Published in final edited form as:
Vaccine. 2014 November 12; 32(48): 6527–6536. doi:10.1016/j.vaccine.2014.08.065.

Multimodality vaccination against clade C SHIV: partial protection against mucosal challenges with a heterologous tier 2 virus

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Abstract

We sought to test whether vaccine-induced immune responses could protect rhesus macaques (RMs) against upfront heterologous challenges with an R5 simian-human immunodeficiency

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virus, SHIV-2873Nip. This SHIV strain exhibits many properties of transmitted HIV-1, such as tier 2 phenotype (relatively difficult to neutralize), exclusive CCR5 tropism, and gradual disease progression in infected RMs. Since no human AIDS vaccine recipient is likely to encounter an HIV-1 strain that exactly matches the immunogens, we immunized the RMs with recombinant Env proteins heterologous to the challenge virus. For induction of immune responses against Gag, Tat, and Nef, we explored a strategy of immunization with overlapping synthetic peptides (OSP). The immune responses against Gag and Tat were finally boosted with recombinant proteins. The vaccinees and a group of ten control animals were given five low-dose intrarectal (i.r.) challenges with SHIV-2873Nip. All controls and seven out of eight vaccinees became systemically infected; there was no significant difference in viremia levels of vaccinees vs. controls. Prevention of viremia was observed in one vaccinee which showed strong boosting of virus-specific cellular immunity during virus exposures. The protected animal showed no challenge virus-specific neutralizing antibodies in the TZM-bl or A3R5 cell-based assays and had low-level ADCC activity after the virus exposures. Microarray data strongly supported a role for cellular immunity in the protected animal. Our study represents a case of protection against heterologous tier 2 SHIV-C by vaccine-induced, virus-specific cellular immune responses.

Keywords

HIV vaccine; SHIV-C; heterologous tier 2 virus; rhesus monkey; cellular immunity; ADCC

1. Introduction

According to UNAIDS global estimates, each year of the last decade added >2.5 million new HIV-1 infections. Considering the recent success of non-vaccine prevention tools, such as microbicides [1], pre-exposure chemoprophylaxis [2], male circumcision (reviewed in [3]), and treatment of infected individuals [4], it may be expected that their increased use will slow the spread of HIV in coming years (reviewed in [5]). However, maintaining control over the HIV epidemic through drug prevention is unconvincing due to suboptimal compliance. Therefore, a prophylactic HIV-1 vaccine is still needed. The pessimistic view regarding prophylactic HIV-1 vaccines has been replaced by cautious optimism due to the RV144 trial outcome [6-8] and recent primate model data indicating prevention of virus acquisition by active immunization against upfront heterologous virus challenges [9-13]. The RV144 vaccine trial showed a moderate efficacy (31.2%) and engendered “no acquisition” as a new ambition for HIV vaccine development [14]. However, reaching this goal is still one of the greatest challenges and requires experimental evaluation of novel vaccine concepts and candidates.

Considering the complexity and challenges involved in testing vaccine candidates in human efficacy trials, biologically relevant animal models that mimic as many aspects of HIV-1 transmission as possible are needed. Due to host restrictions for HIV-1 replication in macaques, the simian immunodeficiency virus (SIV) / rhesus macaque (RM) model has been widely used. However, SIV differs significantly from HIV-1 and therefore cannot be used as a challenge virus to evaluate the efficacy of anti-HIV-1 vaccine candidates. To overcome this difficulty, recombinant simian-human immunodeficiency viruses (SHIVs) that carry

HIV-1 envelope have been developed. Such SHIVs can be used as challenge viruses to evaluate the efficacy of HIV-1 envelope-based immunogens in RMs. Of note, envelope is the most important target for antibody-mediated immune defenses, and antibodies are the best correlates of protection in most licensed vaccines (reviewed in [15]). Therefore, SHIVs can significantly contribute to the preclinical evaluation of HIV-1 vaccine candidates. However, earlier SHIV strains were found to be unsuitable due to X4 tropism and overly too rapid disease progression. New SHIV strains that mimic most aspects of HIV-1 transmission and disease have been developed [16-22].

We constructed a series of exclusively CCR5-tropic clade C SHIVs (SHIV-Cs) [18-20], which offer the advantage of reflecting the world's most prevalent HIV-1 subtype. Among our newly developed SHIV strains, SHIV-2873Nip [19] carries a primary pediatric HIV-1 clade C (HIV-C) *env* isolated from a recently infected Zambian infant who showed rapid disease progression and died within one year of birth. SHIV-2873Nip is a tier 2 virus (less sensitive to neutralizing antibodies), similar to the majority of acutely transmitted HIV-1 strains [23] and causes AIDS in RMs with clinical parameters and disease progression rates similar to those in humans (unpublished data). Hence, we sought to induce immune responses in RMs that would protect against our biologically relevant challenge virus.

In our earlier vaccine efficacy study, simultaneous induction of cellular immunity and challenge virus-specific neutralizing antibodies (after immunization with SIV Gag-Pol particles, HIV-1 Tat and multimeric HIV-1 gp160) were significantly associated with protection against multiple low-dose challenges with the tier 1 SHIV-1157ipEL-p [13, 24]. However, these immune responses were induced only in a fraction of vaccinees. Variable levels of cellular responses may be due to differential protein processing by outbred RMs. To overcome this issue, we immunized a group of RMs with overlapping synthetic peptides (OSP) that were 15 amino acids (aa) in length with an overlap of 11 aa (for Gag, Tat, and Nef proteins). The 15-mer peptides stimulate antigen-specific $CD4^+$ and $CD8^+$ cells in commonly used in vitro assays (ELISPOT assay, intracellular cytokine staining) and represent all potential $CD4^+$ and $CD8^+$ T cell epitopes. These peptides may bind directly to MHC class II molecules of antigen presenting cells (APC) and need only partial processing for binding to MHC class I molecules. In our earlier studies, this approach generated peptide-specific cellular immune responses in all vaccinated outbred mice and also in different strains of inbred mice [25, 26]. The number of peptides made available to MHC molecules after antigen processing is limited [27, 28], but MHC molecules are potentially very promiscuous and can bind to more than million different peptides with significant affinity [29]. Our approach was to make a large number of 15-mer peptides available to APC through direct administration.

For the induction of humoral immune responses against HIV-1 Env, we used our earlier successful strategy of protein-only immunization [13, 30, 31] but used two different (heterologous) Env proteins in a prime-boost strategy. Sequential immunization with different HIV-1 Env versions can lead to more antibody maturation and broadening of neutralizing antibody (nAb) responses [32]. We present immunogenicity and efficacy data of our novel vaccination strategy against a biologically relevant heterologous challenge virus: SHIV-2873Nip [19].

2. Materials and methods

2.1 Immunogens and vaccination

The OSP (15-mers with an 11 aa overlap between sequential peptides) for SIVmne Gag, HIV-1 Tat Oyi [33] and SIVsmE543-3 Nef were commercially synthesized (RS synthesis, Louisville, KY). The peptides represented entire proteins (124, 23 and 63 peptides for Gag, Tat and Nef, respectively). Positively or negatively charged peptides were dissolved in phosphate buffer saline (PBS), whereas neutral peptides were dissolved in DMSO. For Gag peptides, four pools were prepared (pools #1 to #4 consisting of peptides 1-31, 32-62, 63-93, and 94-124, respectively); for Nef peptides, two pools were prepared (pools #1, #2 consisting of peptides 1-32 and 33-63, respectively); for Tat peptides, a single pool was prepared. The concentration of each peptide in the pool was 1.5 mg/ml. Each peptide pool (200 μl, i.e., 300 μg of each peptide) was administered subcutaneously along with an equal volume of Incomplete Freund's adjuvant (IFA). Three doses were given at intervals of five or six weeks (Fig. 1).

The multimeric HIV-C gp160 was derived from a recombinant vaccinia virus expressing *env* of HIV1084i, a recently transmitted HIV-C isolate from a Zambian infant [34]. The same technology was also used for the preparation of SIVmne Gag-Pol particles [35]. The CHO cell-expressed HIV-1 gp145 was produced from the HIV-C06980v0c22 *env*, cloned from the plasma of an acutely viremic but seronegative individual from a Tanzanian heterosexual cohort. The Tat Oyi protein was synthesized based on the *tat* gene of HIV-1 Oyi [33, 36]. HIV-1 Oyi is an isolate from a transiently viremic African (Gabon) patient, who was later described as highly exposed, persistently seronegative (HEPS) [37]. Immunization with Tat Oyi induced immune responses in rabbits [38] and also provided partial protection to RMs against SHIV challenge [39]. For each protein immunization (Fig. 1), 100 μg of protein in IFA was administered by the intramuscular (i.m.) route.

2.2 Animals

Indian-origin RMs (*Macaca mulatta*) were housed at the Yerkes National Primate Research Center (YNPRC), Atlanta, Georgia, USA. YNPRC is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Approval for all procedures was received from the Institutional Animal and Care and Use Committees of Dana-Farber Cancer Institute (DFCI) and Emory University.

A group of 8 RMs was immunized with OSP and recombinant proteins as shown in Fig. 1. The control group consisted of 10 RMs that received IFA only. All RMs were negative for Mamu- B*008 and B*017 alleles. Six Mamu-A*001^{+} RMs were equally distributed among vaccinees and controls (Fig. 1).

2.3 Challenge virus

The SHIV-2873Nip stock was grown in concanavalin A (Con A)-stimulated RM peripheral blood mononuclear cells (PBMC) and the 50% tissue culture infectious dose ($TCID_{50}$) was determined using TZM-bl cells. All RMs were challenged intrarectally (i.r.) with 5,000 $TCID_{50}$ of the virus (low-dose challenge). The challenge was given once a week up to a

maximum of five inoculations. A titration by the i.r. route using low-dose inocula had been performed to verify the virus dose before the challenges of the current experimental groups started. Animals that became systemically infected (>10,000 viral RNA copies/ml) during these multiple challenges were excluded from the subsequent virus inoculations. SHIV-2873Nip is relatively neutralization resistant (tier 2); the 50% inhibitory concentration (IC_{50}) for each of the broadly neutralizing antibodies VRC01, IgG1b12, 2G12, 2F5 and 4E10 was >25 μg/ml [19, 40].

2.4 Measurement of plasma viral RNA (vRNA)

Plasma vRNA was isolated by QiaAmp Viral RNA Mini-Kits (Qiagen, Germantown, MD, USA); vRNA levels were measured by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) for SIV *gag* sequences [41]. Additionally, primers/probes according to Lifson were used [42]. Assay sensitivity was 50 vRNA copies/ml [41]. Time to first detection of viremia was analyzed by Kaplan-Meier analysis.

2.5 Virus-specific cellular immune responses

Interferon-γ (IFN-γ) ELISPOT assays were performed as described earlier [12] using SIVmne Gag, HIV-1 Tat Oyi or SIV Nef peptides. T-cell proliferative responses were measured by CFSE dilution assay [12]. The responses were measured against pools of SIVmne Gag or HIV-1 Tat Oyi peptides.

2.6 Virus-specific humoral immune responses

Binding antibody titers against viral proteins were determined by ELISA as described earlier [31]. The viral proteins used were SIVmac251 p27 (Immunodiagnostics, Inc., Woburn, MA), HIV-1 Tat and HIV-$C_{96ZM651}$ gp120 (both obtained from NIH AIDS Research and Reference Reagent Program, ARRRP).

Sera collected on the day of the first virus challenge were tested for nAb titers against three tier 1 (clade C: SHIV-1157ipEL-p, GS015.EC12PV and clade B: SF162PV) viruses and the tier 2 challenge virus (SHIV-2873Nip) using TZM-bl cells [43]. We used challenge virus-related replication-competent molecular clones of HIV-1 expressing *Renilla* luciferase (NL-LucR.2873Ni and NL-LucR.2878Nipd14) to measure virus-neutralizing activity in A3R5 cell-based assays. These molecular clones were generated by swapping the *env* ectodomain of NL-LucR.T2A with the corresponding regions of SHIV-2873Nip-related molecular clones as described [44]. A3R5 cells, a human lymphoblastoid cell line engineered to express CCR5, are substantially more sensitive than TZM-bl cells to detect virus neutralization [45]. The nucleotide sequences of HIVC *env* used to construct NL-LucR. 2873Ni and NL-LucR.2878Nipd14 have been deposited in GenBank under accession numbers KJ941152 and KJ941153, respectively. Sera were also tested against SHIV-2873Nip in human PBMC-based neutralization assays as described earlier [12]. Virus neutralization was also tested using Con A-stimulated RM PBMC.

Serum antibody-dependent cellular cytotoxicity (ADCC) was tested using CEM-NK^R cells coated with HIV-C gp120 [46]. For antibody-dependent cell-mediated viral inhibition (ADCVI), SHIV-2873Nip-infected CEM-NK^R-CCR5 cells were incubated with 1:100

diluted serum + human PBMC effectors (effector: target = 10:1) and virus replication was monitored [47].

2.7 Dissection of antibody responses linked to the protection or non-protection

Plasma samples from the vaccine-protected RMs was screened for antibody responses specific for protection using a peptide phage display-based approach as described earlier [24]. In brief, total IgG was purified from plasma samples of the protected and a non-protected animal, having comparable binding antibody titers against HIV-1 gp120. Equal amounts of purified polyclonal IgGs were coated on paramagnetic protein G beads (Dynabeads Protein G; Life Technologies). The biopanning was performed using different phage display peptide libraries (7-mer, cyclic 7-mer, 12-mer, and 16-mer). For the protection-linked biopanning, IgG from the protected RM was used for positive selection and IgG from a non-protected RM was used for negative selection. For the inverted biopanning, positive selection was done using IgG from non-protected RM and negative selection using IgG from the protected RM.

2.8 Gene expression microarray analysis

Blood was collected before vaccination, on the day of first virus exposure and six weeks after last virus challenge. Lymph node and rectal pinch biopsies were performed before vaccination and six weeks after last virus challenge. Blood was collected in Tempus tubes, processed immediately according to the manufacturer's instructions, and stored at −80°C. The biopsy specimens were cut into small pieces and immediately placed into RNA*later* solution (Qiagen, Valencia, CA) and also stored at −80°C. Total RNA from blood, lymph node and rectal biopsies was extracted using RNAeasy extraction kits (Qiagen, Valencia, CA). cDNA labeling, hybridization, staining and scanning were performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA) for rhesus gene expression arrays. Array quality was assessed using the R/Bioconductor package [48]. Affymetrix CEL files were processed and normalized using the robust multiarray average (RMA) algorithm [49]. Results were adjusted for multiple testing using the Benjamini and Hochberg (BH) method [50], and significance was determined using a false-discovery-rate cutoff of <5% and a log fold-change cutoff of >2 as compared to baseline. Gene interaction network analysis and visualization were performed on significant probe sets using the Ingenuity pathway analysis (IPA) software package. Microarray data were deposited into the gene expression omnibus database (Accession number: GSE60368).

2.9 Statistical analysis

Log-rank comparisons were used for time-to-infection analyses between vaccinees vs. controls. The Wilcoxon rank sum test was used to compare continuous variables (number of virus challenges, peak viremia and area-under-the-curve) between groups. The reported P-values are based on two-sided testing.

3. Results

3.1. Vaccine safety and immunogenicity

OSP immunization consisted of subcutaneous administration of 300 μg of each peptide (210 peptides representing SIV Gag, HIV-1 Tat and SIV Nef). Recombinant protein immunization consisted of i.m. administration of 100 μg of each protein (HIV-1 gp160, SIV Gag-Pol particles, HIV-1 Tat, and HIV-1 gp145). Peptides as well as proteins were administered in IFA. None of the vaccinees developed untoward side effects, and no local reactogenicity was noted.

Cellular immune responses were measured at two weeks after the 2^{nd}, 3^{rd} and 4^{th} administrations of Gag, Tat and Nef immunogens by IFN-γ ELISPOT assay (Fig. 2A). On the day of first virus exposure (two weeks after the last immunization, referred to as day or week 0), the total number of virus-specific IFN-γ secreting cells among vaccinees ranged between 20 to 570 Spot Forming Units (SFU)/10^6 PBMC. We also measured antigen-specific proliferation of $CD4^+$ and $CD8^+$ T cells at day 0. Two RM showed >10% Gag or Tat-specific proliferating T cells (Fig. 2B and 2C).

Sera collected on day 0 were tested by ELISA to determine binding antibody titers against SIVmac251 Gag, HIV-1 IIIB Tat and heterologous clade C $HIV_{96ZM651}$ gp120 (Fig. 3A). The median binding antibody titers (highest reciprocal serum dilution showing binding by ELISA) against Gag and Tat were 2000 and 400, respectively. As expected, binding antibody titers induced by peptide immunizations against Gag and Tat were substantially lower than those in our earlier study [30] where Gag-Pol particles and Tat protein had been used for immunization. The Env binding titers ranged from 2,000 to 50,000 (Fig. 3A).

Sera collected on day 0 were tested for neutralizing activity against SHIV-2873Nip (tier 2 challenge virus), SHIV-1157ipEL-p (tier 1, SHIV-C), GS015.EC12PV (tier 1, HIV-C) and SF162PV (tier 1, HIV-1 subtype B) by TZM-bl assay (Fig. 3B). None of the vaccines sera showed neutralization of challenge virus (SHIV-2873Nip), but four out of eight vaccinees showed ≥50% neutralization of all three tier 1 viruses (Fig. 3B). The A3R5 cell-based assay is more sensitive to detect virus neutralization [45]. In this assay, nAb responses against challenge virus-related molecular clones (NL-LucR.2873Ni and NL-LucR.2878Nipd14) were measured. Similar to the TZM-bl assay results, virus inhibition was not shown by sera of any vaccinee in the A3R5 cell-based assay (data not shown). ADCC responses were also undetectable against gp120-coated target cells on day 0 (tested using serum as well as purified IgG). In the ADCVI assay, four out of eight vaccinees (REo-12, RMd-12, ROb-12 and RVq-11) showed >50% inhibition of SHIV-2873Nip replication at 1:100 serum dilution.

3.2 Vaccine efficacy against multiple, low-dose challenges with heterologous tier 2 SHIV-C

To recapitulate HIV-1 transmission as closely as possible, the animals were challenged with heterologous SHIV-2873Nip, a virus with exclusive CCR5 tropism, tier 2 neutralization profile, and gradual pathogenicity [19]. Repeated (5x, one dose/week), low-dose (5000 $TCID_{50}$) challenges were given by the mucosal (i.r.) route.

The animals were monitored weekly for plasma vRNA levels (Fig. 4A-B). All controls became viremic and showed peak viremia ranging from 1.1 x 10^4 to 1.3 x 10^7 (median: 2 x 10^5) vRNA copies/ml (Fig. 4C). However, one vaccinee (ROb-12) showed complete protection (no detectable plasma viremia). Among vaccinees with breakthrough infection, peak plasma vRNA ranged from 6.9 x 10^3 to 9 x 10^5 (median: 2 x 10^5) copies/ml (Fig. 4C). There was no difference in the time for onset of viremia (Fig. 4D) or the number of virus challenges before detection of viremia (Fig. 4E) among the vaccinees compared to the controls. All animals were monitored for seven weeks after first detection of viremia to determine AUC; no significant difference was noted among controls and vaccinees (Fig. 4F).

To confirm complete protection of ROb-12, RT-PCR analysis was performed to detect vRNA in a peripheral lymph node as well as in rectal biopsies taken 6 weeks after the last virus challenge; no vRNA copies were detected. Importantly, PBMC of ROb-12 supported SHIV-2873Nip replication in vitro (Fig. 5), which indicates that the cells of ROb-12 were not intrinsically resistant to SHIV-C replication.

3.3. Immune responses shown by the completely protected vaccinee

The one protected, persistently aviremic vaccinee, ROb-12, had developed the highest level of virus-specific cellular responses during immunization (two weeks after second OSP dose; Fig. 2A & Fig. 6) and also showed the highest number of virus-specific ELISPOT responses (570 SFU/10^6 PBMC) on the day of first virus exposure (Fig. 2A & Fig. 6). ROb-12 also exhibited the highest level of HIV-1 Tat-specific proliferating $CD8^+$ T cells on the day of first virus challenge (Fig. 2B). This RM maintained virus-specific cellular responses throughout all virus challenges and indeed the response increased from 570 (week 0) to 1590 SFU/10^6 PBMC during virus exposures (Fig. 6) suggesting antigenic stimulation delivered by the challenges. Among the remaining seven unprotected vaccinees, such anamnestic cellular responses were not observed. It is important to note that the protected RM (ROb-12) was negative for Mamu-A*001, B*008, and B*17 alleles, the MHC genotypes associated with improved control of SIV replication in RMs with these alleles compared to those without.

On day 0, the protected RM (ROb-12), did not show nAb responses against the challenge virus in the TZM-bl as well as A3R5 cell-based assays. The animal did not show lysis of the HIV-C gp120-coated cells in the ADCC assay. Although ROb-12 showed >50% challenge virus inhibition in the ADCVI assay, similar virus inhibition was also shown by three unprotected animals (data not shown). The serum samples of this protected animal collected during virus exposures and two weeks after last virus exposure were also analyzed for nAb, ADCVI, ADCC and gp120 binding Ab titers. Unlike cellular responses, strong boosting of any humoral responses was not observed during virus exposures (data not shown). The findings are summarized in the Table 1. Mucosal samples (rectal washes or biopsies) could not be collected from any vaccinees just prior to virus exposure as this would have breached mucosal integrity.

The differential biopanning strategy designed to identify antibody responses that are present in the protected animal (ROb-12), but absent in the non-protected animal (RVq-11) [24] did

not reveal any protection-linked antibody responses in RObb-12. Similarly, the biopanning strategy designed to identify antibody responses that might be present in the non-protected animal (RVq-11), but absent in the protected animal (RObb-12) did not reveal any specific non-protection-linked antibody responses in the RVq-11.

3.4. Gene expression microarray analysis for the completely protected vaccinee

To understand differentially expressed genes in RObb-12, we performed gene expression microarray analysis for the total blood, lymph node (LN) and rectal pinch biopsies collected before and after 5X low dose challenges (Fig. 7). Supervised analysis identified 353, 28 and 57 individual genes in blood, LN and gut biopsies, respectively, that were differentially regulated. We noted a clear upregulation of several genes involved in cell-mediated immunity, cell signaling, immune cell trafficking and cell cycle pathways. Most notably, CCL8 (C-C motif ligand 8; also known as monocyte chemoattractant protein 2 (MCP-2)) was upregulated in the LN. MCP-2 binds to CCR5 with high affinity and blocks HIV-1 replication [51].

4. Discussion

Here, we report a case of complete protection of a vaccinated RM against repeated mucosal challenges with a biologically relevant immunodeficiency virus. The protected animal showed the highest level of vaccine-induced virus-specific cellular immune responses among the group of vaccinees. Although no vRNA was detected in blood, lymph node and rectal biopsies, the animal showed strong anamnestic immune responses after repeated virus exposures. The role of anamnestic cellular responses in controlling virus replication has been documented [13, 52, 53]. We believe that the anamnestic cellular responses were generated due to a cryptic infection and these responses rapidly cleared infected cells before establishment of systemic infection could occur. Although all vaccinees, including the protected RM RObb-12, carried TRIM5α genotypes that were most resistant to SIVsmE543-3 (TRIM TFP/TRIM TFP or TRIM TFP/ TRIM CYPA or TRIM CYPA/ TRIM CYPA), the PBMC of the protected animal supported robust virus replication ex vivo. Thus, this animal was not intrinsically resistant to replication of the challenge virus but presents a case of prevention of viremia by vaccine-induced immune responses.

We have previously shown prevention of SHIV-C acquisition in vaccinated animals [12, 13, 31]. Unlike in the present study, the challenge virus used in all our earlier studies was relatively neutralization sensitive (tier 1). Prevention of virus acquisition was seen in multiple vaccinees that had developed the highest levels of challenge virus-specific cellular as well as nAb responses (Table 2). Due to the bimodal nature of the protective immune responses, the relative contribution of nAb and cellular immune responses could not be assessed. In the present study, challenge virus-specific nAbs did not develop in any vaccinated animal, which was not surprising considering the tier 2 nature of our challenge virus, SHIV-2873Nip. Despite the absence of nAb as well as ADCC responses on day 0, one vaccinee was protected. The ADCVI mechanism could not be linked to the protection. Moreover, no difference was detected in the epitope specificity of the humoral responses of the protected versus unprotected animal by our extensive biopanning efforts using day 0

polyclonal IgG samples. This suggests that cellular responses played a major role in the protection of this animal. Furthermore, microarray data of ROb-12 showed upregulation of several genes involved in cell-mediated immunity, cell signaling, immune cell trafficking, and cell cycle pathways. Most notably, CCL8 (also known as MCP-2) was upregulated in the LN. It is known that MCP-2 is a potent inhibitor of HIV-1, since it binds to CCR5 with high affinity [51]. This mechanism may also have played a role in preventing the establishment of persistent systemic infection in our protected animal.

In one of our earlier vaccine studies [12], strong virus-specific cellular responses were detected in some animals immediately after vaccination but these animals did not show anamnestic cellular responses after repeated virus exposures. The animals became viremic and also did not control virus replication. In contrast, the protected animal in this study showed strong anamnestic cellular responses. This highlights the importance of anamnestic cellular responses in preventing systemic spread of cryptic infection. Other vaccinees from this study did not show anamnestic cellular responses and also lacked nAb responses. This is in agreement with the finding that no significant differences were seen in the peak viremia and AUC of viral load of vaccinees and controls. The protected RM showed a low level of ADCC activity after the virus exposures but was repeatedly negative before the first virus challenge. We previously reported an alteration in the antibody repertoire even in the absence of viremia in vaccine-protected RMs [54]. It is possible that ADCC mediating antibodies were induced after virus exposures and contributed to the prevention of viremia.

In our earlier mouse studies, OSP were able to induce immune responses in different strains of inbred mice as well as in the outbred mice [25, 26]; the strategy was found to be only modestly successful in RMs. This is not a stand-alone case where a mouse-tested vaccine concept could not be translated with equal success into primates/humans. The discrepancy in immunogenicity and efficacy in mice versus primates has been observed for DNA vaccination (reviewed in [55]) and in our Listeria-vector prime, adenovirus boost strategy [12]. Administration of autologous PBMC pulsed with 15-mer viral peptides to SIV-infected macaques [56-58] and administration of 15-mer HIV-1 p24-like peptides to infected patients [59] boosted virus-specific cellular responses. However, 15-mer peptides administered to the naïve animals in our study, did not effectively induce immune responses.

To induce anti-Env responses, multimeric HIV-C gp160 protein derived from a recently transmitted HIV1084i was administered three times at intervals of six to seven weeks. To broaden anti-Env nAb specificities, we decided to boost the animals with a different HIV-C Env. The animals were given HIV-1 CO6980 gp145 that was 20.9% and 23.1% divergent compared to Env of HIV1084i and the challenge virus, respectively. The strategy of sequential immunizations with heterologous Env has been reported to mimic in vivo development of broad nAb responses [32, 60]. However, this strategy was not successful in the present study to induce nAbs against our tier 2 challenge virus.

Nonetheless, this study reports a case of prevention of viremia by vaccine-induced immune responses. Considering the challenges associated with the induction of nAbs and the recent recognition of the contribution of vaccine-induced cellular immunity to protection [61-64], it

may be important to harness cellular immune defenses as well as ADCC mechanisms for HIV vaccine development.

Acknowledgements

We thank Stephanie Ehnert, Chris Souder and Kalpana Patel from the YNPRC for assistance with the primate studies. We acknowledge ABL, Inc. for the production of the clade C gp145 protein. This work was supported by NIH grants R01 AI100703 to RMR and P01 AI048240 to RMR, SLH, RAR and JLE. This project was also funded in part by the Intramural Research Program of the NIH, National Cancer Institute and by the Office of Research Infrastructure Programs/OD P51 OD011132 to the YNPRC and OD P51 OD011133 (previously NCRR grant P51 RR13986) to the Southwest National Primate Research Center. The work conducted by WEJ was supported by NIH grant AI095092.

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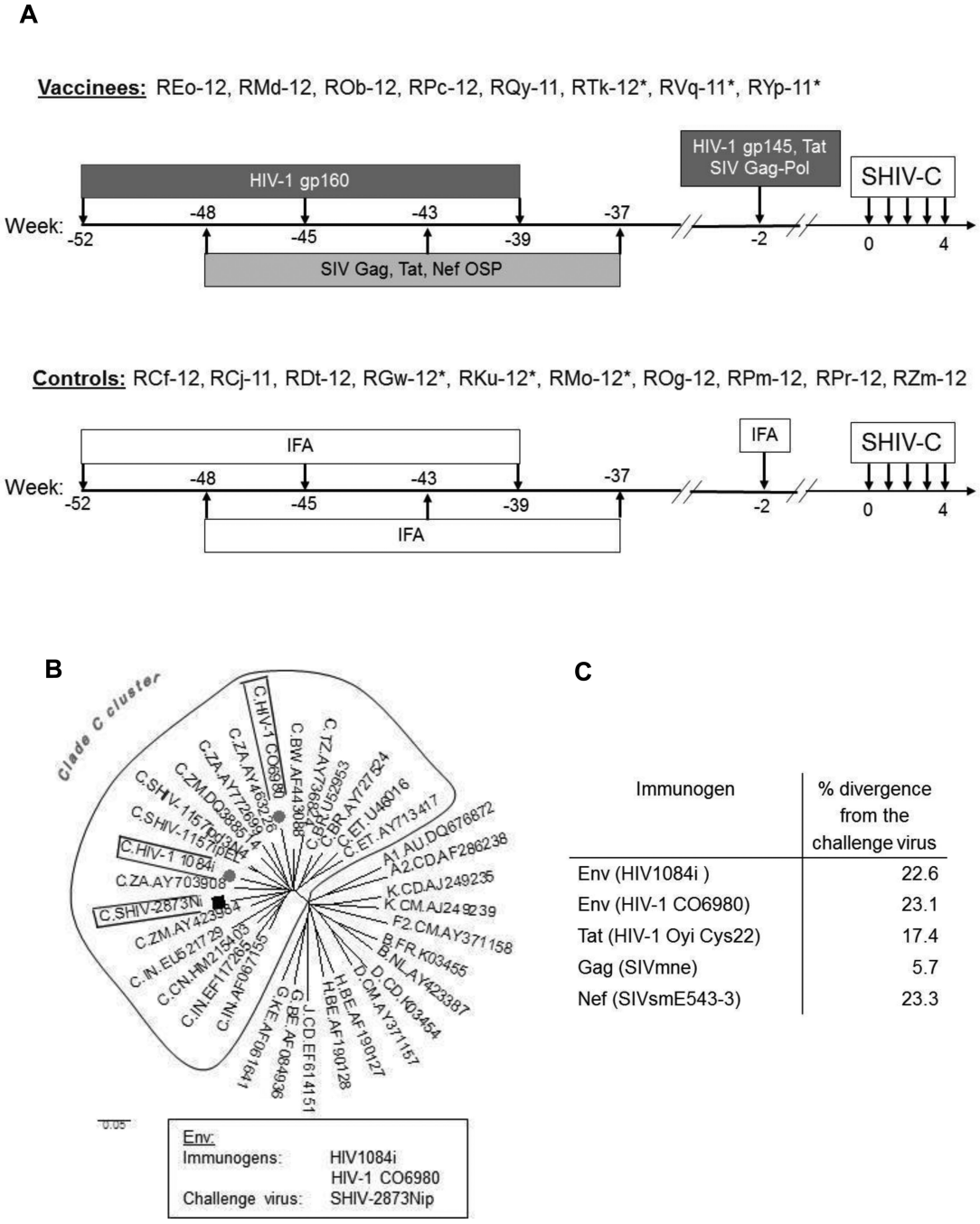
Highlights

1. Vaccine-induced protection against heterologous tier 2 SHIV-C challenge
2. Protection was associated with anamnestic cellular and ADCC responses
3. Protection was achieved in the absence of measurable challenge virus-specific neutralizing antibodies



Immunogen	% divergence from the challenge virus
Env (HIV1084i)	22.6
Env (HIV-1 CO6980)	23.1
Tat (HIV-1 Oyi Cys22)	17.4
Gag (SIVmne)	5.7
Nef (SIVsmE543-3)	23.3

Fig. 1.

Experimental time line and HIV-1 Env phylogenetic analysis. (A) RMs were immunized with 100 μg of recombinant HIV-1 1084i gp160 in IFA at weeks −52, −45 and −37 by the intramuscular (i.m.) route. The animals were also immunized with overlapping synthetic peptides (OSP) representing the entire Gag (SIVmne), Tat (Oyi), and Nef (SIVsmE543-3) at weeks −48, −43 and −37. For OSP immunization, 300 μg of each peptide with IFA was administered by the subcutaneous (s.c.) route. All animals received a final boost with HIV-1 CO6980 gp145, SIVmne Gag-Pol particles and HIV-1 Tat Oyi two weeks before the first

SHIV-C exposure; the recombinant protein immunogens were administered with IFA. Control RMs received IFA alone at corresponding time points. All animals were challenged at weeks 0, 1, 2, 3, and 4 with low-dose SHIV-2873Nip by the intrarectal (i.r.) route. *, Mamu A*001^{+} RMs. (B) Phylogenetic tree of HIV-1 clade C Env sequences of immunogens and challenge virus. Sequences of HIV-1 reference strains were obtained from the Los Alamos HIV-1 sequence database. The evolutionary tree was inferred using the Neighbor-Joining method by MEGA4 software. (C) The percent divergence between amino acid sequences of immunogens and challenge virus.

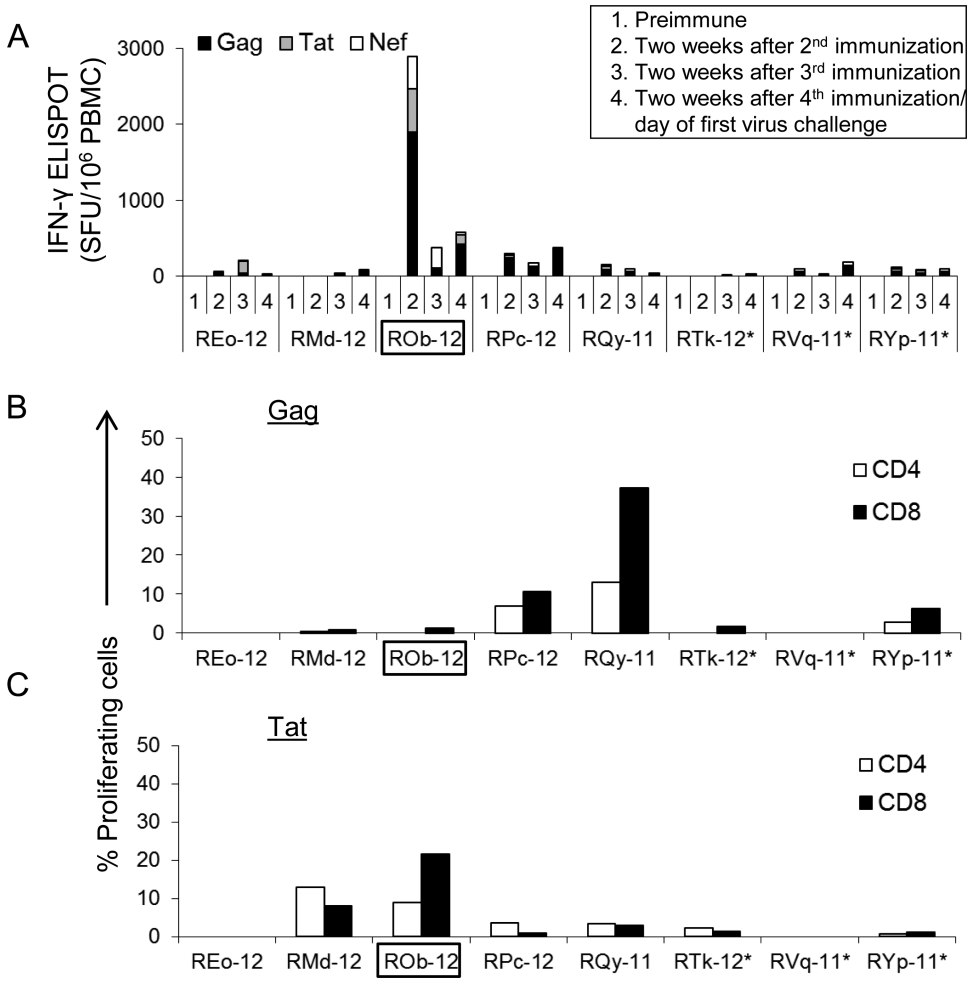


Fig. 2.

Cellular immune responses after vaccination. (A) SIVmne Gag, HIV-1 Tat Oyi and SIVsmE543-3 Nef-specific, IFN-γ-secreting cells (spot-forming units (SFU) / 106 PBMC) as determined by ELISPOT assay at different time points. (B & C) Ex vivo proliferation of CD4+ and CD8+ T cells after stimulation with SIVmne Gag, or HIV-1 Tat Oyi peptides. The assay was performed after the final immunization, i.e., on the day of the first SHIV-C challenge. The RM ROb-12 (boxed) remained aviremic after all heterologous SHIV-C challenges. *, Mamu A*001+ RMs.

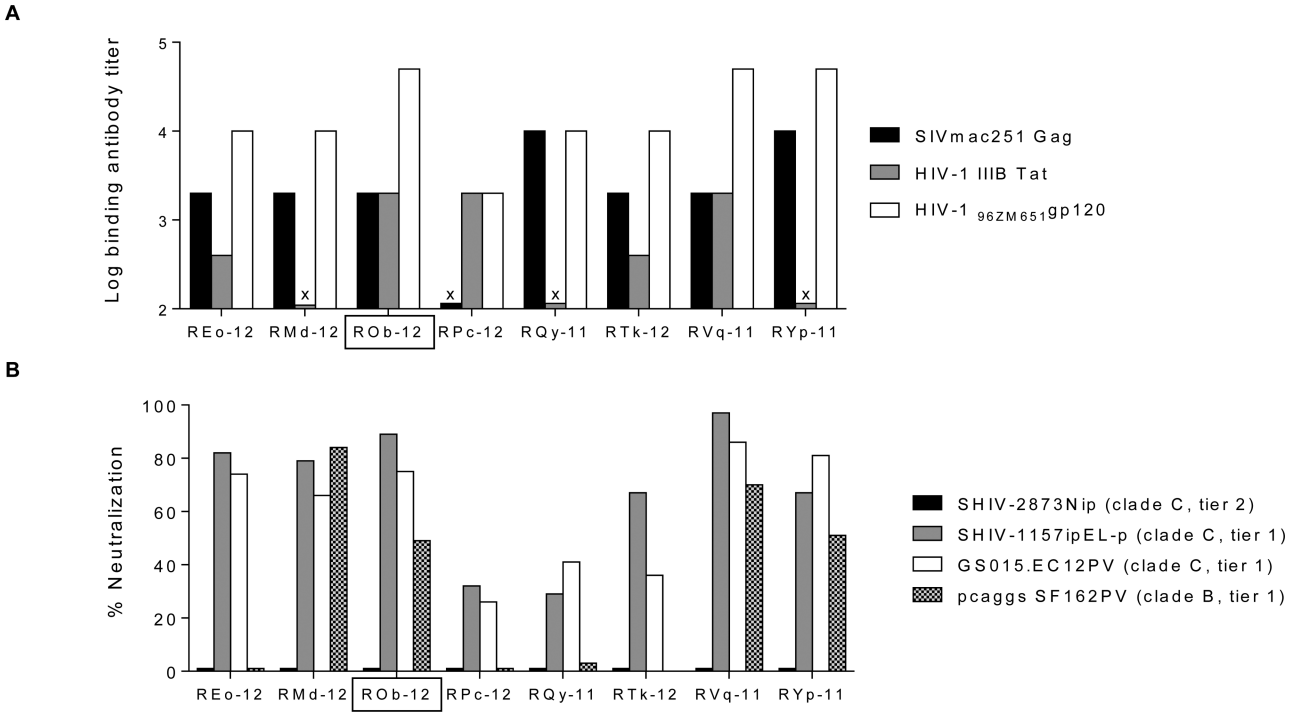


Fig. 3.

Humoral immune responses after vaccination (on the day of first virus exposure). (A) Reciprocal serum antibody ELISA titers against SIVmac251 Gag, HIV-1 IIIB Tat and heterologous clade C HIV96ZM651 gp120. (B) NAb activity against tier 1 (two clade C and one clade B) strains and against the tier 2 challenge virus (SHIV-2873Nip), as measured by TZM-bl assay. The % virus neutralization by sera collected on the day of first virus challenge (at 1:20 dilution with regard to autologous pre-immune serum) is presented. X, binding titer < 200. The RM ROb-12 (boxed) remained aviremic after all heterologous SHIV-C challenges. None of the sera showed neutralizing activity against SHIV-2873Nip.

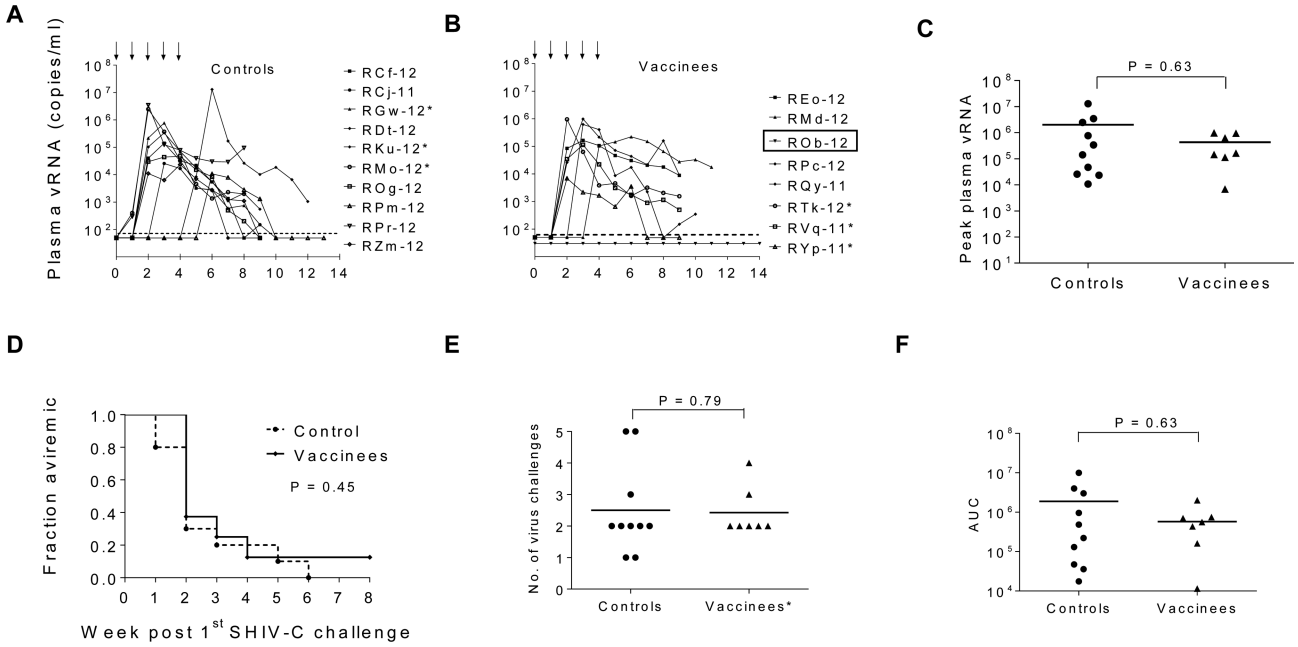


Fig. 4.

Post challenge viremia data. (A–B) Five low-dose i.r. challenges of SHIV-2873Nip were given at weeks 0, 1, 2, 3, & 4 (as shown by black arrows) and plasma vRNA levels were measured. (C) Comparison of peak plasma vRNA levels, (D) Kaplan–Meier plots depicting the fraction of RMs remaining aviremic, (E) comparison of the number of virus challenges before detection of viremia, and (F) comparison of area-under-the-curve (AUC) for viremic RMs. RM ROb-12 (boxed) remained aviremic after all heterologous SHIV-C challenges. Horizontal dashed line in A-B, the lower limit of plasma vRNA detection (50 vRNA copies/ml); horizontal solid lines in C, E, & F, mean peak plasma vRNA, the mean number of virus challenges before detection of viremia, & mean AUC, respectively. *, Mamu A*001+ RMs.

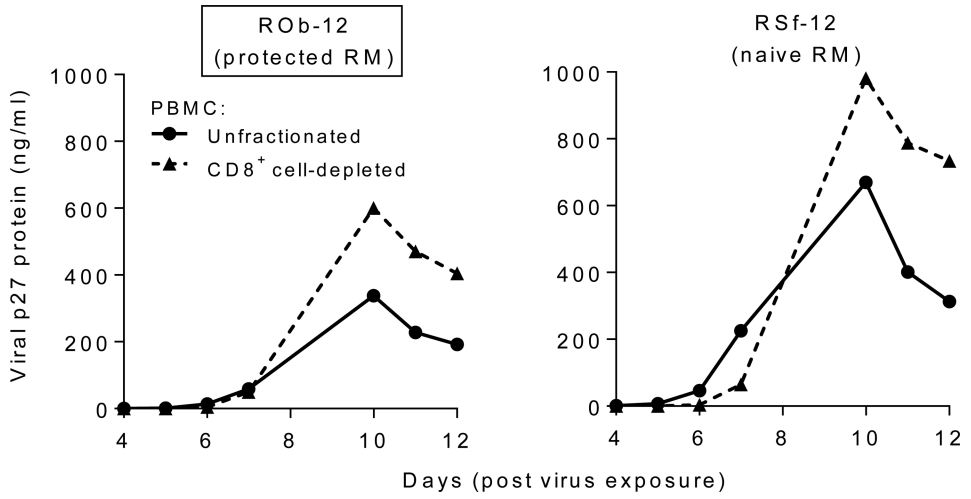


Fig. 5.

Ex vivo replication of SHIV-2873Nip in cultured PBMC of ROb-12, the protected RM (boxed). PBMC were stimulated with Con A in the presence of IL-2. Unfractionated PBMC or CD8+ cell-depleted PBMC were exposed to SHIV-2873Nip, and replication was monitored by p27 ELISA of culture supernatants. As control, virus replication in PBMC (unfractionated or CD8+ cell-depleted) of one naïve RM (RSf-12) was also measured.

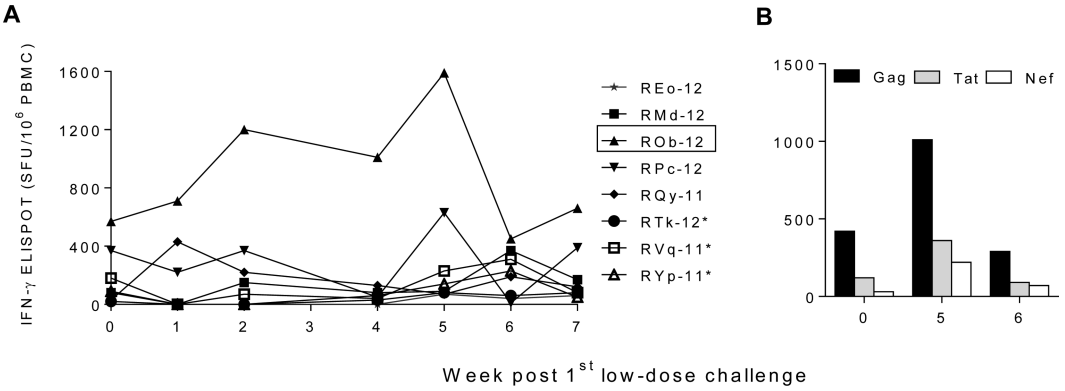


Fig. 6.

Virus-specific cellular immune responses during and after virus challenges. (A) The sum of Gag, Tat and Nef-specific, IFN-γ-producing cells measured by ELISPOT assay is shown for the vaccinees. The RM ROb-12 (boxed) remained aviremic after all heterologous SHIV-C challenges. (B) Anamnestic cellular responses against individual vaccine component shown by ROb-12.

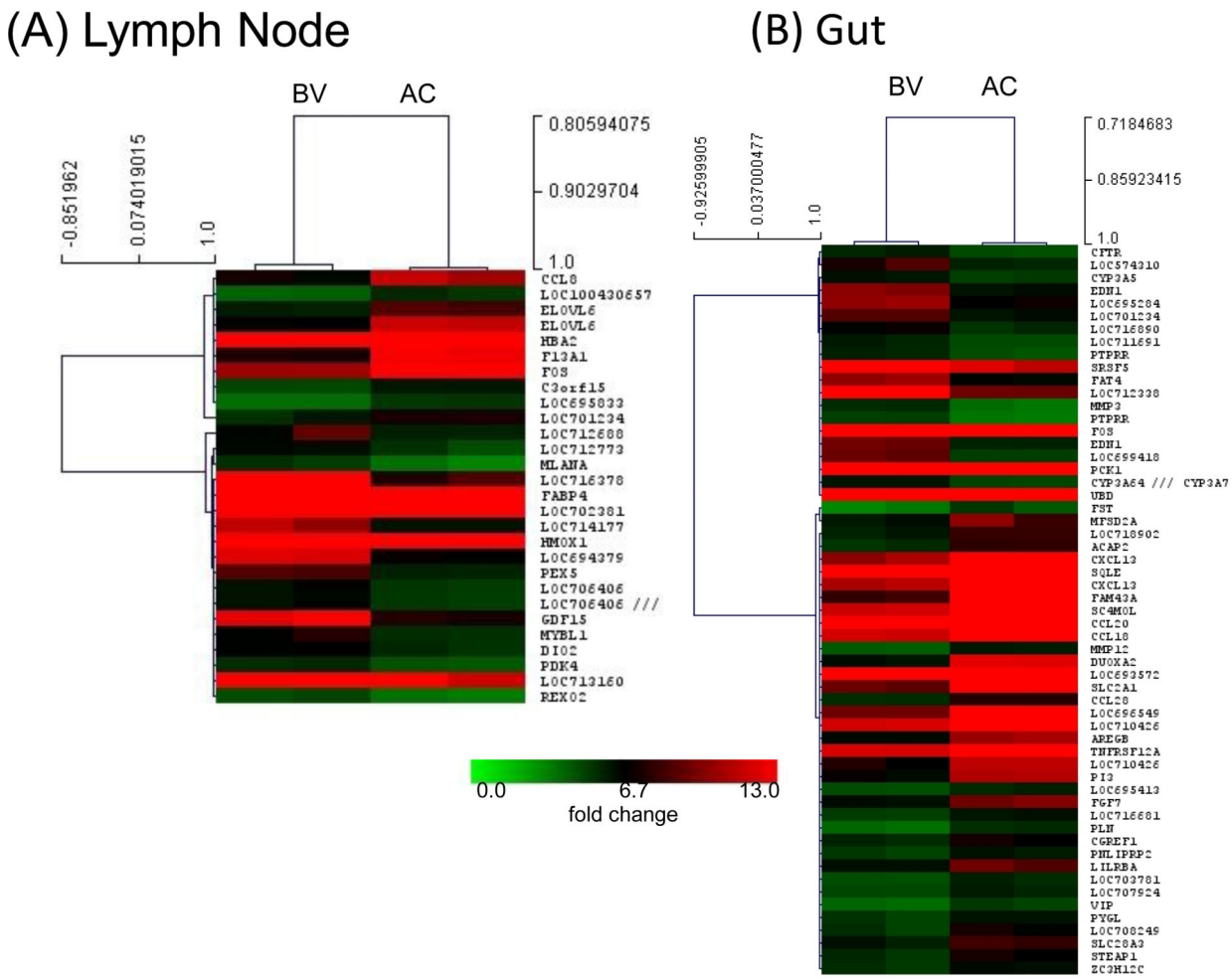


Fig. 7.

Microarray analysis for ROb-12, a RM protected against multiple low-dose challenges with a heterologous R5 SHIV-C. Hierarchical clustering and heat map analysis of differentially expressed genes in the lymph node (A) and gut (B). There were 28 and 57 genes in the lymph node (LN) and gut biopsies, respectively, that were differentially expressed after vaccination and virus challenges (AC) compared to the before vaccination (BV). The analysis uses a significance threshold of false discovery rate <5% and $\log_2$ fold-change of >2.

Table 1

Virus-specific humoral responses.

	Week 0 (for all vaccinated RM)	**Week 6 (for protected RM, RObb-12)**
nAb response (TZM-bl assay)	Not detected	Not detected
nAb response (A3R5 assay)	Not detected	Not detected
Binding Ab titer against HIV-C Env	Did not correlate with peak viremia	Boost (~2 fold) observed
ADCC	Not detected	Low level
ADCVI titer	Did not correlate with peak viremia	No boost observed

Table 2

The immune responses of vaccine-protected (aviremic) vaccinees from present and our earlier published studies.

Vaccine-protected RM (Undetectable viremia)	Challenge virus	Virus-specific immune responses (week 0)		Ref
		Cellular IFN-γ ELISPOT (Total SFU/10^6 PBMC)	Humoral Challenge virus-specific nAb titer (IC_{50}, TZM-bl assay)	
RAt-9	SHIV-1157ip (tier 1)	3830	130	32
RRi-11	SHIV-1157ipEL-p (tier 1)	3560	3157	13
RTr-11	SHIV-1157ipEL-p (tier 1)	920	4382	13
RQe-10	SHIV-1157ipEL-p (tier 1)	520	48	12
RQb-12	SHIV-2873Nip (tier 2)	570	<20	Present study