Vaccine protection against acquisition of neutralization-resistant SIV challenges in rhesus monkeys

Dan H. Barouch1,2, Jinyan Liu1, Hualin Li1, Lori F. Maxfield1, Peter Abbink1, Diana M. Lynch1, M. Justin Iampietro3, Adam SanMiguel1, Michael S. Seaman1, Guido Ferrari1, Donald N. Forthath4, Ilnoun Ourmanov5, Vanessa M. Hirsch5, Angela Carville6, Keith G. Mansfield6, Donald Stablein7, Maria G. Patu6, Hanneke Schuitemaker6, Jerald C. Sadoff8, Erik A. Billings9, Mangala Rao9, Merlin L. Robb9, Jerome H. Kim10, Mary A. Marovich10, Jaap Goudsmit10* & Nelson L. Michael10*

Preclinical studies of human immunodeficiency virus type 1 (HIV-1) vaccine candidates have typically shown post-infection virological control, but protection against acquisition of infection has previously only been reported against neutralization-sensitive virus challenges1–3. Here we demonstrate vaccine protection against acquisition of fully heterologous, neutralization-resistant simian immunodeficiency virus (SIV) challenges in rhesus monkeys. Adenovirus/poxvirus and adenovirus-vector-based vaccines expressing SIVSME660 Gag, Pol and Env antigens resulted in an 80% or greater reduction in the per-exposure probability of infection4,5 against repetitive, intrarectal SIVMAC251 challenges in rhesus monkeys. Protection against acquisition of infection showed distinct immunological correlates compared with post-infection virological control and required the inclusion of Env in the vaccine regimen. These data demonstrate the proof-of-concept that optimized HIV-1 vaccine candidates can block acquisition of stringent, heterologous, neutralization-resistant virus challenges in rhesus monkeys.

Despite the recent demonstration of partial HIV-1 vaccine efficacy in humans6, the immune responses required to protect against acquisition of infection have remained unclear. Preclinical studies of HIV-1 vaccine candidates have begun to elucidate immunological correlates of protection against neutralization-sensitive viruses7–9, but no study has to date reported vaccine protection against acquisition of heterologous, neutralization-resistant virus challenges10. Mucosal SIVMAC251 infection of rhesus monkeys represents a stringent preclinical model of a highly pathogenic, neutralization-resistant virus swarm11–13, and repetitive mucosal challenges more closely mimic sexual HIV-1 transmission in humans than do single high-dose challenges14. We therefore performed two studies to evaluate the protective efficacy of optimized adenovirus/poxvirus and adenovirus/vector-based vaccines against repetitive, heterologous, intrarectal SIVMAC251 challenges in rhesus monkeys.

In the first study, 40 Indian-origin rhesus monkeys (Macaca mulatta) that did not express the class I alleles Mamu-A*01, Mamu-B*08, and Mamu-B*17 associated with spontaneous virological control11–13 were immunized by the intramuscular route with the following vaccine regimens expressing SIVSME660 Gag-Pol and Env immunogens (N = 8 per group): (1) DNA prime, modified vaccinia Ankara (MVA) boost; (2) MVA prime, MVA boost; (3) adenovirus serotype 26 (Ad26) prime, MVA boost; (4) MVA prime, Ad26 boost; and (5) sham controls. Groups were balanced for susceptible and resistant TRIM5 alleles14. Monkeys were primed once at week 0 with 2 × 10^10 viral particles of Ad26 vectors or 10^9 plaque-forming units (p.f.u.) of MVA vectors, or three times at weeks 0, 4, and 8 with 5 mg of DNA vaccines. Animals were then boosted once at week 24 with 2 × 10^10 viral particles of Ad26 vectors or 10^9 p.f.u. of MVA vectors.

The vaccine regimens elicited distinct profiles of cellular and humoral immune responses, as measured by IFN-γ ELISPOT assays (Fig. 1a and Supplementary Fig. 1), multiparameter intracellular cytokine staining (ICS) assays15–17 (Fig. 1b and Supplementary Fig. 2), cellular immune breadth (Supplementary Fig. 3), SIVMAC251 Env-specific binding antibody ELISAs (Fig. 1c), tier 1 neutralizing antibody (NAb) assays against tissue culture laboratory adapted (TCLA) tier 1 SIVSME660 (CP3C-P-A8) and SIVMAC251 pseudoviruses (Fig. 1d), and antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated virus inhibition (ADCVI) assays (Supplementary Fig. 4). Tier 2 NAb responses against neutralization-resistant SIVSME660 (CR54-2A5) and SIVMAC251 (SIVMAC251.30) pseudoviruses, however, were below the 50% neutralization cutoff for positivity, although positive trends were observed in all vaccinated groups (Supplementary Fig. 4).

To evaluate the protective efficacy of these vaccine regimens, all monkeys were challenged repetitively beginning at week 52 (six months following the boost immunization) with six intrarectal inoculations of the heterologous virus SIVMAC251 using a 1:1,000 dilution (930 half-maximal tissue-culture infectious dose (TCID50)) of our challenge stock8. After the first challenge, 75% of sham control monkeys became infected, compared with only 12–25% of the animals that received the heterologous vector regimens DNA/MVA, Ad26/MVA, and MVA/Ad26 (Fig. 1e). The percentage of uninfected animals declined proportionally with each challenge, and the majority of vaccinees and all controls were infected by the end of the challenge protocol. Monkeys that received the Ad26/MVA and MVA/Ad26 vaccines required three challenges to infect 50% of animals in each group, whereas only one challenge was required to infect 50% of animals in the control group (P = 0.004 and P = 0.006, respectively, Wald tests, proportional hazard model). The heterologous vector regimens also showed decreased hazard ratios of 0.17 (95% confidence interval (CI) 0.05–0.57) to 0.20 (CI 0.06–0.63) compared with the controls, corresponding to an 80–83% reduction in the per-exposure probability of infection (Fig. 1f; vaccine efficacy VE = 1 – hazard ratio), as previously described4,5. These data demonstrate vaccine protection against acquisition of infection following repetitive, heterologous, intrarectal SIVMAC251 challenges.

Control monkeys showed peak viral loads on day 14 following infection and then relatively stable mean set point viral loads of 5.85 log SIV RNA copies per millilitre (Supplementary Fig. 5). The Ad26/MVA and the MVA/Ad26 vaccines resulted, respectively, in at least 2.32 and 1.08 log reductions of mean set point viral loads compared with sham controls for over 250 days (P = 0.0037 for each vaccine versus sham, Wilcoxon rank-sum tests) (Fig. 1g and Supplementary Fig. 5). Moreover, half the animals in the Ad26/MVA
group either demonstrated rapid and durable virological control to undetectable levels (Fig. 1g; \( N = 3 \)) or remained uninfected (Fig. 1e; \( N = 1 \)). The Ad26/MVA and MVA/Ad26 vaccines also afforded a survival advantage as compared with the controls (\( P = 0.025 \), log-rank test) (Supplementary Fig. 6).

We next evaluated the immunological correlates of protection against acquisition of infection, defined as the number of challenges required to establish infection, and virological control, defined as set point viral loads. Our prespecified primary immunological correlates analysis (Supplementary Table 1) demonstrated that protection against acquisition of infection was best correlated with Env-specific binding ELISA antibody responses (Fig. 2a; \( P = 0.0001 \), Spearman rank-correlation test) and tier 1 NAb titres (Fig. 2b; \( P = 0.0034 \)) immediately before challenge. Protection against acquisition of infection also correlated with V2-specific antibodies that presumably represented a subset of total Env-specific binding antibodies (Fig. 2e, f; \( P = 0.0001 \)).

Virological control was correlated with Gag ELISPOT breadth (Fig. 2c; \( P = 0.0002 \)) and magnitude (Fig. 2d; \( P = 0.0058 \)) immediately before challenge, consistent with our previous observations18.

In our exploratory immunological correlates analysis, we evaluated 35 humoral and cellular immune parameters at both peak and memory time points before challenge as possible immunological correlates of acquisition and virological control following challenge. No additional immune parameters were significantly correlated with protection.
against acquisition of infection in this analysis after multiple comparison adjustments (Supplementary Table 2). Gag-, Pol- and Env-specific effector memory CD8\(^+\) T-lymphocyte responses exhibited trends towards protection against acquisition, but did not achieve statistical significance according to our pre-specified criteria. In contrast, multiple humoral and cellular immune responses were significantly correlated with virological control (Supplementary Table 3), including Env ELISA, NAb and ADCC responses as well as Gag ELISPOT magnitude and breadth, Pol ELISPOT magnitude and Env CD4\(^+\) effector memory responses. These data support a model in which protection against acquisition of infection is correlated with vaccine-elicited Env antibody responses, whereas virological control is correlated with both T-lymphocyte and antibody responses. These distinct immunological correlates probably reflect fundamentally different biologic requirements for blocking establishment of infection at the mucosal site of inoculation compared with controlling viral replication after infection has become disseminated\(^{15}\). However, the actual mechanisms of protection remain to be determined.

We next evaluated directly the hypothesis that Env was critical for blocking acquisition of infection in this system. In the second study, 40 rhesus monkeys that did not express the class I alleles Mamu-A*01, Mamu-B*08 and Mamu-B*17 were immunized by the intramuscular route with Ad35 prime\(^{21}\), Ad26 boost\(^{21}\) vaccine regimens expressing (1) SIV\(_{SME543}\) Gag-Pol (N = 16), (2) SIV\(_{SME543}\) Gag-Pol and Env (N = 16) and (3) sham controls (N = 8). Groups were balanced for susceptible and resistant TRIM5\(\alpha\) alleles\(^{1,14}\). Monkeys were primed once at week 0 with \(2 \times 10^{10}\) viral particles of Ad35 vectors and boosted once at week 24 with \(2 \times 10^{10}\) viral particles of Ad26 vectors. Cellular immune responses were assessed by IFN-\(\gamma\) ELISPOT assays (Fig. 3a and Supplementary Fig. 7) and multiparameter ICS assays in both the periphery (Fig. 3b and Supplementary Fig. 8) and in colorectal mucosa (Supplementary Fig. 9). Env-specific humoral immune responses were assessed by ELISAs in both the periphery (Fig. 3c) and in colorectal mucosa (Supplementary Fig. 10), tier 1 NAb assays (Fig. 3d) and ADCC assays (Supplementary Fig. 11). Only marginal tier 2 NAb responses were observed (Supplementary Fig. 11).

We assessed protective efficacy of these vaccine regimens against repetitive, heterologous, intrarectal SIV\(_{MAC251}\) challenges as described in the first study. After the first challenge, 50% of sham control monkeys became infected, compared with only 12% of the animals that received the Gag-Pol-Env vaccine (Fig. 3e). The monkeys that received the Gag-Pol-Env vaccine required four challenges to infect 50% of animals in each group, whereas only one challenge was required to infect 50% of animals in the control group (Fig. 3f; \(P = 0.002\), Wald test, proportional hazard model). Moreover, the Gag-Pol-Env vaccine resulted in a decreased hazard ratio of 0.20 (CI 0.07–0.55), corresponding to an 80% reduction in the per-exposure probability of infection. In contrast, the Gag-Pol vaccine afforded only a marginal protective effect, demonstrating the critical role of Env in blocking acquisition of infection in this model. The Gag-Pol and Gag-Pol-Env vaccines resulted in, respectively, at least 1.59 log and 2.18 log reductions of set point viral loads compared with controls (Fig. 3g and Supplementary Fig. 12; \(P = 0.0006\) and 0.0002, respectively, Wilcoxon rank-sum tests). Immunological correlates of protection against acquisition of infection were consistent with the first study, and both peripheral (Fig. 4a–c) and rectal (Fig. 4d) Env-specific IgG correlated with reduced acquisition risk.

Taken together, these data demonstrate that optimized adenovirus/poxvirus and adenovirus/adenovirus vector-based vaccines afforded significant protection against acquisition of infection following highly pathogenic, heterologous, neutralization-resistant SIV\(_{MAC251}\) challenges in rhesus monkeys (Figs 1e, 3e and Supplementary Fig. 13). Although several studies have previously shown partial protection against acquisition of neutralization-sensitive virus challenges\(^1–3\), no HIV-1 vaccine candidate has to date blocked acquisition of heterologous, difficult-to-neutralize virus challenges, including Ad5 (ref. 7), DNA/Ad5 (ref. 1) and cytomegalovirus\(^8\) vaccines. In particular, a recent study demonstrated that a DNA/Ad5 vaccine afforded partial protection against acquisition of SIV\(_{SMIN600}\) which is a neutralization-sensitive tier 1A virus in TZM-bl neutralization assays, but the same vaccine afforded no efficacy against neutralization-resistant SIV\(_{MAC251}\) (ref. 1), highlighting important differences in the stringencies between these two SIV challenge models as well as potentially important phenotypic differences between adenovirus serotypes\(^{17}\). However, we note that the acquisition effect in the present study was relative rather than absolute, and that the majority of vaccinees became infected by the end of the challenge protocol.

Our studies also demonstrate that inclusion of Env in the vaccine was required for the acquisition effect (Fig. 3e), despite an 18% difference in the Env amino acid sequences between the vaccine strain and challenge virus. Moreover, our immunological correlates analyses (Figs 2, 4 and Supplementary Tables 1–3) suggest that Env-specific antibodies are critical for blocking acquisition of infection, whereas multiple cellular and humoral immune responses correlate with virological control, although the actual mechanisms of protection remain to be determined. In addition, the RV144 immunological correlates analyses raised the hypothesis that vaccine-elicited V1/V2-specific antibodies may reduce HIV-1 acquisition risk in humans\(^{22}\). Our data (Figs 2f, 4c) are consistent with this hypothesis, although it remains unclear whether V2-specific antibodies actually protect or simply represent a marker for other Env-specific antibodies or other protective factors.
Considerable efforts are currently underway to identify and to reverse engineer potent, broadly reactive monoclonal antibodies. Although the induction of such NAb responses by a vaccine would presumably be highly desirable, no Env immunogens have to date been developed that can elicit these responses. Our findings suggest that a substantial degree of protection can be achieved against stringent virus challenges even in the absence of high titres of tier 2 NAbs, perhaps reflecting the importance of antibody effector functions that may not be fully measured by traditional virus neutralization assays. Of note, the partial protection in the present study was observed with vectored Env and without a purified Env protein subunit boost. The degree to which an Env protein boost may further improve the protective efficacy afforded by these vaccine regimens remains to be determined.

In summary, our data demonstrate the proof-of-concept that vaccination can protect against acquisition of stringent, heterologous, neutralization-resistant SIVMAC251 challenges in rhesus monkeys. These findings, together with the observations of a critical requirement for Env and the distinct immunological correlates of protection against acquisition of infection and virological control, pave novel paths forward for HIV-1 vaccine development.
METHODS SUMMARY

For each study, 40 Indian-origin rhesus monkeys (Macaca mulatta) were vaccinated with DNA, MVA25, Ad26 (ref. 21) or Ad35 (ref. 20) expressing SIVSMEDA. Correlates analyses included the 16 Gag-Pol-Env-vaccinated monkeys and did not include the Gag-Pol-vaccinated monkeys or the sham controls. P-values reflect Spearman rank-correlation tests.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.
METHODS

Animals, immunizations and challenges. Indian-origin, outbred, young adult, male and female, specific pathogen-free (SPF) rhesus monkeys (Macaca mulatta, 80 animals) that did not express the class I alleles Mamu-A*01, Mamu-B*08 and Mamu-B*17 associated with spontaneous virological control11–13 were housed at the New England Primate Research Center (NEPRC), Southborough, Massachusetts, USA. A total of 40 animals was used for each study. Groups were balanced for susceptible and resistant TRIM5α alleles14. Immunizations were performed by the intramuscular route in the quadriceps muscles with 2 × 1010 viral particles of Ad35 vectors20, 2 × 1010 viral particles of Ad26 vectors21, 106 p.f.u. of MVA vectors22, or 5 mg of DNA vaccines expressing SIVSMEE53 Gag-Pol and/or Env gp140. Monkeys were primed at week 0 and boosted at week 24, except DNA vaccine priming that was performed at weeks 0, 4 and 8. To evaluate for protective efficacy and immunological correlates, all monkeys were challenged repetitively beginning at week 52 with six intrarectal inoculations of the heterologous virus SIVMAC251 using a 1:1,000 dilution (930 TCID50) of our challenge stock9. Monkeys were bled weekly for viral loads (Siemans Diagnostics), and the date of infection was defined as the last challenge time point before the first positive SIV RNA level. Animals were followed to determine set point viral loads. All animal studies were approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC).

Cellular immune assays. SIV-specific cellular immune responses were assessed by IFN-γ ELISPOT assays18 and multiparameter intracellular cytokine staining (ICS) assays19,20 essentially as described. ELISPOT assays used pools of SIVSME53 and SIVMAC251 Gag, Pol and Env peptides. Analyses of cellular immune breadth used sub-pools of 10 peptides covering each antigen. Peptides were 15 amino acids in length and overlapped by 11 amino acids. Nine-colour ICS assays used monoclonal antibodies (Becton Dickinson) against CD3 (SP34; Alexa700), CD4 (L200; AmCyan), CD8 (SK1; allophycocyanin-cyamine7 (APC-Cy7)), CD28 (L293; phycoerythrin-Texas Red (energy-coupled dye; ECD); dinin chlorophyll-A-cyanine5.5 (PerCP-Cy5.5)), CD95 (DX2; phycoerythrin) and TNF-α (Mab11; fluorescein isothiocyanate (FITC)). IFN-γ backgrounds were consistently <0.01% in peripheral blood mononuclear cells and <0.05% in colorectal biopsy specimens.

Humoral immune assays. SIV-specific humoral immune responses were assessed by SIVMAC251 Env ELISAs27, TZM-bl luciferase-based virus neutralization assays28 against tier 1 SIVSMEE60 (CP3C-P-A8) and SIVMAC251 (TCLA) pseudoviruses, TZM-bl virus neutralization assays against tier 2 SIVSMEE60 (CR54-PK-2A5) and SIVMAC251 (SIVMAC251.30) pseudoviruses, antibody-dependent cellular cytotoxicity (ADCC) assays29, and antibody-dependent cell-mediated virus inhibition (ADCVI) assays30. V2-binding assays were performed by surface plasmon resonance with a Biacore 2000 or T200 using a 1:50 serum dilution and a cyclic SIVSMEE54 V2 peptide containing an amino-terminal biotin tag (CIKNNSCAGLEQPMIGCKFNMTGLKRDKKIEYNETWYSRDLICEQPANGSESKCY) and immobilized on streptavidin-coated CMS chips. Mucosal antibodies were assessed using rectal secretions collected with Weck-Cel sponges. Approximately 100 µl rectal secretions were eluted and diluted sixfold, and total IgG and IgA as well as SIV Env-specific IgG and IgA (Immune Technology Corporation) were measured by ELISA using a biotin-conjugated anti-monkey IgG and IgA (Alpha Diagnostics) secondary antibody. Mucosal titres were defined as endpoint ELISA titres multiplied by the dilution of the eluted secretions. Samples showed comparable levels of total IgG.

Statistical analyses and immunological correlates. Protection against acquisition of infection was analysed using Wald tests with a proportional hazard model and the exact conditional likelihood method for breaking ties. A discrete time model provided similar estimates. The number of challenges required for 50% infection of each group, hazard ratios with 95% confidence intervals, per-exposure vaccine efficacy and per-exposure risks of infection were quantified. Vaccine efficacy was defined as the reduction in the per-exposure probability of infection as previously described41. Analyses of virological and immunological data were performed by Wilcoxon rank-sum tests and analysis of survival by log-rank tests. For these tests, P < 0.05 was considered significant and two-tailed tests were performed. Immunological correlates were evaluated by a focused primary analysis and a detailed exploratory analysis using Spearman rank-correlation tests. In the primary analysis, P < 0.01 was considered significant, whereas in the exploratory analysis, P < 0.0014 was considered significant to adjust for multiple comparisons.