ADCC Develops Over Time during Persistent Infection with Live-Attenuated SIV and Is Associated with Complete Protection against SIV\textsubscript{mac}251 Challenge

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Abstract

Live-attenuated strains of simian immunodeficiency virus (SIV) routinely confer apparent sterilizing immunity against pathogenic SIV challenge in rhesus macaques. Understanding the mechanisms of protection by live-attenuated SIV may provide important insights into the immune responses needed for protection against HIV-1. Here we investigated the development of antibodies that are functional against neutralization-resistant SIV challenge strains, and tested the hypothesis that these antibodies are associated with protection. In the absence of detectable neutralizing antibodies, Env-specific antibody-dependent cell-mediated cytolysis (ADCC) emerged by three weeks after inoculation with SIV\textsubscript{Δnef}, increased progressively over time, and was proportional to SIV\textsubscript{Δnef} replication. Persistent infection with SIV\textsubscript{Δnef} elicited significantly higher ADCC titers than immunization with a non-persistent SIV strain that is limited to a single cycle of infection. ADCC titers were higher against viruses matched to the vaccine strain in Env, but were measurable against viruses expressing heterologous Env proteins. In two separate experiments, which took advantage of either the strain-specificity of the time-dependent maturation of immunity to overcome complete protection against SIV\textsubscript{mac}251 challenge, measures of ADCC activity were higher among the SIV\textsubscript{Δnef}-inoculated macaques that remained uninfected than among those that became infected. These observations show that features of the antibody response elicited by SIV\textsubscript{Δnef} are consistent with hallmarks of protection by live-attenuated SIV, and reveal an association between Env-specific antibodies that direct ADCC and apparent sterilizing protection by SIV\textsubscript{Δnef}.

Introduction

The development of a vaccine against HIV-1 continues to be hampered by our limited understanding of the types of immune responses needed for protection. Although safety considerations preclude the use of live-attenuated HIV-1 in people [1–5], live-attenuated strains of simian immunodeficiency virus (SIV) afford the most reliable protection achieved to date in non-human primate models, often providing apparent sterilizing immunity against closely related challenge viruses [6–9]. Thus, identifying the immune responses that mediate protection by live-attenuated SIV and understanding how to elicit them by vaccination may provide important insights for the development of a safe and effective HIV-1 vaccine [10].

Antibody, T cell, and innate immunity have evolved to operate synergistically as an integrated system [11–13], and a combination of these immune responses may be necessary for complete protection by live-attenuated SIV. However, the efficacy of at least one of these immune responses increases over time, since animals challenged with pathogenic SIV\textsubscript{mac}251 months after inoculation with live-attenuated SIV are protected from infection, whereas animals challenged at early time points become infected [7,8]. Although live-attenuated SIV elicits virus-specific T cells [14–16], and the quality of these T cell responses may change over time, the frequency of virus-specific CD8\textsuperscript{+} T cells declines after the acute peak of live-attenuated SIV replication [17]. In contrast, antibodies capable of neutralizing virus infectivity develop over time through affinity maturation [18–21]. An essential role for the
Author Summary

Live-attenuated vaccines can prevent simian immunodeficiency virus (SIV) infection upon experimental challenge of rhesus macaques. Although safety considerations preclude vaccinating humans with live-attenuated HIV-1, it may be possible to replicate the types of immunity induced by live-attenuated SIV through an alternative approach. Thus, identifying the immune responses underlying protection by live-attenuated SIV and understanding their induction would provide guidance for HIV-1 vaccine design. An important role for the maturation of virus-specific antibody responses could explain the time-dependent development of protection by live-attenuated SIV. However, antibodies that block the entry of the challenge virus into cells are usually undetectable. Antibodies can also direct the killing of virus-infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC). Here we show that live-attenuated SIV induces progressive increases in ADCC over time, and that the development of these antibodies is dependent upon the persistent replication of the vaccine strain. In two different experiments, the animals immunized with live-attenuated SIV that remained uninfected after pathogenic SIV challenge had higher measures of ADCC than those that became infected. Our results suggest that antibodies contribute to protection by live-attenuated SIV, and that persistent stimulation of antibody responses may be essential for HIV-1 vaccines to induce high ADCC activity.

affinity maturation of antibody responses could account for the time-dependent development of protection by live-attenuated SIV [22]. However, SIVmac251 is inherently resistant to neutralization [23], and antibodies capable of neutralizing this challenge virus are typically undetectable among completely protected animals [8,9]. We therefore reasoned that functions of antibodies other than neutralization may contribute to protection by live-attenuated SIV.

In addition to virus neutralization, the antiviral functions of antibodies include complement fixation and numerous consequences of Fc receptor crosslinking, such as ADCC [13,24–29]. Since ADCC represents a potential effector mechanism and a proxy for other activities of the same antibodies, we developed a novel assay for quantifying the ability of antibodies to direct ADCC. This assay measures ADCC against virus-infected target cells expressing native conformations of the viral envelope glycoprotein (Env), and is therefore more physiologically relevant than methods based on coating target cells with recombinant gp120, gp140, or peptides [30–37]. We used this assay to investigate the induction of antibodies with ADCC activity, and to test the hypothesis that higher ADCC activity against cells infected by the challenge virus is associated with protection. Our results indicate that persistent infection with SIVΔ nef elicits Env-specific ADCC titers that develop over time, are cross-reactive with Env proteins expressed by heterologous SIV strains, are proportional to vaccine strain replication, and are higher among animals protected against SIVmac251 infection.

Results

Time-dependent maturation of antibody responses

Plasma samples collected at longitudinal time points after inoculation with SIVmac239Δ nef were tested for their ability to neutralize SIVmac239 and to direct ADCC against SIVmac239-infected cells. Only four of ten macaques developed neutralizing antibody titers, and these were not detectable until thirteen weeks after inoculation with SIVmac239Δ nef (Figure 1A). In contrast, ADCC titers were detectable in all animals just three weeks after inoculation with SIVmac239Δ nef (Figure 1B). These ADCC titers were Env-specific, since none of the plasma samples had detectable ADCC activity against target cells infected with SHIV89.6P, which expresses the Env protein of HIV-1AF252. To quantify ADCC titers, we calculated the plasma dilution that reduces the luciferase signal from virus-infected cells by 50%, and to measure differences in the extent of target cell elimination over all dilutions tested, we calculated values for the area under the curve (AUC). By both measures, progressive increases in ADCC were observed over 21 weeks. Thus, antibody titers capable of directing ADCC against SIVmac239-infected cells increased over time, but unlike neutralizing antibodies, emerged early and were detectable in all animals.

Neutralizing antibodies were only detectable in the plasma samples with high ADCC titers. A 50% ADCC titer of approximately 10^4 emerged as a threshold, below which neutralization of SIVmac239 was not detectable (Figure 1C). Among all of the plasma samples collected after inoculation with SIVmac239 Δ nef, the odds of detecting neutralization were 1,966-fold higher in 50% ADCC titer (95% CI 1.8 to 2.192,451, P = 0.034), as estimated by logistic regression (Figure 1C). Likewise, there was a trend towards a higher probability of detecting neutralization by plasma with higher AUC values for ADCC (odds ratio: 67.78-fold higher per 1 AUC unit, 95% CI 0.835–5.5 × 10^9, P = 0.054) (Figure 1D). ADCC titers therefore predicted and were correlated with neutralization.

Persistent replication required to elicit high ADCC titers

The contribution of ongoing vaccine strain replication to the development of antibody responses was evaluated by comparing ADCC in animals immunized with SIVmac239Δ nef to ADCC in animals immunized with an SIV strain that is limited to a single cycle of infection. Plasma samples collected two or twelve weeks after a series of inoculations with single-cycle SIV [38] were tested for ADCC against SIVmac239-infected cells (Figure 2A). Since the geometric mean peak viral RNA loads in plasma for SIVmac239 Δ nef and single-cycle SIV were within two-fold of each other, 1.3 × 10^5 and 7.4 × 10^4 copies per ml respectively (Figure 2B), differences in antibody responses relate to differences in the persistence of SIVmac239Δ nef versus single-cycle SIV. Five weeks after inoculation with SIVmac239Δ nef, median 50% ADCC titers were 51-fold higher than those elicited by single-cycle SIV, and this difference expanded to 233-fold by week 21 (Figure 2C). The 50% ADCC titers (Figure 2C) and the AUC values for ADCC (Figure 2D) at any time point after inoculation with SIVmac239Δ nef were significantly higher than at either time point after inoculation with single-cycle SIV (2-tailed Mann-Whitney U tests, P = 0.0062 to P<0.0001). Thus, in contrast to persistent infection with SIVmac239Δ nef, repeated stimulation of antibody responses with SIV limited to a single cycle of infection did not elicit high ADCC titers.

ADCC titers measured after immunization with SIVmac239Δ nef and single-cycle SIV were compared with antibody titers that bind recombinant forms of SIVmac239 Env in enzyme-linked immuno-adsorbent assays (ELISAs). Relationships among these measures of Env-specific antibody responses were evaluated by calculating Spearman correlation coefficients (Rs). ELISA titers against SIVmac239 gp120 correlated with 50% ADCC titers against SIVmac239-infected cells (Rs = 0.8190, P<0.0001) (Figure 2E), and with AUC values for ADCC (Rs = 0.7809, P<0.0001) (Figure 2F). Although a linear relationship was observed between
ADCC and gp120-binding titers in the animals persistently infected with SIVmac239Δnef. Plasma samples from the animals immunized with single-cycle SIV belonged to an out-group, which was displaced towards higher gp120-binding titers, relative to ADCC (Figure 2E and F). ELISA titers against gp140 also correlated with 50% ADCC titers (R_S = 0.9015, P < 0.0001) (Figure 2G), and with AUC values for ADCC (R_S = 0.8836, P < 0.0001) (Figure 2H). However, any displacement of the single-cycle SIV-immunized animals towards higher gp140-binding, relative to ADCC titers, was more subtle than that observed for gp120-binding titers (Figure 2E–H). This may reflect the occlusion of surfaces in gp140 oligomers that are exposed on gp120 monomers [39]. In comparison to persistent infection with SIVmac239Δnef, immunization with single-cycle SIV may therefore stimulate a higher proportion of gp120-specific antibodies with low or undetectable ADCC activity against virus-infected cells, due to recognition of epitopes that are occluded in the native Env trimer.

Recognition of heterologous Env proteins

The ADCC activity against SIV strains that were matched or mismatched with the vaccine strain in Env was compared. Sera were collected from twelve macaques inoculated with SIVmac239Δnef (Figure 3A), and twelve inoculated with a recombinant form of SIVmac239Δnef containing the env gene of SIVsmE543-3 [40], designated SIVmac239Δnef/E543-3env (Figure 3B). Sera from all 24 animals were tested for ADCC activity against target cells infected with SIVmac239Δnef.
with SIVmac239 or SIVmac239/E543-3 env. On average, the 50% ADCC titers were seven-fold higher when the vaccine and test viruses were matched in Env than when they were mismatched (2-tailed Wilcoxon matched pairs test, P < 0.0001). The 50% ADCC titers were also approximately seven-fold higher at week 22 than at week 6 (2-tailed Wilcoxon matched pairs test, P < 0.0001). Thus, the 50% ADCC titers against the Env-matched virus at week 6 and the Env-mismatched virus at week 22 were comparable. Therefore, ADCC titers against Env-mismatched viruses were lower and required more time to develop than ADCC titers against Env-matched viruses.

ADCC activity is proportional to the extent of vaccine strain replication

The extent of vaccine strain replication was estimated by calculating AUC values for log_{10} transformed SIVΔ nef viral RNA loads in plasma over the first 21 or 22 weeks after inoculation. AUC values for viral loads among animals inoculated with SIVmac239Δ nef and SIVmac239Δ nef/E543-3 were similar, averaging 65 and 67 log_{10} transformed RNA copies per ml×weeks, respectively. The extent of vaccine strain replication by the end of this time period correlated with 50% ADCC titers against Env-matched (RS = 0.68, P < 0.0001) and Env-mismatched (RS = 0.55, P = 0.006) viruses (Figure 4A), and also with AUC values for ADCC (RS = 0.64, P < 0.0001) and Env-mismatched viruses at week 22 were comparable. Therefore, ADCC titers against Env-mismatched viruses were lower and required more time to develop than ADCC titers against Env-matched viruses.

Higher ADCC among animals uninfected after SIVmac251 challenge

Twelve animals were challenged intravenously with SIVmac251NE 46 weeks after inoculation with SIVΔ nef. The SIVΔ nef strain in six of these twelve animals was SIVmac239Δ nef, whereas the other six were inoculated with SIVmac239Δ nef/E543-3. All twelve of these animals resisted two intravenous challenges with SIVmac239 on weeks 22 and 33, while three naive control animals challenged on

Figure 2. ADCC titers elicited by SIVmac239Δ nef versus single-cycle SIV. Plasma samples collected on weeks 2 and 12 after a series of inoculations with single-cycle SIV were titrated for ADCC against SIVmac239-infected cells (A). Target cells infected with SHIVSF162P3 served as a negative control (gray). Dashed lines indicate 50% activity. Geometric mean vaccine strain viral loads reflecting virus particles produced in vivo after inoculation with SIVmac239Δ nef or single-cycle SIV are shown (B). Animals in Group A were inoculated 3 times with single-cycle SIV that was trans-complemented with the vesicular stomatitis virus glycoprotein (VSV G), whereas the animals in Group B were inoculated 6 times with single-cycle SIV that was not trans-complemented [38]. The 50% ADCC titers (C) and the AUC values for ADCC (D) elicited by SIVmac239Δ nef were significantly higher than those elicited by single-cycle SIV (2-tailed Mann-Whitney U tests, P = 0.0062 to P < 0.0001). Binding titers measured by ELISA against SIVmac239 gp120 were correlated with 50% ADCC titers (E), and with AUC values for ADCC (F). Binding titers against SIVmac239 gp140 were also correlated with 50% ADCC titers (G), and with AUC values for ADCC (H). doi:10.1371/journal.ppat.1002890.g002
week 22 and two challenged on week 33 all became infected. When
the twelve SIVΔnef-immunized animals were subsequently re-
challenged with SIVmac251NE on week 46, three became infected,
as did both naive control animals challenged at the same time.

Although all three immunized animals that became infected were
among those inoculated with SIV mac239Δnef/E543-3env, the trend
toward more infections in this group was not significant (2-tailed
Fisher’s exact test, P = 0.18). Comparisons of SIVΔnef viral loads for
animals that became infected versus remained uninfected, or animals
immunized with SIV mac239Δnef versus SIVmac239Δnef/E543-3env,
did not reveal significant differences (Figure S1A–D). Likewise, sera
collected on the day of challenge from the animals that remained
uninfected by SIVmac251NE did not have significantly higher binding
titers against gp120 (2-tailed Mann-Whitney U test, P = 0.2091)
(Figure S1E), or gp140 (2-tailed Mann-Whitney U test, P = 0.3727)
(Figure S1F), in comparison to the animals that became infected.

Sera drawn the day of intravenous challenge with SIV mac251NE
were tested for neutralization of SIV mac251NE and for ADCC
against SIV mac251NE-infected cells. Neutralizing antibody titers
were low to undetectable (Figure 5A), and differences among the
infected versus uninfected animals were not significant at the
highest serum concentration tested, a 1:8 dilution (2-tailed Mann-
Whitney U test, P = 0.3727). However, all of these samples had
measureable ADCC activity (Figure 5B). Whereas differences in
50% ADCC titers were not significant (Figure 5C), the animals
that remained uninfected by SIV mac251NE had higher AUC values
for ADCC than those that became infected (2-tailed Mann-
Whitney U test, P = 0.0091) (Figure 5D). Thus, more complete
elimination of the SIVmac251NE-infected target cells, as measured
by AUC values for ADCC, was associated with protection against
infection by intravenous challenge with SIV mac251NE.

To address the temporal association between the development of
antibody responses and protective immunity, we studied animals
that were challenged at different time points after inoculation with
SIV mac239Δnef. Groups of six female macaques were challenged by
high-dose vaginal inoculation with SIVmac251UCD at weeks five,
twenty, or forty after immunization with SIVmac239Δnef/Reeves et

Figure 3. ADCC against viruses matched or mismatched to the vaccine strain in Env. Sera drawn 0, 6, or 22 weeks after inoculation with SIVmac239Δnef (A) or with the recombinant vaccine strain SIVmac239Δnef/E543-3env (B) were tested for ADCC against target cells infected with SIVmac239 (black), SIVmac239/E543-3env (green), or SHIVSF162P3 (gray). Dashed lines indicate 50% activity.
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Figure 4. Relationship between the extent of vaccine strain replication and ADCC activity. The extent of SIVmac239Δnef or SIVmac239Δnef/E543-3env replication was estimated from the area under the curve (AUC) of log_{10}-transformed vaccine strain viral loads over weeks 0 through 21 or 22, and compared to ADCC activity at week 21 or 22. Vaccine strain viral load AUC values were correlated with 50% ADCC titers (A) against Env-matched (R_s = 0.68, P<0.0001) and Env-
mismatched (R_s = 0.55, P = 0.006) SIV strains, and also with AUC values for ADCC activity (B) against Env-matched (R_s = 0.64, P<0.0001) and Env-
mismatched (R_s = 0.42, P = 0.0421) SIV strains. Linear regression lines are drawn.
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al., manuscript in preparation). All six animals challenged at week five became infected, as did three of six animals challenged at week twenty, and four of six animals challenged at week forty. Three naïve control animals challenged at each time point all became infected, except one animal challenged at week twenty. Peak SIVmac239Denv viral loads were unrelated to the outcome of challenge (Figure S2A). Although the total extent of vaccine strain replication, as estimated from AUC values for SIVmac239Denv viral loads, tended to be higher among the animals that remained uninfected compared to those that became infected, this trend was not significant (2-tailed Mann-Whitney U test, P = 0.0939) (Figure S2B). The animals that remained uninfected by SIVmac251UCD also did not have significantly higher binding antibody titers than those that became infected, as measured by ELISA against gp120 (2-tailed Mann-Whitney U test, P = 0.4304) (Figure S2C) or gp140 (2-tailed Mann-Whitney U test, P = 0.1148) (Figure S2D).

Sera collected on the day of challenge with SIVmac251UCD were evaluated for neutralization of SIVmac251UCD (Figure 6A–C). However, neutralization of SIVmac251UCD was not detectable for any of these serum samples (Figure 6A–C). The absence of detectable neutralizing antibodies is a consequence of the inherent resistance of SIVmac251UCD to neutralization (Figure S3). Indeed, SIVmac251UCD is even more resistant to neutralization than SIVmac239NE by plasma from animals chronically infected with SIVmac239 (2-tailed Wilcoxon matched pairs test, P = 0.0098) (Figure S3A), and by soluble CD4 (Figure S3B). The relative resistance of SIVmac251UCD to antibody may have been a factor in the small number of animals that were protected against infection with this challenge strain.

The capacity of the same sera to direct ADCC against SIVmac251UCD-infected cells was evaluated (Figure 6D–F). In contrast to neutralization, all had measurable ADCC activity (Figure 6D–F). Statistically significant outcomes could not be reached at individual time points, or for a group of animals that combines just those challenged on weeks twenty and forty (Table S1). However, when the animals challenged five, twenty and forty weeks after inoculation with SIVmac239Denv were analyzed together, those that remained uninfected had higher 50% ADCC titers on the day they were challenged than those that became infected (2-tailed Mann-Whitney U test, P = 0.0487) (Figure 6G). A similar trend was observed for AUC values for ADCC, although these differences were not significant (Figure 6H). Also, among the animals that became infected, there was a trend towards higher ADCC activity in animals with lower peak SIVmac251UCD viral loads (Figure 6I and J). Therefore, higher 50% ADCC titers present at later challenge time points after inoculation with SIVmac239Denv were associated with protection against infection by high-dose vaginal challenge with SIVmac251UCD.

**Discussion**

Identifying the immune responses that mediate protection by live-attenuated SIV and understanding their induction may inspire strategies for engineering a safe and effective vaccine against HIV-1. We hypothesized that antibody functions other than neutralization contribute to the protective immunity provided by live-attenuated SIV against wild-type pathogenic SIV challenge. Here we demonstrate that properties of the antibody response reflected...
in ADCC titers mirror hallmarks of protection by live-attenuated SIV. The protective immunity conferred by live-attenuated SIV increases over time [7,8], is usually incomplete against heterologous challenge [41,42], and is greater for vaccine strains that replicate at higher levels [7,9]. In accordance with these observations, our data indicate that ADCC titers increase progressively over time, are lower against viruses expressing heterologous Env proteins, and are proportional to the extent of vaccine strain replication. Furthermore, in two different challenge experiments, measures of ADCC activity were associated with protection against infection by SIV\textsubscript{mac251}.

In one experiment, macaques inoculated with SIV\textsubscript{mac239}\textsubscript{Δ nef} or SIV\textsubscript{mac239}\textsubscript{Δ nef/E543-3mw} that remained uninfected after intravenous challenge with SIV\textsubscript{mac251\textsubscript{NE}} had higher AUC values for ADCC than those that became infected. In another experiment, animals that remained uninfected after high-dose vaginal challenge with SIV\textsubscript{mac251\textsubscript{UCD}} had higher 50% ADCC titers than those that became infected (2-tailed Mann-Whitney U test, P = 0.0487) (G). A similar but non-significant trend was observed in AUC values for ADCC (2-tailed Mann-Whitney U test, P = 0.0761) (H). Non-significant trends were in the direction of lower peak SIV\textsubscript{mac251\textsubscript{UCD}} viral loads for animals with higher 50% ADCC titers ($R_S = -0.4615$, $P = 0.1124$) (I) and higher AUC measurements of ADCC activity ($R_S = -0.4560$, $P = 0.1173$) (J).

Linear regression lines are shown. The macaque with the lowest ADCC titers among those remaining uninfected was the only animal possessing the protective combination of MHC class I alleles Mamu-A*01 and -B*17 [70].

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Figure 6. Neutralization and ADCC on the day of high-dose vaginal challenge with SIV\textsubscript{mac251\textsubscript{UCD}}. At 5, 20, or 40 weeks after inoculation with SIV\textsubscript{mac239}\textsubscript{Δ nef}, groups of 6 macaques each were challenged by high-dose vaginal inoculation with SIV\textsubscript{mac251\textsubscript{UCD}}. Serum collected the day of challenge was evaluated for neutralization of SIV\textsubscript{mac251\textsubscript{UCD}} (A–C) and ADCC against SIV\textsubscript{mac251\textsubscript{UCD}}-infected cells (D–F). Symbols appear in color for immunized macaques that remained uninfected by SIV\textsubscript{mac251\textsubscript{UCD}}, and in black for the immunized animals that became infected. Target cells infected with SHIV\textsubscript{SF162P3} served as a negative control for ADCC assays (gray). Dashed lines indicate 50% activity. The groups challenged on weeks 5, 20, and 40 were combined for statistical analysis (G–J). The SIV\textsubscript{mac239}\textsubscript{Δ nef}-immunized macaques remaining uninfected by SIV\textsubscript{mac251\textsubscript{UCD}} had higher 50% ADCC titers than those that became infected (2-tailed Mann-Whitney U test, $P = 0.0487$) (G). A similar but non-significant trend was observed in AUC values for ADCC (2-tailed Mann-Whitney U test, $P = 0.0761$) (H). Non-significant trends were in the direction of lower peak SIV\textsubscript{mac251\textsubscript{UCD}} viral loads for animals with higher 50% ADCC titers ($R_S = -0.4615$, $P = 0.1124$) (I) and higher AUC measurements of ADCC activity ($R_S = -0.4560$, $P = 0.1173$) (J). Linear regression lines are shown. The macaque with the lowest ADCC titers among those remaining uninfected was the only animal possessing the protective combination of MHC class I alleles Mamu-A*01 and -B*17 [70].

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SIV\textsubscript{mac}251\textsubscript{NE} (46 weeks) than SIV\textsubscript{mac}251\textsubscript{UCD} (5, 20, and 40 weeks), and the effect of mismatches in Env between SIV\textsubscript{mac}239 Δ\textsubscript{ nef}/E543-3\textsubscript{env} and SIV\textsubscript{mac}251\textsubscript{NE}, may also have contributed to the detection of differences in AUC values for ADCC against SIV\textsubscript{mac}251\textsubscript{NE}. Although differences between the two SIV\textsubscript{mac}251 challenge experiments may have favored one method of data analysis over the other, measures of ADCC activity were associated with complete protection in both experiments.

While the relationship between ADCC activity and the outcome of challenge suggests that these antibodies contribute to protection, correlation does not establish causation. In addition to ADCC, Fc receptor crosslinking stimulates the secretion of molecules that promote lymphocyte homing and activation, and that may inhibit virus replication [26,27]. The antibodies that direct ADCC may also mediate effector functions through complement fixation [25,28]. Furthermore, ADCC assays may measure antibodies that block virus infection at concentrations present \textit{in vivo}, but are undetectable using conventional neutralization assays. Therefore, although ADCC may be an important effector mechanism [43], ADCC could also be a surrogate for other effector mechanisms that contribute to protection. Mechanisms of immunity not mediated by antibodies may also covary with ADCC activity. For instance, T cell, antibody, and innate immune responses may all be affected by the extent of antigenic stimulation. It is conceivable that the observed relationships are due to differences that exist among animals inoculated with SIV\textsubscript{mac}239Δ\textsubscript{ nef} versus SIV\textsubscript{mac}239Δ\textsubscript{ nef}/E543-3\textsubscript{env}, or among animals challenged five, twenty, and forty weeks after inoculation with SIV\textsubscript{mac}239Δ\textsubscript{ nef}. Thus, while our findings implicate antibodies in protection by live-attenuated SIV, they do not preclude a role for other immune responses.

More than one type of immune response elicited by live-attenuated SIV may be necessary for complete protection against SIV\textsubscript{mac}251 challenge. Passive transfer experiments in different live-attenuated SIV vaccine models have yielded mixed results regarding the ability of antibodies alone to protect against SIV infection, demonstrating complete protection in one study [44], and no protection in another [45]. In contrast to the absence of detectable neutralizing antibodies in the completely protected animals in this study, relatively high concentrations of neutralizing monoclonal antibodies were necessary to protect macaques against SHIV infection in passive transfer experiments [43,46–48]. T cell responses present in macaques inoculated with SIVΔ\textsubscript{ nef} [14–16], but absent in macaques that received antibodies passively, may help to explain these differences in neutralizing antibody titers required for complete protection.

Our observations are in agreement with other reports that have associated antibody responses with protection. The Robert-Guroff laboratory, and others, have associated lower viral loads after infection with higher ADCC activity measured using target cells that were coated with monomeric gp120 [32,36,37], recombinant gp140 [33], or infected with T cell line-adapted SIV [49]. However, in these studies, ADCC was not associated with protection from infection, or measured using target cells infected with neutralization-resistant viruses. Nevertheless, antibodies that bound recombinant forms of gp120 by ELISA and that neutralized neutralization-sensitive SIV strains were associated with a reduced rate of infection [37]. Consistent with these observations on vaccine protection, a recent study on mother-to-child transmission of HIV-1 found that the breast milk of mothers whose newborns remained uninfected contained antibodies with higher ADCC activity against gp120-coated target cells [50]. In the context of vaccination with different live-attenuated strains of SIV, antibody avidity was also associated with resistance to infection and lower post-challenge viral loads after vaginal challenge with SIV\textsubscript{mac}251\textsubscript{NE} [9]. Likewise, neutralization of SIV\textsubscript{mac}251\textsubscript{NE} at a 1:4 dilution of serum was associated with protection in a combined group of animals that remained uninfected or strongly controlled SIV\textsubscript{mac}251\textsubscript{NE} viral loads [7]. Taken together, these studies support a role for antibodies in protective immunity.

Interest in antibody functions other than neutralization has recently increased as a result of the RV144 trial, in which a modest reduction in the rate of HIV-1 infection was reported among recipients of a recombinant canarypox vector prime and gp120 protein boost vaccine [31]. Virus-specific CD8\textsuperscript{T} cell responses were not measurably different between vaccinated and unvaccinated trial participants. Whereas antibodies capable of neutralizing primary HIV-1 isolates were also undetectable among vaccinated individuals, gp120-binding titers were consistently detectable by ELISA. Functions of antibodies other than neutralization have therefore been postulated to potentially be responsible for protection in the RV144 trial [52]. Among six primary variables in the immune correlates analysis of the RV144 trial, IgG titers to the V2 region of gp120 were associated with protection, whereas Env-specific IgA antibodies were associated with a higher risk of infection [53]. There was also a non-significant trend towards a lower risk of HIV-1 infection among vaccine recipients with higher ADCC activity using the assay described here. This relationship reached borderline statistical significance after excluding subjects with Env-specific IgA in plasma [53]. These observations further support a role for antibodies in vaccine protection against immunodeficiency virus infection.

Persistent expression of Env may be essential to elicit protective antibody responses. The progressive increases in ADCC activity over time, and the considerably higher ADCC activity elicited by SIV\textsubscript{mac}239Δ\textsubscript{ nef} versus single-cycle SIV, imply that the persistent antigenic stimulation provided by ongoing SIV\textsubscript{mac}239Δ\textsubscript{ nef} replication is important for the development of high ADCC titers. Differences in the maturation of antibody responses may also contribute to the better protection provided by SIVΔ\textsubscript{ nef} in comparison to single-cycle SIV [38]. Furthermore, a longer period of persistent infection with SIVΔ\textsubscript{ nef} was required for ADCC titers against SIV strains expressing heterologous Env proteins to reach the levels observed at an earlier time point against the Env-matched strain. Persistent Env expression may therefore be required to elicit antibodies with high and broadly reactive ADCC activity against circulating HIV-1 strains with diverse neutralization-resistant Env proteins.

A vaccine against HIV-1 must contend with a degree of sequence variation that typically renders neutralizing sera ineffective against heterologous HIV-1 strains isolated from other people [19,54]. The Env proteins of SIV\textsubscript{mac}239 and SIV\textsubscript{mac}E543-3 differ in amino acid sequence by 18%, which approximates the median difference between the Env proteins of individual HIV-1 isolates within a clade [34,55]. Therefore, the ADCC titers observed against cells infected with neutralization-resistant Env-mismatched target viruses suggest that antibodies may have broader efficacy against heterologous HIV-1 isolates than is generally revealed by neutralization assays.

In summary, we show that properties of the antibody response elicited by SIVΔ\textsubscript{ nef} mirror hallmarks of protection by live-attenuated SIV, and that ADCC activity is associated with apparent sterilizing protection against SIV\textsubscript{mac}251. These observations support a role for antibodies in protection by live-attenuated SIV, despite the paradoxical absence of detectable neutralizing antibody titers against the challenge virus in most fully
protected animals. The temporal analyses of ADCC activity against both Env-matched and Env-mismatched viruses, and the significantly higher ADCC titers observed in SIVΔnef-infected animals than in animals repeatedly immunized with single-cycle SIV, suggest that persistent Env expression may be necessary to drive the maturation of high-titer, broadly reactive antibody responses. Therefore, strategies designed to persistently stimulate Env-specific antibodies may significantly improve the efficacy of vaccines against HIV-1.

Materials and Methods

ADCC assay

Due to the variability and limited scalability of assays dependent upon primary cells, we engineered a pair of cell lines to serve as targets and effectors in ADCC assays. The target cells were derived from CEM.NKR-CCR5 5T cells [56,57] (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, contributed by Dr. Alexandra Trkola). These were transduced with a pLNSX-derived retroviral vector to express firefly luciferase under the transcriptional regulation of the SIV LTR promoter [23]. Target cells were maintained in “R10” culture media consisting of RPMI (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.1 mg/ml Primocin (InvivoGen). Effector cells were derived from the CD16-negative human NK cell line KHYG-1 [58] (Japan Health Sciences Foundation) by stable transduction with a pQCXIN-derived retroviral vector expressing rhesus macaque CD16 (FcgRIIA variant 7) [59]. KHYG-I effector cells were maintained at a density of 1x10⁵ to 4x10⁵ cells per ml in R10 media supplemented with IL-2 at 10 U per ml (Roche) and cyclosporine A at 1 µg per ml (Sigma).

Four days prior to each ADCC assay, the target cells were infected by spinoculation [60]. On the day of the assay, infected target cells were washed three times with RPMI media before use. ADCC assays were conducted in round-bottom 96-well plates. Each well contained 10⁵ effector cells and 10⁴ target cells. Effector and target cells were incubated together for eight hours in the presence of triplicate serial two-fold dilutions of serum or plasma before the luciferase activity was measured using the luciferase substrate reagent BriteLite Plus (Perkin Elmer). Relative light units (RLU) indicate luciferase expression by infected target cells. Wells containing uninfected target cells plus effector cells defined 0% RLU, and well containing infected target cells plus effector cells with no serum or plasma defined 100% RLU. The %ADCC activity was defined as 100 minus %RLU.

Antibodies from some macaques bound uninfected CEM.NKR-CR5 cells. To deplete these antibodies, 10⁵ uninfected target cells were resuspended in the sample and incubated for twenty minutes at room temperature. This process was repeated twenty times for the animals immunized with single-cycle SIV, and twelve times for the animals in the Env-mismatch and vaginal challenge studies.

Neutralization assays

Neutralization was measured as previously described [23,61]. The sensitivity of the virus neutralization assay was maximized by minimizing the amount of virus input required to obtain a consistent level of infected C8166-secreted alkaline phosphatase (SEAP) reporter cells. These amounts were 0.5 ng p27 SIVmac239, 5 ng p27 SIVmac251NE, and 0.5 ng p27 SIVmac239ΔUCD per well. However, the resistance of SIVmac251NE, SIVmac251ΔUCD, and SIVmac251ΔTCLA to neutralization by sCD4-IgG was evaluated using 5 ng p27 per well for all 3 viruses. Each well contained 15,000 C8166-SEAP cells. Plasma or serum dilutions were pre-incubated with virus for one hour at 37°C before adding C8166-SEAP cells. After three days, SEAP activity was determined using a luminescent assay (Applied Biosystems).

ELISAs

Recombinant 6-His tagged SIVmac239 gp120 or gp140 protein (Immune Technology) diluted to 0.5 µg/ml in 0.1 M sodium bicarbonate pH 9.5 was coated onto Maxisorb ELISA plates (NUNC). Plates were blocked using PBS containing 0.5% Tween-20 (Sigma) and 5% blocking-grade non-fat dry milk (NFDM) block (BioRad). Antibody dilutions were made in PBS containing 0.5% Tween-20 and 5% NFDM. Bound IgG was detected using a horseradish peroxidase conjugated goat anti-mouse/human IgG antibody (Santa Cruz Biotechnology). The upper limit of a 95% confidence interval calculated using plasma from 10 naive macaques diluted 1:100 served as the endpoint titer [62].

Animals

The animals were Indian-origin rhesus macaques (Macaca mulatta) housed in a biocontainment facility at the New England Primate Research Center (NEPRC), and given care in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. The animal samples used here were collected under experimental protocols approved by the Harvard Medical Area Standing Committee on Animals, and conducted in accordance to the Guide for the Care and Use of Laboratory Animals [63]. Additional analyses using these animals will be published separately by R. C. Desrosiers and by R. K. Reeves.

Viruses

The intravenous SIVmac239 challenge dose used on week 22 consisted of twenty animal-infected doses of virus produced by transfection of 293T cells. The week 33 intravenous challenge with SIVmac239 contained ten animal-infected doses of a rhesus PBMC-derived virus stock used previously [38]. The intravenous SIVmac251NE challenge was ten animal-infected doses (32 pg p27) of a rhesus PBMC stock prepared in February of 1991, used in other studies [7-9]. Vaginal challenges consisted of two inoculations on one day of 1 ml undiluted SIVmac239ΔUCD [64] (100 ng p27), prepared at the California National Primate Research Center in June of 2004. Neutralization and ADCC assays were done using SIVmac239 and SIVmac239/E543-3Δnef produced by transfection of 293T cells, and SIVmac251NE and SIVmac251ΔUCD expanded from the corresponding uncloned challenge stocks in rhesus macaque PBMC. SIVmac251Δnef was also expanded in rhesus PBMC (AIDS Research and Reference Reagent Program, NIAID), NIH, contributed by Drs. Janet Harouse, Cecilia Cheng-Mayer, Ranajit Pal and the DAIMS, NIAID). The rhesus PBMC used to expand virus stocks were depleted of CD8+ cells using Dynal anti-CD8 magnetic beads (Invitrogen), activated with phytohemagglutinin (PHA) (Sigma), and then cultured in IL-2 (Roche). SIVmac239ΔTCLA was grown in MT4 cells.

Plasma viral RNA load measurements

Challenge viruses were detected using primers specific for the nef sequences of SIVmac239 or SIVmac251 within the deletion in SIVmac239Δnef [65]. The primers specific for wild-type SIVmac239

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were GAATACTCCATGGAGAAACCCAGC and ATTCGC-
CATTGGTAACTCATTGCTTTTTCAG, and the labeled probe
had the sequence CTTTTGGCCTCATACTGATACCCTCCTAC. To
reflect the polymorphic nature of the uncloned SIVmac251 virus
stocks, the primer set designed to amplify SIVmac251 contained
degenerate bases P and K, which mimic mixtures of G and T or A
and G, respectively (GlenResearch). The SIVmac251-specific
primers were GAATACTCCATGGAGAAACCCAGC and
TGGCATTGTTAACC,TGTCATTGPTCTTACAG, and the
SIVmac251-specific probe sequence was TAGPAGGGAGAT-
GATGACATTGATGGG. Complete or apparent sterilizing
protection was defined as the absence of detectable wild-type viral
RNA from plasma at every post-challenge time point using the
above primer/probe sets in a real-time RT-PCR assay with a
nominal threshold of detection of 10-30 copies of RNA per ml [66].

Statistical analysis
Fifty percent titers were calculated as the dilution at which a line
connecting the values above and below 50% RLU would intercept
the 50% RLU line. AUC values for ADCC were calculated such
that they would be proportional to 50% ADCC titers, and
represent the areas between 100% RLU and the titration curves as
they appear in the figures. Whereas %ADCC, defined as 100%
minus %RLU, appears asymptotic as it approaches 100%,
minimum %RLU values are inversely proportional to 50%
ADCC titers. Therefore, AUC values for ADCC were calculated
from values for log10100 minus log10%RLU, which were summed
over all dilutions. This sum was multiplied by the log1010-
transformed dilution factor of two to find an area. The ability of
ADCC activity to predict neutralization was evaluated by logistic
regression using SPSS (IBM). The statistical significance of other
comparisons was evaluated in Prism version 4.1b (GraphPad
Software) using 2-tailed Mann-Whitney U tests, 2-tailed Fisher’s
exact tests, 2-tailed Wilcoxon matched pairs tests, and Spearman
regression using SPSS (IBM). The statistical significance of other
comparisons was evaluated in Prism version 4.1b (GraphPad
Software) using 2-tailed Mann-Whitney U tests, 2-tailed Fisher’s
exact tests, 2-tailed Wilcoxon matched pairs tests, and Spearman
correlation coefficients.

Supporting Information
Figure S1 SIVΔnef viral loads and ELISA titers among
animals challenged with SIVmac251ΔNe. There were no
significant differences in vaccine strain viral loads among the
animals that became infected versus those that remained
uninfected after intravenous challenge with SIVmac251ΔNe in terms
of peak log10 RNA copies per ml (2-tailed Mann-Whitney U test,
P = 0.8636) (A), or AUC log10 RNA copies per ml×weeks for the
period of weeks 0–46 after inoculation (2-tailed Mann-Whitney U
test, P = 0.2091) (B). SIVmac251Δnef and SIVmac251ΔNe/E543-
3em did not differ significantly in peak vaccine strain viral loads (2-
tailed Mann-Whitney U test, P = 0.8182) (C), or in AUC values for
vaccine strain viral loads over weeks 0–46 (P = 0.9372) (D). The
outcome of challenge was not significantly related to binding
antibodies measured by ELISA against recombinant SIVmac239
gp120 (2-tailed Mann-Whitney U test, P = 0.2091) (E), or gp140
(2-tailed Mann-Whitney U test, P = 0.3727) (F).

Figure S2 SIVmac251Δnef viral loads and ELISA titers
among animals challenged with SIVmac251ΔUCD. Peak log10
SIVmac251Δnef viral loads appeared unrelated to protection
against infection by SIVmac251ΔUCD (2-tailed Mann-Whitney U
test, P = 0.8437) (A). SIVmac251Δnef AUC log10 RNA copies per
ml×weeks for the period through the day of challenge with
SIVmac251ΔUCD at 5, 20, or 40 weeks after inoculation appeared
higher among the animals that remained uninfected, but this
difference was not significant (2-tailed Mann-Whitney U test,
P = 0.0939) (B). The SIVmac251Δnef-immunized animals that
remained uninfected did not have significantly higher ELISA
titers in sera collected on the day of challenge than those that
became infected against SIVmac239 gp120 (2-tailed Mann-
Whitney U test, P = 0.4304) (C), or gp140 (2-tailed Mann-Whitney U
test, P = 0.1148) (D).

Table S1 Significance of differences among animals
infected versus uninfected after SIVmac251ΔUCD
challenge. Differences among infected versus uninfected animals
in peak SIVmac251Δnef viral loads, total SIVmac251Δnef replication
(estimated from AUC values for log10 RNA copies per ml×weeks),
gp120 ELISA titers, gp140 ELISA titers, 50% ADCC titers, and
AUC values for ADCC were evaluated for significance by 2-tailed
Mann-Whitney U tests. Significance was not determined (ND) for
comparisons that included less than 3 animals in one group.

Author Contributions
Conceived and designed the experiments: MDA RKR RCD RPJ DTE.
Performed the experiments: MDA JDH. Analyzed the data: MDA WL
RPJ RCD DTE. Wrote the paper: MDA DTE. Provided technical
assistance: WAL. Performed the viral load measurements: MP JDL.
Performed veterinary procedures: AC KGM.

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