



Research Paper

Correlates of Protection Against SIV_{mac251} Infection in Rhesus Macaques Immunized With Chimpanzee-Derived Adenovirus Vectors



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ABSTRACT

We report on prime-boost vaccine regimens with two simian adenovirus (Ad) vectors (SAdV) or two human serotype Ad vectors (HAdV) expressing Gag and gp160 of simian immunodeficiency virus (SIV)_{mac239} tested in HAdV-seropositive rhesus macaques (RMs) repeatedly challenged rectally with low doses of SIV_{mac251}. Both vaccine regimens reduced set point and peak viral loads (PVL) and accelerated viral clearance. In SAdV-vaccinated controller genotype RMs resistance against infection correlated with levels of envelope (Env)-specific antibody (Ab) titers. In both vaccine groups CD8⁺ T cells controlled viral loads (VL) upon infection. Circulating CD4⁺ and CD8⁺ T cells showed significant changes in their transcriptome over time following vaccination, which differed between the vaccine groups. T cells from SIV-resistant RMs had unique transcriptional profiles indicating that both follicular T helper (T_{FH}) cell responses and highly activated CD8⁺ T cells may play a role in protection. © 2018 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Worldwide, the acquired immunodeficiency syndrome (AIDS) is the leading cause of death in humans, aged 15–59. HIV-1 vaccines that have undergone testing in large-scale clinical trials either lacked efficacy or only provided transient protection to a fraction of vaccine recipients (Buchbinder et al., 2008; Huang et al., 2015; James, 2003; Rerks-Ngarm et al., 2009).

Here we tested the efficacy of two different adenovirus (Ad) vaccine regimens in preventing virus acquisition or promoting rapid viral control upon breakthrough infections in a preclinical rhesus macaques (RM) model of repeated low dose intra-rectal simian immunodeficiency virus (SIV)_{mac251} challenges. Both regimens, one based on simian adenovirus vectors (SAdV) vectors to which humans lack pre-existing neutralizing antibodies (nAbs) (Xiang et al., 2006), the other on human serotype Ad vectors (HAdV) vectors to which pre-existing immunity is common, were designed to induce SIV-specific T and B cell responses.

The vaccines were tested in RMs with or without controller genotypes. Our results show that, although both vaccine regimens induce immune responses of comparable magnitude, they show marked differences in antibody functions, efficacy, correlates of protection and changes in the transcriptome of circulating T cells. Differences in the CD4⁺ T cells' transcriptomes of resistant vs. susceptible RMs indicate that enhanced activity of T helper (T_{FH}) cells is protective while regulatory T cells (Tregs) promote infections. For CD8⁺ T cells, which are not thought to prevent virus acquisition but rather limit virus spread, resistant RMs show enhanced activity of TNF-regulated gene expression prior to SIV challenges.

2. Materials and Methods

2.1. Vaccine Vectors

Ad vectors were derived from serotypes SAdV23, SAdV24, HAdV5 and HAdV26. The E1- and E3-deleted Ad vectors express Gag or gp160 of SIV_{mac239}. To induce nAbs to the vaccines, HAdV vectors expressing the rabies virus glycoprotein (rab.gp) were used. Vectors were generated, rescued, expanded, purified, titrated and quality controlled as described (Zhou et al., 2010).

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2.2. Non-Human Primates

Two to three-year-old, healthy Indian origin *Macaca mulatta* were housed at Bioqual, Inc. (Rockville, MD). Animals were typed for Mamu-A*01, A*02, A*08, A*11, B*01, B*03, B*04, B*08 and B*17 alleles (UW AIDS Vaccine Research Lab, Madison, WI).

2.3. Ethics Statement

Animals were housed in accordance with the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care International Standards and in the Guide for the Care and Use of Laboratory Animals of the US-NIH. The Institutional Animal Use and Care Committee of BIOQUAL approved the study (09-3437-25). When immobilization was necessary, RMs were sedated i.m. with 10 mg/kg of Ketamine HCl (Parke-Davis, Morris Plains, N.J.). Details of animal welfare and steps taken to ameliorate suffering were in accordance with the recommendations of the Weatherall report, “The use of non-human primates in research”. RMs were housed in an air-conditioned facility with ambient temperature of 21–25 °C, relative humidity of 40%–60% and 12 h light/dark cycle. RMs were housed in suspended, stainless steel, wire-bottomed 6 sq. ft. cages and provided with a commercial primate diet and fresh fruit and vegetables 2× daily and water ad libitum. Social housing, toys, foraging equipment and mirrors were provided. RMs were monitored at least 2× daily for behavior, food intake, activity, and overall health. Sick RMs were euthanized using methods consistent with recommendations of the American Veterinary Medical Association Panel on Euthanasia.

2.4. Analyses of Ad-Specific Antibodies

RMs were screened prior to enrollment for nAbs to SAdV24 and SAdV23 vectors (Xiang et al., 2006) and found to be seronegative. Using the same assay they were screened for nAbs to HAdV vectors after immunizations with the HAdV-rab.gp vectors.

2.5. Immunization Regimen

RMs were injected intra-tracheally with 1×10^{11} vp of HAdV5rab.gp and HAdV26rab.gp vectors twice in a monthly interval. RMs were bled 2 weeks later to determine HAdV-specific nAb titers. RMs were then distributed according to genotypes and HAdV-specific nAbs titers into 3 groups of 12 animals each. Twelve RMs were primed i.m. with 5×10^{10} vp of SAdV24gag mixed with 5×10^{10} vp of SAdV24gp160; they were boosted 6 months later with the same dose of SAdV23 vectors expressing the same inserts. Another 12 animals were primed with HAdV26 vectors and boosted 6 months later with HAdV5 vectors expressing the same inserts and used at the same doses. The remaining 12 animals were not immunized.

2.6. Viral Challenge

Six months after the boost, RMs were challenged rectally 10 times in weekly intervals with 1 TCID₅₀ of SIV_{mac251} (most kindly provided by Nancy Miller, DAIDS, Bethesda, MD). Animals that developed viral loads (VL) above 1000 RNA copies/ml received not further challenges.

2.7. Plasma VL

Plasma SIV VL was determined by quantitative real-time RT-PCR (Lewis et al., 2010). Peak viral loads (PVL) reflect the highest VL within an animal. Set point VL reflect median loads maintained for 4 weeks as of the week after the PVL.

2.8. Virus Integration

Genomic DNA was extracted from CD3⁺CD4⁺ live PBMCs with DNeasy Blood and Tissue Kit (Qiagen). Ten nanograms of DNA were amplified by PCR using a mix of forward primers for simian and human Alu sequences, and reverse primers for SIVgag. The following primers were used: first PCR: simian Alu, 5′-TTCCGGTGGCTCACGCTG-3′; human Alu, 5′-TAGTCGGGAGGCTGAGGCAGGAGAA-3′; SIVgagR1, 5′-TCTCTTCTGCGTGAATGCACC-3′; SIVgagR2, 5′-AAGCTTTTTAAATTTCTGAGCC TG-3′ under the following conditions: 94 °C for 1 min, 20 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, final elongation at 72 °C for 1 min. GapDH was used under the same conditions, with primer sequences 5′-TGCCACCCAGAAGACTGTGG-3′ and 5′-ACCAGGAAATGAGC TTGACAAAG-3′. Two microliters of the amplicon were digested with 10 units of *NotI* for 30 min at 37 °C, followed by enzyme inactivation at 65 °C for 20 min. The product was used as template for a nested real-time PCR (50 °C for 20s, 95 °C for 10 min, and 35 cycles of 95 °C for 15 s and 60 °C for 1 min), which was performed utilizing the mix of reverse SIVgag primers, and a forward primer specific for the LTR region of SIV: 5′-AGGAAGAGGCCTCCGGTTG-3′. All real-time PCR samples were quantified by normalization in comparison to GapDH sequences. Sample were tested by qPCR using 40–60 replicates. Outliers were excluded by median absolute deviation tests.

2.9. CD8⁺ Cell Depletion

RMs were injected with depleting anti-CD8α Ab (cM-T807R1, NIH Reagent Source, NIH) first subcutaneously at 10 mg/kg, then intravenously at 5 mg/kg on days 0, 3, 7 and 10.

2.10. Elisa

Sera of RMs were tested for gp160-specific Abs by an ELISA on plates coated with a baculovirus-derived gp160 protein (Emmer et al., 2016).

2.11. ADCC Assay

The ADCC assay was performed as described (Alpert et al., 2012a) with SIV_{mac251}-infected RM CD16⁺ luciferase⁺ CEM Nkr target cells and HKYG1 effectors cells.

2.12. Isolation and Preservation of Lymphocytes

Purified PBMCs were tested immediately after isolation or frozen in 90% FBS and 10% dimethyl sulfoxide (Sigma, St. Louis, MO) at −80 °C till testing.

2.13. Synthetic Peptides

Peptide pools of 15-mers (overlapping by 11 amino acids) spanning the SIV_{mac239} Gag and envelope (Env) proteins were reconstituted in dimethyl sulphoxide (DMSO) and pools were prepared from individual peptide stocks obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

2.14. Intracellular Cytokine Staining (ICS)

The function of SIV-specific CD8⁺ T cells was assessed by ICS for IFN-γ, IL-2, and TNF-α production as well as expression of the degranulation marker CD107 after stimulation with SIV Gag or Env peptide pools as described (Dunham et al., 2006).

2.15. Statistical Analysis

Differences in SIV acquisition and VL declines were determined by Mantel-Cox test. Normality of data was tested by D'Agostino & Pearson

omnibus normality test. Not-normal data were analyzed by non-parametric tests, i.e., Wilcoxon-Mann-Whitney test for 2 groups comparison or Kruskal-Wallis test with Dunn correction for multiple comparisons. Differences in VL and set point VL were determined by Kruskal-Wallis test with Dunn correction. Differences in relative levels of integrated SIV genome at 2 and 12 weeks were tested by ANOVA with Holm-Sidak correction. Differences in Ab titers and T cell responses were determined by Wilcoxon-Mann-Whitney test. Correlations were determined using Spearman correlation with Bonferroni correction. For multiple comparisons adjusted *p*-values are shown. Analyses were conducted using GraphPad Prism 6, SAS 9.2, and SPICE v5.1.

2.16. Cell Purification

Stained PBMCs were sorted into CD3⁺CD4⁺ and CD3⁺CD8⁺ cells on a MoFlo AsteriosEQ cell sorter.

2.17. RNA Purification

RNA was extracted with the RNeasy micro kit (Qiagen, Hilden, Germany) according to manufacturer's specifications; genomic DNA was removed by on-column DNase digestion. RNA integrity was assessed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) capillary electrophoresis on a RNA Pico chip.

2.18. Microarray Hybridization

cDNA synthesis and amplification was performed with the NuGEN Ovation One-Direct System (NuGEN, San Carlos, CA, USA). Total RNA was used for cDNA synthesis followed by whole transcriptome amplification by NuGEN's Ribo-SPIA® technology. The amplified single stranded DNA was purified with AMPure XP beads (Beckman, Indianapolis, IN, USA). Qualitative and quantitative analyses were performed on the Bioanalyzer and NanoDrop, respectively. 5 µg of the amplified DNA was used for biotinylation and fragmentation using the NuGEN Ovation Encore Biotin Module (Nugen, San Carlos, CA, USA). All samples were hybridized to Affymetrix GeneChip® Rhesus Macaque Genome Arrays (Affymetrix, Santa Clara, CA, USA). The probe arrays were washed, stained and scanned as described in the Affymetrix GeneChip® Expression Analysis Technical Manual. CEL files were extracted from the raw scanned images using the Affymetrix GeneChip® command console Software. Quality Control metrics were monitored on the Affymetrix Expression console software; discordant arrays were excluded from further downstream analyses.

2.19. Microarray Transcript Differential Expression Analyses

Transcript expression values were modeled and normalized using the Robust Multi-array average (RMA) method, and log₂ transformed (AffyPLM, R Bioconductor.) X-linked genes were excluded from subsequent analysis. Differentially expressed transcripts were identified using one- or two-way ANOVA. Transcripts differentially expressed between challenge virus-susceptible and resistant animals were identified using a moderated *t*-test. Heatmaps represent log₂ transformed expression values, and are supervised on the abscissa (samples) and organized by unsupervised hierarchical clustering on the ordinate (transcripts). Expression values of each transcript (rows) are mean centered and scaled to unity standard deviation. Unsupervised hierarchical clustering of transcripts (rows) used Euclidean distance and Complete Linkage methods.

2.20. Microarray Pathway Analysis

Most differentially expressed transcripts between vaccine groups, and between susceptible and resistant RMs were selected for pathway

analysis using the Ingenuity Pathway Analysis Upstream Regulator Analysis.

Principal component analysis was performed on the expression values of transcripts differentially expressed between susceptible and resistant animals and subsequently filtered by pathway analysis. Principal components 1 and 2 account for 36% and 12% respectively of variance for the CD4⁺ T cell analysis, and 38% and 12% respectively of variance for the CD8⁺ T cell analysis.

Data available through the National Center for Biotechnology Information and the Gene Expression Omnibus (GEO) database: accession number GSE102115, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102115>.

3. Results

3.1. SIV Acquisition, VL, and Levels of SIV Integration

To assess effects of pre-existing Abs to the HAdV vectors, we first injected 36 Indian-origin RMs with HAdV vectors expressing an unrelated transgene. RMs developed moderate nAb titers to HAdV5 and higher titers to HAdV26 (Table 1). RMs with high or low nAb titers were distributed equally into 3 groups. Each group had 8 Mamu-A*01⁺ RMs and groups 1 and 3 had one Mamu-B*17⁺ RM each (Table 1). RMs of group 1, the SAdV group, were vaccinated 4 weeks after the last HAdV exposure with 5 × 10¹⁰ virus particles (vp) of SAdV24 expressing Gag (SAdV24gag) mixed with 5 × 10¹⁰ vp of SAdV24 expressing gp160 (SAdV24gp160). Antigen were derived from SIV_{mac239}. RMs of group 2, the HAdV group, were vaccinated with the same doses of HAdV26 vectors expressing the same inserts. Six months later RMs of the SAdV24 and HAdV26 groups were boosted

Table 1
Characteristics of RMs.

| Group | NHP ID | Genotype | Gender | HAdV5 Titer | HAdV26 Titer | |
|---------------|------------|----------|--------|-------------|--------------|-------|
| SAdV group | 4825 | —** | M | 20 | 640 | |
| | 4826 | — | M | 20 | 640 | |
| | 4889 | — | M | 0 | >1280 | |
| | 4372 | A01, A02 | F | 20 | 1280 | |
| | 4395 | A01, A02 | M | 0 | 320 | |
| | 4400 | A01 | M | 160 | 640 | |
| | 4552 | A01 | M | 20 | 160 | |
| | 4553 | A01 | M | 40 | >1280 | |
| | 4770 | A01 | M | 40 | 10 | |
| | 4773 | A01 | M | 80 | 40 | |
| | 4774 | A01 | M | 160 | 20 | |
| | 4894 | A02, B17 | M | 0 | 160 | |
| | HAdV group | 4389 | A02 | F | 0 | 160 |
| | | 4892 | A02 | M | 0 | 1280 |
| | | 4893 | B01 | M | 20 | 80 |
| | | 4916 | — | M | 40 | >1280 |
| 4381 | | A01, A02 | M | 40 | 1280 | |
| 4394 | | A01 | M | 20 | 1280 | |
| 4547 | | A01 | M | 160 | 1280 | |
| 4776 | | A01 | F | 40 | 20 | |
| 4777 | | A01 | M | 160 | 40 | |
| 4778 | | A01 | M | 80 | 80 | |
| 4779 | | A01 | M | 20 | 640 | |
| 4781 | | A01 | M | 10 | 320 | |
| Control group | 4824 | — | M | 0 | 160 | |
| | 4886 | — | M | 0 | 0 | |
| | 4891 | — | M | 0 | 20 | |
| | 4365 | A01 | F | 10 | 1280 | |
| | 4398 | A01, B01 | F | 10 | 0 | |
| | 4546 | A01 | M | 80 | 640 | |
| | 4769 | A01 | M | 80 | 20 | |
| | 4771 | A01 | M | 40 | 1280 | |
| | 4772 | A01 | F | 160 | 80 | |
| | 4775 | A01 | M | 640 | 40 | |
| | 4780 | A01 | M | 20 | 160 | |
| 4392 | A02, B17 | F | 40 | 0 | | |

—**Animals were negative for A01, A02, B01 or B17.

with SAdV23 and HAdV5 vectors, respectively, expressing the same inserts and used at the same doses as the priming vectors. The control group was not vaccinated. Starting 6 months after the boost, RMs were challenged rectally in weekly intervals for up to 10 times with 1 mean tissue infective dose (TCID₅₀) of SIV_{mac251}. RMs with VL ≥ 1000 viral RNA copies per ml of plasma received no further challenges.

All control RMs develop plasma VL ≥ 40 within 8 challenges (Fig. 1a, b). In the SAdV and HAdV groups 2 and 1 RMs remained uninfected, respectively, after 10 challenges. RMs of the SAdV and HAdV groups developed lower PVL (SAdV: $p < 0.0001$, HAdV: $p = 0.0006$ by Mann-Whitney) and set point VL (SAdV: $p < 0.0001$, HAdV: $p = 0.0008$) compared to control RMs (Fig. 1c). Viral loads were affected by genotype; Mamu-A*01-/B17-RMs of the SAdV but not the HAdV group developed significantly lower peak and set-point viral loads (adjusted [adj.] $p = 0.0209$ by Kruskal-Wallis test with Dunn correction) compared to those of the control group. The Mamu-A*01-/B17- RMs of the SAdV but not the HAdV group showed a potential trend towards increased resistance against sustained SIV_{mac251} infection, compared to those of Mamu-A*01-/B17- RMs control RMs (Fig. 1d,e). By 4 months after the 1st challenge, all of the HAdV- and 8/10 of the infected SAdV-vaccinated RMs controlled VL below levels of detection (Fig. 1f). Differences in decline of VL were significant comparing the SAdV ($p = 0.026$) and HAdV groups ($p = 0.009$) to the control group. In the vaccine groups weeks time till control of plasma virus titers to <40 correlated

directly with PVL (Fig. 1g). Pre-existing HAdV-specific nAb titers did not affect sensitivity to infection or virus spread.

To assess if RMs with undetectable VL had cleared the infection RMs were treated with a CD8⁺ cell-depleting Ab regimen at 28 weeks after the last challenge. We selected 5 RMs from the SAdV group and 4 RMs from the HAdV group; one of the SAdV RMs (#4825) had low viral loads at that time, one of the HAdV RMs (#4781) had never been infected, the other RMs had had undetectable viral loads for at least 3 months prior to CD8 depletion. All RMs, except #4781, increased VL following CD8⁺ cell depletion (Fig. 1h) confirming that virus had not been cleared. Once the treatment was stopped, VL rapidly declined in all but RM #4825.

Relative amounts of integrated SIV in circulating CD4⁺ T cells were tested at 2 and 12 weeks after infection in a subset of RMs (SAdV group: $n = 7$, HAdV group: $n = 11$, control group: $n = 4$) (Fig. 1i). We found levels of integrated SIV genome by 2 weeks after detectable viremia, which significantly increased in the HAdV group ($p = 0.02$) by 12 weeks after infection. By then relative amounts of integrated SIV were significantly lower in RMs of the SAdV than the control group ($p = 0.02$). Levels of virus integration or their changes over time were not affected by PVL, time to control, Mamu genotype, pre-existing HAdV-specific nAb titers or Ab titers to Env. HAdV-vaccinated RMs with lower levels of SIV integration by 2 weeks after infection (<50,000) had higher frequencies of Gag-specific CD8_{EM} T cells responses before challenges than those with higher levels ($p = 0.02$),

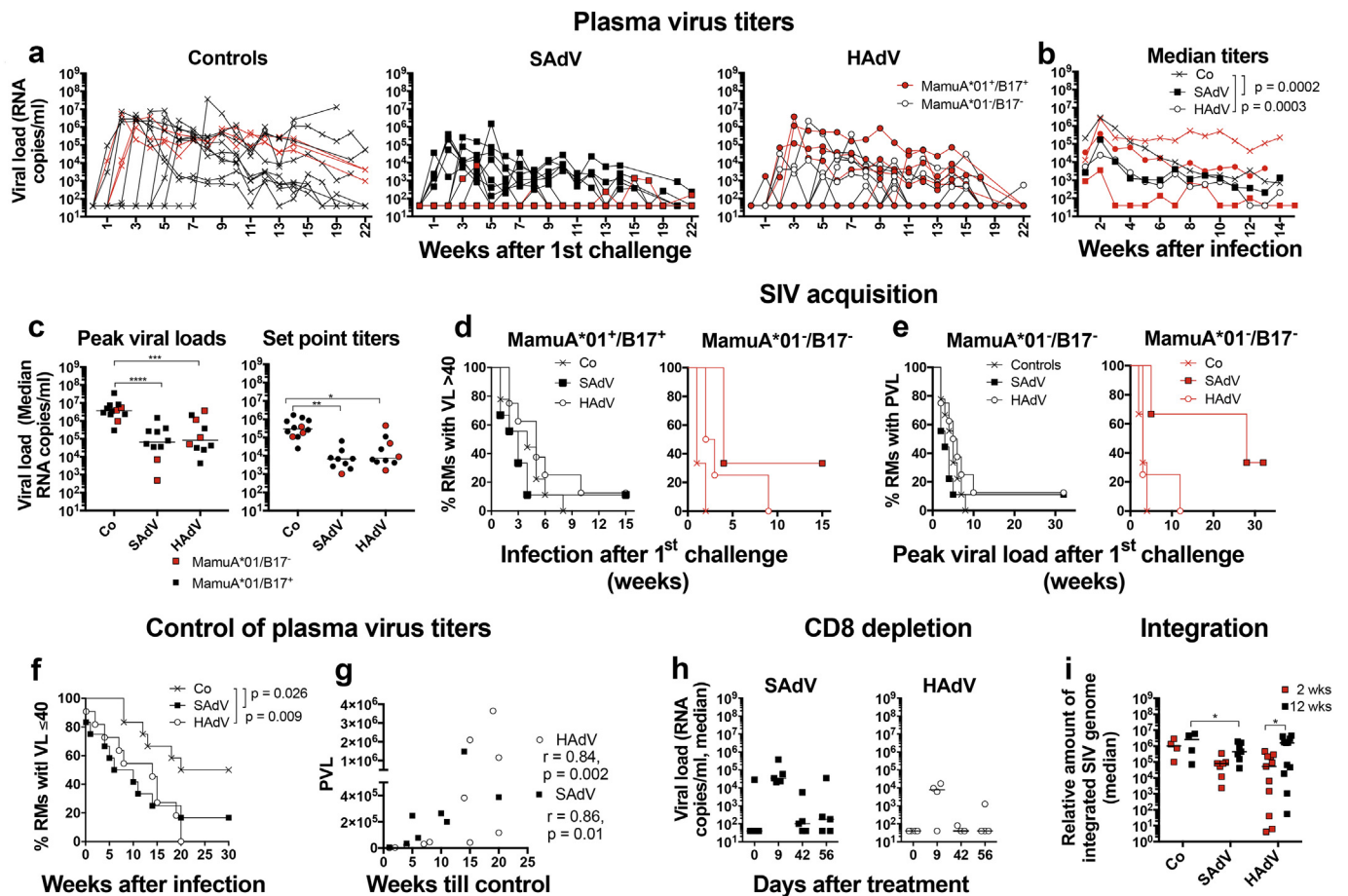


Fig. 1. Virus Acquisition, VL, and SIV Integration. (a) Viral titers over time. Mamu-A*01-/B17- animals in this and other graphs are highlighted in red. (b) Median VL with data synchronized as of beginning of infection. (c) PVL and set point VL the latter defined as the average VL for the 4 time points following PVL. Significant differences between groups are indicated by lines. Stars in this and subsequent graphs show level of significance: * $p = 0.01$ – <0.05 , ** $p = 0.001$ – <0.01 , *** $p = 0.0001$ – <0.001 , **** $p < 0.0001$. (d) Virus acquisition. (e) Time after first challenge till time of PVL. (f) Clearance of VL according to week after infection. (g) Correlations between PVL and weeks from infection till virus control. (h) VL of subcohorts of SAdV- (right, black squares, $n = 5$) and HAdV-vaccinated (left, grey circles, $n = 4$) RMs after CD8 Ab treatment. The RM with detectable titers (RM #4825) before depletion maintained titers after treatment ended. (i) Relative levels of integrated SIV genome at 2 (red symbol) and 12 (grey symbol) weeks after infection of RM subcohorts ($n = 7$ for SAdV, $n = 11$ for HAdV, $n = 4$ for controls). Differences between time points were determined by paired *t*-tests. Differences between the three groups were determined by ANOVA.

while in the same group RMs with low levels of integrated virus by week 12 after infection ($<10^6$) had higher frequencies of Gag-specific $CD8_{CM}$ ($p = 0.003$) and $CD8_{EM}$ T cells ($p = 0.04$) before challenges.

3.2. Ab Responses

Env-specific Ab responses were low post-priming (PP), increased post-boost (PB) and contracted by the time of challenges (Fig. 2a). After challenges (AC), infected but not protected RMs increased Ab titers. Ab titers in control RMs were higher after infection than in vaccinated RMs (SAdV: $p = 0.003$, HAdV: $p = 0.007$) (Fig. 2b), which presumably reflects their higher and more sustained SIV loads. Sera from MamuA*01/B17⁺ vaccinated RMs and from 6 control RMs were tested for antibody-dependent cell-mediated cytotoxicity (ADCC) (Fig. 2c). Sera from control RMs were negative. In the SAdV group 5/8 RMs scored positive at a 1:500 and 4/8 at a 1:1000 dilution of sera. Only 2/7 HAdV-vaccinated RMs showed low activity ($<10\%$ killing) at the 1:500 dilution. In MamuA*01/B17⁺ RMs of the SAdV, but not the HAdV group, or MamuA*01/B17⁻ RMs of either groups Ab titers tested shortly before challenges (BC) directly correlated with number of challenges required to achieve infection ($r = 0.72$, $p = 0.03$ by Spearman correlation, Fig. 2d). Pre-existing HAdV-specific Ab titers had no significant effect on magnitude of Ab responses.

3.3. T Cell Responses

$CD4^+$ and $CD8^+$ T cell responses to Gag were tested 4 weeks after each vaccine dose, shortly BC, one week after RMs became viremic or

one week after the 10th challenge in protected RMs by intracellular cytokine staining (ICS) for production of IFN- γ , IL-2, or TNF- α , or surface expression of CD107. In MamuA*01⁺ RMs responses were also analyzed prior to challenges upon staining with the Gag-CM9 tetramer. Responses to Env in the SAdV and HAdV groups and responses to both antigens in control animals were analyzed at the 2 later time points; they were marginal (Supplementary Fig. S1).

Both vaccine regimens induced Gag-specific $CD4^+$ and $CD8^+$ T cell responses (Fig. 3a). Responses to the immunodominant MamuA*01 epitope of Gag were higher in HAdV than in SAdV-immunized RMs PP and early PB (Fig. 3b). They became comparable by the time of 1st challenge and showed no correlation with parameters indicative for resistance to SIV infection. Frequencies of Gag-specific $CD4^+$ T cells tended to decline AC in HAdV-immune RMs; those of $CD8^+$ T cells tended to increase in this group. In the SAdV group frequencies of Gag-specific $CD8^+$ T cells PB inversely correlated with number of challenges till infection (Fig. 3c). In the HAdV group frequencies of Gag-specific total $CD8^+$ T cells AC inversely correlated with PVL (Fig. 3d); those BC and AC also inversely correlated with numbers of weeks between infection and control of VL (Fig. 3e,f).

Functional profiles varied over time and differed between the two cell subsets and the two vaccine groups (Supplementary Fig. 2a). Both T cell subsets showed comparable profiles in the SAdV and HAdV groups PP. Early PB, a large fraction of $CD4^+$ and $CD8^+$ T cells from the HAdV group were only positive for CD107 or IL-2; IFN- γ^+ or TNF- α^+ T cells were more prevalent in the SAdV group. The SAdV group showed other changes by the time of challenge; most notable were increases in TNF- α^+ $CD8^+$ T cells. Late PB and AC changes were more pronounced

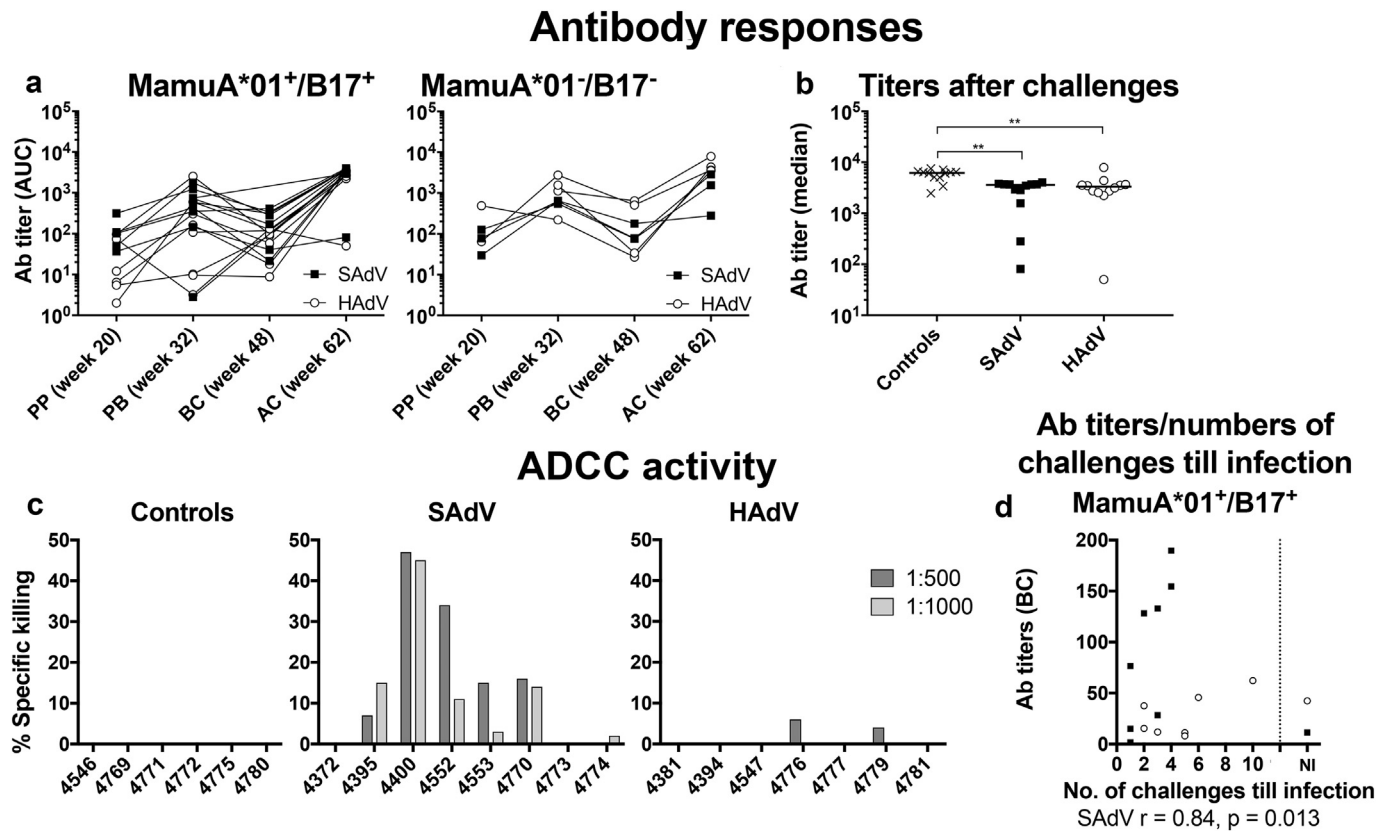


Fig. 2. Env-Specific Abs. Env-specific Abs were measured by ELISA at 20 (PP), 32 (PB), 48 (BC) and 62 (AC) weeks after the 1st vaccine. (a) Titters are shown as area under the curve calculated from serial dilutions of sera for SAdV- (closed squares) and HAdV-vaccinated (open circles) MamuA*01⁺/B17⁺ and MamuA*01⁻/B17⁻ RMs. (b) Titters after challenge at 62 weeks after priming. Connecting lines indicate significant differences by Mann-Whitney tests. (c) ADCC activity in sera of RMs of the 3 groups tested at serum dilutions of 1:500 and 1:1000. Data for pre-vaccination serum samples were subtracted. At the 1:500 dilution data for the SAdV groups were significantly different from those of the control group ($p = 0.019$ by 2-way Anova) (d) Correlations (by Spearman) between Ab titers at week 32 and number of challenges needed to achieve infection of SAdV (black squares) or HAdV (open circles)-immune MamuA*01/B17⁺ RMs. R- and unadjusted p-values are shown below the graphs. Data from RMs that did not become infected (NI) after 10 challenges are shown to the right.

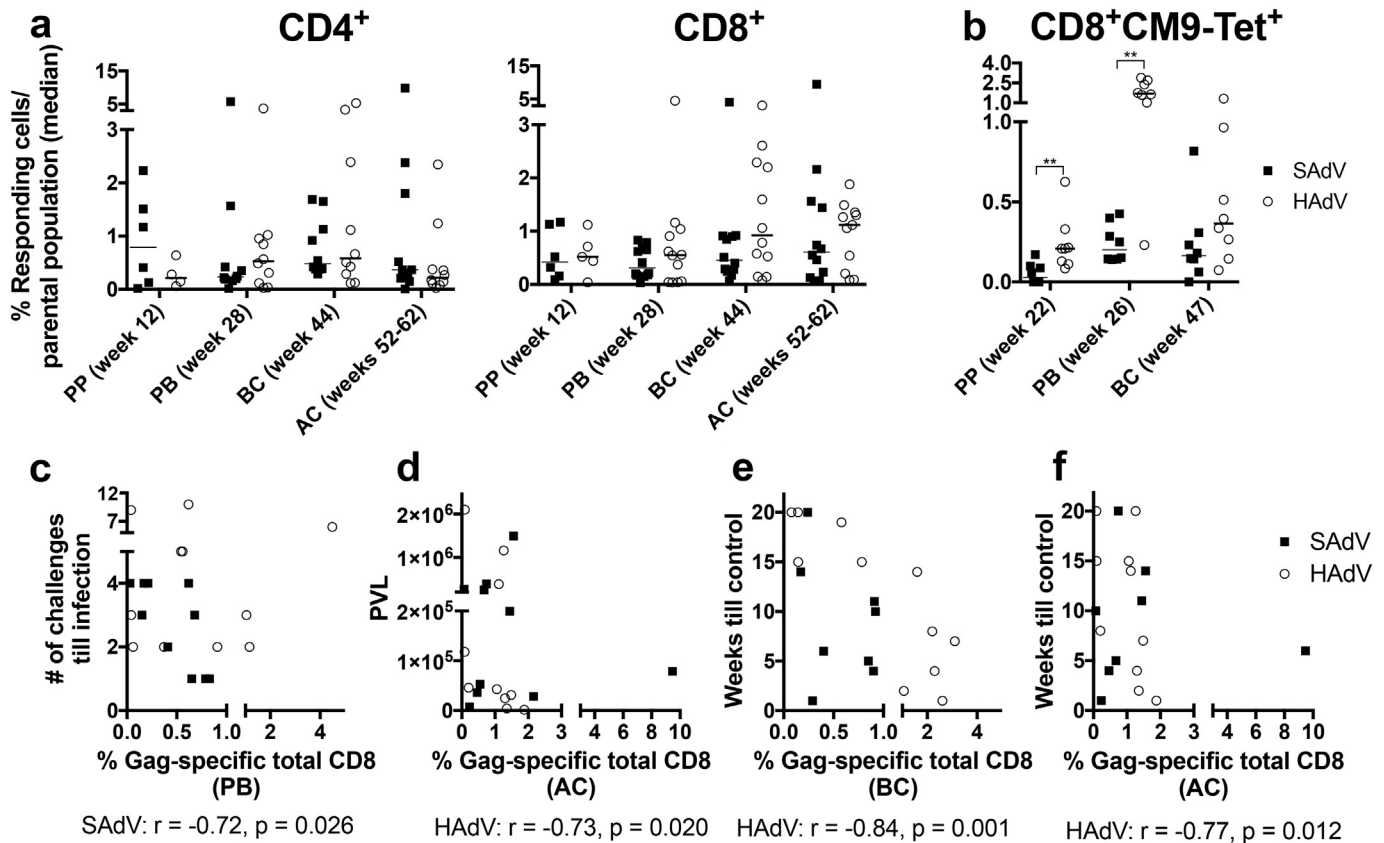


Fig. 3. Gag-Specific T Cell Responses. (a) Frequency of Gag-specific CD4⁺ and CD8⁺ T cells at 12 (PP), 28 (PB), 44 (BC) and 52–62 weeks after the 1st vaccine dose. AC: RMs were tested 1 week after the last SIV_{mac251} dose. Lines indicate medians. (b) MamuA*01⁺ RMs were tested at 22 (PP), 26 (PB) and 47 (BC) weeks after the 1st vaccine dose for CM9-tetramer (Tet)⁺ CD8⁺ T cells. Significant differences were determined by Mann Whitney. (c–f) Correlations between T cell frequencies and parameters of viral infection. (c) Number of challenge till infection vs. % Gag total CD8⁺ T cells PB. (d) PVL vs. % Gag total CD8⁺ T cells AC. (e,f) Weeks from infection till control of SIV titers vs. (e) % Gag total CD8⁺ T cells BC or (f) % Gag total CD8⁺ T cells AC. Significant r and p values are below the graphs. See also Fig. S1, S2.

in the HAdV group where for both T cell subsets percentages of TNF- α ⁺ and IFN- γ ⁺ cells increased BC followed by a shift towards IL-2⁺ cells in CD8⁺ T cells and by an increase in CD4⁺ T cells that were also positive for CD107.

We compared frequencies and functions of central (T_{CM}) and effector memory (T_{EM}) CD4⁺ and CD8⁺ T cells isolated from susceptible RMs that became infected upon challenges to those of protected RMs that remained SIV-free upon challenges at the two PB time points and AC combining data from the two vaccine groups. Frequencies of the two CD4⁺ T cell subsets were comparable at all time points (Fig. 4A). Early PB most CD4⁺_{EM} and CD4⁺_{CM} T cells of protected RMs were mono-functional for IL-2 while functions were more diverse in susceptible RMs. Shortly BC profiles of the two CD4⁺ T subsets became similar. Changes in functions were subtle AC (Supplementary Fig. 2b). Shortly BC CD8⁺_{EM} frequencies tended to be lower in protected RMs while CD8⁺_{CM} cells showed the opposite trend. Frequencies of CD8⁺ T_{EM} cells increased in susceptible RMs AC but remained stable or declined in resistant RMs (Fig. 4a). In protected RMs, CD8⁺_{EM} T cells were dominated by IL-2⁺ cells PB. This population declined over time and was replaced by IFN- γ ⁺ cells. The proportion of TNF- α ⁺ cells increased markedly in protected RMs AC. In susceptible RMs, CD8⁺_{EM} cells were mainly positive for CD107 early PB; later IFN- γ ⁺ cells became more prevalent. Changes in the susceptible RMs' CD8⁺ T_{EM} functional profiles were subtle AC. In resistant RMs most CD8⁺_{CM} T cells were mono-functional for IL-2 early PB; shortly before challenges CD107⁺ cells became dominant. The profile of CD8⁺_{CM} T cells changed further AC in resistant RMs, the proportions of IFN- γ ⁺ or TNF- α ⁺ cells increased and cells became more poly-functional. In susceptible RMs CD8⁺_{CM} cells mainly expressed CD107 either alone or in combination with IL-2

early PB; cells that also produced IFN- γ became more prevalent shortly BC. Most CD8⁺_{CM} cells were positive for IL-2 either alone or in combination with CD107 with or without IFN- γ AC. Cells positive for TNF- α were virtually absent at all 3 time points in susceptible RMs (Supplementary Fig. S2b).

Gag-specific CD8_{EM} and CD8_{CM} responses of the SAdV group early PB correlated with PVL (CD8_{CM}: $r = 0.84$, $p = 0.003$, CD8_{EM}: $r = 0.66$, $p = 0.04$). In the HAdV group CD8_{EM} cells BC inversely correlated with weeks from infection till control ($r = -0.55$, $p = 0.035$). In MamuA*01/B15⁺ but not MamuA*01/B15⁻ RMs of the SAdV group number of challenges till infection inversely correlated with % of Gag-specific CD8_{CM} cells BC ($r = -0.8$, $p = 0.024$) (Fig. 4b–e).

Overall these data show that differences in T cell functions between groups segregated according to level of protection were marginal for CD4⁺ T cells shortly before or after challenges. Differences were more evident for CD8⁺ T cells, which also showed more pronounced changes after challenges. Unexpectedly, such changes were also observed with samples from RMs that never became overtly viremic.

3.4. T Cell Gene Expression Profiles Over Time

To dissect differences in T cells according to time after vaccination we analyzed gene expression profiles of circulating CD4⁺ and CD8⁺ T cells from vaccinated RMs at 22 weeks PP and at 2 and 23 weeks PB. Between 22 weeks PP and 2 weeks PB changes were minor (Fig. 5a,b; Supplementary Table S1); they were pronounced between 2 vs. 23 weeks PB, and 22 weeks PP vs. 23 weeks PB. This was unexpected as it shows that the T cells' transcriptional response was stronger in the post-acute PB stage. Differentially expressed genes induced during

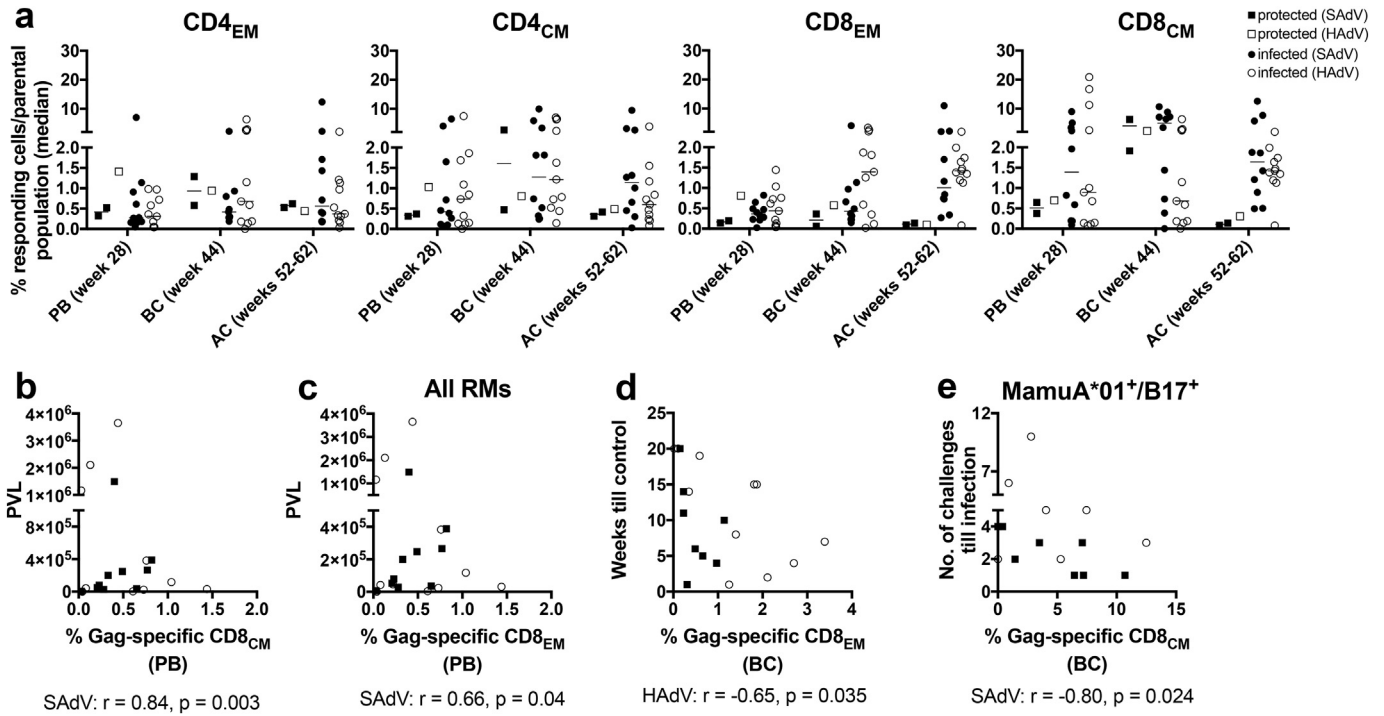


Fig. 4. Gag-specific T Cell Subset Responses. (a) Frequency of Gag-specific CD4_{EM}, CD4_{CM}, CD8_{EM} and CD8_{CM} T cells in RMs at the indicated time points as in Fig. 3. Samples from the two vaccine groups (SAdV – closed symbols, HAdV – open symbols) and protected (filled squares) vs. susceptible RMs (open squares) are shown separately. (b–e) Correlations between T cell frequencies and parameters of viral infection. (b,c) PVL vs. %Gag-specific (b) CD8_{CM} or (c) CD8_{EM} PB. (d) Weeks from infection till virus control vs. %Gag-specific CD8_{EM} BC. (e) For MamuA*01/B17+ RMs only: number of challenges till infection vs. %Gag-specific CD8_{CM} BC. See also Fig. S2.

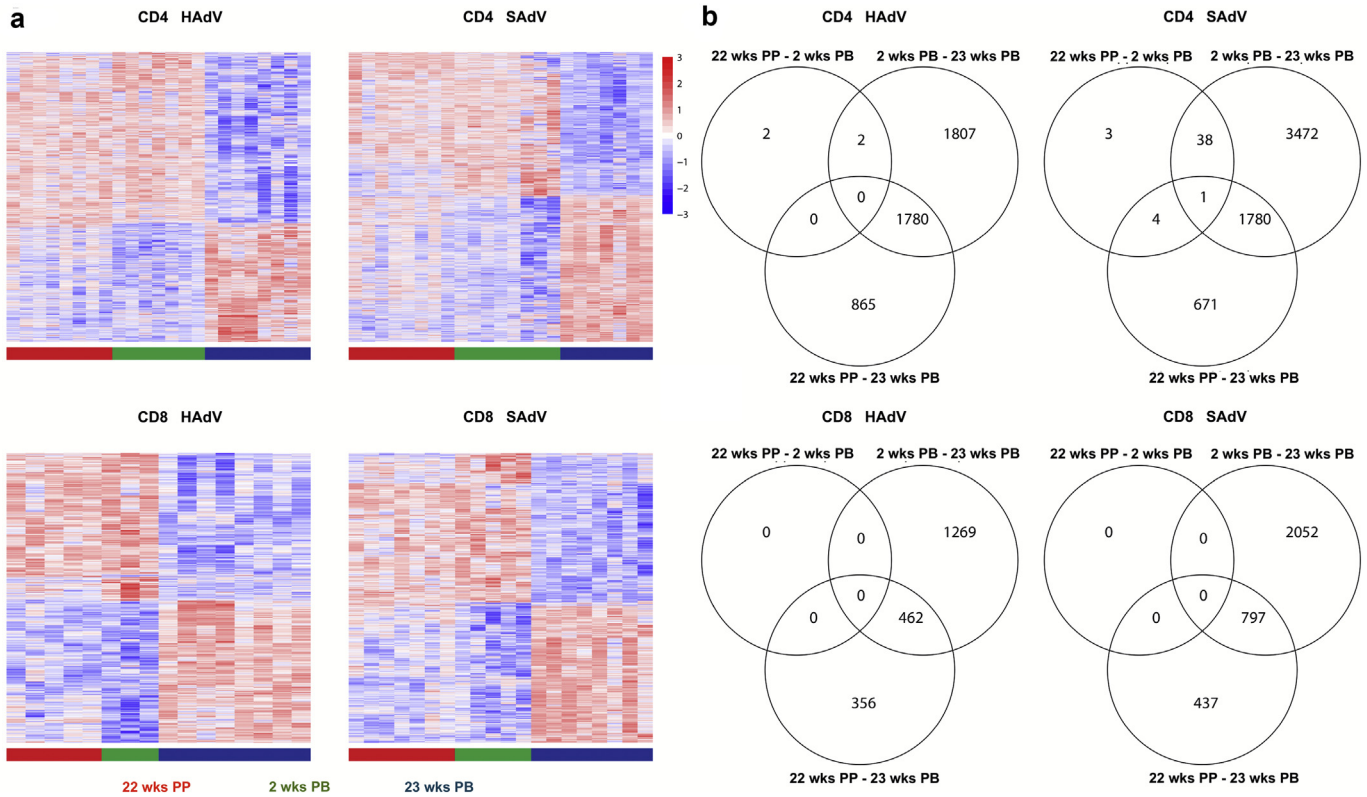


Fig. 5. Kinetics of Changes in T Cell Gene Expression Profiles Following Vaccination. (a) Heatmaps of transcripts significantly differentially expressed between 22 weeks PP, 2 weeks PB and 23 weeks PB, for each T cell subset and vaccine. Expression values represent regularized log₂ transformed normalized read counts, and are scaled across columns to normalize expression between transcripts. Samples are organized by time point. (b) Venn diagrams representing the numbers of transcripts significantly differentially expressed between each pair of time points by moderated *t*-test, Benjamini and Hochberg corrected $p \leq 0.05$. See also Fig. S3, Tables S1–3.

the post-vaccination phase were more common in the SAdV than the HAdV group, and in CD4⁺ than CD8⁺ T cells (Fig. 5b).

3.5. Differences in T Cell Transcriptomes Between Vaccine Groups

We used the same set of data to determine differences between the vaccine groups (Supplementary Fig. 3, Table S1). Focusing on genes that give insight into T cell activation such as genes encoding factors of cell cycling, metabolism, surface markers or functions revealed the following patterns for CD4⁺ T cells. More transcripts involved in cell cycling and the PI3K/Akt pathway were differentially expressed in the SAdV than the HAdV group. Changes for transcripts of glycolysis expressed at their highest levels at 23 weeks PB were more pronounced in HAdV-immune RMs, suggesting that their CD4⁺ T cells were more activated at the time of challenges. Changes in factors involved in lipid metabolism or mitochondrial functions were more common in the SAdV group, suggesting transitioning towards a more resting stage (Taub et al., 2013). Changes for IL-10 transcripts were observed only in the SAdV group while those for other T cell cytokines only changed in the HAdV group. Runx1, which blocks Th2 development, increased in both groups at 23 weeks PB and, IDO1, a factor that promotes Treg induction showed a similar pattern in the HAdV group.

In CD8⁺ T cells, changes in cell cycling genes were more common in the SAdV than the HAdV group (Supplementary Table S1). Transcripts of the PI3K/Akt pathways declined PB while SLC2A1, encoding the main glucose transporter Glut 1, increased as did transcripts encoding enzymes of glycolysis and the tricarboxylic acid cycle in the SAdV group and, less pronounced, in the HAdV group. Lipid metabolism and mitochondrial transcripts changed mainly in the SAdV group. Changes in cell surface markers were unique to each group. In the SAdV group, CTLA-4 and LTB increased by 23 weeks PB. IFNG and IL12A, the latter only in the SAdV group, decreased early PB and then increased. Increases at 23 weeks PB were seen for IL-10 in the SAdV group, and TNF and IL-8 in the HAdV group. A direct comparison of T cell surface markers and transcription factors showed higher increases PB of Ki-67 and the transcription factors Runx 1 and 2 in the SAdV than the HAdV group (Supplementary Table S2).

To further identify differences in gene expression profiles of T cells from the vaccine groups, we performed pathway analysis on transcripts that were most differentially modulated between the vaccine groups over time (Supplementary Table S3). CD4⁺ T cells showed the most pronounced differences for the IL-5, IL-4 and IFN- γ pathways mainly between 2 and 23 weeks PB. Expression levels of most genes regulated by IL-4 and IFN- γ increased more over time in the HAdV than the SAdV group.

For CD8⁺ T cells the most significant differences between vaccine groups were seen for genes controlled by HIF-3 α , which negatively regulates HIF-1 α (Ravenna et al., 2016) (Supplementary Table S3). Expression of these genes increased over time in the HAdV group and decreased in the SAdV group. Expression of genes regulated by the stress response kinase MKNK1 (Joshi et al., 2009), and Myc, an early regulator of T cell activation, increased from 22 weeks PP to 2 weeks PB, and then strongly decreased by 23 weeks PB in the HAdV group and to a lesser extent in the SAdV group.

These results indicate that CD4⁺ T cells remain more activated upon HAdV immunizations while the SAdV regimen maintains a higher activation status of CD8⁺ T cells.

3.6. Differences in T Cell Transcriptomes Between Resistant and Susceptible RMs

We compared gene expression in T cells of protected to susceptible RMs combining RMs of the two vaccine groups (Fig. 6). CD4⁺ T cell gene expression profiles from the 3 resistant RMs and from an additional two HAdV group RMs (#4776 and #4779), which both had low PVL (#4776 = 4280, #4779 = 31,300 RNA copies/ml) and controlled

VL rapidly, clustered separately from those of more susceptible RMs (Supplementary Fig. S4). CD4⁺ T cells with higher expressions of transcripts involved in cell cycling, the PI3K/Akt pathways and lipid metabolism were linked to increased susceptibility to infection (Supplementary Table S4). Resistant RMs had lower expression of the HIV-1 co-receptor CX3CR1. Co-stimulators, such as PD-L2 (PDCD1LGL), ICOS-L (ICOSLG) were higher in CD4⁺ T cells from protected animals as were CXCL13, a T_{FH} cell-produced B cell attractant and the T_{FH} markers CXCR5 and ICOS (Mesquita et al., 2016; Schaerli et al., 2000); PRDM1, which encodes Blimp1, a factor that prevents T_{FH} cells differentiation, was lower (Choi et al., 2011; Johnston et al., 2009). GATA3, a key transcription factor for Th2 differentiation (Yagi et al., 2011) was increased in protected RMs, while Runx1 was higher in susceptible RMs, which also showed increases in TGFBR1 and 3 and SMAD5, which are linked to Treg responses (Oh and Li, 2013).

More than 150 pathways showed differences. Those driven by NFATC2, IFN- γ , IFNB1, LIF and IL-27 were most significant (Supplementary Table S5); differences were also observed in IL-4 and IL-5 signaling. With the exception of the IFNB1 pathway, most transcripts of the other pathways, such as LIF, which inhibits Th17 development (Gao et al., 2009), or IL-27, which blocks Th1 development (Iwasaki et al., 2015), or transcripts that promote Th2 differentiation and provide essential help to B cell responses (IL-4 and IL-5) exhibited higher expression levels in protected RMs.

The same comparison for CD8⁺ T cells showed separate clustering of resistant and susceptible RMs (Supplementary Fig. S3). CD8⁺ T cells from resistant RMs had higher expression of most transcripts indicative of cell cycling, glucose and lipid metabolism with reduced activity of inhibitors of PI3K/Akt signaling, and lower expression in factors of translation, mRNA processing and protein synthesis (Supplementary Table S4). Resistant RMs showed higher expression of the activation marker CD95 (Fas) while transcripts for IL-7R, a memory T cell survival marker, were lower. Transcripts for several chemokines and TNF family members were higher in resistant RMs. Two transcription factors, i.e., BACH2, which promotes memory formation and NFATC1, which plays a role in inducible cytokine production, were higher in CD8⁺ T cells from susceptible animals.

Pathway analyses showed that TNF signaling and downstream pathways such as IL-1B and NF- κ B were more active in CD8⁺ T cells of resistant RMs, as were pathways initiated by TcR ligation such as CAVI and HIF-1 α . CREB1, a transcription factor that binds to NFATc1 and 2 in activated T cells (Gabriel et al., 2016) and RE1 Silencing Transcription Factor (REST), which reduces the activity of NFATs (Ohba et al., 2006) (Supplementary Table S5).

Overall, with the caveat that these results are based on small numbers of protected RMs, they point towards a crucial role of CD4⁺T_{FH} cells for protection against infection. They also suggest that more activated CD8⁺T cells may prevent systemic spread of SIV presumably by rapidly exterminating infected cells at the port of viral entry.

4. Discussion

The search for an efficacious AIDS vaccine remains a challenge after several large-scale clinical trials showed either no protection against virus transmission (James, 2003; Buchbinder et al., 2008; Huang et al., 2015) or only limited protection (Rerks-Ngarm et al., 2009).

Here we present a study in which sequential immunization with two serologically distinct SAdV vectors expressing SIV Gag and Env conferred significant protection against viral spread upon low dose rectal SIV_{mac251} challenges in RMs. We chose a prime-boost regimen to increase the overall magnitude of immune responses prior to challenges. Repeated immunization also had major effects on the T cells' gene expression profiles, especially late after the boost, suggesting that repeated immunizations affect the T cells' long-term differentiation pathways.

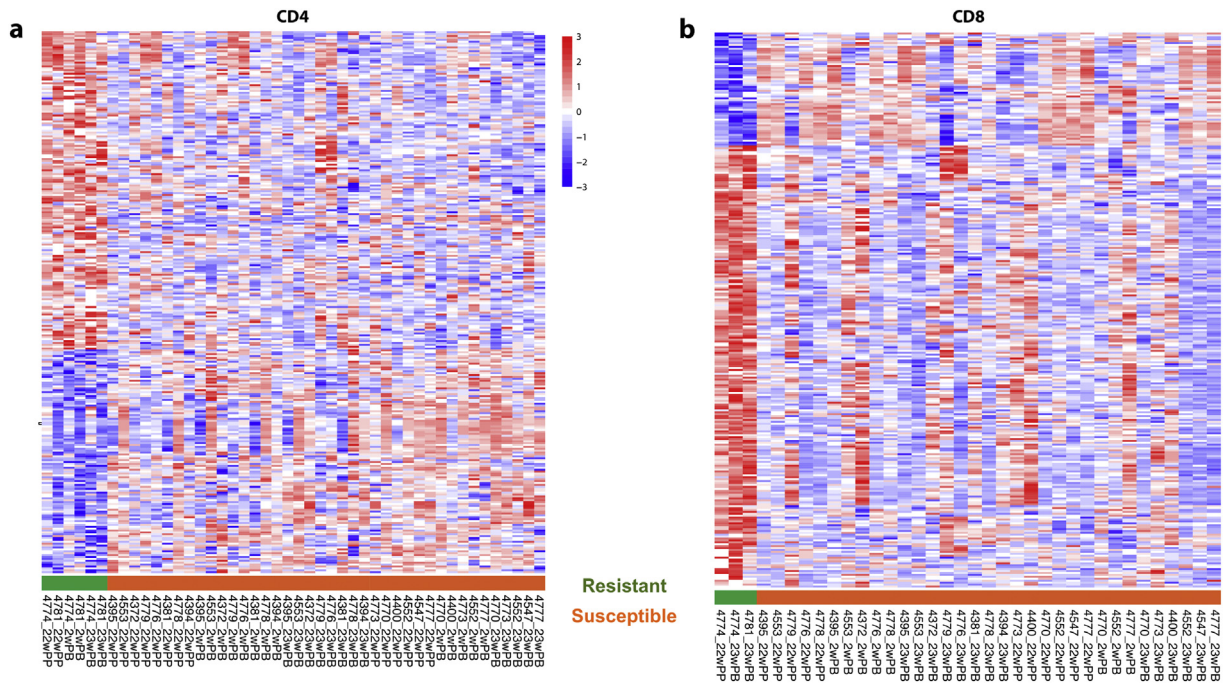


Fig. 6. Transcript Signatures Associated with Protection. Transcripts differentially expressed between challenge resistance vs. susceptibility RMs. Both vaccine groups at all time points were ranked by significance (moderated *t*-test *p* value). Top 2000 ranked transcripts (lowest *p* values), were analyzed using the Ingenuity Pathways upstream regulator analysis module. Heatmaps represent expression values of those transcripts found in the most significantly enriched upstream regulator gene sets. See also Supplementary Fig. S4, Tables S4.5.

SAdV vaccination allowed for rapid control of virus replication and prevented increases of levels of SIV integration over time. The HAdV vaccine regimen in agreement with previous studies (Letvin et al., 2011) also conferred significant protection against VL upon breakthrough infections, but this vaccine regimen was only effective in controller genotype RMs. Pre-existing nAbs to the HAdV vectors failed to accelerate SIV acquisition.

Several of the unvaccinated RMs with controller genotypes spontaneously controlled SIV_{mac251}, indicating that the course of infection upon low dose mucosal challenges may differ from that upon high dose SIV challenges, which typically leads to a rapidly progressing infection. In RMs receiving either of the vaccine regimens, nAb titers to HAdV5 or HAdV26 did not affect resistance to infection or viral clearance and had no discernible effects on the magnitude of vaccine-induced immune responses.

Sera of most RMs of the SAdV but not the HAdV group were positive for ADCC shortly before challenges. The mechanism underlying this difference is unclear. Previous studies showed that a DNA vaccine prime/HAdV5 vector boost given to HIV-1 infected individuals on retroviral therapy failed to enhance ADCC activity (Gach et al., 2016). A chimpanzee study showed that replication-competent HAdV vector vaccine are superior to replication-defective Ad vectors at inducing ADCC responses (Peng et al., 2005). These data suggest that the distinct biology of HAdV as compared to SAdV vectors, which results in distinct cytokine responses (Hensley et al., 2005) may have contributed to differences in antibody functions. Alternatively, pre-existing neutralizing antibodies to the HAdV vectors, which have been shown to affect T cell functions upon HAdV5 immunization in humans (Pine et al., 2011), may have affected antibody functions. Unlike in other vaccine models (Bradley et al., 2017; Fouts et al., 2015) ADCC titers did not correlated with protection against virus acquisition or VL. In the SAdV-immunized RMs with controller genotypes unlike in those vaccinated with the HAdV vaccines, numbers of rectal SIV challenges needed to achieve infection were directly correlated with circulating Env-specific Ab titers after the boost. As SIV_{mac251} is extremely resistant to neutralization, we assume that non-nAbs lowered the risk of infection in agreement with results of the RV144 trial. Also in agreement with previous reports (Hansen

et al., 2011) SIV-specific CD8⁺ T cells, mainly directed against Gag, played a significant role in allowing for rapid viral control. HAdV group animals with higher frequencies of Gag-specific CD8⁺ T cells controlled the virus faster and better than those with lower frequencies. These correlations were not significant for the SAdV-immune RMs. As both vaccine groups developed similar viral loads and controlled virus with similar kinetics this could suggest that a potentially less effective Gag-specific CD8⁺ T cell response in the SAdV group was compensated for by other mechanisms such as ADCC (Alpert et al., 2012b). Unexpectedly, CD8⁺ T cells also seemed to play a role in the protected RMs. Three of the 24 vaccinated RMs did not become viremic during the course of challenges. Another RM, #4825, showed several low viral blips (<1000 copies/ml) as of the 5th challenge, which upon initial challenge developed into a full-blown infection 6 weeks after the last challenge with albeit moderate VL (6000–35,000 RNA copies/ml). The other 3 RMs appeared to have developed sterilizing immunity. Nevertheless, functions of their circulating CD8⁺ T cells changed after challenges. This could reflect longitudinal changes in cytokine production profiles driven by differentiation, or, alternatively, changes could have been driven by a local infection that was rapidly controlled before the animals developed detectable viral titers in blood. The latter would be compatible with the infection course of #4825.

Magnitudes of B and T cell responses were largely comparable between SAdV- and HAdV-vaccinated RMs prior to challenges although there was a general, albeit insignificant, trend towards higher Gag-specific T cell responses at the time of challenge in Mamu-A*01⁺/B*17⁺ RMs of the HAdV group. In the HAdV but not the SAdV group, levels of integration increased between week 2 and 12 after infection; this difference could again reflect the trend towards lower SIV titers in blood of the SAdV group.

There were marked differences in functions and gene expression profiles of circulating T cells of the SAdV and HAdV groups. CD4⁺ T cells in SAdV-immune RMs were more resting by the time of challenges than those from HAdV-immune RMs as indicated by differences in gene expression profiles. The SAdV-immune RM's CD4⁺ T cell transcriptome was indicative of a Th2 profile while that HAdV-immune RMs showed evidence of heightened Treg activity, as has been reported previously

upon HAdV5 vaccination (Larocca et al., 2016). CD8⁺T cells showed the opposing pattern; according to their metabolic profile those from the SAdV group were more activated.

Functional differences between protected RMs and susceptible RMs were subtle for CD4⁺ T cells. The CD4⁺ T cells' pre-challenge gene expression profiles of protected RMs showed increases in transcripts indicative of heightened T_{FH} activity while susceptible RMs had increases in transcripts typically linked to Tregs. With the caveat that number of protected animals were small and that the transcriptomes were not analyzed prior to vaccination, which precludes a distinction between the role of genetic vs. vaccine-induced resistance factors, the results nevertheless suggest benefits by CD4⁺ T cells able to promote affinity matured, switched antibody responses and deleterious effects of those that are immunoinhibitory.

Protected RMs had higher frequencies of CD8_{CM} than CD8_{EM} cells (ratio of 10:1, $p = .002$ by t -test) just prior to challenges compared to susceptible RMs. CD8⁺ T_{EM} and T_{CM} cells from resistant RMs were largely mono-functional prior to challenges while those from susceptible RMs had robust frequencies of T cells with 2–4 functions. CD8⁺ T cells from resistant RMs became more poly-functional after challenges and at that stage both subsets differed from those of susceptible animals by higher proportions of TNF- α ⁺ and lower proportions of IL-2⁺ cells. The CD8⁺ T cell gene expression profile indicates increased metabolic activity and cell cycling in protected RMs suggesting that, although their Gag-specific CD8⁺ T cells were skewed towards higher proportions of T_{CM} than T_{EM} cells, their whole circulating CD8⁺ T cell population was more activated. Transcriptional analysis showed increased TNF signaling in CD8⁺ T cells of resistant RMs prior to challenges. TNF- α inhibits entry of HIV-1 into permissive cells and reduces HIV-1 replication in freshly but not chronically infected cells and may thus have reduced SIV acquisition and spread (Kumar et al., 2013).

Overall, our results differ substantially from those obtained pre-clinically with the clinically ineffective HVTN505 vaccine (Letvin et al., 2011). Indeed, this DNA-HAdV5-based vaccine regimen failed to protect RMs from SIV_{mac251} challenges, but conferred partial protection against acquisition of the less stringent SIV_{smE660} virus; reductions of set-point VL were only seen in RMs with controller genotypes. The STEP/Phambili trial vaccine regimen based on immunizations with HAdV5 vectors lacking Env sequences reduced PVL in RMs subsequently infected with SHIV89.6P but had no effect on a SIV_{mac239} infection (Casimiro et al., 2005). A regimen that mimicked the RV144 vaccine caused some modest protection against rectal low dose SIV_{mac251} acquisition in RMs without reducing VL in infected RMs (Pegu et al., 2013).

Several vaccine candidates have shown protection of RMs against acquisition of neutralization-sensitive strains of SIV such as SIV_{smE660} (Lai et al., 2012; Patel et al., 2013) or delayed acquisition of SIV_{mac251} (Barouch et al., 2012). One study showed that 50% of nonhuman primates immunized repeatedly with HAdV26 vectors expressing SIV Env, Gag and Pol followed by protein boosts were protected against intra-rectal challenges with a heterologous strain of SIV (Barouch et al., 2015). As in our study protection correlated with titers of binding Abs. It remains to be tested if our regimen, based on sequential use of two SAdV vectors, could be improved further by addition of an Env protein boost, which, as we reported (Emmer et al., 2016), promotes induction of nAbs.

In summary, this study shows that a vaccine platform, to which humans in general lack pre-existing nAbs that were linked to increased HIV-1 acquisition in previous HAdV5 vaccine trials (Buchbinder et al., 2008), induces SIV-specific immune responses in RMs that provide significant protection against chronic SIV infection.

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Conflict of Interests

The authors declare no competing interests.

Author Contributions

ST, LHH, RKK, DC, SG, TMS, ZX, YL, MZ, XYZ conducted experiments, wrote paper. QI: statistical analysis. JMB, SB, RA: microarray analysis, wrote paper. MGL: RM study. GS, HCJE: data interpretation, wrote paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.02.025>.

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