pig-tailed macaques, while minimal changes occurred in ΔGY-controlling animals. Consistent with increased CD8+ T cell turnover in both ΔGY-progressing macaques, a trend toward increased HLA-DR expression was observed at late time points prior to necropsy (Fig. 5D). A similar, although less striking, increase was observed for CD4+ T cells (Fig. 5E).

BrdU incorporation in T cells and monocytes in ΔGY- and SIVmac239-infected pig-tailed macaques was compared to results previously reported for these viruses in rhesus macaques (24) (Fig. 5F, G, and H). Although viral set points for ΔGY were reduced in rhesus macaques compared to those for SIVmac239, replication continued, and all chronically infected animals ultimately progressed (n = 4) (24). Despite the small number of animals, CD8+ and CD4+ T cell turnover was increased in ΔGY-infected rhesus macaques compared to ΔGY-infected pig-tailed macaques (Fig. 5F and G). However, in both macaque species there was little if any evidence of elevated monocyte turnover during ΔGY infection, in contrast to that in SIVmac239 infection (Fig. 5H).

Microbial translocation was evaluated in ΔGY-controlling (n = 14) and SIVmac239-infected (n = 4) animals by measuring the levels of soluble CD14 (sCD14) and lipopolysaccharide (LPS) in plasma. As is typical of pathogenic SIV infection (38), sCD14 progressively increased during SIVmac239 infection (Fig. 6A and B), while no change from preinfection levels occurred for ΔGY-infected animals (Fig. 6). Although not statistically significant, similar trends were found for plasma LPS, where a modest increase from preinfection levels was seen in SIVmac239-infected animals while ΔGY-infected animals appeared to remain unchanged (Fig. 6C and D).

ΔGY controllers exhibit robust SIV-specific CD4+ and CD8+ T cell responses in peripheral blood and intestinal mucosa. To assess correlates of ΔGY control, we examined SIV-specific CD4+ and CD8+ T cell cytokine responses at week 66 after
FIG 3  Localization and immunophenotype of ΔGY-infected cells in the small intestine during acute infection in pig-tailed macaques. (A to C) Localization of SIV RNA in jejunum from ΔGY-infected pig-tailed macaques by in situ hybridization (ISH). Representative ISH images from biopsy specimens collected from three animals at day 7, two animals at day 15, and two animals at day 28 are shown. Black arrows indicate infected cells. Infected cells were undetectable at day 7 (A), sparsely distributed in epithelium, lamina propria, and organized lymphoid nodules at day 15 (B), and present only in organized lymphoid nodules at day 28 (C). (D to F) Immunophenotyping by multilabel confocal microscopy of SIV-infected cells in jejunum from a ΔGY-infected animal at day 15 (D and E) and from an SIVmac239-infected animal at day 28 (F). For each panel, the individual channels are shown on the left and the larger merged image is shown on the right. (D) All ΔGY-infected cells were CD3⁺, indicated by a yellow color in the merged image (white arrow and arrowhead). No SIV-infected macrophages were present (blue); however, a white arrowhead shows a CD68⁺ macrophage phagocytosing an infected CD3⁺ cell. (E) Memory T cells (CD45RO⁺) (white arrow) and proliferating cells (BrdU⁺) (white arrowheads) are infected with ΔGY. (F) SIVmac239-infected macrophages (CD68 and CD163) are clearly detectable (white arrowhead).
GY infection in blood and gut from 6 pig-tailed macaques that had been controlling GY to levels of 15 to 30 copies/ml for approximately 1 year. Results for intracellular cytokine expression of IFN-γ, IL-2, and TNF-α for peripheral blood or gut CD4+ and CD8+ T cells following stimulation with pools of SIV Gag, Pol, or Env peptides (36,37) are shown (Fig. 7).

Although there was variability among specific cytokine responses and in the viral components targeted, in peripheral blood, CD4+ T cells were reactive, with responses ranging from 0 to 2.3% to Gag, Pol, and/or Env (Fig. 7). However, in all animals there were remarkably strong and broad responses in gut CD4+ T cells, especially toward Gag and Pol (Fig. 7). GC72 exhibited the strongest responses, with up to 19% of cells producing IFN-γ to Pol peptides. GG10 also had strong gut CD4+ T cell responses, with approximately 1 to 2% of cells producing IFN-γ to Env and Pol peptides. CD8+ T cells in blood and gut were generally lower than CD4+ T cell responses, although animal GC72 displayed strong responses to Gag peptides in peripheral blood (2.7%, IFN-γ) and gut (15%, IL-2) and strong responses to Pol peptides in gut (1.5 to 6%, IFN-γ and TNF-α) (Fig. 7). Thus, despite having undetectable viral loads for >70 weeks, GY-infected controlling animals exhibited robust SIV-specific T cell responses that were greater for CD4+ than for CD8+ T cells and greater in cells from gut mucosal tissue than in those from peripheral blood. In contrast, animals infected with SIVmac239 had consistently lower CD4 and CD8 T cell responses (<0.4%) to Gag, Pol, and Env in peripheral blood (not shown) despite higher viral loads (see Fig. S1C in the supplemental material). The rapid and profound depletion of mucosal CD4+ T cells (Fig. 2) precluded an assessment of mucosal responses in these animals.

Role of CD8 cellular responses in GY control. In 4 pig-tailed macaques that had maintained undetectable levels of plasma virus (<15 to 50 RNA copies/ml), CD8+ cells were depleted using an anti-CD8 monoclonal antibody. Two animals (AT36 and AT4D) were depleted at week 97 following infection, and two animals (HC67 and HJ98) were depleted at week 82. Plasma viral RNA increased in all 4 animals to peaks of 2.5 × 10^4, 5.4 × 10^3, 1.2 × 10^5, and 1.7 × 10^5 copies/ml within 2 weeks of antibody treatment, corresponding to the decline in CD8+ T cells. With recovery of these cells, viral control was reestablished in all 4 animals (Fig. 8A, B, and C). In two animals, HC67 and HJ98, CD8+ T cell cytokine responses to Gag, Pol, and Env peptides after CD8+ T cell recovery increased markedly compared to predepletion levels (Fig. 8D versus E), consistent with a strong anamnestic response. Thus, although GY was controlled for 1.6 to 1.8 years to levels

FIG 4 Localization of SIVmac239- and GY-infected cells at necropsy in pig-tailed macaques with disease progression. SIV RNA in situ hybridization was performed at necropsy on jejunum (A and D), mesenteric lymph nodes (B and E), and brain tissue (C and F) from pig-tailed macaques IK37 infected with SIVmac239 with AIDS (top row) and GR26 infected with GY (bottom row) with disease progression. Representative ISH images from necropsy samples collected from the four animals infected with SIVmac239 (IK37, IP82, GR06, and GR08) and the two animals that progressed to disease with GY (GR26 and HA94) are shown. In SIVmac239 infection, numerous infected cells are diffusely scattered throughout the jejunum, lymph node, and brain (week 25). In the GY-progressing animals, infected cells were not detectable in jejunal or brain and were only rarely present in mesenteric lymph nodes (arrows; week 74).
that were at or below the limits of detection, this virus was clearly present and remained highly replication competent and susceptible to CD8\(^+\) cell control.

**Neutralizing antibody responses to ΔGY.** SIVmac239, from which ΔGY was derived, is highly resistant to neutralization and typically does not elicit high titers of neutralizing antibodies during infection (64). To determine if humoral immune responses were contributing to ΔGY control, neutralizing antibody titers to ΔGY were determined, as was the neutralization sensitivity of ΔGY relative to that of parental SIVmac239. Sera from 6 ΔGY-controlling pig-tailed macaques and the two ΔGY-infected animals that progressed to disease (GR26 and HA94) were evaluated prior to infection and at 28 and 46 weeks after infection. ID\(_{50}\) values were determined for ΔGY as well as a panel of neutralization-sensitive (SIVmac251.6 and SIVsmE660.2A5,VTRN) and neutralization-resistant (SIVsmE660.2A5 and SIVmac239) SIVs. As shown in Table 2, all animals generated high ID\(_{50}\) titers to neutralization-sensitive SIVs, while no or extremely weak neutralization of SIVsmE660.2A5 or SIVmac239 was observed. Importantly, ΔGY-controlling macaques showed poor neutralization of ΔGY up to week 46, even though these animals controlled ΔGY by week 28. No differences were found in neutralization sensitivity of ΔGY-infected pig-tailed macaques and previously reported rhesus macaques (24). Values for SIVmac239-infected animals were determined at necropsy; values for ΔGY-infected animals were determined at week 22. Statistically significant differences are indicated. P values were determined using the Mann-Whitney nonparametric test.

**Microbial translocation of SIV-infected macaques.** Plasma soluble-CD14 (sCD14) and lipopolysaccharide (LPS) in pig-tailed macaques, as indicators of microbial translocation, are plotted over time (mean ± SEM) for ΔGY-infected controllers (black diamonds) and SIVmac239-infected animals (red diamonds). Values are expressed as a percent change from preinfection levels and show trends for increases in SIVmac239 infection, although they are not statistically significant.
between ΔGY and SIVmac239 when assayed using sera from SIVmac-infected rhesus macaques (not shown). Thus, ΔGY was not more neutralization sensitive than SIVmac239 and did not elicit autologous neutralizing titers during infection, indicating that neutralizing humoral immune responses were unlikely to have contributed to ΔGY control.

**Viral evolution in ΔGY-infected progressing and controlling animals.** To assess evolution of ΔGY in the two progressing pig-tailed macaques, both of which remained persistently viremic (see Fig. S1B in the supplemental material), single-genome amplification (SGA) and direct sequencing were performed on Env peptides (Gag, Pol, and Env) in CD4+ and CD8+ T cells in peripheral blood (PBMCs) and small intestinal lamina propria in six ΔGY-controlling and -progressing animals at week 66 after infection. The percentage of gated cells positive for a particular peptide after stimulation with the relevant peptide pool are those that were >0.05% after subtracting the unstimulated control responses for each peptide pool. Animal identifiers are indicated at the top right.

ΔGY mutation was maintained, and the R722G and R751G mutations were identified in all amplicons. Although mutations in this animal at positions 715 (A715T for HA94 and A715D for GR26) and 818 (T818I) were shared with GR26, mutations at positions 745 (T745I), 854 (E854G), and 879 (L879S) were unique, but none created a Tyr-based sorting motif (i.e., YxxØ).

As described above, in 4 pig-tailed macaques that controlled ΔGY to undetectable levels, CD8+ cell depletion led to a rapid reappearance of plasma viremia. SGA was performed on rebounding viruses in plasma from animals HC67 and HJ98 (CD8 depleted at week 82) to determine the extent of viral evolution during the prolonged period of elite host control (see Fig. S2A in the supplemental material). In contrast to the ΔGY-infected pig-tailed macaques with progressive infection, changes in the Env cytoplasmic tail in animal HC67 were minimal (Fig. 9B; see Fig. S2D in the supplemental material). The ΔGY mutation was retained in all 30 amplicons, and only a G860R mutation appeared in a minority (i.e., 4 of 30 amplicons). The R751G optimizing mutation was not found, consistent with rapid and durable control of ΔGY in this animal. Interestingly, following CD8 depletion in HJ98, the ΔGY mutation was maintained, and R722G and R751G were found in all amplicons, as was V837A (also seen in GR26) and a new mutation, S792L (Fig. 9B; see Fig. S2E in the supplemental material). Relative to that in HC67, viral control in HJ98 occurred more slowly in that the time to <100 RNA copies/ml was 20 weeks, compared to 8 weeks for HC67 (see Fig. S2A in the supplemental material). When SGA was performed on plasma RNA from HJ98 during its acute peak at week 2 (1.6 × 10⁶ RNA copies/ml), the R722G mutation was found in 1 of 10 clones, suggesting that acquisition of this putative compensatory change contributed to the delay in ΔGY control in this animal.

We next sought to compare genetically the relative diversity over the entire Env protein for ΔGY-controlling and -progressing pig-tailed macaques and their divergence from the clonal viral inoculum. Amino acid highlighter plots for each animal show the positions of fixed mutations over time, underscoring the accumulation of mutations in the gp41 cytoplasmic domain in progressing animals (see Fig. S3A to D in the supplemental material). A nucleotide-based phylogenetic tree revealed 4 distinct lineages, representing each of the 4 infected macaques (Fig. 10A). The mean Env diversity and divergence from the parental clone were quantified by pairwise comparisons for each animal and represent the average accumulated changes that emerged after infection (Fig. 10B). Since viral sequences were obtained from samples originating from different weeks after infection, no direct comparisons were made, but it is clear that progressing animals, although sampled earlier after infection, had considerably more viral diversity and divergence, corresponding to the greater total replication in these animals than in controlling animals. This difference was substantial for GR26 at week 58 postinfection, with at least 7 times the diversity and nearly 3 times the divergence of viruses from either HJ98 or HC67 at week 82. For HA94, this difference was less, but it was still 3 times the diversity and nearly 2 times the divergence of controlling animals. For GR26, where longitudinal samples were analyzed, the means of both parameters increased over time in the context of poor early viral control. The endpoint means for diversity and divergence for both HJ98 and HC67 were more consistent with several weeks of infection, corresponding to acute viral replication and then immune control.

Taken together, these findings indicate that, relative to ΔGY-
FIG 8 CD8⁺ cell depletion in ΔGY-controlling pig-tailed macaques. (A) Four ΔGY-controlling pig-tailed macaques (AT4D, AT36, HC67, and HJ98) underwent CD8⁺ cell depletion using monoclonal antibody cM-T807 or MT807R1, as described in Materials and Methods (orange arrows). Sampling times for determining intracellular cytokine responses to SIV peptides are indicated (green arrows). Following treatment, a transient burst of viral replication was observed in all four animals. Shaded areas indicate the limit of sensitivity for the plasma SIV RNA assay. (B and C) For HC67 and HJ98, graphs show the decline in peripheral CD8 T cells starting with the first of 4 anti-CD8 antibody injections. (D and E) Anti-SIV intracellular cytokine analyses (IFN-γ, IL-2, and TNF-α) for CD8⁺ T cell PBMCs at week 31 after ΔGY infection prior to CD8⁺ cell depletion (D) and at week 8 following CD8⁺ depletion (E).
Sporadic mutations occurred in these regions in the ectodomain and frequently involved glycosylation sites, consis-
ting in regions of the V1/V2 and V4 variable loops and the gp41
animals (see Fig. S3 in the supplemental material).

function even in the absence of SIV infection (17,20).
activation resulting from compromised gut epithelial barrier
macaques
responses (66). The host-pathogen relationship is further exacer-
ted by diverse SIV isolates is a particularly robust model for
bility to rapidly infect and deplete CD4+ T cells in gut lymphoid
tissue. An ensuing disruption in epithelial barrier function is as-
associated with microbial translocation and chronic immune acti-
tent with ongoing viral replication and immune escape, while only sporadic mutations occurred in these regions in ΔGY-controlling
animals (see Fig. S3 in the supplemental material).

DISCUSSION
Pathogenic infections by CD4-tropic primate lentiviruses, HIV-1 in humans and SIV in Asian macaques, have in common the ability to rapidly infect and deplete CD4+ T cells in gut lymphoid tissue. An ensuing disruption in epithelial barrier function is associated with microbial translocation and chronic immune activation that contributes to generalized immune dysfunction, a loss of CD4+ T cell regenerative capacity, and an exhaustion of cellular responses (66). The host-pathogen relationship is further exacerbated in favor of the virus by a high mutation rate that enables HIV and SIV to escape from adaptive cellular and humoral immune responses (15,67–73). Among SIV models, infection of pig-tailed macaques by diverse SIV isolates is a particularly robust model for disease, with animals typically progressing rapidly and uniformly to AIDS (8, 17, 18, 20). This more virulent outcome in pig-tailed macaques has been attributed to increased basal levels of immune activation resulting from compromised gut epithelial barrier function even in the absence of SIV infection (17, 20).

In the current report we show that relative to that of rhesus macaques, infection of pig-tailed macaques by ΔGY, an Env cyto-
plasmic domain mutant of SIVmac239 that lacks a preserved GYxxΩ trafficking motif, paradoxically leads to markedly enhanced host control. We previously reported that in rhesus macaques, ΔGY replicates acutely to levels comparable to those for SIVmac239 but with the onset of host immune responses is suppressed to plasma levels 2 to 3 logs less than those for SIVmac239. Although gut CD4+ T cells were only transiently infected and there was no detectable microbial translocation, ongoing ΔGY replication occurred in rhesus macaques, associated with chronic immune activation, the appearance of possible compensatory mutations in the Env cytoplasmic tail, and progression to AIDS (24). In contrast, in pig-tailed macaques, ΔGY, although replicating acutely to levels identical to those for SIVmac239, was rapidly and profoundly controlled in the majority of animals to levels below the limit of detection. As in rhesus macaques (24), ΔGY largely spared CD4+ T cells in gut lamina propria and did not cause microbial translocation. However, in contrast to rhesus macaques, these animals did not have chronic immune activation, and they retained normal numbers of CD4+ T cells in gut and blood for up to 100 weeks after infection. Remarkably robust SIV-specific CD4+ T cell responses were generated and sustained in the intestine, although ΔGY persistence was demonstrated by anti-CD8 depletion at 82 to 97 weeks after infection. Given the high replication competence of ΔGY during acute infection and following CD8+ cell depletion, these findings indicate that this virus, while highly replication fit, was rendered susceptible to host immune control and that this control was more effective in pig-tailed than in rhesus macaques.

TABLE 2 Neutralizing antibody responses in ΔGY-infected pig-tailed macaques

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*ID50 values determined on TZM-bl cells for ΔGY-infected controlling and -progressing pig-tailed macaques using sera obtained before or at 28 or 46 weeks after infection. Neutralizing antibody responses of autologous sera to ΔGY and neutralization sensitivity of a panel of neutralization-sensitive and neutralization-resistant viruses are indicated. Neutralization-sensitive SIVsmE660-2A5.VTRN was generated by introducing 4 amino acid changes into neutralization-resistant SIVsmE660-2A5 (46).*
A critical question is what host responses contribute to ΔGY’s enhanced control. The rapid reappearance of virus following CD8<sup>+</sup> cell depletion is consistent with control by cytotoxic T lymphocytes (CTL), a view supported by the prompt suppression of viremia during CD8<sup>+</sup> T cell recovery and anamnestic antiviral cytokine responses. However, the anti-CD8<sup>+</sup> chain antibody used to deplete CD8<sup>+</sup> cells is not specific for CTL (74,75), and it is possible that virus control could have been exerted by CD8<sup>+</sup> NK cells through antibody-dependent cell-mediated cytotoxicity (ADCC) or other cell types expressing this molecule. In addition, strong SIV-specific CD4<sup>+</sup> T cell responses detected in blood and particularly in gut could have contributed, since in HIV infection these responses have been correlated with both enhanced antiviral CD8<sup>+</sup> T cell activity and viral control (76–78). It is possible that sparing of CD4<sup>+</sup> T cells in lamina propria during acute ΔGY infection enabled potent mucosal responses to occur. Although in humans and in macaques, control of HIV or SIV, respectively, has been correlated with particular MHC class I alleles (15,54, 79, 80), the high proportion (19 of 21 animals) of ΔGY-controlling pig-tailed macaques in this outbred population and the absence of any association between putative restrictive alleles and outcome suggests that non-HLA-dependent or multiple host immune effector mechanisms are involved. High titers of neutralizing antibodies to neutralization-sensitive SIVs were generated in ΔGY-infected pig-tailed macaques. However, these antibodies failed to neutralize ΔGY (Table 2), indicating that the ΔGY mutation neither rendered this virus more neutralization sensitive nor elicited more potent neutralizing antibodies. Taken together, our results indicate that the ΔGY mutation, while having no early effect on viral replicative capacity, rendered SIVmac239 highly susceptible to cellular control, with CD8<sup>+</sup>, CD4<sup>+</sup>, and possibly innate cellular responses playing a role (18,81).

Transcriptomic profiling of SIV-infected pig-tailed macaques has revealed intense early proinflammatory and type I interferon responses in peripheral blood, lymph nodes, and gut that are sustained during chronic infection (18, 81).
associated with DNA damage pathways, T and B cell proliferation, apoptosis, and signatures of innate immune activation that are in marked contrast to those in natural hosts of SIV, which mount acute responses that are often greater than those seen in macaques but are uniformly downregulated during chronic infection (82, 83). Although a similar spectrum of responses occurs in SIVmac-infected rhesus macaques (40, 81), accelerated or more robust responses may occur in pig-tailed macaques, which could account for ΔGY’s rapid control in this species. Although ΔGY-controlling pig-tailed macaques resemble natural hosts of SIV in remaining clinically well with normal levels of CD4 T cells in blood and gut and no systemic immune activation, natural hosts tolerate high levels of viremia following downregulation of their acute immune responses, while ΔGY-infected pig-tailed macaques exhibit a high degree of viral control (84). Further studies of the transcriptomic responses of pig-tailed macaques during acute and chronic ΔGY infection, along with a longitudinal assessment of their host immune response, will provide a valuable probe to assess the magnitude and breadth of host responses that correlate with ΔGY control and the extent to which these responses persist during chronic infection.

It remains to be determined how the Gly-720/Tyr-721 amino acid deletion within the GYxxØ trafficking motif renders SIVmac more susceptible to control in pig-tailed macaques. A YxxØ motif within 6 or 7 amino acids of the predicted membrane spanning domain is universally conserved in all primate lentiviruses and, in association with a proximal glycine, mediates binding to cellular clathrin adaptor complexes and highly efficient clathrin-dependent endocytosis of Env from the plasma membrane (85–90). Although for HIV and SIV, additional endocytic signals exist (85–87, 91), loss of this most membrane-proximal motif by the ΔGY mutation has been associated with modest increases in Env expression on the plasma membrane (85). It is possible that such an increase could enhance the susceptibility of virus-producing cells to ADCC (91). In addition to the AP2 endocytic adaptor complex, this GYxxØ domain also binds to the AP1 complex (87, 89), and alterations in intracellular trafficking in biosynthetic pathways from the Golgi apparatus or postendocytic sorting of Env from endosomes could alter Env processing or its loading and presentation on MHC class I or II molecules to increase target cell susceptibility to immune attack or the priming of cellular responses by antigen-presenting cells (92–94).

The ΔGY mutation does not affect CD4 or CCR5 tropism in standard cell-cell fusion assays (95) (not shown). However, a less well-defined region that overlaps the GYxxØ motif is a potent basolateral sorting signal (96–99). It is possible that alteration of this directional signal for viral assembly could affect viral budding or cell-to-cell spread in specific anatomical compartments. Alternatively, mucosal tissues are known to express abundant proinflammatory and interferon-stimulated genes (5, 100), and a suppression of ΔGY replication at this site could have occurred had the ΔGY mutation rendered SIVmac239 more susceptible to these responses.

One intriguing and striking alteration associated with the ΔGY mutation was a marked reduction in infected macrophages, as determined by in situ hybridization and multilabel confocal microscopy. This finding correlated with the absence of detectable virus in the central nervous system (CNS), where monocyte/mac-
ophagocyte trafficking of infected cells is thought to play a critical role in neuro-AIDS (101, 102). ΔGY-infected animals also exhibited a lack of BrdU incorporation in monocytes during chronic infection, which we have shown correlates with macrophage turnover and progression to AIDS (34). While there has been recent controversy as to whether myeloid cells isolated from tissues are actually infected or have simply phagocyted debris from neighboring SIV-infected T cells (103), previous analyses of tissues from SIV-infected macaques (24, 35, 101, 104, 105) and the current study show SIV antigens and nucleic acids in tissues from SIVmac239-infected pig-tailed macaques, which were distinguishable from phagocyted SIV antigens and CD3+ T cell fragments (Fig. 3). Although it is unclear how the ΔGY mutation led to this outcome, macrophages express a number of interferon-stimulated genes, including SAMHD1 (106) and IFITM family members (107), and it is possible that the ΔGY mutation rendered this virus more susceptible to these or other cellular factors expressed in macrophages.

Two ΔGY-infected pig-tailed macaques (GR26 and HA94) developed persisting viremia and progressed to disease by 74 and 76 weeks. Although their disease was atypical for AIDS in that CD4+ T cells in blood and lamina propria were only mildly depleted (Fig. 2) and macrophages were not clearly infected, both animals developed chronic immune activation, as reflected in BrdU incorporation in CD4+ and CD8+ T cells and HLA-DR expression on CD8+ T cells. These animals also developed thrombocytopenia, which is commonly associated with SIV disease progression in pig-tailed macaques (108), and tissues at necropsy showed pulmonary arteriopathy uniquely associated with SIV infection (56–58). Single-genome amplification of the Env cytoplasmic domain in both animals showed conservation of the ΔGY mutation, as well as R751G, which is a well-described mutation for the SIVmac239 clone (65). Of particular interest were novel mutations that likely reflected selection pressure to restore defects caused by the ΔGY mutation (Fig. 9). Among these, an R722G mutation flanking the ΔGY mutation became fixed in all amplicons at necropsy in both animals. In GR26, R722G was not present during peak viremia at week 2 but was detectable in 6 of 10 amplicons at week 6, suggesting that any role it played in fitness occurred after the onset of host immune responses and the decline in viremia. Although this change did not create a recognizable Tyr-based trafficking motif, it was also noted in at least some amplicons in all 4 rhesus macaques that progressed to AIDS (24). Two changes seen previously in rhesus macaques, an S727P mutation, which in small numbers of rhesus macaques partially restored gut tropism and pathogenicity to ΔGY (28), and a deletion of QTH from amino acid positions 734 to 736, were not observed in ΔGY-progressing pig-tailed macaques. Although additional changes were seen (Fig. 9), these were largely nonoverlapping, with the exception of a conservative T818I mutation in 80 to 90% of amplicons. Given the possibility that some or all of these mutations could be compensating for a defect caused by the ΔY deletion, assessing their impact on the ΔGY virus in vitro and in vivo should provide an approach to probe the virological determinants for ΔGY’s altered tropism and enhanced susceptibility to host control.

Although ΔGY viremia was suppressed below the limits of detection in the majority of pig-tailed macaques, viral persistence was clearly demonstrated by anti-CD8 depletion (Fig. 8). This finding raises intriguing questions concerning the cellular and anatomic reservoirs that harbored this virus and whether there was ongoing viral evolution in these sites. As demonstrated by ΔGY’s robust acute replication and rapid kinetics of rebound following CD8+ cell depletion, this virus remained highly replication fit despite strong host control. For HIV-1, viral persistence continues during antiretroviral therapy, since viremia recurs following cessation of therapy even after years of suppression (109, 110). It remains controversial whether HIV-1 that emerges in this setting is truly latent or if low levels of replication and evolution continue during therapy (111, 112). When SGA was performed following CD8 depletion on two ΔGY-infected animals that had maintained low to undetectable levels of viremia for several months, there was markedly less divergence and diversity than in animals that progressed to disease (Fig. 10). For HC67, which controlled plasma viremia by 10 weeks, little to no evolution occurred in the Env cytoplasmic domain. Moreover, the absence of the R751G fitness mutation likely indicated that rapid and potent viral suppression occurred and was maintained. In contrast, for HJ98, which did not control ΔGY viremia until 20 weeks postinfection, mutations present following CD8 depletion included both R751G and R722G. The latter mutation, previously implicated in ΔGY-infected rhesus macaques as a possible compensatory mutation (24), was also present in 1 of 10 amplicons at the peak of acute viremia, suggesting that it may have delayed host control. It remains unclear if the additional changes acquired in virus from HJ98 occurred during this delayed period of control or during the aviremic period that followed. However, the paucity of changes in gp120 and the gp41 ectodomain for both HC67 and HJ98 is in marked contrast to the number of changes in viruses identified in ΔGY-progressing animals (see Fig. S3 in the supplemental material) and suggests that there was little viral turnover in these animals following control. Given intense interest in the cells and anatomic compartments where HIV and SIV reservoirs reside and in their susceptibility to pharmacological (113, 114) and immunological (115) interventions, the ΔGY model of host control in pig-tailed macaques could provide an opportunity to explore novel approaches to eradicate virus that persists in the face of immune responses that control but do not eradicate infection.

In summary, deletion of Gly-720 and Tyr-721 from the conserved GYxxØ (GYRPV) trafficking motif in the SIVmac239 Env cytoplasmic domain results in a novel phenotype in pig-tailed macaques in which this virus replicates to wild-type levels during acute infection, spares mucosal CD4+ T cells and macrophages, and is rapidly and profoundly controlled in the absence of chronic immune activation. The finding that elite control of ΔGY paradoxically is seen in pig-tail macaques, a species that progresses more rapidly to AIDS following SIV infection, suggests that the ΔGY mutation has rendered SIVmac highly susceptible to immune responses in this species. A critical future question will be to determine whether immune responses that are effective in controlling ΔGY will enable pig-tailed macaques to resist pathogenic challenge viruses. Although the SIV ΔNef model, in which SIVmac239 is attenuated by deletion of its nef gene, remains the best model for protection from homologous SIV challenges (116–118), ΔNef provides less effective protection from heterologous SIV or simian-human immunodeficiency virus (SHIV) challenges (119–121). Studies in pig-tailed macaques that assess the ability of ΔGY infection to elicit protective immune responses are in progress. As a model in which a highly replication-fit but immunologically controllable virus has been generated by only a 2-amino-acid deletion in a cellular trafficking signal, the ΔGY/pig-tailed
macaque model will provide an opportunity to elucidate viral, cellular, and host factors that are critical determinants in the delicate balance between viral persistence, host control, and progression to AIDS.

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