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Vaccine-Elicited Antibodies Mediate Antibody-Dependent Cellular Cytotoxicity Correlated with Significantly Reduced Acute Viremia in Rhesus Macaques Challenged with SIV_{mac251}[1]

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Effector cells armed with Abs can eliminate virus-infected target cells by Ab-dependent cellular cytotoxicity (ADCC), an immune mechanism that has been largely overlooked in HIV vaccine development. Here, we show that a prime/boost AIDS vaccine approach elicits potent ADCC activity correlating with protection against SIV in rhesus macaques (*Macacca mulatta*). Priming with replicating adenovirus type 5 host range mutant-SIV recombinants, followed by boosting with SIV gp120, elicited Abs with ADCC activity against SIV_{mac251}-infected cells. In vitro ADCC activity correlated with in vivo reduced acute viremia after a mucosal challenge with pathogenic SIV. Our findings expose ADCC activity as an immune correlate that may be relevant in the rational design of an efficacious vaccine against HIV. ***The Journal of Immunology,*** **2005, 174: 2185–2189.**

FcR-bearing cells armed with Abs can kill virus-infected cells by Ab-dependent cellular cytotoxicity (ADCC),[3] an immune mechanism known to occur in vitro and in vivo (1–3). Passive transfer of Abs with in vitro ADCC activity against HSV-infected cells, for example, can protect complement-deficient mice against lethal challenge with HSV, despite the inability of the Abs to neutralize the virus (4). ADCC responses may also affect the efficacy of experimental vaccines against genital herpes (5), influenza virus infections (6), and schistosomiasis (7). In addition, an in vivo protective role of ADCC has been described in a mouse model of genital infection with *Chlamydia trachomatis* (8), suggesting that ADCC can be relevant in protection against sexually transmitted pathogens.

In humans and in non-human primates, the significance of ADCC in protection against immunodeficiency viruses is less clear. Abs with anti-HIV ADCC activity appear early in acute infection, often preceding a neutralizing Ab response (9), but they can also be detected at other stages of disease progression, including asymptomatic infection and AIDS (for reviews, see Refs. 10–12). ADCC activity has been associated with lower viral loads and higher $CD4^+$ T cell counts in subtype B HIV-infected humans (13, 14) and with slower disease progression in SIV-infected rhesus macaques (15). Potent ADCC activity against viral envelope-coated targets has also been observed in sera of HIV-1 infected long-term non-progressors (16) and in some cohorts of healthy children born to HIV-infected mothers (17), suggesting that ADCC may contribute to the in vivo control of viremia during immunodeficiency virus infections. The induction of anti-HIV or anti-SIV ADCC responses would therefore seem a reasonable aim of vaccination, yet this immune response has not been well-addressed in either clinical or preclinical studies. Notably, a significant contribution of ADCC to AIDS vaccine efficacy has not been established in vaccine challenge studies with HIV in the chimpanzee model or with SIV or chimeric simian-HIV in macaques.

We have been pursuing an AIDS vaccine approach that involves priming with replication-competent adenovirus (Ad)-HIV and Ad-SIV recombinant vaccines, followed by boosting with viral envelope protein. In non-human primates, this regimen has elicited potent and long lasting viral-specific immunity in all components of the immune system: humoral, cellular, and mucosal (18–25). The vaccine approach has resulted in protection of chimpanzees against homologous and heterologous i.v. challenge with HIV (18). Protective efficacy has also been observed in rhesus macaques that have been challenged vaginally or rectally with pathogenic SIV (19, 20, 22, 25). In our most recent Ad-SIV/gp120 vaccine efficacy study, a strong level of protection against an intrarectal SIV_{mac251} challenge was achieved (24, 25). Notably, gp120-binding Ab titers and anti-envelope and anti-Rev cellular immune responses significantly correlated with reduced acute and setpoint viral loads, respectively (25). However, the vaccine regimen failed to elicit Abs capable of neutralizing primary $SIV_{mac251,}$ even in protected macaques that had undetectable plasma viremia after the pathogenic challenge. We therefore contemplated the hypothesis that the vaccine-elicited Abs mediated ADCC as an alternative functional immune mechanism of protection. Here we demonstrate that vaccination elicited potent ADCC activity correlating with reduced acute viral loads. Our observations underscore the relevance of ADCC activity in the design of an efficacious vaccine against HIV.

Materials and Methods

Sera and Ab purification

Sera, obtained at the indicated timepoints and stored at −70°C, were studied retrospectively and represented samples from a previous vaccine experiment involving 47 rhesus macaques (Fig. 1*a*; Refs. 24 and 25). Samples were thawed at room temperature, diluted 10-fold in R-10 (RPMI 1640 containing 10% FCS, 2 mM L-glutamine, and antibiotics), and filtered through a 0.2-μm polyethersulfone syringe filter (Nalgene) before a 30-min incubation at 56°C for complement inactivation. Samples were stored at 4°C and used at the indicated dilutions for ADCC assays. In some cases, serum IgG was purified using immobilized protein G-agarose (Pierce Biotech), as recommended by the manufacturer. Rhesus IgG from naive macaques (Nordic) was used as a negative control for ADCC assays using purified IgG.

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[3] Abbreviations used in this paper: ADCC, Ab-dependent cellular cytotoxicity; Ad, adenovirus; Ad5hr, Ad type 5 host range mutant; RFADCC, rapid fluorometric ADCC.

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Target and effector cells

Human cells were used as both target and effector cells to focus on the ability of the rhesus sera to mediate ADCC. It has been shown that rhesus PBMC effector cells, in the absence of serum, can effectively kill target cells pulsed with inactivated SIV or other retroviral envelope proteins (26), greatly decreasing the sensitivity with which ADCC can be measured. We have confirmed this observation, showing killing of SIV-gp120-coated rhesus $CD4^+$ T cells or human CEM-NK^r cells by rhesus PBMC at levels as high as 25 and 40%, respectively. Two different target cells were used. Envelope-coated CEM-NK^r target cells were prepared by incubating 5×10^6 CEM-NK^r cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health) with 15 μg of native SIVgp120 (Advanced Bioscience Laboratories) for 1 h at room temperature, followed by washing twice in ice-cold R-10. Chronically SIV_{mac251}-infected H9 T cell targets were prepared by infecting H9 cells with cell-free SIV_{mac251} (Advanced Bioscience Laboratories) and culturing in R-10 with twice-weekly passage and addition of fresh uninfected H9 cells once per week. The cells were monitored weekly for SIV expression using indirect immunofluorescence assays on fixed cells with a mouse anti-SIV_{mac251} p27 mAb (no. 13-113-100; Advanced Biotechnologies) and on live cells using a mouse anti-SIV gp120 mAb (no. 13-115-100; Advanced Biotechnologies). The secondary Ab was a fluorescein-conjugated goat anti-mouse $F(ab')_2$ (BioSource International). Both assays established that equivalent numbers of SIV Gag- and Env-expressing cells were present in chronically SIV_{mac251}-infected H9 cultures. The cells were routinely monitored for SIV Gag expression and used in the rapid fluorometric ADCC (RFADCC) assay within 24 h of confirming an infected cell frequency of ≥90%.

Human PBMCs were used as effector cells. The PBMCs were isolated by Ficoll-Hypaque density centrifugation of buffy coats obtained from healthy, HIV-seronegative volunteer donors (Department of Transfusion Medicine, Clinical Center, National Institutes of Health) and stored viably frozen until use. The buffy coats, irreversibly anonymized before distribution and a discardable by-product of another type of donation, are exempt from Institutional Review Board review as determined by the Office of Human Subjects Research, Clinical Center, National Institutes of Health.

RFADCC assay

The RFADCC assay was adapted from the fluorometric assessment of T lymphocyte Ag-specific lysis assay (27) and has been described in detail elsewhere.[4] Here, we maintained the dual labeling of target cells with PKH-26 (Sigma-Aldrich), a membrane dye to uniformly label the entire target cell population, and with CFSE (Molecular Probes), a vital dye that is rapidly lost when cell membranes are damaged. In this way, the target cell population could be easily monitored for percentage of lysed cells. The procedure was altered as appropriate to detect ADCC activity, rather than Ag-specific $CD8^+$ T lymphocyte killing as originally designed. Briefly, five million target cells were double-labeled with 2.5×10^{-6} M PKH-26 and 2.5×10^{-6}M CFSE. Effectors and targets were resuspended in R-10 and dispensed into each well of a microtiter plate. CEM-NK^r targets coated with gp120 were used at an E:T ratio of 10:1. SIV_{mac251}-infected H9 cells were used at an E:T ratio of 50:1, except for experiments with purified IgG and those in which the kinetics of induction of ADCC activity was examined where the E:T ratio was 10:1. Purified IgG (100 μl) or serum diluted as indicated was added to duplicate wells, and plates were centrifuged for 3 min at 1000 rpm to promote cell-to-cell interactions. After 4 h at 37°C, the cells were washed once with PBS and fixed in 3.7% paraformaldehyde-PBS (v/v). Non-gated events (25,000–50,000) were acquired within 18 h using a FACSCalibur instrument (BD Biosciences). Flow cytometry data were acquired using CellQuest software. Analysis and density plots were generated with WinMDI 2.0.

Statistical methods

Median acute and setpoint viral loads were calculated as described previously (25). Differences in percent killing between immunized animals and mock-immunized controls at specified times and dilutions were assessed using the Wilcoxon rank sum test. Differences between immunization groups 1–4 were tested using the Kruskal-Wallis test. Correction for multiple comparisons over times and dilutions was made by the Hochberg method. The Wilcoxon signed rank test was applied to the enhancement of percent killing after boosting. Correlations of ADCC (percent killing at the time of challenge at 1/10,000 serum dilution) with viral loads and with binding Ab titers were calculated using Jonckheere-Terpstra trend analysis.

Results

ADCC activity against SIV-infected cells

We pursued a combination AIDS vaccine regimen based on priming rhesus macaques with replicating Ad type 5 host range mutant (Ad5hr)-SIV recombinants, followed by boosting with SIV gp120 (Fig. 1*a*). Sera collected from Ad5hr-SIV/gp120 prime/boosted animals at the time of challenge mediated potent in vitro killing of SIV_{mac251}-infected H9 cells in a dose-dependent fashion (Fig. 1*b*, groups 1–4). In contrast, group 6 control animals had no ADCC activity against SIV-infected H9 cells. The difference in mean ADCC activity for groups 1–4 combined vs that of group 6 controls was highly significant over dilutions of 1/10 to 1/10,000 ($p < 0.00001$) and of 1/100,000 ($p = 0.0056$). Sera from animals in group 5, boosted with a synthetic SIV polypeptide "peptomer" (25) rather than gp120, also exhibited ADCC activity compared with controls, but only at a 1/10 dilution ($p = 0.038$) and were not included in additional experiments.

ADCC activity correlates with efficacy

Although a primary endpoint of AIDS vaccine efficacy is complete protection from challenge with HIV/SIV, a secondary endpoint has been defined as reduction in viral load and prolonged survival without AIDS (28). To determine whether vaccine-induced in vitro ADCC responses were relevant to protective efficacy in vivo, we used the Jonckheere-Terpstra trend test to compare ADCC activity across postchallenge acute viremia groups. Vaccine-elicited ADCC activity at the time of challenge significantly correlated with low viral burdens during the acute phase of infection (Fig. 2; $p = 0.029$). An ADCC activity of 30% or higher was consistently observed in animals with the lowest viral loads, including those with persistently undetectable SIV RNA in plasma and those with only transient viremia after rectal challenge. Not surprisingly, the lowest ADCC activity and highest viremia was exhibited by the control macaques, and if eliminated from the trend analysis,

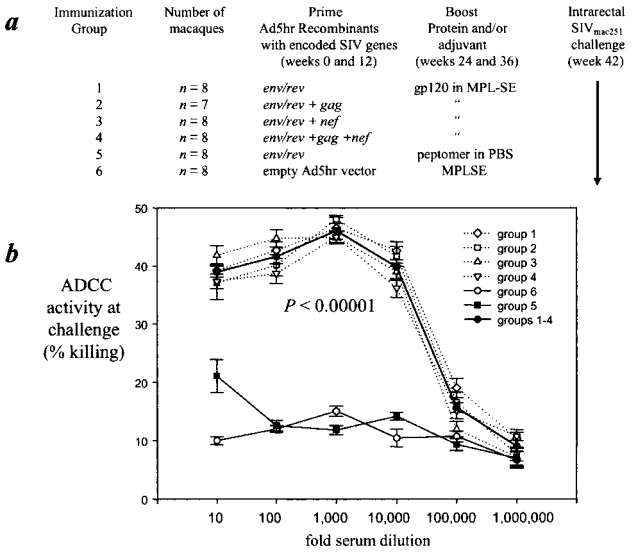

FIGURE 1. A replicating Ad5hr-SIV recombinant priming/protein boosting regimen elicits ADCC activity. *a*, Immunization and challenge protocol from which sera under study were obtained, summarized from Ref. 25. *b*, Titration of ADCC activity against SIV_{mac251}-infected H9 cells. Sera from individual macaques in each group were separately tested. Mean values for each immunization group are shown, in addition to an overall mean for groups 1–4. Bars denote SEM.

[4] V. R. Gómez-Román, R. Florese, L. J. Patterson, B. Peng, D. Venzon, K. Aldrich, and M. Robert-Guroff. A simplified method for the rapid fluorometric assessment of antibody-dependent cell-mediated cytotoxicity. *Submitted for publication.*

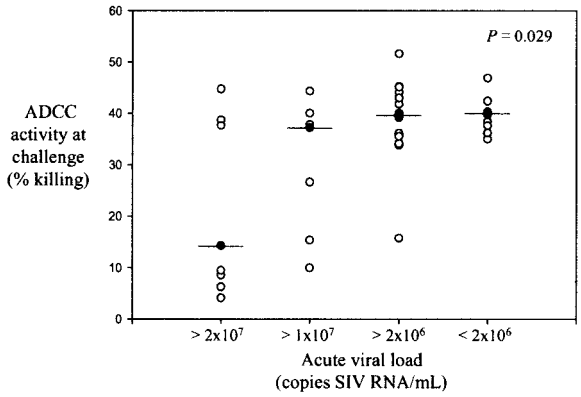

FIGURE 2. ADCC correlates with reduced acute viremia. ADCC activity against SIV_{mac251}-infected H9 cells is negatively correlated with acute phase viremia after mucosal challenge with pathogenic SIV ($p = 0.029$). ○, The mean of duplicate determinations of ADCC activity by sera from each animal at the time of challenge. Horizontal bars denote the median ADCC value for each category. Viremia groupings are as previously described (25). Sera diluted 1/10,000.

statistical significance was not reached. However, the control group of eight animals exhibited a significant internal correlation, with higher ADCC activity associated with lower viremia ($p = 0.036$). Thus, the controls contributed importantly and appropriately to an unrestricted analysis of the overall range of possible ADCC activity levels before challenge.

Envelope-specific ADCC activity

Our data are consistent with our previous observation that envelope-binding Ab titers at the time of challenge correlate strongly with reduced acute viremia (25), suggesting that non-neutralizing gp120-binding Abs, together with other vaccine-induced immune mechanisms, may help limit the spread of SIV-infected cells in the periphery. In support of this hypothesis, there was a significant correlation between ADCC activity and anti-gp120 binding Ab titers in the sera of these animals at the time of challenge (Fig. 3; $p < 0.00001$). To explore this correlation further, we tested whether the SIV envelope was a target of ADCC activity. Using SIV_{mac251} gp120-coated $CD4^+$ CEM-NK^r cells as targets, macaques in immunized groups 1–4, but not group 6 controls, exhibited SIV envelope-specific ADCC activity at the time of challenge (Fig. 4; $p < 0.00001$). No significant differences in ADCC activity across immunization groups 1–4 were observed here with gp120-coated targets, similar to the results above with SIV-infected targets, indicating that co-priming with Ad5hr-SIV*gag* or Ad5hr-SIV*nef* or both did not affect the level of induced ADCC activity. That the main target of ADCC in these macaques is the SIV envelope is supported by the geometric mean binding Ab titers in sera of macaques primed with the various immunogens: at the time of challenge, the geometric mean titer of anti-gp120 Abs in groups 1–4 was 65,308, whereas the geometric mean titer of anti-Nef in groups 3 and 4 and of anti-Gag in groups 2 and 4 was 12 and 85, respectively.

ADCC is IgG mediated

To confirm that the observed ADCC activity was Ab-mediated, we purified IgG from the sera of five rhesus macaques from different immunization groups and assessed ADCC activity. IgG from the immunized animals mediated killing of SIV-infected H9 cells in a dose-dependent manner that was highest in the nanomolar range (Fig. 5). In contrast, IgG from a mock-immunized animal had a background ADCC activity that was similar to the activity of commercially available rhesus IgG from naive rhesus macaques. In agreement with previous reports describing an ADCC "prozone" effect in the presence of excess Ab (29), we observed a decrease in ADCC activity at high concentrations of IgG.

Kinetics of vaccine-elicited ADCC activity

To examine the kinetics of the vaccine-elicited ADCC response, we assessed ADCC activity at different time points during the course of immunization. Priming with replication-competent Ad5hr-SIV_{smH4}*env/rev*, with or without Ad5hr-SIV*gag* or Ad5hr-SIV*nef*, was sufficient to elicit a heterologous ADCC response against SIV_{mac251}-infected H9 target cells (Fig. 6). Statistically significant differences were seen between sera of the immunized macaques compared with controls at week 14 ($p < 0.0001$) and between sera of immunized macaques at week 14 and sera obtained before immunization ($p < 0.00001$). Following boosting with native SIV_{mac251} gp120, ADCC activity at week 42 remained significantly higher in groups 1–4 than in the controls ($p < 0.0001$) and was enhanced compared with postpriming levels at week 14 ($p = 0.000096$). Notably, group 5 macaques also exhibited significant ADCC activity (mean of 20.74%) relative to controls following priming at week 14 ($p = 0.0002$), but this activity was not boosted by the peptomer immunization (data not shown). After rectal challenge, the immunized macaques in groups 1–4

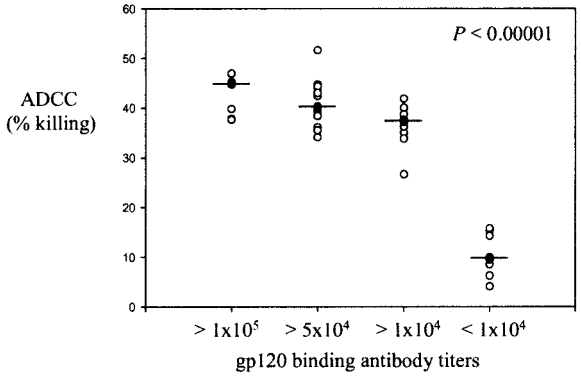

FIGURE 3. ADCC correlates with gp120-binding Ab titers. ADCC activity against SIV_{mac251}-infected H9 cells at the time of challenge is positively correlated with anti-SIV gp120 serum Ab-binding titers ($p < 0.00001$). ○, The mean of duplicate determinations of ADCC activity by sera from each animal at the time of challenge. Horizontal bars denote the median ADCC value for each category. Sera diluted 1/10,000.

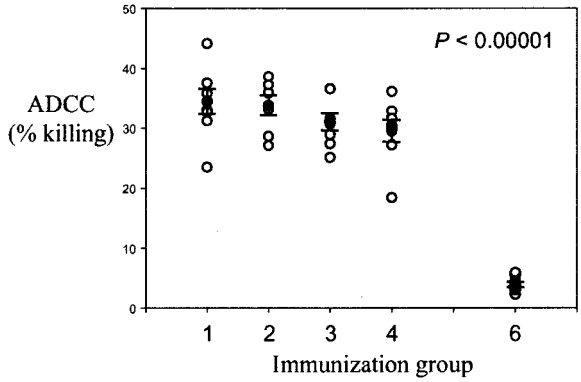

FIGURE 4. The Ad-SIV/gp120 vaccine regimen elicits ADCC with SIV envelope specificity. The mean percent killing from duplicate experiments of gp120-coated CEM-NK^r cells by each animal serum at time of challenge is shown by open circles (○). Group means are represented by filled circles (●). Bars denote SEM. Sera diluted 1/10,000.

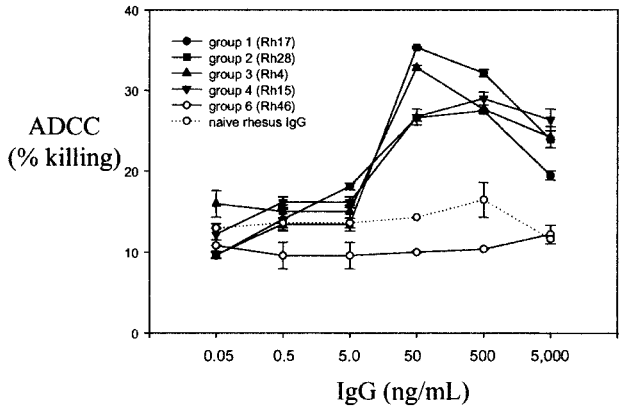

FIGURE 5. ADCC is IgG mediated. ADCC killing of SIV_{mac251}-infected H9 targets by IgG purified from sera obtained at the time of challenge from each of five rhesus macaques from different immunization groups is shown. Purified IgG from naive rhesus macaques served as an additional negative control. Bars denote the deviation from the mean in duplicate experiments.

exhibited a significant anamnestic response in ADCC activity. Two weeks postchallenge, ADCC activity in the immunized animals was significantly elevated above control levels ($p < 0.0001$) and above the ADCC level at the time of challenge ($p < 0.00001$). Increases in ADCC activity were seen in both immunized and control macaques by 4 wk postchallenge, with the former animals continuing to show higher activity ($p < 0.0001$). The appearance of ADCC activity in the control animals at 4 wk is in agreement with previous reports describing anti-SIV ADCC responses arising in naive nonhuman primates within 30 days of SIV infection (30–32).

Discussion

To our knowledge, this is the first demonstration that high-titered SIV-specific ADCC activity can be elicited by vaccination and is correlated with an early marker of protection against an SIV challenge. Although it has been assumed that ADCC activity would correlate with protective efficacy, an association between ADCC and reduced viral loads has never been demonstrated. Previous studies using SIV/macaque or cynomologus monkey AIDS vaccine models have failed to either induce or associate ADCC responses with protection (30–32). However, several recent studies have shown that vaccine regimens incorporating envelope immunogens conferred better protection than similar regimens lacking the envelope component (33–35). In these reports, anti-envelope binding, but not neutralizing, Abs were elicited. Although increased protection in the envelope-containing arm was attributed to increased cellular responses or more rapid development of neutralizing Abs postchallenge, each study raised the question of possible involvement of ADCC activity. Our results should stimulate further exploration of the role of ADCC in vaccine-induced protective efficacy.

Although it is tempting to speculate that ADCC was the protective humoral immune mechanism suppressing acute viremia in the immunized animals, this can only be proven in vivo and in the absence of other immune responses induced by vaccination. As in the case of the aforementioned HSV-ADCC murine model (4), passive transfer of these SIV-specific non-neutralizing and ADCC-mediating Abs will have to address this question in future studies. Further, a limitation of the assay system used here is the reliance on human effector and target cells due to the known difficulty of using rhesus cells in ADCC assays (26). Although the use of human cells allowed assessment of functional Ab-mediating ADCC activity without directly assaying the sera using autologous target and effector cells obtained from each individual macaque, it cannot be established with certainty that ADCC was operative in vivo. For this reason too, future passive transfer experiments will be important in clarifying the in vivo situation.

The precise manner in which the systemic ADCC activity described here might have contributed to protection against the intrarectal SIV challenge is unclear, because we have been unable to detect ADCC activity in rectal secretions collected from these macaques. However, importantly, the intrarectal challenge was conducted using cell-free virus, and ADCC acts against infected cells. Therefore, ADCC activity would not be expected to influence the course of infection until the challenge virus has crossed the rectal barrier and infected local cells in the mucosa. In the macaque model for SIV pathogenesis, intrarectal or vaginal inoculation of SIV results in virus dissemination, followed by rapid trafficking of SIV-infected cells from the mucosa to lymph nodes and blood (36). It is plausible, therefore, that the strong ADCC responses elicited by this vaccine regimen, present in serum at the time of challenge, may have contributed to protective efficacy by limiting the early spread of SIV-infected cells trafficking to the periphery shortly after mucosal challenge. It remains to be established whether the ADCC activity observed here against T cell line-adapted SIV-infected cells can also mediate killing of primary SIV-infected cells, including dendritic cells and peripheral or intraepithelial SIV-infected lymphocytes, which are more relevant targets of in vivo ADCC after an intrarectal challenge.

Several practical features make ADCC responses relevant to the development of an effective HIV vaccine. Most importantly, ADCC is a non-MHC-restricted process broadly applicable to the human population. In addition, unlike MHC-restricted CTL activity, ADCC is mediated by NK cells, macrophages, neutrophils, γδ T cells and other FcγR-bearing granular effectors. Therefore, the induction or expansion of a specific precursor T cell population before killing is not necessary, making ADCC an early and rapid immune response if relevant Abs have already been elicited by vaccination, as seen in Fig. 6. Several studies have reported dysfunction of NK and ADCC effector cells over the course of HIV infection (37, 38). However, this effect does not occur during the acute phase of infection. In the SIV rhesus macaque model, NK effector cells are the first to be activated, and peak around 2 wk post-SIV infection before returning to prechallenge levels (39). NK cell activity also peaks 2 wk following SIV infection (40). Therefore, it is quite reasonable to assume that vaccine-induced Ab

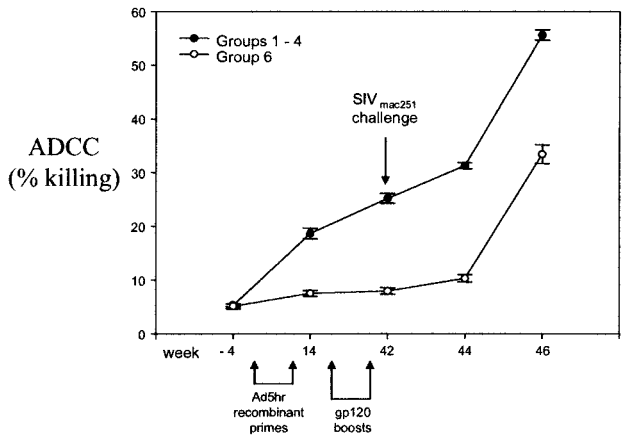

FIGURE 6. Kinetics of ADCC response. Serum ADCC activity against SIV_{mac251}-infected H9 cells is shown after priming with heterologous Ad5hr-SIV_{smH4}*env/rev*, boosting with SIV_{mac251}gp120, and challenging intrarectally with SIV_{mac251}. Mean values are shown at each time point for immunized macaques in groups 1–4 (●, $n = 31$) and for mock-immunized controls (○, $n = 8$). Bars denote the SEM. Sera diluted 1/10.

present at the time of challenge could mediate ADCC activity and impact initial viral burdens before any major decline in potential effector cell populations.

Recognizing the importance of ADCC responses in vivo, the World Health Organization has long acknowledged ADCC as one of the main immune mechanisms relevant to vaccine development against viral infectious diseases in man (41). However, most research on viral vaccines to date continues to focus on the induction of neutralizing Abs and MHC-restricted T cell-mediated immunity, with little or no regard to the induction of cytotoxic responses such as ADCC. Here, we have established an association between ADCC activity and vaccine efficacy in rhesus macaques partially protected against pathogenic SIV. Our findings should stimulate further studies on the role of ADCC in protective efficacy and the development of vaccines aimed at eliciting ADCC together with other humoral and cellular immune responses.

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