Lymph node T cell responses predict the efficacy of live attenuated SIV vaccines

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Live attenuated simian immunodeficiency virus (SIV) vaccines (LAVs) remain the most efficacious of all vaccines in nonhuman primate models of HIV and AIDS, yet the basis of their robust protection remains poorly understood. Here we show that the degree of LAV-mediated protection against intravenous wild-type SIVmac239 challenge strongly correlates with the magnitude and function of SIV-specific, effector-differentiated T cells in the lymph node but not with the responses of such T cells in the blood or with other cellular, humoral and innate immune parameters. We found that maintenance of protective T cell responses is associated with persistent LAV replication in the lymph node, which occurs almost exclusively in follicular helper T cells. Thus, effective LAVs maintain lymphoid tissue-based, effector-differentiated, SIV-specific T cells that intercept and suppress early wild-type SIV amplification and, if present in sufficient frequencies, can completely control and perhaps clear infection, an observation that provides a rationale for the development of safe, persistent vectors that can elicit and maintain such responses.

It has been two decades since the first report that LAV administration can completely protect rhesus macaques from subsequent challenge with highly pathogenic, wild-type SIV, a degree of efficacy that is in sharp contrast to that achieved with a wide variety of alternative HIV and SIV vaccine strategies1-6. However, LAV-mediated protection is inversely proportional to the degree of attenuation, such that the most effective designs retain considerable pathogenic potential, precluding their development as vaccines in humans6-8. Although it has long been appreciated that delineation of a common immunologic mechanism for LAV efficacy would be a major advance in the field of HIV and AIDS vaccines—providing an immune response target for the development of safe and effective vaccine designs8—the basis of LAV efficacy, particularly the durable, complete, apparently sterilizing control characteristic of the best LAVs, remains controversial. Research using various types of LAVs and nonhuman primate (NHP) species, as well as different pathogenic challenge viruses and varying timing and routes of challenge, has variously implicated, or argued against, T cell immunity, humoral immunity, innate immunity and viral interference in LAV-mediated protection without the emergence of a unifying, consensus protective mechanism or a clear-cut definition of multiple different mechanisms acting in combination to achieve protection5,6,8-16.

In this study, we sought to clarify the immunologic (or other) basis of LAV-mediated protection against highly pathogenic SIV by statistical correlation of fundamental measures of LAV-elicited immunity with efficacy after challenge in a large cohort of LAV-vaccinated rhesus macaques. Our approach was to vaccinate groups of macaques with different LAVs that varied in their level of attenuation, their degree of amino acid sequence homology with the wild-type SIVmac239 challenge virus or both with the intent of producing sufficient heterogeneity in both immune responses and protective efficacy at the level of the overall cohort to create a statistically powerful immune parameter versus efficacy matrix. We used the most stringent route of SIV challenge—high-dose intravenous administration—because LAVs are unique among SIV vaccines in their ability to completely control such a systemic SIV challenge1-3 and hematogenous dissemination is a common step in all primary HIV and SIV infections, including those arising from mucosal surfaces17. In this regard, a vaccine that intercepts and completely controls hematogenous HIV spread would be applicable to both mucosal and intravenous routes of HIV entry, the latter of which is a clinically relevant way of acquiring infection that is not addressed by vaccines that are designed to elicit mucosal immunity. Notably, this experimental approach strongly and specifically identified characteristics of the SIV-specific T cell response in the lymph node (including its magnitude, effector differentiation, functional properties and kinetics of activation after challenge) as cross-platform predictors of efficacy.
of LAV-mediated protection against high-dose intravenous SIV challenge. We further linked the maintenance of these protective lymph-node–based T cells to the ability of replication-competent LAV to persist in follicular helper CD4+ T cells in secondary lymphoid tissues. Together, these findings provide further support for the concept that early SIV infection, even the systemically distributed infection that follows high-dose intravenous challenge, is vulnerable to the high-frequency, effector-differentiated memory T cell responses that are uniquely elicited and maintained long term by persistent vectors.

RESULTS

Heterogeneous protection with different LAV types
To achieve the heterogeneity in both immune responses and protective efficacy that is necessary for robust statistical delineation of immune correlates, we selected LAV designs for study that vary in their level of attenuation and degree of amino acid sequence homology with the wild-type SIVmac239 challenge virus. These LAVs were expected to differ in the amount and persistence of SIV antigens that they provide to the immune system for elicitation of SIV-specific T cell and antibody responses (leading to differences in the magnitude, differentiation and durability of the responses resulting from each vaccine), as well as in the ability of the resulting vaccine-elicited adaptive immunity to cross-react with the challenge virus. The prototype LAV, SIVmac239(Δnef), is only moderately attenuated and is sequence matched to the challenge virus. SIVmac239(Δ3) and single-cycle SIVmac239 are also sequence matched to the challenge virus but are progressively more attenuated than SIVmac239(Δn)13,18,19. SHIV89.6 is also only moderately attenuated, but it differs from the SIVmac239(Δn) prototype by virtue of highly heterologous, HIV-derived env, tat, vpu and rev sequences and its ability to use chemokine (C–X–C motif) receptor 4 (CXCR4) as a co-receptor5. SIVsmE543(ΔnB) is variably attenuated (because of its sensitivity to restrictive tripartite motif containing protein 5 (TRIM5) alleles) and is heterologous in its amino acid sequence to SIVmac239 (~15% overall divergence)13,20. We administered each LAV to six to eight macaques to create an overall study group of 32 vaccinated animals (Fig. 1a). We excluded macaques with class I major histocompatibility complex (MHC I) alleles that are associated with enhanced CD8+ T cell–mediated SIV control (Mamu-A*01, Mamu-B*08 and Mamu-B*17)13 to focus the analysis on new correlates of protection.

Consistent with the expected characteristics of the selected LAVs, the SHIV89.6-vaccinated macaques in our study group had the highest peak LAV viremia, highest peak number of LAV RNA copies within peripheral blood mononuclear cells (PBMC) and most acute-phase effector-site CD4+ T cell depletion, followed by the macaques vaccinated with SIVmac239(Δn), SIVmac239(Δ3), SIVsmE543(ΔnB) and single-cycle SIVmac239 (Fig. 1b,c and Supplementary Fig. 1). Differences in these parameters were less apparent in chronic-phase, as the replication of even the least attenuated LAVs was brought under stringent control by week 10 after vaccination, and the modest acute-phase effector-site CD4+ T cell depletion was transient. Notably, however, the peak numbers of PBMC-associated LAV DNA copies established during acute-phase viremia decayed very slowly over time, providing a persistent measure of the amount of peak LAV replication (Fig. 1c).

After a 50-week vaccination period to allow full maturation and stabilization of immunity to SIV2, we intravenously challenged the 32 LAV-inoculated and 6 unvaccinated control macaques with 10^3 infectious units of wild-type SIVmac239 (Fig. 1a). The LAV-vaccinated macaques manifested three distinct outcomes after this challenge: (i) complete protection (n = 19), characterized by no discernable net increase in overall plasma viral load (pvl) and either no wild-type SIVmac239, as detected by discriminatory quantitative RT-PCR (n = 16), or transient, low amounts of wild-type virus (<10^3 copies ml^-1; n = 3); (ii) partial protection (n = 7), defined as a clear establishment of wild-type SIV infection (>10^6 copies ml^-1 of wild-type SIVmac239 RNA by discriminatory quantitative RT-PCR and persistently elevated overall pvl) but with an overall pvl that was reduced by a factor of at least 2log in the first 70 days after challenge compared to unvaccinated controls; or (iii) no protection (n = 6), in which the viral dynamics after challenge were indistinguishable from those of the unvaccinated controls (Fig. 1d). We confirmed the biologic validity of these different outcome groups by showing that completely protected macaques lacked both an anamnestic T cell response to wild-type SIV challenge (a sensitive measure of increased SIV replication) and any evidence of SIV pathogenesis (for example, loss of mucosal CD4+ memory T cells), whereas partially protected and nonprotected macaques manifested both unequivocal anamnestic responses and mucosal CD4+ memory T cell depletion that was commensurate with their respective pvl (Fig. 1e,f). Protection was highest in macaques vaccinated with LAVs that were the least attenuated and the most homologous to the challenge virus (Fig. 1d). The peak plasma LAV load (initial 4 weeks after vaccination) and the chronic-phase plasma LAV load immediately before challenge, as well as the number of LAV DNA copies in PBMC immediately before challenge (which, as indicated above, closely correlates with peak LAV load in plasma)—all reflections of LAV attenuation—statistically delineated macaques at each end of the protection spectrum (in particular, completely protected compared to nonprotected macaques; Fig. 1g,h). Notably, however, the amount of PBMC-associated LAV RNA at the time of challenge did not significantly correlate with outcome (Fig. 1h).

Lymph node SIV-specific T cell responses predict outcome
Before challenge, we quantified 11 primary parameters of SIV-specific immunity for assessment as potential correlates of protection, including three humoral immune parameters—SIVmac239 neutralization titers, native SIV env binding titers (neutralization of tissue-culture–adapted SIVmac251) and SIV env-directed antibody-dependent cell-mediated cytotoxicity (all in plasma samples)—and eight T cell response parameters—the magnitude of SIV proteome-specific CD4+ and CD8+ T cells in PBMC, bronchoalveolar lavage (BAL) fluid and lymph node tissue, as well as the breadth of these responses in PBMC. LAV-vaccinated macaques manifested a spectrum of values for each of these parameters, with the exception of SIVmac239 neutralizing antibody titers, which were detectable at low amounts in only a very small proportion of LAV-vaccinated macaques (Supplementary Fig. 2). It is also noteworthy that SHIV89.6-vaccinated macaques lacked antibody responses to the heterologous SIV env.

We next assessed each of these parameters for correlation with the outcome groups described above using the nonparametric Kruskal-Wallis test and adjusting for multiple comparisons using the Bonferroni method. Notably, only two parameters—the magnitude of SIV proteome-specific CD4+ and CD8+ responses in the lymph node—statistically delineate the outcome groups using this method (Table 1 and Fig. 2a). Moreover, the SIV proteome-specific CD4+ and CD8+ T cell responses in the lymph node were the only parameters to statistically delineate each of the three outcome groups from each other using the Wilcoxon rank-sum test (Fig. 2a and Supplementary Fig. 3). In partially protected and nonprotected
macaques, the magnitude of the SIV-specific T cell response (CD4+ and CD8+) in the lymph node, but not in the blood, inversely correlated with peak viremia after challenge as determined by Spearman analysis (Fig. 2b), indicating that lymph-node-based T cell responses before challenge also predict the degree of SIVmac239 control once persistent infection with the wild-type virus is established. The majority of SIV-specific CD8+ T cells in the lymph nodes before challenge of subsequently completely protected macaques

![Diagram](https://example.com/diagram.png)

Figure 1 LAV virology and differential efficacy. (a) Schematic illustrating the vaccination and challenge protocol and rhesus macaque groups. IV, intravenous. (b,c) Comparison of the amount of plasma LAV RNA (b) and PBMC-associated LAV RNA and DNA (c) in the blood of the different LAV-vaccinated macaque groups during the vaccine phase. (d) Effect of wild-type SIVmac239 challenge on the overall amount of plasma SIV RNA in LAV-vaccinated and unvaccinated control macaques (the shaded area corresponds to results after challenge). The two whisker plots (right) compare the change from baseline (during the initial 4 weeks after challenge) and the set point (13–19 weeks after challenge) pvl values in the partially protected, nonprotected and unvaccinated groups (completely protected macaques had no net change in pvl after challenge). The significance of the overall differences in these values was determined by Kruskal-Wallis test, and pairwise differences were determined by Wilcoxon rank-sum test when the Kruskal-Wallis P value was <0.05. (e) Analysis of the change in the frequency of total SIV-specific CD4+ and CD8+ T cells in BAL fluid of the designated protection groups after challenge. Significant differences in the maximal change in response frequencies after challenge were calculated by Wilcoxon rank-sum test for each protection group compared to the completely protected group. (f) Analysis of the depletion of CD4+ memory T cells after challenge in the designated protection groups in the BAL fluid and jejunal mucosa (baseline before challenge was normalized to 100%; statistical analysis as in e). (g) Comparison of the peak plasma LAV loads and plasma LAV loads before challenge in the three protection groups (statistical analysis as described in d). (h) Comparison of PBMC-associated LAV RNA and DNA loads before challenge in the three protection groups (statistical analysis as described in d). NS, not significant.
Table 1  Kruskal-Wallis analysis of the 11 primary immune response predictors compared to the protection categories

| Immune predictors | Unadjusted P | Bonferroni-corrected P |
|-------------------|--------------|------------------------|
| PBMC CD4+ T cell response magnitude | 0.0672 | 0.7392 |
| PBMC CD8+ T cell response magnitude | 0.0195 | 0.2148 |
| PBMC CD4+ T cell response breadth | 0.0301 | 0.3313 |
| PBMC CD8+ T cell response breadth | 0.1593 | 1.0000 |
| Lymph node CD4+ T cell response magnitude | 0.0003 | 0.0036 |
| Lymph node CD8+ T cell response magnitude | 0.0006 | 0.0070 |
| BAL fluid CD4+ T cell response magnitude | 0.0450 | 0.4950 |
| BAL fluid CD8+ T cell response magnitude | 0.0221 | 0.2426 |
| SIVmac239 neutralizing antibodies | 0.1458 | 1.0000 |
| SIV env-specific antibodies; TCLA SIVmac251 neutralization | 0.0079 | 0.0872 |
| SIV env-specific antibodies; ADCC | 0.0073 | 0.0808 |

Values in bold are those that reached statistical significance after Bonferroni correction. TCLA, T cell line-adapted; ADCC, antibody-dependent cell-mediated cytotoxicity.

manifested phenotypic evidence of both recent activation (human leukocyte antigen DR (HLA-DR) and programmed cell death protein 1 (PD-1) expression) and effector differentiation (chemokine (C-C) motif receptor 5 (CCR5) expression) (Fig. 2c), suggesting an ability to manifest an immediate antiviral effector response. To ascertain whether these SIV-specific T cells could directly mediate anti-SIV effector activity, we assessed the ability of sorted populations of memory T cells from PBMC and lymph node tissue to suppress SIVmac239 replication in vitro. Indeed, CD8+ (but not CD4+) memory T cells from the lymph nodes (but not from the blood) of completely protected macaques manifested potent SIV suppression, and across the overall cohort, the extent of this suppression strongly predicted each of the three outcome groups and inversely correlated with peak pvl after challenge in the macaques that established wild-type SIVmac239 infection (Fig. 2d).

Persistent LAV maintains T cell responses in lymph nodes

The ability of efficacious LAVs to maintain protective T cell responses in lymph nodes seemed to depend on the preferential persistence of LAV replication in this tissue (Fig. 3a), as we found a highly significant correlation between the magnitude of both the SIV-specific CD4+ and CD8+ T cell responses in lymph nodes and the amount of lymph node cell-associated LAV RNA (a measure of ongoing LAV replication; Fig. 3b). Because, as noted above (Fig. 2a–d), the lymph-node–based SIV-specific T cell responses strongly correlate with protective efficacy, it is not surprising that the amount of LAV RNA in lymph node cells before challenge also correlated with outcome after challenge (Fig. 3c). The preferential persistence of LAV in lymph node can be explained by the highly selective replication of LAV in the CD4+PD–1hi, inducible T cell co-stimulator (ICOS)hiCD200hiCCR7low follicular helper T (TFH) cell subset (Fig. 3d and Supplementary Fig. 4), which is localized specifically in B cell follicles and is, therefore, found almost exclusively in lymph nodes and other secondary lymphoid tissues.21–23

An analysis of diverse tissues obtained at necropsy from ten completely protected LAV-vaccinated macaques confirmed that B cell follicle–rich secondary lymphoid tissues, including lymph nodes, spleen and colonic mucosa, contained significantly higher copy numbers of cell-associated LAV RNA and DNA per 10^6 cells than primary lymphoid tissues (bone marrow) and extra-lymphoid tissues (jejunal mucosa, liver and BAL fluid) that typically lack such B cell follicles (Fig. 3e). Notably, the mean frequencies of SIV-specific CD8+ T cells in each tissue closely correlated with the log mean of the amount of cell-associated SIV RNA in that tissue (Fig. 3f), an observation that provides further evidence that the amount of ongoing LAV replication determines the frequency at which SIV-specific CD8+ T cells are maintained in any given tissue. Thus, CD4+ TFH cells serve as a sanctuary site for LAV persistence23,24 and concentrate LAV replication (and antigen production) in secondary lymphoid tissues, providing an immune stimulus for production and maintenance of SIV-specific T cells that is proportional to the extent of TFH cell infection.

Linkage of protection to early lymph node T cell activation

Collectively, these data suggest that after intravenous challenge, the lymph node and other secondary lymphoid tissues are major sites at which LAV-generated, SIV-specific T cells intercept the nascent wild-type SIV infection, and if these T cells are present in sufficient numbers, the wild-type SIV infection can be completely controlled and possibly cleared. To probe the dynamics and characteristics of the immune response at this putative intercept, we used a microarray analysis to compare the transcriptional profiles of unfractonated lymph node cells obtained from completely protected, nonprotected and unvaccinated control macaques 7 days before and 4 and 14 days after wild-type SIVmac239 challenge. Before challenge, the gene expression profiles of unfractonated lymph node cells from completely protected and nonprotected macaques were indistinguishable from each other but were different than those from the unvaccinated control macaques (Fig. 4a,b). Gene pathways that were significant in distinguishing vaccinated from unvaccinated macaques included metabolic pathways that are associated with stress responses and survival (eukaryotic initiation factor 2 (EIF2), mammalian target of rapamycin (mTOR), oxidative phosphorylation and protein ubiquitination), as well as pathways involved in T cell activation (nuclear factor of activated T cells (NFAT), phospholipase Cγ (PLC-γ), protein kinase A (PKA) and CD28) and are therefore consistent with a low-level immune response to the LAV (Fig. 4c and Supplementary Table 1). Notably, at post-challenge day (PCD) 4 (a time point when cell-associated SIV RNA and DNA were not yet detectable in the lymph nodes of the unvaccinated control macaques), the gene expression profiles of the lymph node cells from completely protected and nonprotected (but not control) macaques, although indistinguishable from each other, differed markedly from the profiles of these cells before challenge, involving multiple pathways related to immune activation (Fig. 4d–f and Supplementary Table 2). A gene-level comparison of lymph node cells at PCD 4 and before challenge from completely protected, nonprotected and control macaques using an immune response gene filter revealed upregulation of T cell, inflammasome and antigen-presenting–cell genes, as well as of genes regulating the interface of innate and adaptive immunity in the vaccinated macaques compared to controls (Fig. 4g and Supplementary Discussion). Because the representation of the major cell subsets in the lymph nodes of completely protected and nonprotected macaques at PCD 4 was unchanged from that at baseline (Supplementary Fig. 5), these differences in gene expression profiles between baseline and PCD 4 probably reflect an in situ immune response. Moreover, given that the lymph node transcriptional profiles changed in vaccinated but not unvaccinated control macaques, they were likely initiated by an adaptive (LAV-elicited) memory response to challenge. From PCD 4...
Figure 2 Immunological correlates of LAV-mediated protection. (a) Comparison of the magnitude of the total SIV-specific CD4+ and CD8+ T cell responses before challenge in the PBMC or lymph node memory compartments of the designated protection groups. The significance of the overall differences in these values was determined by Kruskal-Wallis test, and pairwise differences were determined by Wilcoxon rank-sum test when the Kruskal-Wallis P-value was < 0.05. The horizontal bars show the mean values. (b) Spearman analysis of the correlation between the magnitude of the total SIV-specific CD4+ and CD8+ T cell responses before challenge in the PBMC or lymph node memory compartments and the peak change in pVL after challenge among the partially protected and nonprotected macaques (n = 13 total). (c) Flow cytometric analysis of the phenotype of overall (left) and SIV gag-specific (right) CD8+ T cell populations from the lymph nodes of two representative LAV-vaccinated, completely protected macaques before challenge. The profiles shown were gated on CD3+CD8+ small lymphocytes. In the plots on the left, cell clusters corresponding to the naive (blue) and memory (red) T cell populations and the major differentiation-defined memory T cell subsets (TEM, effector memory; TCM, central memory; TTrEM, transitional effector memory) are shown. (d) Analysis of the ability of isolated memory T cells from LAV-vaccinated macaques to suppress SIVmac239 replication in autologous CD4+ T cells. On the top are results from a representative completely protected macaque (the percentage of gag p27+ cells is shown for each profile). In the middle is a comparison of the degree of viral suppression by sorted CD8+ memory T cells from PBMC and lymph node tissue before challenge for the designated protection groups, with the significance of differences between groups analyzed as described in a. On the bottom is a Spearman analysis of the correlation between the degree of viral suppression by sorted CD8+ memory T cells from PBMC or the lymph node tissue and the change in peak pVL after challenge among partially protected and nonprotected macaques (n = 13 total). NS, not significant.
Figure 3 Association of tissue LAV replication and SIV-specific T cell responses. (a) Whisker plot comparing the amount of cell-associated LAV RNA before challenge in mononuclear cells from the blood, lymph node (LN), BAL fluid and jejunal mucosa in macaques vaccinated with the designated LAV. *P ≤ 0.05, indicating significantly higher amounts of LAV RNA in the lymph node compared to the other three tissues by Wilcoxon signed-rank test. (b) Spearman analysis of the correlation between the amount of cell-associated LAV RNA before challenge in unfractionated lymph node cells and the magnitude of the total SIV-specific T cell responses before challenge in the CD4⁺ and CD8⁺ lymph node memory T cell compartments. (c) Comparison of the amount of cell-associated LAV RNA before challenge in lymph node cells in the designated protection groups, with the statistical analysis performed as described in Figure 2a (horizontal bars show the mean values). (d) Isolation of replicon-competent LAV from the designated sorted lymph node cell populations before challenge (inset at right) from three representative completely protected macaques using CEMx174 coculture (22 days; 10⁴ sorted lymph node cells and 10⁵ CEMx174 target cells per culture) with detection of infection by flow cytometric analysis of intracellular LAV RNA copies per 10⁵ cells in each tissue (right axis). The levels of cell-associated LAV RNA or DNA in secondary lymphoid tissues (B cell–follicle rich; including all lymph nodes, spleen and colonic mucosa) compared to those in nonsecondary lymphoid tissues (B cell–follicle poor; including the bone marrow, BAL fluid, liver and jejunal mucosa) of the completely protected macaques. The P values shown were calculated by Wilcoxon rank-sum test. (f) The mean frequency (+ s.e.m.) of total SIV-specific T cell responses within the CD4⁺ and CD8⁺ memory T cell populations of the designated tissues obtained from necropsy of the same ten completely protected macaques (left). Spearman analysis showing the correlation between the mean frequencies of SIV-specific CD8⁺ T cells in each tissue with the log mean copy number of cell-associated SIV RNA per 10⁵ cells in the corresponding tissue (right). BM, bone marrow; Mes, mesenteric; Ile, ileocecal; Ing, inguinal; Ax, axillary.

to PCD 14, the relationship between the transcriptional profiles of lymph nodes from completely protected, nonprotected and control macaques changed (Fig. 4g and Supplementary Fig. 6). Consistent with the onset of progression of wild-type SIVmac239 infection in nonprotected and control but not completely protected macaques, lymph node cells from the nonprotected and control macaques showed similar patterns of gene expression that were associated with enhanced innate interferon (IFN) and effector T and natural killer cell responses, whereas the expression of immune-related genes in lymph node cells from completely protected macaques was largely unaltered over this period (Supplementary Discussion).

To specifically compare the activity of memory T cells in the lymph nodes of completely protected and nonprotected macaques after wild-type SIVmac239 challenge, we examined the transcriptional profiles of sorted CD8⁺ and CD4⁺ memory T cells from lymph nodes obtained at PCD 4 and PCD 14. This analysis revealed that CD8⁺ memory T cells from PCD 4 lymph nodes of completely protected macaques manifested a relative upregulation of T cell receptor signaling genes and granzymes and a downregulation of the interleukin-7 receptor (IL-7R) gene compared to nonprotected macaques (Fig. 5), reflecting higher T cell activation and earlier establishment of CD8⁺ T cell effector responses in the lymph nodes of these completely protected animals.25–27 (Supplementary Discussion). Although fewer gene expression changes distinguished CD4⁺ memory T cells from the lymph nodes of completely protected compared to nonprotected macaques at PCD 4, there was still significantly higher expression (fold change > 1.3, P < 0.01) of genes associated with antigen-specific activation of CD4⁺ memory T cells in completely protected macaques, including CD40 ligand (CD40LG), lymphocyte-activation gene 3 (LAG3), cytotoxic T lymphocyte–associated protein 4 (CTLA4),
Diferentially expressed canonical pathways by –log10(FDR) between PCD−7 and PCD 4 for control, nonprotected and completely protected macaques (x-comparing the fold change in gene expression from PCD–7 to PCD 4 in unfractionated lymph node cells between completely protected (Yellow and red dashed lines indicate the thresholds for the pathway overrepresentation test 5% and 25% FDR estimates, respectively. (d) versus nonprotected versus completely protected macaques). (b) Scatterplot comparing gene expressions between completely protected (y-axis) and nonprotected (x-axis) macaques by fold change (FC) relative to unvaccinated controls. Dashed lines indicate absolute 2-fold change. (c) Top canonical pathways differentially expressed between vaccinated macaques (completely protected and nonprotected groups averaged) and unvaccinated controls. Yellow and red dashed lines indicate the thresholds for the pathway overrepresentation test 5% and 25% FDR estimates, respectively. (d) Scatterplot comparing the fold change in gene expression from PCD–7 to PCD 4 in unfractionated lymph node cells between completely protected (y-axis) and nonprotected (x-axis) macaques. (e) Scatterplot comparing the fold change in gene expression from PCD–7 to PCD 4 in unfractionated lymph node cells between completely protected and nonprotected macaques (y-axis). (f) Heatmap of the top 15 differentially expressed canonical pathways by –log10(FDR) between PCD–7 and PCD 4 for control, nonprotected and completely protected macaques (unvaccinated controls contributed no significant pathway at >5% FDR). (g) Heatmap of the average fold change in gene expression from PCD–7 to PCD 4 (top) and from PCD 4 to PCD 14 (bottom) in unfractionated lymph node cells for control, nonprotected and completely protected macaques. The genes shown in the heatmap were selected by having absolute fold change >2 at a 5% FDR in at least one protection outcome group and were immune filtered (Online Methods).

Figure 4 Transcriptional profiling of unfractionated lymph node cells before and after wild-type SIV challenge. (a) Comparison of the overall gene expression profiles of unfractionated lymph node cells before challenge (PCD−7) from control (unvaccinated) macaques and LAV-vaccinated macaques that were either completely protected or nonprotected after wild-type SIV challenge. Genes differentially expressed between these three groups were selected by t test with a 5% false discovery rate (FDR), and then multidimensional scaling was used to visualize relatedness of the data sets (control versus nonprotected versus completely protected macaques). (b) Scatterplot comparing gene expressions between completely protected (y-axis) and nonprotected (x-axis) macaques with fold change (FC) relative to unvaccinated controls. Dashed lines indicate absolute 2-fold change. (c) Top canonical pathways differentially expressed between vaccinated macaques (completely protected and nonprotected groups averaged) and unvaccinated controls. Yellow and red dashed lines indicate the thresholds for the pathway overrepresentation test 5% and 25% FDR estimates, respectively. (d) Scatterplot comparing the fold change in gene expression from PCD−7 to PCD 4 in unfractionated lymph node cells between completely protected (y-axis) and nonprotected (x-axis) macaques. (e) Scatterplot comparing the fold change in gene expression from PCD−7 to PCD 4 in unfractionated lymph node cells between completely protected and nonprotected macaques (y-axis). (f) Heatmap of the top 15 differentially expressed canonical pathways by –log10(FDR) between PCD−7 and PCD 4 for control, nonprotected and completely protected macaques (unvaccinated controls contributed no significant pathway at >5% FDR). (g) Heatmap of the average fold change in gene expression from PCD−7 to PCD 4 (top) and from PCD 4 to PCD 14 (bottom) in unfractionated lymph node cells for control, nonprotected and completely protected macaques. The genes shown in the heatmap were selected by having absolute fold change >2 at a 5% FDR in at least one protection outcome group and were immune filtered (Online Methods).

B and T lymphocyte attenuator (BTLA) and regulators of T cell survival (the NF-kB transcriptional pathway)28,29 (Supplementary Fig. 7 and Supplementary Discussion). By PCD 14, CD8+ memory T cells from nonprotected macaques manifested transcriptional evidence of effector functions37, for example, upregulation of genes encoding granzymes, as well as transcriptional activation of innate immunity downstream of type I IFN signaling, protein ubiquitination and cellular proliferation pathways30 (Fig. 5 and Supplementary Tables 3 and 4), which is consistent with the late development of both adaptive and innate immune responses in these macaques. CD4+ memory T cells from the lymph nodes of nonprotected macaques showed similarly increased expression of type I IFN response genes at PCD 14 (Supplementary Fig. 7). In contrast, CD8+ and CD4+ memory T cells from the lymph nodes of completely protected macaques at PCD 14 manifested relative upregulation of genes associated with long-term memory and cellular quiescence and survival30,31 (Fig. 5, Supplementary Fig. 7, Supplementary Table 3 and Supplementary Discussion). Thus, our transcriptional analysis of lymph node memory T cells provides evidence of a larger and/or earlier CD8+ and CD4+ T cell response to challenge in completely protected compared to nonprotected macaques (in keeping with the higher-magnitude, SIV-specific T cell responses in the lymph nodes of completely protected macaques). This early response is associated with control of infection and prevention of the marked innate and adaptive immune activation that characterizes progressive wild-type SIV infection.

DISCUSSION
Correlation analysis does not delineate cause and effect and, therefore, cannot unequivocally establish whether persistent LAV replication, the associated SIV-specific immune response or an uncharacterized covariate are mechanistically related to the observed protection. However, these data, taken together, lead most logically to a model in which persistent LAV replication elicits and maintains differentiated and activated, antiviral effector memory T cells in tissues, and if these T cells are present at a sufficient frequency before challenge, they can completely control (and perhaps clear) wild-type SIV infection in the first days after challenge. Secondary lymphoid tissues seem to be the crucial site of viral intercept after intravenous challenge, and CD8+ memory T cells from lymph nodes of optimally LAV-vaccinated macaques can strongly suppress wild-type SIV replication in autologous CD4+ T cells. Although CD4+ memory T cells from the lymph nodes of the same macaques lacked this capability, the very strong correlation between the frequency of SIV-specific CD4+ T cells in lymph node tissue and outcome suggests their crucial involvement in protection, perhaps through direct effector activity against SIV-infected macrophages, help of SIV-specific, CD8+ T cell functionality
or both12,33. Notably, lymph-node–based, SIV-specific T cell responses also delineated partial controllers from noncontrollers, suggesting that when the LAV-elicited SIV-specific T cell responses are below the threshold for complete control, an anamnestic expansion of the LAV-elicited, SIV-specific memory T cells can ensue, with the outcome being determined, as with conventional prime-boost vaccines, by a competition between the kinetics and antiviral activity of the anamnestic response and viral escape4.

Viral interference probably does not substantially contribute to the observed protection given that LAV replication is restricted largely to CD4+ T_FII cells, a minor subset of the total potential population of CD4+ target cells. Innate immunity elicited by persistent LAV replication is another candidate mechanism for mediating protection. Although our sampling schedule may have missed a transient innate immune response, our detailed transcriptional analysis revealed no evidence of differential innate immune activation in the lymph nodes of protected compared to unprotected macaques before challenge or at 4 days after challenge and thereby argues against this possibility. In addition, the number and activation status of natural killer cells in the lymph node before and after challenge did not correlate with protection, arguing against involvement of these innate immune effectors (Supplementary Fig. 8). Perhaps the most compelling evidence in support of the T cell protection model of LAV efficacy is the analogous immediate establishment of a ‘critical mass’ of replicating virus that is capable of dynamic evolution and immune escape4. To date, this pattern of protection has been consistently observed only with LAVs or SIV vaccines that are based on persistent, nonpathogenic vectors or on other strategies that are capable of eliciting and maintaining such effector memory T cell responses37.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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ONLINE METHODS

Animals. A total of 38 adult male rhesus macaques (Macaca mulatta) of Indian genetic background were used in this study. All macaques were free of coccopithecin herpesvirus 1, simian type D retrovirus, simian T lymphotropic virus type 1 and SIV infection at the start of the study and were used with approval of the Oregon National Primate Research Center's Animal Care and Use Committee under the standards of the NIH Guide for the Care and Use of Laboratory Animals. Macaques with major histocompatibility complex alleles (Mamu-A*01, Mamu-B*08 and Mamu-B*17), which have previously been associated with spontaneous and/or LAV-associated control of wild-type SIV13, were excluded from this study.

Viruses. SIVmac239(Δnef)2, SIVmac239(Δ3)18, SHIV89.6 (ref. 5) and SIVsmE543(Δnef) were derived from SIVmac239). Single-cycle SIVmac239 was produced from bone marrow, BAL fluid, lymph nodes, spleen, liver, jejunum and colon as previously described. Stock of wild-type SIVmac239 challenge virus.

T cell assays. SIV-specific T cells were enumerated in mononuclear cells isolated from blood and tissues by flow cytometric intracellular cytokine analysis, as previously described in detail47. Briefly, mixes of sequential (11-amino-acid overlapping) 15-mer peptides (Anaspec, Inc.) spanning SIVmac239 gag, env, pol, nef, rev, tat, vif, vpr and vpx proteins were used as antigens (rev + tat + vif + vpr + vpx peptide mixes were used in combination) in conjunction with 500 ng ml−1 of both CD28 (28, 2, BD Biosciences) and CD49d (9F10, BD Biosciences) co-stimulatory monoclonal antibodies. Cells were incubated at 37 °C with peptide mixes or pools and co-stimulatory antibodies for 1 h followed by an additional 8 h incubation in the presence of Brefeldin A (5 μg ml−1; Sigma-Aldrich). Stimulation in the absence of peptides served as a background control. Cells were stained with fluorochrome-conjugated monoclonal antibodies, and data were collected on an LSRll (BD Biosciences) and analyzed using the FlowJo software program (version 8.8.6, Tree Star, Inc.). Response frequencies (CD69+ and TNF-α) and co-stimulatory monoclonal antibodies were first determined in the overall CD4+ and CD8+ populations and then memory corrected (memory subset populations were delineated on the basis of CD28 and CD95 expression)48. The sum of net response frequencies determined for each peptide mix described above was considered the overall (pan-proteome) SIV-specific response frequency. To measure the breadth of the SIV-specific T cell responses in PBMC, we determined the frequencies of CD4+ and CD8+ T cells that were responsive to 82 separate pools of ten 15-mer peptides consisting of SIVmac239 proteins (env, pol, gag, nef, rev, tat, vif, vpr and vpx with 22, 26, 13, 6, 2, 3, 5, 2 and 3 pools, respectively). Breadth was reported as the number of ten-mer peptide mixes with an above-threshold (0.05%) response. To determine the cell surface phenotype of SIV-specific CD8+ T cells, mononuclear cells were stimulated as described above, except that the CD28 co-stimulatory monoclonal antibody was used as a fluorochrome conjugate to allow CD28 expression to be assessed by flow cytometry. After incubation, cells were stained on the surface for lineage markers (CD3, CD4 and CD8) and memory and activation markers (CCR5, PD-1 and HLA-DR) before fixation and permeabilization and intracellular staining for response markers (IFN-γ and TNF-α). Viral suppression assays were based on the approach of Martins et al.47. Briefly, autologous CD4+ T cells were isolated from PBMC by NHP CD4+ T cell isolation kits (Miltenyi Biotec), activated in vitro with staphylococcal enterotoxin B (1 μg ml−1; Sigma) and CD3-specific (SP34-2 at 2.5 μg ml−1), CD28-specific (28.2 at 2.5 μg ml−1) and CD49d-specific (9F10 at 2.5 μg ml−1) antibodies, infected with SIVmac239 stock by centrifugation (1,200g for 2 h at 30 °C) and labeled with carboxyfluorescein succinimidyl ester (CFSE) (2.5 μM) for 10 min at 37 °C. These target cells were incubated with (or without) effector cells (sorted CD4+ or CD8+ memory T cells, obtained as described above) at an effector-to-target ratio of 1:1 for 72 h with IL-2 (100 U ml−1; Sigma), and the percentage of SIV gag p27+ cells in the CFSE− subset was determined by a flow cytometric analysis of intracellular SIV gag p27 staining (using the monoclonal antibody 55-2F12, NIH AIDS Research and Reference Reagent Program, used at 200 ng per 105 input cells). The percentage of viral suppression was calculated by the formula 100 – (percentage of p27 with effectors – percentage of p27 in uninfected target cells)/percentage of p27 without effectors – percentage of p27 in uninfected target cells) × 100. T cell phenotype determination was performed by flow cytometry as previously described49 using established criteria for memory T subsets, activated T cells and follicular helper T cells21,22,39,46,50,51.

Antibody response assays. Neutralizing antibodies against SIVmac239 and tissue-culture–adapted SIVmac251 were quantified in luciferase reporter gene assays using the T2M-bl and M7 Luc cell lines, respectively52. SIV env-specific antibodies with antibody-dependent cell-mediated cytotoxic activity were measured as described53.

Microarray analyses. RNA was isolated using RNeasy Micro Kits (Qiagen), and the quantity and quality of the RNA was confirmed using a NanoDrop 2000c (Thermo Fisher Scientific) and an Experion Electrophoresis System. Samples (50 ng) were amplified using Illumina TotalPrep RNA amplification

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kits (Ambion). The microarray analysis was conducted using 750 ng of biotinylated complementary RNA hybridized to HumanHT-12_V4 BeadChips (Illumina) at 58 °C for 20 h. The arrays were scanned using Illumina’s iSCAN and quantified using Genome Studio (Illumina). The analysis of the GenomeStudio output data was conducted using the R56 and Bioconductor55 software packages. Quantile normalization was applied, followed by a log2 transformation. The LIMMA package56 was used to fit a linear model to each probe and per -

tion in those macaques with established wild-type SIVmac239 virus after challenge (partially protected and nonprotected groups), we used the Spearman correlation test to assess predictors across pairs of the three protection categories. In addition, to analyze the degree of protection, we conducted Wilcoxon rank-sum tests to compare predictors across two pairs of tissue types. We used R for all statistical analyses54.

The goal of the primary statistical analysis was to predict outcome after challenge from immune response variables before challenge (responses to the LAVs). The primary measure of outcome was the ordinal series GSE40006).

We conducted all follow-up and secondary or exploratory statistical analyses without multiplicity correction using nonparametric tests: we used Kruskal-Wallis, Wilcoxon and Spearman tests for the categorical, dichotomous and correlation tests, respectively58. When comparing cell-associated viral load across tissue types, we applied Wilcoxon signed-rank tests to the within-macaque differences across tissues. We used R for all statistical analyses54.

Statistical analyses. The goal of the primary statistical analysis was to predict outcome after challenge from immune response variables before challenge (responses to the LAVs). The primary measure of outcome was the ordinal series GSE40006).

The LIMMA package56 was used to fit a linear model to each probe and per -
tion in those macaques with established wild-type SIVmac239 virus after challenge (partially protected and nonprotected—were determined and validated as described in the Results section. The 11 primary independent potential immune response predictors are summarized in Table 1. In all cases, we used the immunologic values obtained from samples collected at the closest available time point before the challenge. To determine which, if any, of these immune response parameters could predict outcome, we used univariate, nonparametric Kruskal-Wallis tests to assess each of the 11 primary predictor (immunologic) variables versus the three protection categories. We considered a parameter to be a correlate of a predictor of outcome if the P value resulting from this analysis was less than 0.05 after Bonferroni correction for multiple comparisons. In follow-up analyses, we conducted Wilcoxon rank-sum tests to compare predictors across pairs of the three protection categories. In addition, to analyze the degree of protection, we conducted Wilcoxon rank-sum tests to compare predictors across two pairs of tissue types. We used R for all statistical analyses54.