Multiple Low-Dose Challenges in a Rhesus Macaque AIDS Vaccine Trial Result in an Evolving Host Response That Affects Protective Outcome

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Using whole-blood transcriptional profiling, we investigated differences in the host response to vaccination and challenge in a rhesus macaque AIDS vaccine trial. Samples were collected from animals prior to and after vaccination with live, irradiated vaccine cells secreting the modified endoplasmic reticulum chaperone gp96-Ig loaded with simian immunodeficiency virus (SIV) peptides, either alone or in combination with a SIV-gp120 protein boost. Additional samples were collected following multiple low-dose rectal challenges with SIVmac251. Animals in the boosted group had a 73% reduced risk of infection. Surprisingly, few changes in gene expression were observed during the vaccination phase. Focusing on postchallenge comparisons, in particular for protected animals, we identified a host response signature of protection comprised of strong interferon signaling after the first challenge, which then largely abated after further challenges. We also identified a host response signature, comprised of early macrophage-mediated inflammatory responses, in animals with undetectable viral loads 5 days after the first challenge but with unusually high viral titers after subsequent challenges. Statistical analysis showed that prime-boost vaccination significantly lowered the probability of infection in a time-consistent manner throughout several challenges. Given that humoral responses in the prime-boost group were highly significant prechallenge correlates of protection, the strong innate signaling after the first challenge suggests that interferon signaling may enhance vaccine-induced antibody responses and is an important contributor to protection from infection during repeated low-dose exposure to SIV.

Since the identification of human immunodeficiency virus (HIV) as the primary cause of AIDS (1, 2), substantial efforts have been put into developing a vaccine against the virus. Different vaccine strategies have been devised to generate specific cellular or humoral responses, or combinations thereof, in order to prevent viral acquisition or disease progression. HIV cellular tropism, rapid escape mutations, and viral latency are major prevention and treatment hurdles (3–6). Here we used transcriptional profiling of host responses to vaccination and subsequent repeated challenges to further understand the protective effects of a novel vaccination strategy based on the use of the endoplasmic reticulum resident chaperone gp96 (7). This heat shock protein, similar to soluble Hsp65 and hsp65 (8, 9), binds to and activates dendritic cells (10) and functions as a chaperone for tumor-secreted peptides. The modified form of gp96, gp96-Ig, secreted by tumors (11), results in an enhancement of tumor antigen cross-priming of CD8 cytotoxic T lymphocytes (CTLs) (12) and induces a mucosal immune response following intraperitoneal injection of transfected cells (13). When chaperoning simian immunodeficiency virus (SIV) peptides, gp96-Ig induces a strong multifunctional memory response in the rectal and vaginal mucosa of rhesus macaques (14).

Starting from these observations, a vaccine approach was designed with the aim of combining the enhancement of CD8 CTL cross-priming elicited by gp96, the particular role of secreted gp96-Ig for loading a broad spectrum of SIV peptides to major histocompatibility complex I (MHC I) molecules, and the CD4-dependent antibody responses elicited by the recombinant envelope protein rSIV-gp120. When used to vaccinate rhesus macaques, live, irradiated vaccine cells secreting a modified gp96-Ig carrying SIV peptides (gp96SIV-Ig) in combination with a SIV-gp120 protein boost provided considerable protection against weekly mucosal challenge with the highly pathogenic strain SIVmac251 (15). After seven challenges, the hazard ratio was 0.27, representing a 73% reduction in risk for viral acquisition. Importantly, vaccination with either gp96SIV-Ig or gp120 protein alone provided no significant protection. However, gp96SIV-Ig vaccination alone provided for potent cross-presentation of SIV antigens, generating CD8 CTLs but no antibody. Conversely, gp120 protein vaccination alone generated antibody but few CTLs. This argues that both cellular and humoral immune responses are required for protection.

In this study, we performed RNA profiling and functional genomic analyses of whole-blood samples obtained from our previous vaccine trial (15). Our analyses revealed that monocyt/macrophage- and neutrophil-mediated inflammatory response...
signatures are predictive of an occurrence of delayed viremia, in contrast to effective protection from viral acquisition, achieved by prime-boost vaccination. Effective protection during multiple weekly low-dose challenges was associated with an evolving immune response specific to the persistently protected animals along the first three challenges. A strong interferon (IFN) response and a lack of inflammatory gene expression upon the first challenge, complemented by elements of humoral immunity, were followed by a resolved IFN response and the onset of adaptive immune responses, with predicted recruitment of phagocytic cells after the second challenge and a further increased inflammatory response after the third challenge. Taken together, the data in the present transcriptional analysis suggest that innate antiviral signaling induced by the first challenge contributes to persistent protection via a hypothesized enhancement of vaccine boost-elicited antibody responses. The delay of viral acquisition is disrupted once proinflammatory signals become predominant during continued weekly challenges.

MATERIALS AND METHODS

Animals, vaccine cells, and study design. The present study used whole-blood samples from a previously described vaccine trial (15). Briefly, 36 Indian-origin, outbred, young-adult male and female rhesus macaques were divided into three groups of 12 animals each. Groups were balanced for Mamu-A*01 (three in each group), Mamu-B*08 (one in each group), and susceptible and resistant TRIM5 alleles. There were no Mamu-B*17 animals. The prime group (PG) received gp96SIV1g vaccine 293 cells that were transfected with plasmids encoding gp96-Ig and SIVmac251 Rev-Tat-Nef, Gag, and gp160 (14). Animals were injected intraperitoneally with 10^7 irradiated gp96SIV1g vaccine cells, which secrete 10 µg of gp96SIV Ig per 24 h, in Hanks’ balanced salt solution. For the prime-boost group (PBG), 100 µg of rSIV-gp120 protein was added to the vaccine cells. The control group received 293-gp96-Ig cells not transfected with SIV antigens. After a 30-week vaccination phase, which consisted of priming in weeks 0, 6, and 25 and (for the prime-boost group only) additional boosts in weeks 6 and 25, all animals were subjected to up to seven weekly low-dose intracutaneous challenges, starting at week 33, with SIVmac251 at a dosage of 120 50% tissue culture infective doses (TCID50). Whenever an animal had a detectable viral load (>50 copies of viral RNA/ml of plasma) at 5 days postchallenge, it was considered viremic, further challenges were suspended, and only viral load screening was continued on a weekly basis. Whole-blood samples (preserved in Paxgene tubes) were collected 2 weeks prior to the first prime, 1 week after the first prime, 2 weeks before and 1 week after the third prime, 1 week before the first challenge, 5 days into the corresponding study week for newly infected animals, and 4 days into the study week for viremic animals (also denoted as challenge 2 and challenge 3) (Fig. 1). The monkeys were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International at Rockville Advanced Bioscience Laboratories (Rockville, MD) (15).

RNA isolation and microarray processing. Total RNA was isolated from Paxgene tubes by use of Paxgene Blood RNAeasy minikits (Qiagen) following the manufacturer’s protocol. RNA quality was assessed on an Agilent 2100 bioanalyzer, using the nanochip format, and only intact RNA was used for microarray analyses. We also used multidimensional scaling to screen for additional extreme sample outliers. One hundred nanograms of each RNA sample was hybridized to one Agilent 4 × 44K rhesus macaque array (GEO accession no. GPL17465), and processing of raw data was performed as previously described (16). The resulting data set was background corrected and quantile normalized using the R package limma (17). We filtered out low-intensity probes that were in the 20th percentile for at least three samples. CD4^+ and CD8^+ T cell counts. CD4^+ and CD8^+ T cell counts were periodically determined from whole blood and by fluorescence-activated cell sorter (FACS) analysis with a FACSSLyte kit (BD Immunocytometry Systems, San Jose, CA), and analyses were performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The antibodies used were anti-CD3 (SP34; BD Biosciences), anti-CD4 (L200; BD Biosciences), anti-CD8 (DK25; DakoCytomation, Carpinteria, CA), and anti-CD20 (B9E9; Beckman Coulter, Fullerton, CA).

Statistical analysis. Differential expression was determined by comparing either baseline (week −2) samples to group-matched samples during the vaccination phase or week 32 samples to group-matched samples during the challenge phase, based on a linear model fit with Bayesian correction for each probe, using the R package limma (17). There were at least 10 samples per group for each of the time points, except at week 32, when only 7 samples for the PG and 9 samples for the PBG passed quality control (QC). Criteria for differential expression were an absolute change of 1.4-fold (given the high absolute signal intensity after filtering) and a P value of <0.05, calculated using a moderated t test.

Likelihood analysis of exposure-induced loss of immunization during repeated low-dose challenges with SIVmac251 was carried out using the R library regoes12pcb as detailed previously (18). The maximum log likelihoods of a geometric infection model fit (no challenge-induced changes in immunity) and a two-phase model fit (challenge-induced changes in immunity between the first and following challenges) showed that in the prime-boost group, multiple exposures to SIV challenge induced a significant loss of immunity between the first and following challenges. The right-tailed χ^2 test for maximal log likelihood differences gave a P value of 0.01 (PBG) for the geometric versus two-phase model. For the rescored survival data based on the assumption that SIV-positive animals with viral titers above the limit of detection had already been infected at the preceding challenge, the P value for the same test was 0.32. For graphical representation, we used the likelihood maximizers for the geometric model fit to illustrate that rescored survival data strongly supported a geometric infection model.

Functional enrichment analysis. Functional analysis of statistically significant gene expression changes was performed using the Ingenuity pathway analysis (IPA) knowledge base (Ingenuity Systems). For all gene set enrichment analyses, right-tailed Fisher’s exact test was used to calculate a P value determining the probability that each biological function or canonical pathway assigned to that data set was due to chance alone. All enrichment scores were calculated in IPA by using the probes that passed our QC filter as the background data set. A Z score was calculated to determine whether gene expression changes for known targets of each biofunction were consistent with what is expected from the literature (for Z values of >2, the biofunction is predicted to be significantly activated, and for Z values of <2, the biofunction is predicted to be significantly inhibited). IPA network scoring was used to determine the highest-ranked networks associated with differentially expressed (DE) genes for prime-boost group animals in challenge 1 and challenge 2. These networks were merged into one graph and overlaid with log_2 fold change differences between challenge 1 and challenge 2 data.

Microarray data accession number. Raw microarray data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO accession number GSE59068.

RESULTS

To further define the correlates of protection against the acquisition of infection following vaccination, we assessed the global host response to vaccination and subsequent challenges through microarray analysis of RNAs isolated from whole blood collected during a previous vaccine trial (15). In that study, rhesus macaques were vaccinated with live, irradiated vaccine cells secreting the modified endoplasmic reticulum chaperone gp96-Ig. The prime group (PG) was vaccinated with gp96-Ig carrying the peptides SIVmac251 Rev-Tat-Nef, Gag, and gp160 (gp96SIV Ig), and the prime-boost group (PBG) was vaccinated with gp96SIV Ig plus boosts with the recombinant envelope protein rSIV-gp120.
control group received gp96-Ig only (no SIV peptides). After a 32-week vaccination phase, all animals were subjected to up to 7 weekly low-dose mucosal challenges with highly pathogenic SIVmac251 at a dosage of 120 TCID50. Transcriptional profiling was performed during the vaccination phase and following the first three challenges (Fig. 1A). Whenever an animal had a detectable viral load (>50 copies/ml of plasma) 5 days after any challenge, it was considered viremic, further challenges were suspended, and only viral load screening was continued on a weekly basis (15).

As noted previously (15), there were unusual patterns in viral titers. Specifically, although 11 of 12 animals vaccinated only with gp96SIV-Ig tested SIV negative after the first challenge, 7 of these animals showed very high viral titers when tested 5 days after the second challenge, suggesting that they had been infected 1 week earlier. This “delayed viremia” between the first and second challenges was also seen in 4 animals from the prime-boost group. Similar occurrences, but with a lower prevalence, took place for further challenges in all three study groups, including the control group. Assuming that these animals had already acquired SIV at the preceding challenge, survival analysis still demonstrated a significantly reduced risk of infection, with a hazard ratio of 0.31 (instead of 0.27), for the prime-boost group compared to the control group (15). We therefore considered the aforementioned animals as having delayed viremia after the first challenge instead of being uninfected, and the animals were grouped accordingly for our transcriptional comparisons (Fig. 1A).

FIG 1 Overview of study design and differential gene expression. (A) Schematic diagram of blood sampling schedule and challenge outcomes. Vaccination of the prime group (PG), prime-boost group (PBG), and control group (CG) was performed at weeks 0, 6, and 25 (orange symbols). Beginning at week 33, animals were subjected to weekly low-dose challenges (orange arrows). Starting with 12 animals in each study group, the numbers in each tree branch indicate how many animals were SIV positive, delayed viremic, or protected after each challenge. Blood draws (red lines) for RNA isolation were performed at weeks −2, 1, 23, 26, and 32 and 5 days after each challenge. There were at least 10 samples per group for each of the time points, except at week 32, where only 7 samples for the PG and 9 samples for the PBG passed QC (see Materials and Methods). (B) Numbers of differentially expressed genes within each study group. Differential gene expression was determined by comparing vaccination-phase samples to their study-group-matched baseline at week −2. For the challenge phase, postchallenge samples were compared to their prechallenge baseline at week 32. Using a moderated, Bayesian-corrected t test in limma (see Materials and Methods), differential expression with a P value of <0.05 and group-averaged fold change to average baseline (fold change) of >1.4 was considered significant. The table shows numbers of DE genes in the three vaccination groups for each comparison. NS*, a nonsignificant number of samples was used for comparison. Colored boxes represent a heat map of the number of DE genes in each box.
observed consistently modest numbers of DE genes during the vaccination phase, with rather inconclusive functional enrichment (Fig. 1B; see Table S1 in the supplemental material for comparisons of DE genes for the PG and PBG at weeks 26 and 32 relative to week −2). Criteria for differential expression were an absolute change of >1.4-fold and a P value of <0.05, calculated using a moderated t test (see Materials and Methods). It has previously been described that the gp96-Ig vaccine approach (delivered by the intraperitoneal route) induces a strong mucosal immune response, while systemic immune responses are not significantly upregulated (13, 14). Therefore, the observed lack of gene expression in whole blood during the vaccination phase was not surprising. Although detection of significant gene expression changes affecting cell populations with low representation (such as dendritic cells) in whole blood is certainly hampered, the significance and novelty of using this noninvasive way to screen host responses in the present vaccine trials are given by the large differences in the host transcriptional response after the first challenge between delayed-viremic and persistently protected animals: postchallenge comparisons to the postvaccination time point week 32 showed hundreds of DE genes for protected animals across all three challenges, as well as for delayed-viremic animals from challenge 1.

Large differences in host transcriptional response after the first challenge between delayed-viremic and persistently protected animals. Given the aforementioned delayed viremia in several animals in both vaccine groups after the first challenge, we aimed to determine whether the presence of virus in delayed-viremic animals from the PG and PBG induced similar host responses and how these responses compared to those of the uninfected and protected animals in the control and prime-boost groups, respectively, after the first challenge. Genes that were differentially expressed compared to group–specific week 32 baseline controls were determined using the statistical cutoffs of an absolute change of >1.4-fold and a P value of <0.05. There were comparable numbers of DE genes across these comparisons: 402 for delayed-viremic animals in the gp96-Ig group, 144 for delayed-viremic animals in the prime-boost group, 235 for protected animals in the prime-boost group, and 681 for uninfected control group animals.

Enrichment in canonical pathways for each set of DE genes was performed. For the delayed-viremic animals in both vaccination groups, there was some enrichment in several pathways leading to or involving the inflammatory response, including granulocyte adhesion and diapedesis, TREM1 signaling, NF-κB signaling, and Toll-like receptor (TLR) signaling (Fig. 2A). However, the pathway enrichment pattern differed between these two groups in that for several pathways involved in the transition between the innate and adaptive responses, the delayed-viremic animals in the prime-boost group had stronger enrichment, with a higher ratio of genes. In contrast, the protected animals in the prime-boost group had essentially no enrichment in pathways involved in a proinflammatory response, but they had a pronounced innate immune response, with enrichment of pathways such as IFN signaling (also observed in delayed-viremic PBG animals, not in delayed-viremic PG animals) and activation of IRF by cytosolic pattern recognition receptors.

To further investigate the differences between groups, we determined the functional enrichment of the DE genes that made up the major pathways that distinguished the groups (Fig. 2B). Quite different biofunctions were triggered as a consequence of the first mucosal challenge. Among the largest disparities between protected animals and those with delayed viremia were functional categories pertaining to viral infection and replication of virus, with a predicted inhibition of these functions in the protected animals and less statistically significant enrichment in the animals with delayed viremia, despite the noted similarity in enrichment of IFN signaling in PBG animals. As already noted from pathway analysis, delayed viremia was associated with a highly significant engagement of macrophage-related inflammatory response signaling. This was consistent with high activation scores for migration and chemotaxis of myeloid cells. Also, the predicted decrease of the quantity of granulocytes (Fig. 2B) for delayed-viremic animals, together with a strong activation of cell migration, points to an engagement of neutrophils in innate cellular defense mechanisms toward the challenge compartment. Based on transcriptional profiling after the first challenge, we identified strong proinflammatory responses as a hallmark of a lack of protection and innate antiviral defense mechanisms as unique signatures associated with protection. Both the functional and canonical pathway enrichment profiles for the protected animals differed greatly from those of the control group, suggesting that the protected profile was a result of the vaccination regimen.

Evolving host responses in the protected group during multiple challenges show strong but quickly resolved IFN signaling. Before addressing the question of which specific vaccination-induced host response factors contributed to persistent protection during the first three weekly viral challenges, we examined whether multiple challenges changed the rate of protection after each challenge. This question is particularly important in the context of vaccine studies. An effective vaccine eliciting memory responses should result in protection rates that remain the same over time, despite repeated pathogen exposure.

Previously, viral copy numbers 5 days after challenge were used to determine if an animal had been infected (defined as >50 copies of viral RNA/ml of blood). Using this criterion, the number of seropositive animals from the prime-boost group changed across the first three challenges, from 0 to 5 to 7 (Fig. 1A) (original survival data are presented in reference 15). On the other hand, as discussed in the preceding section, there is evidence that animals with viral load measurements above the limit of detection 5 days after infection had already acquired SIV at the preceding challenge in terms of their proinflammatory responses. We analyzed both the original and the rescored survival data for the prime-boost group and followed the statistical approach outlined by Regoes (18) to test in which case the survival data showed a statistical difference between a geometric model (no effect of challenge history, corresponding to the null hypothesis) and a two-step model (effect of challenge history between challenges 1 and 2, corresponding to the alternative hypothesis). P values were derived from a χ² test between the log likelihood maximizers of the two models. In the case of the original data, the null hypothesis was rejected (P = 0.01), indicating that there was an effect of challenge history on future challenges. Importantly, when the delayed-viremic animals were rescored as infected, there was not a statistically significant effect of continuing exposure in the prime-boost group. This is graphically represented by a high accordance between the log likelihood maximizer for the geometric model fit (infection probability per exposure of 0.32) (Fig. 3A, dashed line) and the actual survival curve. Also, this maximizer showed that
prime-boost vaccination lowered the probability of infection by nearly half (from 0.52 for the control group to 0.32 for the prime-boost group), with time consistency over several challenges.

Even though the statistical analysis indicated that in the prime-boost group there was no effect of prior exposure, the RNA profiles of the protected animals in this group differed slightly from one another after challenges 2 and 3 and differed vastly from the challenge 1 profiles. There were 330 and 681 DE genes in comparing protected animals in challenges 2 and 3, respectively, to the week 32 baseline sample, in contrast to 235 DE genes with the protected animals in challenge 1 (absolute change of 1.4-fold and P value of <0.05). Additionally, there were significant numbers of unique DE genes in each of the data sets (Fig. 3B). Results from functional enrichment analysis for canonical pathways of these sets of DE genes showed that only the protected animals from the prime-boost group had highly significant expression for

![Diagram](http://cvi.asm.org/Downloaded from http://cvi.asm.org on July 19, 2018 by guest)
EIF2 signaling, activation of IRF by cytosolic pattern recognition receptors, and IFN signaling after challenge 1 (Fig. 3C). These pathways included well-characterized antiviral response transcripts, such as ISG15, ISG20, IFIT1, IFIT3, DDX58, and OAS1, known to affect viral replication in the context of SIV infection (19–22). Further examination of host responses in persistently protected animals showed that after the second challenge, innate viral sensing in these animals was largely abated (Fig. 3C). TLR3 was differentially expressed only after the first challenge, and proinflammatory molecules, such as LCN2, TREM1, and SERPINA1, were upregulated 2-fold after the second challenge.

IPA network analysis further illustrates our findings (Fig. 3D), as the top-ranked challenge-specific networks show a clear contrast between innate antiviral responses after the first challenge and an onset of proinflammatory signatures in conjunction with TLR2/4/6 expression. Functional analysis based on high-ranked Z scores (a |Z| value of ≥2 is required for significant predicted activation) and enrichment scores (Table 1), showed that processes related to adhesion and movement of immune cells absent after the first challenge became more dominant after the second challenge. Among the strongest contrasts in the prime-boost group between the first and second challenges was the predicted decreased quantity of immune cells (downregulation of CD8A, PRKCH, and CCR7 and upregulation of SERPINA1 and LCN2), but also a significant increase in chemotaxis and cell movement of a large class of phagocytes after the second challenge (Table 1, challenge 2). Downregulation of MHC II molecules (HLA-DMB, HLA-DOB, HLA-DRA, and HLA-DR5) is an important hallmark.

FIG 3 Distinct host responses after each challenge in protected animals. (A) Challenge history and immunization for the prime-boost group. The number of infected animals is indicated on the y axis, and the number of challenges is indicated on the x axis. The original survival data were rescored under the assumption that delayed-viremic animals were infected. The P value refers to whether the survival data showed a statistical difference between the geometric model (no effect of challenge history, corresponding to the null hypothesis) and a two-step model (effect of challenge history between challenges 1 and 2, corresponding to the alternative hypothesis). This value was derived from a χ² test between the log likelihood maximizers of the two models. In the case of the original data, the null hypothesis was rejected (P = 0.01) (data not shown). For the rescored data, the P value of 0.32 indicates that there was no difference, and therefore there was no effect of challenge history on immunity. The dashed lines represent the maximum likelihood of the fit to the survival data. The box represents the region of the graph that was enlarged in a comparison of the rescored to original data (graphs on the right). (B) Differential gene expression analysis based on statistical comparisons of study-group-matched baseline and challenge samples (fold change of >1.4-fold and P value of <0.05) showed a high frequency of differentially expressed genes in protected animals specific for each of the three challenges. (C) Canonical pathway analysis was performed using the IPA knowledge base. For each of the three lists of DE genes used for panel B, the top 4 enrichment scores (given by −log₁₀P values from Fisher’s exact test) within each set were considered and merged the resulting tables. The radial represents the enrichment score for each pathway across the three data sets. (D) Merging top-ranked networks between challenges 1 and 2 showed distinct differences in host response, with a highly upregulated antiviral response network at challenge 1 and, in contrast, a highly upregulated network of TLR and TREM signaling at challenge 2.
TABLE 1 Functional enrichment scores for protected PBG animals following the first three challenges

| Functional category                  | Challenge 1 | Challenge 2 | Challenge 3 |
|--------------------------------------|-------------|-------------|-------------|
|                                      | -log₁₀ P    | Z score     | -log₁₀ P    | Z score     | -log₁₀ P    | Z score     |
| Viral infection                      | 13.58838    | -3.156      | 10.7055     | -0.705      | 9.327902    | 0.86        |
| Inflammation of organ                | 6.739929    | -2.827      | 8.696804    | -2.271      | 9.340084    | -1.001      |
| Replication of RNA virus             | 19.73283    | -2.674      | 5.627088    | -0.057      | NA          | NA          |
| Antiviral response                   | 20.6575     | 2.058       | NA          | NA          | 3.657577    | 1.948       |
| Phagocytosis of cells                | NA          | NA          | 5.303644    | 3.76        | 5.974694    | 2.658       |
| Cell death of immune cells           | 5.966576    | 2.234       | 15.5376     | 2.887       | 7.158641    | 2.411       |
| Quantity of leukocytes               | 6.928118    | 0.215       | 15.1107     | -2.528      | 12.98297    | 0.659       |
| Differentiation of leukocytes        | 7.334419    | 1.007       | 14.77989    | -0.08       | 8.617983    | 2.513       |
| Cell movement of myeloid cells       | NA          | NA          | 9.580044    | 3.022       | 11.69037    | 4.554       |
| Cell movement of leukocytes          | 2.61261     | -0.403      | 14.00174    | 2.043       | 11.4908     | 4.539       |
| Function of leukocytes               | 6.032452    | 0.838       | 12.57349    | 0.16        | 16.21042    | 1.264       |
| Function of myeloid cells            | 3.331614    | NA          | 5.647817    | NA          | 14.91009    | NA          |
| Quantity of phagocytes               | 5.476254    | -0.257      | 9.28735     | -2.056      | 14.91009    | -0.164      |

NA, not available.

of viral CTL avoidance (23), and this was observed only for animals that succumbed to infection after the second challenge and was absent in persistently protected animals. By the third challenge, the observed trend from strong innate signaling to adaptive immune response was further pronounced, with a more prevailing predicted activation of chemotaxis and cell movement of phagocytes by upregulation of the proinflammatory markers S100A8, S100A9, and TREM1 and upregulation of the cytopathic molecule MMP9.

As noted, the vaccine regimens were expected to induce a strong cell-mediated response for both vaccine groups, with an additional antibody arm for the prime-boost group. It was previously shown that the presence of antibodies specific to SIV gp120 in blood serum prior to challenge was highly correlated (Pearson correlation coefficient of 0.84) with the number of challenges required to establish infection for this group (15). The functional analysis of the prime-boost DE gene set identified elements that directly supported the anticipated humoral component throughout all three challenges, albeit with much less prevalence than the aspects involving IFN signaling. Differential gene expression showed a specific enrichment in the prime-boost group for B cell receptor signaling, phosphatidylinositol 3-kinase (PI3K) signaling in B lymphocytes, and antigen presentation, with downregulation of CD40 and CD79B and several MHC II molecules, such as HLA-DMB, HLA-DDB, and HLA-DRA. There was also increased expression of TNFSF13B (BLysS), a cytokine of the TNF ligand family that is a B cell activator that promotes maturation and class switching.

In summary, we have established statistical evidence for efficient, time-homogeneous vaccine protection throughout several challenges in terms of viral acquisition. In contrast, transcriptional profiling for protected animals revealed host responses that evolved from challenge to challenge, from an antiviral IFN response after the first challenge to cellular adaptive immune responses after the second challenge and proinflammatory signatures after the third challenge.

Comparative analysis of host responses in protected animals and natural hosts early after infection shows similar innate and anti-inflammatory signaling. The set of 235 genes that were differentially expressed after the first challenge in protected animals (Fig. 4A) showed a distinct cluster of innate antiviral immune genes with upregulation of 4- to 8-fold after the first challenge (ISG15, IFI27, IFIT2, IFI6, IRF7, and MX1). To interrogate which part of this signature was associated with vaccine-related protection, we compared it to SIV-negative control animals after the first challenge and found 171 differentially expressed genes unique to prime-boost-vaccinated animals (Fig. 4A, "PBG only"). Functional enrichment showed highly significant functions related to the predicted activation of cell death of immune cells and inhibition of viral replication and infection only for this cluster. In contrast, for the set of common genes (Fig. 4A, "PBG & Control"), we were not able to determine statistically significant activation predictions.

Natural hosts, such as African green monkeys (AGM), acquire SIV with set-point viral load levels similar to those in macaques, without developing progression to chronic disease and its hallmark of CD4+ T cell depletion. The strong and quickly resolved IFN response has been found to be a hallmark of nonpathogenic infection, despite potent viral acquisition (24–26). In order to determine whether the observed IFN responses in protected PBG animals were specific to acquisition prevention (in both pathogenic and nonpathogenic infection models), we compared transcriptional changes in blood 10 days after infection of AGM and Asian pigtail macaques (PM) (based on statistical analysis of data published in reference 25) to host signatures of protected prime-boost group animals 5 days after the first challenge. Among the common, highly enriched pathways were EIF2K signaling (up-regulation of PKR and downregulation of many ribosomal transcripts) and IFN signaling (Fig. 4B). Although pathogen receptor signaling was enriched for AGM, PM, and protected PBG signatures, closer inspection showed larger disparities. Whereas up-regulation of double-stranded RNA sensors (TLR3, RIG-I, and MD5) was present only in protected animals, TLR1 was solely expressed in natural hosts and in PM. As a control, we also compared these two signatures to that of SIV-negative control group animals. Despite undetectable viral loads 5 days after the first challenge, none of the above-mentioned transcripts were differentially expressed in this group. In contrast, SIV-negative control group animals showed highly predicted activation of cell movement of myeloid cells and significant enrichment for inflammatory responses by virtue of NF-κB signaling. None of these categories
were enriched for AGM, PM, or the protected prime-boost group animals.

**DISCUSSION**

Using the canonical multiple-low-dose-challenge scheme for nonhuman primate vaccine trials, the vaccination regimen studied here showed a reduction of viral acquisition risk of 73% for the prime-boost group. Its unconventional design of using gp96-Ig-secreting cells carrying SIV peptides induced a strong multifunctional memory CTL response in the rectal lamina propria prior to challenge. In addition, elevated SIV gp120-specific antibody titers in blood serum 5 weeks prior to the first challenge are statistically significant correlates of protection for the prime-boost group animals (15). Despite the relatively high vaccine efficacy in the protected group, protection from viral acquisition was only partial.

In the present genomic analyses, we identified specific host response factors that were required to carry prechallenge immune correlates over to protection after multiple challenges. Transcriptional profiling of whole blood demonstrated that persistent protection upon viral challenge coincided with a strong IFN-mediated antiviral response, which abated after the second challenge. Similar patterns of IFN responses in the context of therapeutics against lentivirus infection have been shown to improve viral control (27, 28). It was also noted in a single-challenge nonhuman primate SIV vaccine trial that challenge outcome is correlated with the relative balance between SIV-specific IFN-γ-driven T cell responses and nonspecific IFN-γ-driven inflammation (29). Loss of this balance is in line with the gene expression profiles of delayed-viremic prime-boost group animals, in which we observed an early onset of inflammatory responses despite the presence of IFN signaling. The extent to which innate immunity can modulate or cooperate with adaptive immune activation to mount an antiviral immune response to HIV and SIV infections has been discussed (30). Within the context of an adenovirus type 5 (Ad5)-HIV vaccine, postvaccination transcriptional changes in IFN signaling and myeloid cell trafficking are implicated in the induction and magnitude of HIV-specific CD8+ T cell responses (31). In this study, although there was evidence of SIV-specific responses in terms of INF-γ secretion by CD8+ T cells before challenge in both vaccine groups, the study outcome based on survival after viral challenge showed that these immune correlates of vaccination are not necessarily also immune correlates of protection (15). Also, for prime-boost group animals, the double-stranded RNA receptor TLR3 was expressed exclusively in protected animals after the first challenge, whereas the expression of surface TLR4 was unique to delayed-viremic animals after the first challenge. It was previously shown that only TLR3 enhances CD8+ T cell responses in vivo and that TLR2 and TLR4 activation may suppress pathogen-induced CD8+ T cell responses (32). This suggests that TLR4-mediated signaling may possibly act as an antagonist to CD8+ T cell responses during challenge, despite the presence of these responses prior to challenge. Furthermore, neutrophil and macrophage activation in animals with delayed viremia, in conjunction with upregulation of PD-L1, may be a cause of further inactivation of CD8+ T cells (33).

Comparison of the host response of protected animals in the prime-boost group with that of SIV-infected natural hosts is of particular interest. The latter acquire SIV without developing immunodeficiency and also show a strong type I IFN response at 10
uninfected and infected CD4 T cells, we relied on the argument that a basic mass-action law would facilitate the evolution of infection in terms of the numbers of infected CD4 T cell count data are shown on the right y axis, starting at prechallenge week 32 and continuing throughout three challenges. For both antibody titers and cell counts, colors stand for status within the first two challenges: turquoise represents animals which remained protected throughout the first two challenges, and red stands for animals which were either infected or delayed viremic during the first two challenges.

Given that strong humoral immune correlates in protected animals, we hypothesize that preexisting antibody responses may be enhanced sufficiently by direct stimulation of T and B cells by type I IFN. Also, the strong enrichment of E1F2 signaling after the first challenge in both the protected prime-booster animals and the SIV-infected natural hosts and PM provides further hypotheses for how the initial IFN-mediated antiviral response to SIV affects host restriction factors important for preventing or enhancing viral acquisition or target cell depletion.

Understanding HIV infection from a modeling perspective has relied on the argument that a basic mass-action law would faithfully describe the evolution of infection in terms of the numbers of uninfected and infected CD4 T cells and the number of infective viral particles. Based on postchallenge data, there is evidence that for consistently protected animals, CD4 T cell counts remained stable at an intermediate level throughout several challenges, whereas delayed-viremic or infected animals showed either very high or very low CD4 T cell levels prior to challenge and larger variations over time (Fig. 5). Also, prechallenge antibody data show a distinct pattern of elevated SIV gp120-specific antibodies. Clearly, larger numbers of naive target cells would increase the chance of establishing productive infection, and having smaller numbers of CD4 T cells during the challenge phase would decrease the probability of viral acquisition. Based on these data, we suggest that as long as antibody-secreting cells (ASCs) can be efficiently activated (indicated by early upregulation of TNFSF13B) in a T helper cell-dependent manner, polyclonal antibody responses will delay viral acquisition during repeated challenges. On the other hand, for animals with very small numbers of CD4 T cells, this response may become inefficient in that its induction of epitope-specific antibody production is too slow to prevent viral acquisition after challenge. This observation is corroborated by the stability and maintenance of tissue-resident CD4 T cells in the lamina propria for a long period in persistently protected animals. Figure 6 shows percentages of CD4 CD3 T cells relative to CD3 T cells at 11 weeks postinfection (or after the last challenge) and suggests that balancing CD4 T cell populations during the early challenge phase may counteract the premature exhaustion of resident T cells in the rectal mucosa. As indicated by transcriptomic profiling, a strong early IFN response after the first challenge can potentially act to sustain T helper cell-dependent humoral responses and substantially delay viral acquisition. Similar roles of highly efficient T helper cell function in mediating polyfunctional responses by virtue of IFN produc-

FIG 5 Prechallenge immune correlates and postchallenge CD4 T cell counts for prime-booster group animals during repeated low-dose challenges. Levels of SIV Env-specific antibodies are shown on the left y axis, and CD4 T cell count data are shown on the right y axis, starting at prechallenge week 32 and continuing throughout three challenges. For both antibody titers and cell counts, colors stand for status within the first two challenges: turquoise represents animals which remained protected throughout the first two challenges, and red stands for animals which were either infected or delayed viremic during the first two challenges.

FIG 6 Preservation of rectal lamina propria CD4 T lymphocytes in protected, vaccinated animals with a strong IFN response after the first challenge. Animals were immunized with gp96SIV or gp96Mock (prime group [PG] and control group [CG], respectively) or with gp96SIV and gp120 (prime-booster group [PBG]) by the intraperitoneal route at weeks 0, 6, and 25. Pinch biopsy samples were harvested from each animal 11 weeks after infection (or after the last challenge for animals P439 and P442, which remained SIV negative after seven challenges). Rectal CD4 T cells were analyzed by flow cytometry after gating on live lymphocytes (CD3 T cells), and the percentage of CD4-positive cells is shown. The individual data for each monkey were plotted. Turquoise represents animals which remained protected throughout the first two challenges, and red stands for animals which were either infected or delayed viremic during the first two challenges. Data for animals P439 and P442 from the PBG are highlighted, as they remained SIV negative after seven consecutive challenges. Overall, PBG animals showed a high degree of preservation of CD4 T cells compared to controls (t test P value of 0.0151).
tion have been observed in elite controllers within a human cohort study (41).

In resoring the survival data based on virological and host transcriptional evidence, we have presented statistical arguments in favor of protection rates remaining constant throughout multiple exposures to SIV. In addition, whole-blood transcriptomic profiling demonstrated a short-term delay of viremia, with macrophage-mediated proinflammatory responses as the important hallmark, independently of the vaccine regimen. Macrophage activation and its proinflammatory consequences are associated with efficient viral replication in acute HIV/SIV infection (42–44). On the other hand, early establishment of an anti-inflammatory milieu is predictive of disease nonprogression in the case of natural hosts (45). In the context of the weekly mucosal low-dose challenge design, targeted transcriptional evaluation after SIV challenge may be used to determine whether animals with SIV levels below the limit of detection in blood should be suspended, at least temporarily, from further challenge.

Taken together, our results show that despite promising, vaccination-induced immune correlates, multiple low-dose challenges can result in evolving host responses that affect the protective outcome. Findings from our earlier study (15) indicate that gp96–Ig induces a long-lasting memory response in gut mucosal sites, with the ability to rapidly undergo multiple rounds of proliferation in response to antigen, a hallmark of memory cells. In the context of this vaccine study, there is strong evidence that undetectable viral load was an insufficient characterization of protection from viral acquisition on a subsequent challenge. Postchallenge host response profiling of vaccinated animals showed that whereas short-term seronegativity is characterized by strong proinflammatory responses, protection comes with a strong and quickly resolved IFN-driven antiviral response and subsequent adaptive immune responses. Based on our transcriptomic analysis and known prechallenge correlates of protection, we hypothesize that innate antiviral signaling events may provide important contributions to enhancement of humoral responses, and therefore to higher vaccine efficacy.

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