HIV

Exposure to SIV in utero results in reduced viral loads and altered responsiveness to postnatal challenge

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HIV disease progression appears to be driven by increased immune activation. Given observations that fetal exposure to infectious pathogens in utero can result in reduced immune responses, or tolerance, to those pathogens postnatally, we hypothesized that fetal exposure to HIV may render the fetus tolerant to the virus, thus reducing damage caused by immune activation during infection later in life. To test this hypothesis, fetal rhesus macaques (*Macaca mulatta*) were injected with the attenuated virus SIVmac1A11 in utero and challenged with pathogenic SIVmac239 1 year after birth. SIVmac1A11-injected animals had significantly reduced plasma RNA viral loads ($P < 0.02$) up to 35 weeks after infection. Generalized estimating equations analysis was performed to identify immunologic and clinical measurements associated with plasma RNA viral load. A positive association with plasma RNA viral load was observed with the proportion of $CD8^+$ T cells expressing the transcription factor, FoxP3, and the proportion of $CD4^+$ T cells producing the lymphoproliferative cytokine, IL-2. In contrast, an inverse relationship was found with the frequencies of circulating $CD4^+$ and $CD8^+$ T cells displaying intermediate expression of the proliferation marker, Ki-67. Animals exposed to simian immunodeficiency virus (SIV) in utero appeared to have enhanced SIV-specific immune responses, a lower proportion of $CD8^+$ T cells expressing the exhaustion marker PD-1, and more circulating T_H17 cells than controls. Although the development of tolerance was not demonstrated, these data suggest that rhesus monkeys exposed to SIVmac1A11 in utero had distinct immune responses associated with the control of viral replication after postnatal challenge.

INTRODUCTION

Vaccines are traditionally designed to induce neutralizing antibodies and/or cytotoxic T cells that can specifically recognize and destroy a given target, for example, an infectious agent. Although highly successful against many acute infectious agents, this approach has not only failed to protect against HIV but also, in some instances, been associated with more infections, not fewer (*1*). Unlike other acute infectious agents, HIV persists and results in progressive disease in the setting of an activated immune system, one that is associated with altered homeostasis of $CD4^+$ T cells (*2*, *3*) and with the elicitation of proinflammatory cytokines (*4*, *5*). The fact that viral replication is necessary but not sufficient for disease progression has been revealed by examination of nonhuman primate responses to infection by simian immunodeficiency virus (SIV). Thus, sooty mangabeys and African green monkeys, species that are naturally infected with SIV, exhibit high viral loads but manifest only low levels of chronic inflammation and incur few, if any, clinical complications after infection, whereas rhesus and pigtailed macaques, species not naturally infected with SIV in the wild, have equally high viral loads and display persistent pathological inflammation that is accompanied by disease progression (*6*–*10*). Because traditional vaccines generally induce activation of the immune system (*11*), they may paradoxically favor viral replication and spread (*12*). If this is the case, then protection against HIV may best be achieved by an immune response that is wholly different from those normally induced by traditional vaccines.

An alternative approach to prevent the replication and spread of HIV in vivo would be to create a vaccine that instead suppresses an immunoreactive response against the virus, for example, one that generates tolerance in an antigen-specific manner. If such a response were to dampen the rate of viral spread, then the virus might instead be cleared by the normal processes of cell turnover (*13*). Because lentiviral infection appears to target a number of $CD4^+$ T cell subpopulations, including long-lived memory $CD4^+$ T cells (*14*, *15*), prevention of inflammation during the initial stages of infection may reduce the spread of virus to such cells and create a pool of infected cells that can be cleared by natural homeostatic mechanisms. Such a dynamic might account for the comparatively rapid loss of viral reservoirs found in babies and in some adults treated shortly after infection (*16*, *17*).

Several routes of immunization have been historically associated with the induction of tolerance, including administration of low or high doses of antigen in the absence of costimulation, oral administration of antigen, and exposure to antigen in utero (*18*–*21*). Notably, the latter route is one that occurs as a matter of course during gestation of the human fetus in an untreated, HIV-infected mother. Because more than 50% of those around the world who are infected by HIV are women of childbearing age (who, unfortunately, are often not on suppressive antiretroviral therapy during the course of pregnancy), such exposures are also quite common. Yet, remarkably, only about 5 to 10% of babies born to such mothers are found to have been infected in utero (*22*, *23*).

We wondered whether the apparent protection of the human fetus from HIV infection might be related to the fact that the human fetal immune system is more likely to generate a tolerogenic, as opposed

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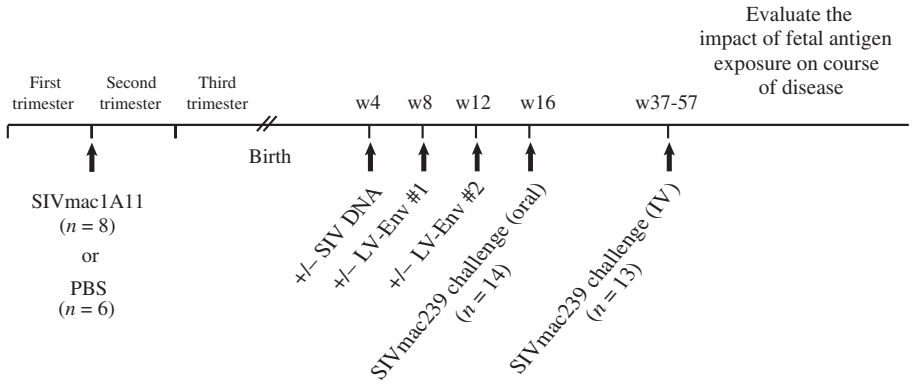
to an immunoreactive, response to exogenous antigen. In previous studies, we have shown that the T and myeloid lineages in utero are derived from a hematopoietic progenitor cell that is distinct from that found in the adult (*24*). When stimulated, fetal naïve $CD4^+$ T cells are polarized toward a $FoxP3^+$ T cell lineage with immunosuppressive properties (*25*), whereas fetal $CD14^+CD16^-$ monocytes are relatively deficient in their ability to up-regulate the surface molecules necessary for antigen presentation and delivery of costimulatory signals (*26*). We have also shown that in utero development of the nonhuman primate (rhesus macaque) immune system has many parallels to that of the human (*27*). A previous report in which fetal macaques were injected with nonpathogenic SIVmac1A11 in utero also found that exposed animals were better able to control viral replication when challenged with pathogenic virus at 1 year of age, although the mechanism of protection was unclear (*28*). We have now extended these studies to evaluate a larger array of immune parameters and to specifically address the possibility that inoculation of nonpathogenic SIV into the developing rhesus macaque fetus might generate a tolerogenic immune response, one that protects against aberrant immune activation and disease progression after postnatal challenge. Our results suggest that in utero exposure to SIV has important effects on the development of the fetal immune system that are associated with, and possibly causal of, partial control of viral replication upon subsequent challenge.

A



B

Study group	Fetal immunization	Neonatal immunization	Animals
A1	PBS	None	#18, #19, #20
A2	PBS	SIV antigens	#21, #22, #23
B1	SIVmac1A11	None	#24, #25, #26, #27
B2	SIVmac1A11	SIV antigens	#28, #29, #30, #31

Fig. 1. Study design. (**A**) Timeline of immunizations and virus challenges. Fetal macaques were injected with SIVmac1A11 or PBS in the late first trimester (55 ± 5 days gestation; term 165 ± 10 days). Animals were then delivered by cesarean section at full term and immunized with either PBS or SIV antigens as neonates. At 16 weeks postnatal age, each animal was orally challenged with SIVmac239. Animals that did not demonstrate productive infection were then challenged intravenously between 37 and 57 weeks of age with SIVmac239 and followed for disease progression. IV, intravenous. (**B**) Summary of study groups.

RESULTS

Experimental design

To determine whether exposure to SIV antigens in utero might have an impact on the immune response to pathogenic SIV challenge after birth, six fetal monkeys (group A) served as sham controls and eight fetal monkeys (group B) were injected with a nonpathogenic molecular SIV clone, SIVmac1A11, under ultrasound guidance during the late first trimester. Newborns were then delivered by cesarean section at term (160 ± 2 days gestation; term 165 ± 10 days) (Fig. 1A). To specifically explore the hypothesis that prenatal exposure to SIV might induce tolerance to SIV antigens, because of clonal deletion, the induction of antigen-specific regulatory cells, and/or the generation of antigen-specific anergy, half of the animals in each group were exposed intramuscularly (IM) to a DNA plasmid encoding SIV Env at 4 weeks after birth, followed by intramuscular injections of a lentivirus (LV) expressing SIV Env at 8 and 12 weeks after birth (LV-Env) (Fig. 1B). All of the monkeys were then orally challenged (at postnatal week 16) with pathogenic SIVmac239 [10^5 $TCID_{50}$ (median tissue culture infectious dose)]. When only 1 of the 14 monkeys was found to be productively infected (even after repeated oral challenges), the remaining 13 monkeys (including 6 in the control group A and 7 in the experimental group B) were challenged intravenously with SIVmac239 (100 $TCID_{50}$) at an age range of 37 to 57 weeks. In addition to monitoring viral loads and $CD4^+$ T cell counts, measures of immune phenotype and function were collected over time.

Lower viral load in SIV-exposed animals after intravenous challenge

At 37 to 57 weeks of age, six monkeys in the control group A and seven monkeys in the experimental group B were successfully challenged by the intravenous route. Thereafter, viral loads in the two groups were found to diverge (Fig. 2A), with those exposed to SIVmac1A11 in utero having lower peak plasma RNA viral loads ($P < 0.04$) at 2 weeks after infection, lower plasma RNA viral load at 4 weeks after infection ($P < 0.006$), and significantly lower plasma RNA viral load over the remainder of the study ($P < 0.02$) (Fig. 2A). Similar reductions in viral loads were also noted for peripheral blood mononuclear cell (PBMC)–associated viral DNA ($P = 0.03$ at peak and $P < 0.04$ at week 4); this difference, however, was not significant over all time points assessed (Fig. 2B). Notably, one monkey that had been exposed to SIVmac1A11 in utero was able to control viral replication to undetectable levels between weeks 16 and 20 after intravenous challenge (Fig. 2A). Finally, changes in the circulating $CD4^+$ T and $CD8^+$ T cell counts were not different between groups after intravenous challenge (Fig. 2, C and D).

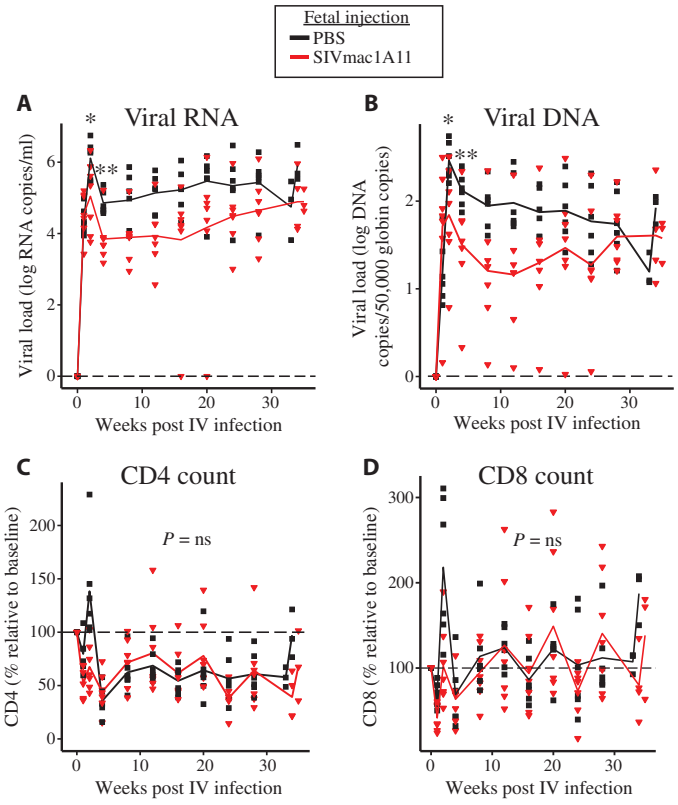


Fig. 2. Animals exposed to SIVmac1A11 in utero have reduced peak and set point viral loads after infection. Animals were intravenously challenged with SIVmac239 at 37 to 57 weeks of age and followed for viral load and immune cell counts up to 35 weeks after infection. (**A**) Plasma RNA viral loads were significantly lower in animals exposed to SIV in utero than controls at 2 weeks (*$P < 0.04$), 4 weeks (**$P < 0.006$), and for all time points ($P < 0.02$) after intravenous SIVmac239 challenge. (**B**) Cell-associated DNA viral loads were significantly lower in animals exposed to SIV in utero than controls at 2 weeks (*$P = 0.03$), 4 weeks (**$P < 0.04$), but not over all time points (P = ns) after intravenous SIVmac239 challenge. P values for a given time point were calculated using t tests. (**C** and **D**) Number of $CD4^+$ T cells (C) and $CD8^+$ T cells (D) after intravenous SIVmac239 challenge. Y axes represent the $CD4^+$ or $CD8^+$ T cell count over the course of infection as a percentage of the $CD4^+$ or $CD8^+$ T cell count on the day of intravenous SIVmac239 challenge. Longitudinal analyses were calculated using generalized estimating equations techniques. Data points represent individual animals. Animals exposed to SIV in utero are shown in red, and controls are shown in black. ns, not significant.

No evidence of immune tolerance before SIVmac239 challenge

To determine whether exposure to SIVmac1A11 in utero generated a state of tolerance to SIV, animals in groups A and B were immunized intramuscularly with either phosphate-buffered saline (PBS) (groups A1 and B1) or with SIV DNA at week 4, and LV-Env (groups A2 and B2) at weeks 8 and 12. At week 12 postnatal age, PBMCs from each animal were stimulated in vitro with aldrithiol-2–inactivated SIV (AT-2 SIV). Proliferative responses of PBMCs, as measured by dilution of carboxyfluorescein diacetate succinimidyl ester (CFSE), were found to be small, variable, and indistinguishable between the groups, irrespective of exposure to SIV antigens in utero or postnatally, and whether in the presence or absence of $CD25^+$ regulatory T cells (T_{regs}) (Fig. 3A). Upon measuring anti-SIV immunoglobulin G (IgG) antibodies in plasma (at week 9 postnatal age), monkeys in the control group (A1) that had not been immunized with LV-Env after birth showed no detectable circulating IgG specific for AT-2 SIV, whereas monkeys in each of the other three groups (A2, B1, and B2) showed levels of circulating SIV-specific IgG that were both detectable and equivalent (Fig. 3B). These observations suggest that exposure to SIVmac1A11 in utero induced an IgG response that was detectable 9 weeks after birth, and was not associated with intrinsic or extrinsic (for example, $CD25^+$ T_{reg}-mediated) T cell tolerance, at least as measured using these assays under the conditions described.

Immunologic measures associated with viral load

Measures of immune phenotype and function (including those of cell activation and proliferation as well as of cytokine production by different T cell subsets) were studied as a function of time after intravenous challenge with SIVmac239. To explore which of these parameters might be associated with the observed changes in viral load between the two groups after intravenous SIVmac239 challenge, generalized estimating equations were used to identify significant associations with viral load. Because most of the divergence in viral load between the groups occurred after the set point (4 weeks after infection), we restricted our analyses to between 4 and 35 weeks after infection. Given the small number of animals in this study and the fact that these analyses were exploratory, multivariate models of measured parameters and multiple comparison corrections were not included. Of 192 measures that were analyzed, 15 were found to be associated with viral load. These measurements fell into four main categories (Table 1). Although the generalized estimating equations analysis did not find an association between the frequency of total $CD4^+$ T_{regs} and viral load (see also fig. S1), it did identify positive associations between plasma viral load and $CD8^+$ T cells with a central memory phenotype (both $CD95^+CD28^+$ and $CD45RA^-CD27^+$) expressing the transcription factor FoxP3 and low levels of the interleukin-7 (IL-7) receptor, CD127 (*29*) (Table 1). A similar pattern was found for total, naïve (both $CD95^-CD28^+$ and $CD45RA^+CD27^+$), and central memory $CD8^+$ T cells that expressed FoxP3 and high levels of the proliferation marker, Ki-67. None of these measurements were significantly different between groups (fig. S2).

The only clinical measurements that were identified by our generalized estimating equations analysis to be associated with viral load were the ratio of $CD4^+$ to $CD8^+$ T cells, the number of circulating monocytes, expression of alanine aminotransferase (ALT; a measure of liver health), and serum cholesterol. All measurements were made on peripheral blood samples. The CD4/CD8 T cell ratio was the only measurement found to be inversely proportional to viral load; increases in all other measures were associated with higher viral loads. However, when analyzed over the course of infection, no significant difference in these measurements was found between animals exposed to SIVmac1A11 in utero and unexposed (sham) controls (fig. S3).

As a measure of immune function, PBMCs were stimulated in vitro to assess their ability to produce cytokines [interferon-γ (IFNγ), tumor necrosis factor–α (TNFα), IL-2, IL-4, and/or IL-17] after polyclonal stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Only $CD4^+$ T cells capable of producing the lymphoproliferative cytokine, IL-2, were found to be associated with viral load. This association was positive, such that a stronger IL-2 response was associated with higher viral loads. IL-2 production was lower in PBMCs from animals exposed to SIVmac1A11 in utero when compared to controls (fig. S4). No differences in IL-2 production were observed immediately before infection.

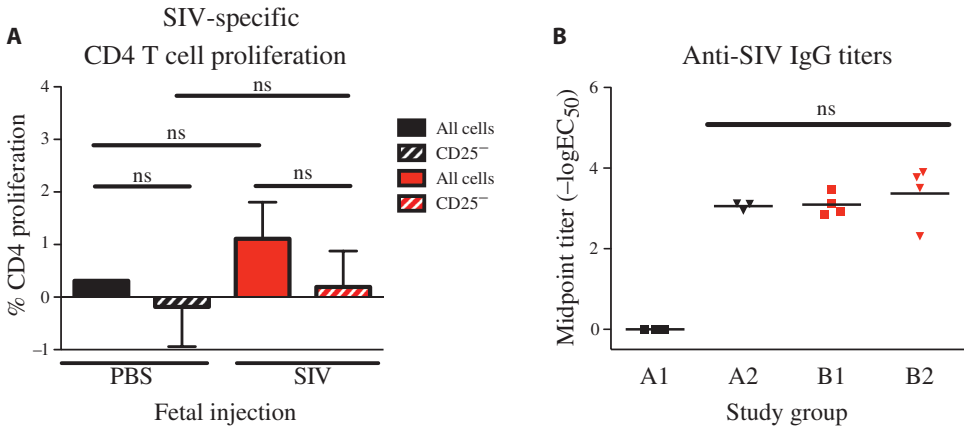


Fig. 3. No evidence of antigen-specific tolerance before oral challenge. Fetal exposure to SIVmac1A11 followed by neonatal vaccination elicited detectable humoral but not cellular responses before oral challenge. (**A**) Proliferating CD4$^+$ T cells in total PBMCs from 12-week-old rhesus monkeys were labeled with CFSE and incubated with AT-2 SIV for 6 days. (**B**) IgG titers against AT-2 SIV in plasma from 9-week-old animals were not detectable in group A1, but were detectable in groups A2, B1, and B2. No significant differences were found between the three groups with detectable titers. Differences were assessed using *t* tests with significance defined as $P < 0.05$. ns, not significant.

Table 1. Plasma RNA viral loads are associated with multiple immunologic and clinical measurements. A panel of 192 immunologic and clinical measurements, taken at multiple time points after intravenous SIVmac239 challenge, was analyzed for associations with log plasma RNA viral load by generalized estimating equations. Fifteen measurements were identified as being significantly associated with viral load ($P < 0.05$).

Parameter	Log RNA *P* value	Viral load estimate
CD8_CM_CD127l ow_FoxP3+	0.0419	0.1003
CD8_CM_Ki-67hi_ FoxP3+	0.0029	0.2764
CD8_Ki-67hi_FoxP3+	0.0030	1.7308
CD8_Naïve_Ki-67hi_FoxP3+	0.0096	7.8656
CD4:CD8	0.0475	−0.4095
Monocytes	0.0230	0.0002
ALT	0.0097	0.0041
Cholesterol	0.0301	0.0091
CD4_IL2+	0.0481	0.0137
CD4_EM_Ki-67+	0.0166	−0.0052
CD4_EM_Ki-67int	0.0005	−0.0155
CD4_Ki-67int	0.0001	−0.0588
CD8_CM_Ki-67int	0.0135	−0.0272
CD8_EM_Ki-67+	2.03×10^{-5}	−0.0082
CD8_EM_Ki-67int	0.0372	−0.0308

The frequency of both CD4$^+$ and CD8$^+$ T cells expressing Ki-67 was inversely correlated with viral load over the course of infection, except in the case where Ki-67$^+$ cells coexpressed FoxP3 (as discussed above). When plotted longitudinally, it became apparent that Ki-67–expressing CD4$^+$ T cells were more abundant in rhesus monkeys exposed to SIVmac1A11 in utero compared to control monkeys not exposed to virus in utero ($P < 0.004$) (Fig. 4A). No difference between experimental groups was found in CD8$^+$ T cells (Fig. 4B). Ki-67 was found to be expressed in CD4$^+$ T cells from SIVmac1A11-exposed monkeys in a unique manner, with many more cells expressing intermediate levels of Ki-67 protein relative to those observed in CD4$^+$ T cells from sham control rhesus monkeys (Fig. 4C). Such Ki-67 intermediate (Ki-67int) cells were not differentially abundant at the time of intravenous SIVmac239 challenge, but did increase in abundance in the SIVmac1A11-exposed animals as a function of time thereafter when compared to the sham-injected control animals ($P < 0.01$) (Fig. 4D, left panel). By contrast, there was no difference between the groups in CD4$^+$ T cells expressing high levels of Ki-67 (Fig. 4D, right panel).

Including Ki-67 high-expressing (Ki-67hi) and Ki-67 intermediate-expressing (Ki-67int) CD4$^+$ and CD8$^+$ T cells into the generalized estimating equations analysis revealed multiple (and potentially overlapping) subsets that were significantly inversely associated with viral load over the course of infection. Linear regression models were then used to analyze these associations for every time point measured after infection. Plots for each measurement of viral load with each measurement of CD4$^+$ Ki-67int T cells are shown in Fig. 5A, with significant associations found at weeks 2, 4, 8, 16, and 24 after intravenous SIVmac239 challenge. These associations remained significant even after removing the animal that controlled viral replication to undetectable levels (animal #27). By contrast, it was only at week 2 after intravenous SIVmac239 challenge that the frequency of CD4$^+$ Ki-67hi T cells had an association with viral load (Fig. 5B).

Immunologic measures not associated with viral load but different between groups

Multiple previous studies with SIV-infected rhesus macaques and HIV-infected humans have raised the possibility that viral replication and spread can be suppressed by adaptive immunity (for example, virus-specific neutralizing antibodies and effector T cells) and augmented by the immunologic effects of chronic activation (for example, resulting in the release of proinflammatory cytokines and the generation of functionally "exhausted" T cells) (*30*–*32*). Although the generalized estimating equations models used in Table 1 did not show that any such measures predicted lower viral loads in the SIVmac1A11-exposed rhesus monkeys at any time point after intravenous challenge with SIVmac239, several merit mention.

Although monkeys from control group A1 (with no exposure to SIVmac1A11 in utero and no postnatal vaccination) did not develop detectable antibody responses against AT-2 SIV until after the peak of viremia (2 weeks after infection), monkeys in each of the other groups had detectable anti-SIV antibody titers by 2 weeks after infection (and a single animal had detectable antibody titers on the day of intravenous infection) (fig. S5). In a generalized estimating equations model, these titers showed no significant relationship to viral RNA levels or to the observed differences in viral load between group A and group B monkeys.

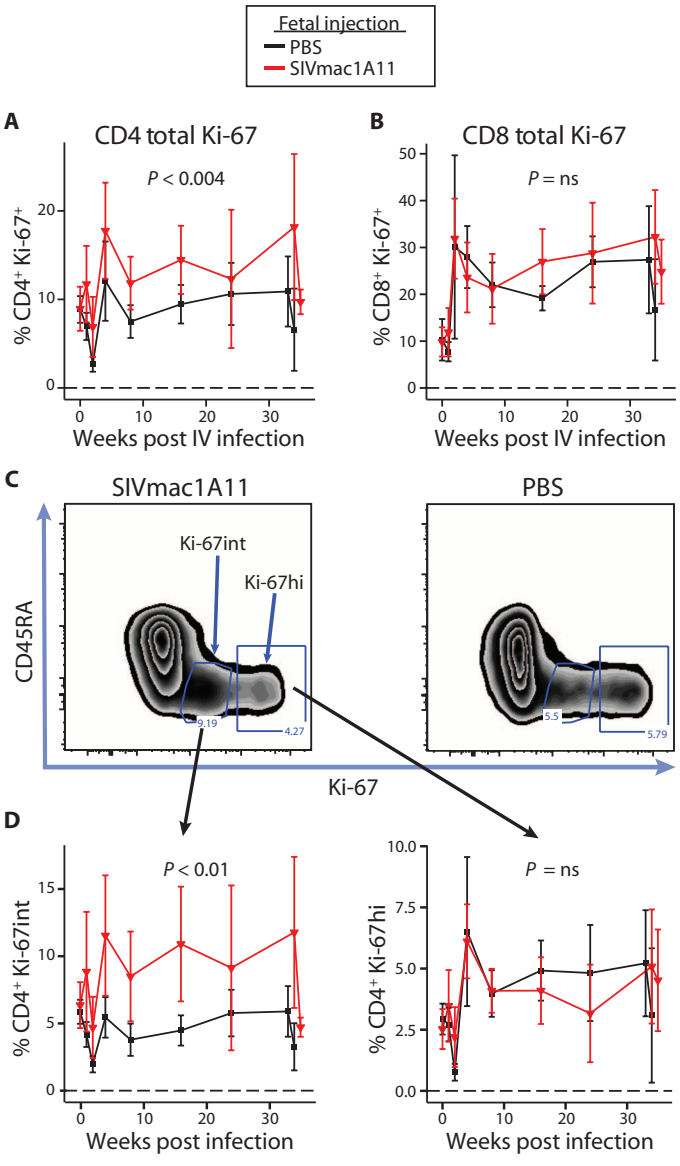


Fig. 4. Ki-67 expression is altered in animals exposed to SIVmac1A11 in utero. (**A** and **B**) After intravenous SIVmac239 challenge (37 to 57 weeks after birth), the frequency of Ki-67$^+$ cells in the (A) CD4$^+$ T cell population or (B) CD8$^+$ T cell population was measured over time. (**C**) Representative cytogram of the distribution of Ki-67 in memory (CD45RA$^-$) CD4$^+$ T cells from animals exposed prenatally to SIVmac1A11 (left panel) or PBS as a control (right panel). (**D**) Frequencies of Ki-67int (left panel) or Ki-67hi (right panel) expression on CD4$^+$ T cells after intravenous SIVmac239 challenge.

However, it is noteworthy that the only animal with anti-SIV antibody titers detected on the day of infection (#27) was able to completely control viremia at weeks 16 and 20 after infection.

The frequency of circulating CD4$^+$ T cells producing IL-17 was measured after polyclonal stimulation of PBMCs and staining for intracellular IL-17. The frequency of such cells was not different between groups on the day of intravenous challenge, but when the frequency of IL-17–producing CD4$^+$ T cells was plotted longitudinally, animals injected with SIVmac1A11 in utero were found to have significantly more of these cells than controls after intravenous challenge with SIVmac239 ($P = 0.01$) (Fig. 6A, right panel). To further interrogate an observed trend in the relationship between T helper 17 (T_H17) cell frequency and viral load that had not reached significance in the generalized estimating equations analysis, a linear mixed-effects regression model was used. Animal ID and time point after infection were included as random effects to account for the impacts of both inter-individual variability and time since infection on log plasma RNA viral load. Each 1% increase in T_H17 cell frequency was found to reduce plasma RNA viral load by 0.1 log, resulting in a significant ($P < 0.0001$) relationship between the frequency of T_H17 cells and log plasma RNA viral load (Fig. 6B). Additionally, T_H17 cell frequencies were found to be positively associated with the proliferation marker Ki-67 on CD4$^+$ (fig. S6A) but not CD8$^+$ (fig. S6B) T cells.

To measure SIV-specific cellular responses, PBMCs were cultured with a peptide pool spanning the SIV Gag protein p27, and responses were assessed by cytokine flow cytometry (CFC) (Fig. 7A) or an IFNγ-capture ELISpot (enzyme-linked immunospot) assay. Compared to controls, animals exposed to SIV in utero had significantly higher antigen-specific production of the cytokine TNFα from CD8$^+$ T cells by CFC ($P < 0.02$) (Fig. 7B) and more IFNγ-producing cells by ELISpot ($P < 0.05$) (Fig. 7C) after intravenous challenge with SIVmac239. Although the variance was high because not every animal responded to these stimulations, the results were consistent within a given animal and increased over the course of infection in both assays. However, the generalized estimating equations model did not detect a significant association between these responses and viral load. Therefore, although animals exposed to SIVmac1A11 in utero mounted greater antigen-specific responses, it is unclear whether these responses had an impact on the observed differences in viral load.

Expression of the negative regulators, PD-1 and Tim-3, was measured on splenic CD4$^+$ and CD8$^+$ T cells at the time of tissue harvest. Rhesus monkeys exposed to SIVmac1A11 in utero were found to have significantly fewer CD8$^+$ T cells expressing PD-1 than did control animals ($P < 0.04$) (fig. S7A) with an even more substantial difference found in the fraction of PD-1–expressing central memory cells ($P < 0.002$) (fig. S7B). Because of limited cell numbers, we were not able to measure the expression of these proteins at any time point other than the time of tissue harvest, and thus, these were not included in the generalized estimating equations analyses.

Evidence of persistent SIVmac1A11 infection postnatally

Previous reports of live attenuated SIV vaccines have described the persistence of live attenuated vaccine replication in lymph nodes, associated with a more robust antigen-specific T cell response in lymph node cells and lower viral replication after pathogenic challenge (*33*). To investigate whether exposure of rhesus macaques to SIVmac1A11 in utero had a similar effect, cryopreserved inguinal lymph node cell suspensions from animals in group B2 were screened for the presence of SIV RNA and DNA at 14 weeks of age (before oral or intravenous SIV challenge). Two of four animals had detectable SIV RNA, and three of four animals had detectable SIV DNA at this time point (table S1).

DISCUSSION

We report here a preliminary study in nonhuman primates that asks the question: Might exposure to nonpathogenic SIV in utero have a determinative impact on the response to challenge with pathogenic SIV after

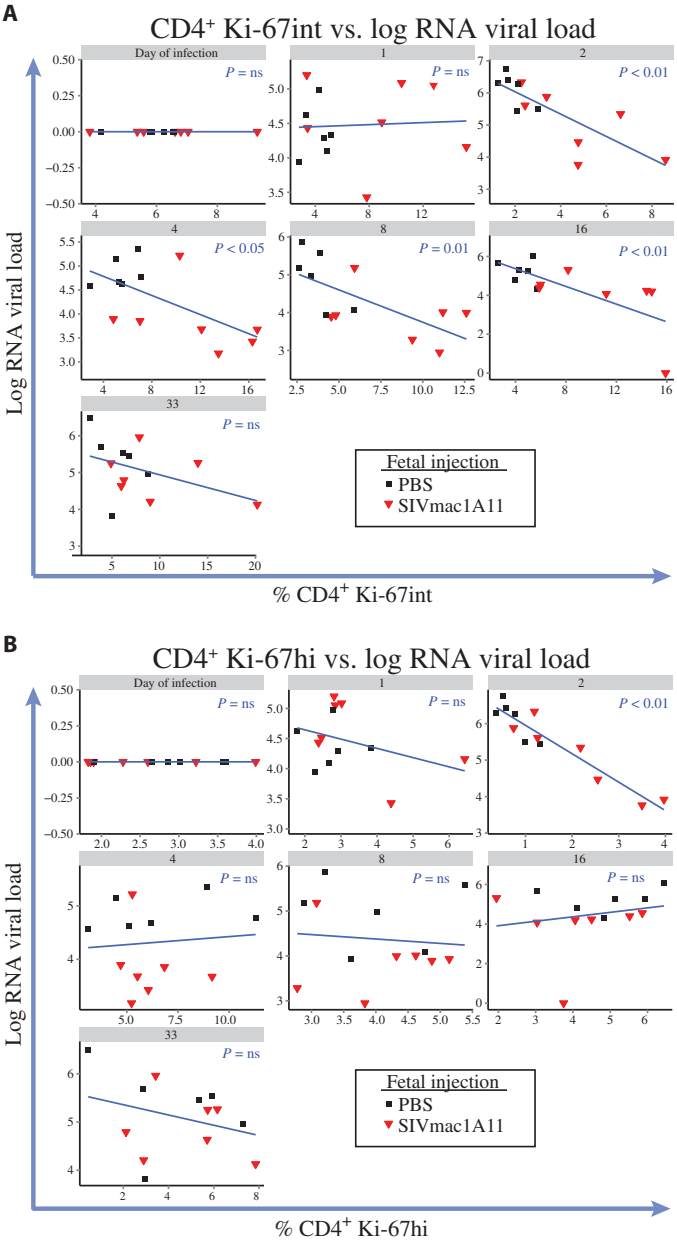


Fig. 5. Ki-67int cell number is inversely correlated with viral load after infection. To investigate the impact of Ki-67int cells on viral load, linear regression was used to test for associations between the frequencies of Ki-67int CD4$^+$ T cells and log plasma RNA viral load for each time point measured after intravenous SIVmac239 challenge. (**A** and **B**) Ki-67int (A), but not Ki-67hi (B), CD4$^+$ T cells (*x* axes) were found to be significantly inversely correlated with log plasma RNA viral loads (*y* axes) at many of the time points tested. Numbers in gray bars represent weeks after intravenous SIVmac239 challenge. Significance was defined as $P < 0.05$.

birth? Given prior data examining such in utero antigenic exposure in mice, nonhuman primates, and humans, it was anticipated that nonresponsiveness to SIV antigens might ensue, for example, because of clonal deletion, the induction of antigen-specific regulatory cells, and/or the generation of antigen-specific anergy. If so, and should an adaptive immune response normally be protective against SIV infection and/or disease progression, monkeys challenged after birth with pathogenic SIV would predictably fare poorly; by contrast, and should infection and/or disease progression be driven by the effects of chronic immune activation, then such nonresponsiveness to SIV might provide a measure of protection instead. The latter possibility is supported by a recent study in macaques showing that a vaccination regimen engineered to produce antigen-specific tolerance to SIV protected monkeys from multiple infectious challenges with a pathogenic SIV isolate (*34*).

The results of the current study support the hypothesis that exposure to SIV antigens during the late first trimester in utero has an effect on the response of the newborn to subsequent challenge with pathogenic SIV. Most remarkably, circulating peak plasma RNA viral loads as well as set point viral loads were 1 log lower in the infants exposed to SIVmac1A11 in utero compared to unexposed controls. This difference in viral load was not clearly related to the generation of antiviral T or B cell effector responses, as described by Otsyula *et al.* (*28*), who showed that fetal or neonatal infection with SIVmac1A11 induced a small but detectable cytotoxic T lymphocyte (CTL) response in PBMCs from SIVmac1A11-exposed animals stimulated in vitro. However, Otsyula *et al.* were only able to study a small number of animals ($n = 3$ injected in utero) for a relatively short period of time (12 weeks after infection) and were not able to further characterize the immune responses that may have contributed to the differences in viral loads. In our study, strong IFNγ (T_H1-like) responses and higher antibody titers against SIV were (with the exception of one monkey) neither associated with nor predictive of the lower viral loads observed in the animals exposed to SIVmac1A11 in utero. Exposure to SIVmac1A11 in utero was also not associated with any demonstrable measure of antigen-specific "tolerance" to SIV, for example, as assayed by responses to SIV before or after challenge in the presence or absence of CD25$^+$ T_{regs}. Of a total of 192 parameters of clinical health and immune phenotype and function that were measured, only four general categories were associated with plasma viral load. These were the fraction of CD8$^+$ T cells expressing the transcription factor FoxP3; the peripheral blood measurements of four clinical parameters (CD4/CD8 T cell ratio, monocyte abundance, ALT, and serum cholesterol); the fraction of CD4$^+$ T cells producing IL-2; and different cell subsets expressing the proliferation marker Ki-67. In particular, animals exposed to SIVmac1A11 in utero and challenged with SIVmac239 after birth were much more likely to generate multiple CD4$^+$ and CD8$^+$ T cell phenotypes with an intermediate level of expression of Ki-67, and the appearance of such cells was associated with differences observed in viral load between treatment groups.

These observations raise the possibility that exposure of the fetal immune system to antigen results in the generation of responses that are not yet clearly defined and that are, at the very least, different than those expected in adults. Classical adaptive immune responses (for example, T_H1 effector cells producing IFNγ or SIV-specific antibodies) might be generated but are not able to contain viral replication and spread under these circumstances. On the other hand, it is intriguing that SIVmac1A11-exposed monkeys showed a high representation of CD4$^+$ T cells with intermediate expression of Ki-67 after pathogenic intravenous challenge and that the frequency of such cells was predictive of the observed lower peak and set point viral loads found in this group. Sieg *et al.* (*35*) have reported that cells from a subset of HIV-infected patients have an inability to up-regulate Ki-67 and progress through the cell cycle after T cell receptor stimulation. Defects in IL-2 signaling were proposed to play a role, but the exact mechanism remains

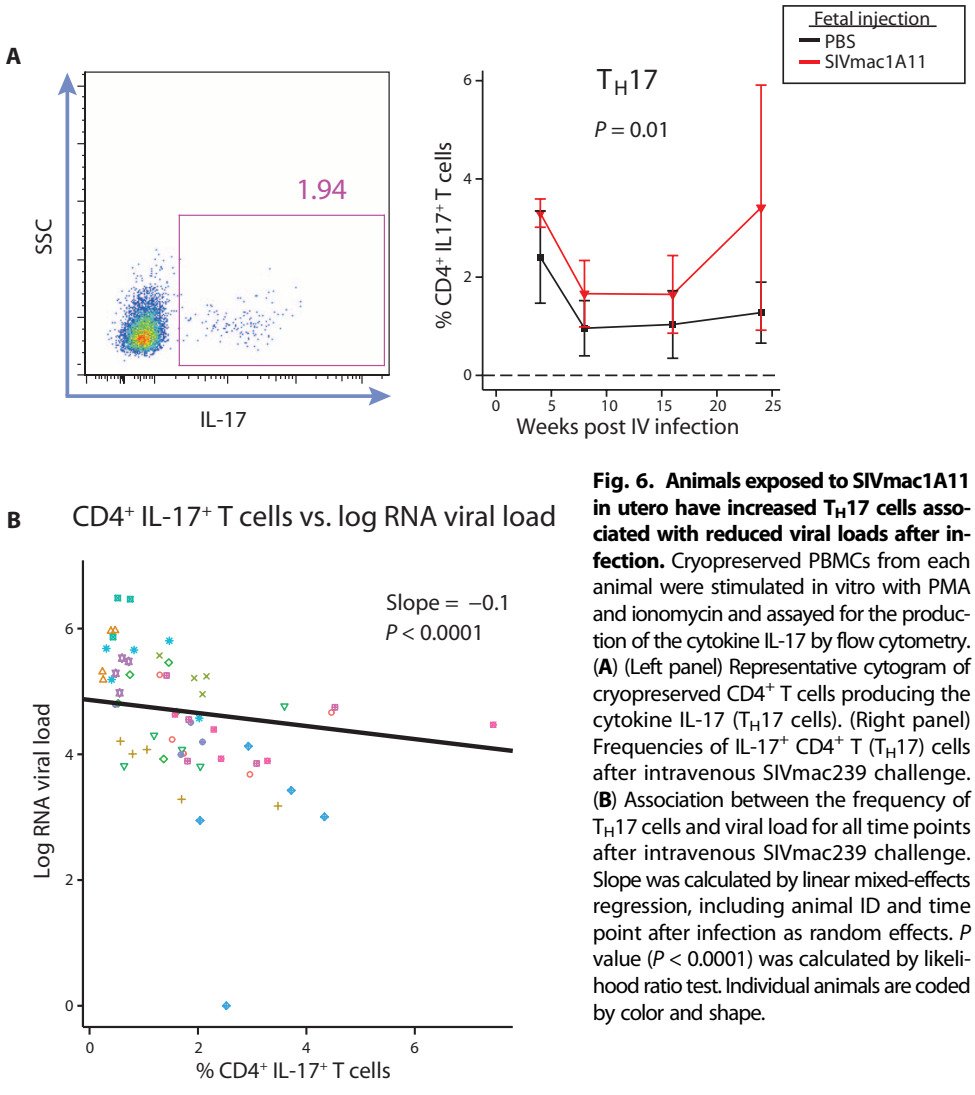


Fig. 6. Animals exposed to SIVmac1A11 in utero have increased T_H17 cells associated with reduced viral loads after infection. Cryopreserved PBMCs from each animal were stimulated in vitro with PMA and ionomycin and assayed for the production of the cytokine IL-17 by flow cytometry. (**A**) (Left panel) Representative cytogram of cryopreserved $CD4^+$ T cells producing the cytokine IL-17 (T_H17 cells). (Right panel) Frequencies of IL-17^+ $CD4^+$ T (T_H17) cells after intravenous SIVmac239 challenge. (**B**) Association between the frequency of T_H17 cells and viral load for all time points after intravenous SIVmac239 challenge. Slope was calculated by linear mixed-effects regression, including animal ID and time point after infection as random effects. *P* value ($P < 0.0001$) was calculated by likelihood ratio test. Individual animals are coded by color and shape.

been involved in dampening cellular immune responses, thereby enabling viral replication and spread. It is also possible that high levels of such cells may have been induced in response to increasing viral loads found at peak viremia. Our generalized estimating equations analysis, however, showed no association between the antigen-specific immune responses and viral load, although our power was limited by the small number of animals in our study.

$CD4^+$ T cells and other cells producing the cytokines IL-17 and IL-22 are important for the maintenance of gut barrier integrity (*7*, *38*–*43*). Studies in rhesus macaques have revealed that the frequencies of T_H17 and T_{reg} cells, and the relative ratio between these two cell populations, can distinguish pathogenic from nonpathogenic SIV infection and may also predict disease progression (*7*, *39*, *44*). In this light, the increased frequency of T_H17 cells observed in our study could be important for maintenance of mucosal epithelial integrity. Although not directly measured here, we have previously proposed a model of chronic inflammation whereby the loss of T_H17 cells, especially in the gastrointestinal tract, compromises gut barrier integrity and can allow luminal contents, including microbial products, into the lamina propria or potentially into the bloodstream (*39*). It has been proposed that this breakdown sets off a cycle of inflammation where intestinal immune cells respond to microbial insult by producing proinflammatory cytokines (such as type I interferons) (*45*–*49*). T_H17 cell frequency was found to be significantly inversely related to viral load in this study, supporting the hypothesis that the maintenance of T_H17 cells may have had a positive impact on disease progression. To further clarify the role of cells producing IL-17 and/or IL-22 in the context of in utero exposure to virus, future studies are aimed at also measuring parameters of microbial translocation in treated animals and controls.

Because of the limited availability of tissue samples from these animals, we were only able to interrogate four animals for the presence of virus in lymph nodes at 14 weeks after birth (about 29 weeks after SIV exposure in utero), giving us little power to detect associations between the presence of virus in the lymph nodes and any of the outcome variables measured. In addition, SIV-specific T cell responses in these lymph nodes were low and indistinguishable between groups, and we cannot rule out the possibility that the persistence of SIVmac1A11 was able to prime a lymph node antiviral response capable of reducing the viral load after challenge (*33*). It is worth noting, however, that Fukazawa *et al.* (*33*) found that the presence of active viral replication (as measured by the presence of viral RNA) from a live attenuated

unclear (*35*). The Ki-67int phenotype observed in this study may represent a similar population of unresponsive cells, and it is intriguing that we also measured reduced frequencies of IL-2–producing $CD4^+$ T cells. Another possibility is that Ki-67int cells are arrested in one stage of the cell cycle (*36*) or in a state of anergy, as previously described in the setting of "aborted activation" (*37*).

Had tolerance been induced in this study, we might have also predicted an increase in peripheral T_{regs} in animals exposed to SIVmac1A11 in utero. Although this study did not identify a role for total $CD4^+$ $CD127^{low}FoxP3^+$ T_{regs}, there were observed associations between $CD8^+$ T cells with a central memory phenotype (both $CD95^+CD28^+$ and $CD45RA^-CD27^+$) that expressed FoxP3 and low levels of CD127, and viral load at 4 weeks after infection. Although not functionally analyzed in the present study, $CD8^+$ T cells expressing FoxP3 have been previously reported to possess potent suppressor activities (*29*). Notably, noncytolytic MHC1b/E-restricted $CD8^+$ T regulatory cells appear to be playing a role in the protective responses against SIV observed after oral feeding of rhesus macaques with a combination of inactivated SIV and the bacterium, *Lactobacillus* (*34*). Thus, these cells could have

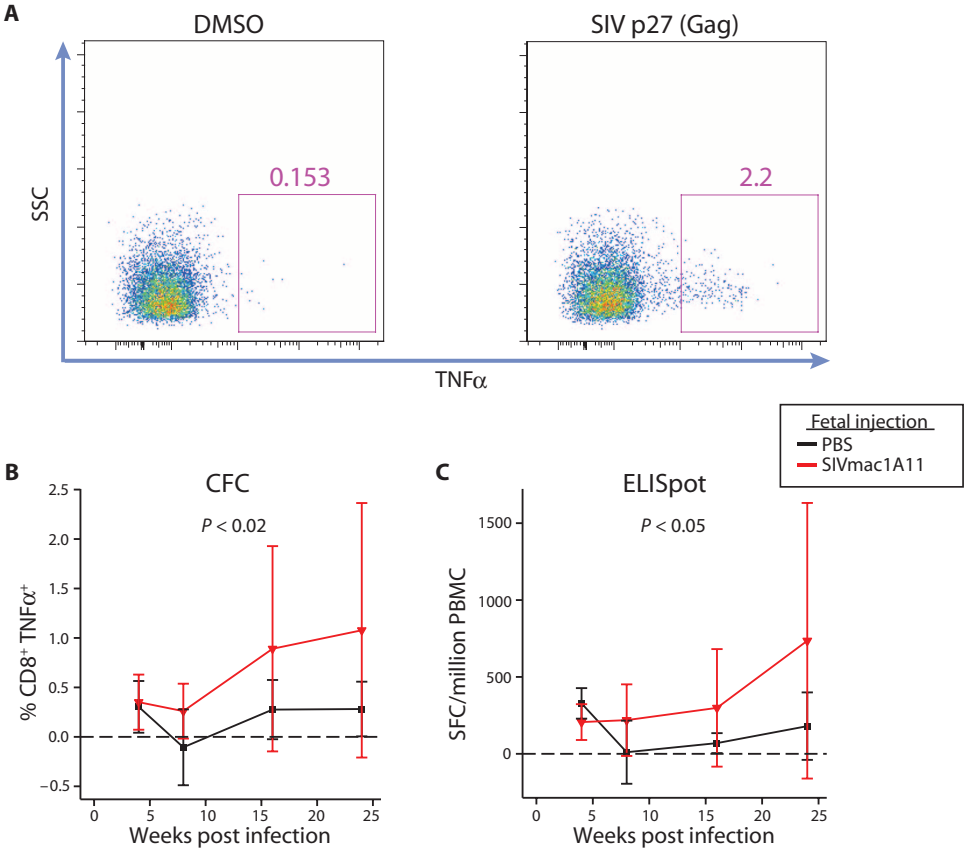


Fig. 7. Animals exposed to SIVmac1A11 in utero have increased antigen-specific immune responses after infection. (**A**) Representative cytograms of cryopreserved $CD4^+$ T cells producing the cytokine TNFα after stimulation with dimethyl sulfoxide (DMSO) (negative control, left panel) or an SIV Gag (p27) peptide pool (right panel). (**B** and **C**) Frequencies of SIV-specific responses measured by (B) CFC or (C) ELISpot after intravenous SIVmac239 challenge. *P* values were calculated by generalized estimating equations.

vaccination had the most marked impact on viral control after challenge, with levels of viral RNA that were orders of magnitude higher than those observed in this study.

Unexpectedly, 13 of 14 animals in this study remained uninfected despite multiple oral challenges with SIVmac239. Although oral infection with SIVmac239 has been described previously (*50*), to our knowledge, this has not been attempted before in juvenile macaques. We chose this challenge virus because of its homology with the available antigens for in vitro tolerance assays (for example, AT-2). Although it is possible that the oral challenges may have induced tolerance to SIV, we observed no impact of the number of oral challenges on viral load or on the magnitude of the antigen-specific responses. Additionally, it is possible that the administration of SIV antigens between weeks 4 and 12 postnatal age could have hindered our ability to observe the direct impact of in utero exposure on the outcome of disease after challenge. Further experiments without oral challenges with SIVmac239 or neonatal immunization are currently under way to address each of these possibilities.

It is interesting to consider the observations of this study in the context of mother-to-child transmission of HIV. It will be important to know to what extent exposure of the fetus and/or newborn to HIV alters the developing immune system and the subsequent ability to either clear HIV in the face of effective antiretroviral therapy and/or fend off the virus upon subsequent exposure. This line of inquiry might illuminate a long-standing question in HIV biology: Why is it that so few fetuses are infected with the virus in utero? Might it be the case, for instance, that a strong adaptive immune response is engendered (by the mother and/or the fetus) upon exposure to HIV in utero, resulting in the clearance of the virus before birth? Alternatively, might it be that altered responsiveness to HIV in utero results in less immune activation and slower rates of viral replication and spread, allowing the fetus to eliminate infected cells and to replace them with uninfected counterparts instead?

Cohorts of exposed uninfected children have been reported for a variety of other infectious diseases (*51*). In studies of filarial infections during pregnancy, children born to infected mothers with a high worm burden had reduced cellular responses to filarial antigens and were at higher risk of filarial infection during childhood (*52*). Similarly, studies of children born to mothers who had placental malaria infection during pregnancy showed increased frequencies of T_{regs} associated with decreased malaria-specific cytokine production by T cells (*53*). HIV-exposed uninfected children have been shown to have detectable levels of T cell responses to HIV antigens after in vitro stimulation (*54*, *55*). These data were corroborated in a study by Legrand *et al.* where researchers found a population of antigen-specific T cells in umbilical cord blood from HIV-exposed uninfected children (*56*). This study also found that removal of $CD4^+CD25^+$ T_{regs} resulted in a significant increase in antigen-specific $CD4^+$ and $CD8^+$ T cell responses, revealing the presence of a circulating population of T_{regs} that suppressed HIV-specific immune responses (*56*).

These data suggest that prenatal exposure can lead to the induction of tolerance to infectious pathogens in neonates. However, it remains unclear how immunologic tolerance to HIV may influence the outcome of subsequent HIV infection. Perhaps, the outcome would be similar to the lack of disease progression observed in SIV infection in natural host species. Although other mechanisms, such as the low numbers of CCR5-expressing $CD4^+$ T cells (*57*, *58*), certainly play a role in the lack of disease progression in sooty mangabey infection, the control of immune activation during infection is strongly associated with a lack of disease progression in SIV-infected sooty mangabeys and African green monkeys and has also been noted in the context of HIV infection (*59*–*61*). To date, human cohort studies on the long-term impact of in utero exposure to HIV are lacking.

The study described here was designed to be exploratory in nature and thus was composed of a relatively small number of animals (three to four per group), limiting statistical power to detect small differences in some outcomes between the groups. Additionally, the young age of the

animals provided some challenges in detecting SIV-specific responses, both related to the magnitude of the responses, and because the quantity of PBMCs was limited since small volumes of blood were collected at each time point based on body weight. Despite this, we believe that these studies, although preliminary, provide a relevant and interesting avenue of research to pursue.

Although these studies, like those of Andrieu and colleagues (*34*, *62*), should be viewed as preliminary, it is striking that interventions designed to promote the generation of a tolerogenic immune response are in each case associated with partial or complete protection against pathogenic SIV challenge. If our findings can be confirmed and extended, then one might envisage developing vaccines that induce a tolerogenic response in people at risk for HIV infection. Similar vaccines might be useful against other chronic infectious agents where, as in HIV, pathology and spread of the pathogen are often associated with an activated immune system. Further work on tolerogenic vaccines might find application in the prevention or treatment of inflammatory autoimmune diseases.

MATERIALS AND METHODS

Study design

Female rhesus macaques ($n = 14$) negative for the MHC (major histocompatibility complex) alleles Mamu-A*01 and Mamu-A*02 as well as for Mamu alleles associated with "elite control" of SIV viremia (for example, Mamu-B*08 and Mamu-B*17) (*63*) were time-mated with males who were heterozygous for Mamu-A*01 and Mamu-A*02. Animals were identified as pregnant by ultrasound, using established methods (*64*), and randomly assigned to two treatment groups. Six fetal monkeys (group A) served as sham controls, and eight fetal monkeys (group B) were injected with a nonpathogenic molecular SIV clone, SIVmac1A11, under ultrasound guidance during the late first trimester. Newborns were then delivered by cesarean section at term (160 ± 2 days gestation; term 165 ± 10 days) (Fig. 1A). To specifically explore the hypothesis that prenatal exposure to SIV might induce tolerance to SIV antigens, because of clonal deletion, the induction of antigen-specific regulatory cells, and/or the generation of antigen-specific anergy, half of the animals in each group were exposed intramuscularly to a DNA plasmid encoding SIV Env at 4 weeks after birth, followed by intramuscular injections of an LV expressing SIV Env at 8 and 12 weeks after birth (LV-Env) (groups A2 and B2, Fig. 1B). All of the monkeys were then orally challenged (at postnatal week 16) with pathogenic SIVmac239 (10^5 $TCID_{50}$). When only 1 of the 14 monkeys was found to be productively infected (even after repeated oral challenges), the remaining 13 monkeys (including 6 in the control group A and 7 in the experimental group B) were challenged intravenously with SIVmac239 (100 $TCID_{50}$) at an age range of 37 to 57 weeks. In addition to monitoring viral loads and $CD4^+$ T cell counts, measures of immune phenotype and function were collected over time. An outline of the study is shown in Fig. 1A.

Rhesus monkeys

All animal procedures conformed to the requirements of the Animal Welfare Act, and protocols were approved before implementation by the Institutional Animal Care and Use Committee at the University of California, Davis. Activities related to animal care were performed according to California National Primate Research Center (CNPRC) standard operating procedures. Newborns were delivered by cesarean section at term (160 ± 2 days gestation; term 165 ± 10 days) using standardized protocols, then nursery-reared through 3 months postnatal age. Infant health, food intake, and body weights were recorded daily in the nursery and then on a regular basis when moved into juvenile housing and according to established protocols. Blood samples (~3 to 6 ml, dependent on age) were collected from a peripheral vessel to monitor complete blood counts, clinical chemistry panels, and viral loads after inoculation. At defined time points, an aliquot (1 to 3 ml) was also used for immunologic assays.

SIV antigens

Lentiviral vectors expressing the ectodomain of the SIV envelope protein (gp140) were produced using plasmids by the Penn Vector Core, University of Pennsylvania, Philadelphia, PA. A second plasmid containing a gene insert for SIV gp140 was used for naked DNA injections (SIV DNA). In vitro assays were stimulated with whole, AT-2–inactivated SIVmac239 or microvesicle control (MV) (Biological Products Core, AIDS and Cancer Virus Program, Leidos Biomedical Research Inc., Frederick National Laboratory), or SIVmac239 15-mer peptide pools corresponding to p27 (Gag) or gp120 (Env) proteins [National Institutes of Health (NIH) AIDS Reagent Resource Program].

Immunizations

In the late first trimester, eight fetuses were injected under ultrasound guidance using an intraperitoneal approach with nonpathogenic SIVmac1A11 (100 $TCID_{50}$) and established methods. Controls were administered PBS. Beginning at 4 weeks postnatal age, four infants that had received SIVmac1A11 prenatally and three sham controls were immunized with SIV plasmid DNA (1.4 mg, IM). At 8 and 12 weeks postnatal age, they were administered the LV-Env (LV#1, LV#2) (~10^7 infectious units) (Fig. 1A). A summary of the treatment groups is shown in Fig. 1B.

Virus preparations

All virus stocks were obtained through the CNPRC Immunology and Pathogen Detection Resources Core, prepared and titered by endpoint dilution in CEMx174 cells according to standard protocols (*65*), and stored frozen at ≤−135°C until use. Aliquots were thawed immediately before inoculation.

Virus challenge

At 16 weeks postnatal age, all animals were challenged orally with 1 ml of SIVmac239 (10^5 $TCID_{50}$). Only one animal (group B2) had detectable plasma viral loads after infection. Consequently, all remaining animals were injected intravenously with SIVmac239 (100 $TCID_{50}$) at a range of 37 to 57 weeks.

Sample preparation

Freshly isolated peripheral blood was spun at 300*g* in a benchtop centrifuge to separate cells from plasma. The plasma fraction was removed, spun again at 500*g* to pellet any contaminating cells, placed in aliquots, and frozen at ≤−80°C. PBMCs were isolated from the cellular fraction by diluting samples 1:2 in PBS, layered onto Ficoll-Hypaque (Sigma), and centrifuged at 800*g* for 20 min. The leukocyte layer was removed by pipette and diluted in PBS containing 2% fetal bovine serum (FBS), and cells were pelleted by centrifugation at 350*g* for 5 min. Cells were washed twice with PBS containing 2% FBS and

resuspended in fresh RPMI 1640 supplemented with 10% FBS, 2 nM L-glutamine, and penicillin (100 U/ml) and streptomycin (R10), and left overnight at 4°C.

ELISpot assay

ELISpot plates (Millipore) were washed with PBS and coated with anti-IFNγ capture antibody (clone GZ-4, Mabtech, 16 μg/ml) for 1 hour at room temperature. Plates were washed four times with PBS and blocked with R10 medium for 1 hour at 37°C and 5% CO_2. AT-2 SIV (10 μg capsid/ml), MV (dose matched to total protein content of AT-2 antigen), p27 peptides (10 μg/ml), or gp120 peptides (10 μg/ml) were added in triplicate wells, along with the costimulatory antibodies, CD28 (BD Biosciences, 4 μg/ml) and CD49d (BD Biosciences, 4 μg/ml). Cells (1×10^5 per well) in R10 medium were added, and plates were incubated at 37°C and 5% CO_2 for 16 to 18 hours. A mixture of PMA (20 ng/ml) and ionomycin (1 μg/ml) was used as a positive control. Plates were then washed twice in PBS, washed two more times in PBS with 0.05% Tween 20 (PBST), and incubated with a biotinylated anti-IFNγ secondary antibody (clone 7B6-1, Mabtech, 1 μg/ml) and incubated for 1 hour at 37°C and 5% CO_2. Plates were next washed twice in PBST and incubated with streptavidin–alkaline phosphatase for 1 hour at room temperature, washed with PBST, and soaked in a bath of PBST for 1 hour at room temperature. PBST was then removed, and spots were developed with Vector Blue substrate in the dark for 5 to 15 min, after which the reaction was stopped by rinsing plates with water. When plates were dry, spots were counted using an S5 Analyzer (CTL Analyzers LLC). Results were reported as spot-forming cells (SFC) per million PBMCs after subtraction from background (DMSO-only wells).

Phenotypic analysis of lymphocyte populations

Freshly isolated PBMCs (5×10^5 cells) were surface stained with the viability dye, Aqua amine reactive dye (Invitrogen), as well as monoclonal antibodies directed against CD3 (clone SP 34-2, BD Biosciences), CD4 (NIH Nonhuman Primate Reagent Resource Program), CD8 (clone 3B5, Invitrogen), CD25 (clone M-A251, BD Biosciences or clone 4E3, Miltenyi), CD95 (clone DX2, BD Biosciences) or CD45RA (clone 2H4, Beckman Coulter), CD27 (clone M-T271, BD Biosciences) or CD28 (clone CD28.2, BD Biosciences), and CD127 (clone hIL-7R-M21, BD Biosciences) for 20 min at room temperature, fixed, and then permeabilized with Affymetrix FoxP3 Fix/Perm Buffers (Affymetrix) per the manufacturer's instructions. Permeabilized cells were then stained intracellularly for Ki-67 (clone B56, BD Biosciences) and FoxP3 (clone PCH101, Affymetrix) for 30 min at 4°C, washed in PBS–2% FBS, and analyzed by flow cytometry. A minimum of 150,000 events were acquired on a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.).

T_{reg} depletion assays

T_{reg} cells were depleted from PBMC cultures using anti-CD25–labeled paramagnetic microbeads and MS columns, according to the manufacturer's instructions (Miltenyi Biotec).

Antibody production

Antigen-specific antibody production was measured by enzyme-linked immunosorbent assay (ELISA); 96-well ELISA plates (Nunc Inc.) were coated overnight with AT-2 SIV (1 μg capsid/ml) or PBS. Plates were then washed three times with PBST and blocked with blocking buffer (PBS containing 2.5% bovine serum albumin) for 1 hour at room temperature. Frozen plasma samples were thawed, and eight 4-fold serial dilutions were made in blocking buffer. Diluted plasma (100 μl) was then added to duplicate wells and incubated at room temperature for 2 hours. Wells were washed three times in PBST and incubated with an anti-nonhuman primate IgG secondary antibody conjugated to horseradish peroxidase (12.5 μg/ml; Rockland Immunochemicals). Plates were then incubated for 1 hour at room temperature and developed using a TMB substrate kit (BD Biosciences), and the reaction was stopped with dilute sulfuric acid (2N). Plates were read at 450 nm on an ELISA plate reader and at 690 nm for the background. Midpoint titers were calculated from sigmoidal dilution curves using Prism Software (GraphPad Inc.).

Predictors of viral load

Regression analyses were used to examine potential associations of log-transformed plasma viral load measurements with a panel of 192 clinical and immunologic parameters. Measurements were taken from each animal at multiple time points after infection; because measurements from the same animal are more likely to be correlated than measurements taken from different animals, we used generalized estimating equations techniques to conduct repeated-measures regression analyses. Generalized estimating equations have the effect of adjusting SEs to account for both within-animal and between-animal variability, while averaging over all animals to generate mean estimates of the outcomes. We limited the analyses to time points following the viral load set point (4 weeks after infection), the time at which viral load measurements were observed to increase and diverge between the two groups. Because this analysis was exploratory, adjustments were not made for multiple comparisons.

Statistical analyses

Two-sided *t* tests (results of which appear in Fig. 2) were performed with GraphPad Prism version 5.0 (GraphPad Software, www.graphpad.com). Longitudinal analyses and associations with viral load were generated using R software (http://CRAN.R-project.org) with a generalized estimating equations package (http://cran.r-project.org/web/packages/gee/index.html). Linear regression models were generated in R using ln commands. Linear mixed-effects regression was generated in R using lmer commands. *P* values for lmer analysis were generated using the likelihood ratio test by generating two lmer models with log plasma RNA viral load as an outcome and including the random effects of animal ID and time point after infection with or without the fixed effect of T_H17 cell frequency. The two models were then compared using analysis of variance (ANOVA) to assess how T_H17 cell frequency helps to explain the variability in plasma RNA viral load. Significance was defined as $P < 0.05$.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Animals exposed to SIVmac1A11 in utero show no difference in the frequency of T_{regs} after infection.

Fig. S2. $CD8^+$ T cell subsets associated with viral load are not different between groups.

Fig. S3. Clinical measurements associated with viral load are not different between groups.

Fig. S4. Animals exposed to SIVmac1A11 in utero have a lower frequency of IL-2–producing $CD4^+$ T cells after infection.

Fig. S5. Animals exposed to SIVmac1A11 in utero have reduced humoral responses after infection.

Fig. S6. T_H17 cells are directly correlated with $CD4^+$ but not $CD8^+$ T cell proliferation at multiple time points after infection.
Fig. S7. Splenocytes from animals exposed to SIVmac1A11 in utero have lower expression of exhaustion markers on $CD8^+$ T cells.
Table S1. Residual SIVmac1A11 nucleic acid was found in lymph nodes postnatally.
Reference (*66*)

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Exposure to SIV in utero results in reduced viral loads and altered responsiveness to postnatal challenge

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Getting an early start in HIV protection

HIV disease is characterized by a state of chronic immune activation that appears to contribute to the dysfunction of the immune system. Control of immune activation in infected patients may help to protect against immune dysfunction and delay the progression of the disease. In a new study, Baker *et al*. exposed fetal rhesus macaques to simian immunodeficiency virus (SIV), a close relative of HIV, during a prenatal period, hypothesizing that this would cause the macaque's immune system to become less reactive to, or tolerant of, material derived from SIV. When experimentally infected after birth, the authors hypothesized that exposed macaques might mount less aggressive immune responses against the virus, have lower immune activation, and may have less severe disease than unexposed animals. They found that macaques exposed to SIV in utero did not display any direct evidence of tolerance to SIV; however, the animals did have significantly lower viral loads after infection and altered immune responses that were associated with the control of viral replication. This study supports a new avenue for HIV vaccine design.