Published in final edited form as:

Vaccine. 2014 September 08; 32(40): 5198–5205. doi:10.1016/j.vaccine.2014.07.046.

Apoptosis of antigen-specific CTLs contributes to low immune response in gut-associated lymphoid tissue post vaccination

Masaru Shimada^{a,*}, Shinji Yoshizaki^a, Motohide Ichino^b, Dennis M. Klinman^c, and Kenji Okuda^a

^aDepartment of Molecular Biodefense Research, Yokohama City University, Graduate School of Medicine, Yokohama 236-0004, Japan

^bDepartment of Immunology, Yokohama City University, Graduate School of Medicine, Yokohama 236-0004, Japan

^cCancer and Inflammation Program, National Cancer Institute, Frederick, MD 21702, United States

Abstract

The gut-associated lymphoid tissue (GALT) represents a major reservoir of HIV in infected individuals. Vaccines can induce strong systemic immune responses but these have less impact on CD4 T cells activity and numbers in GALT. In this study, we vaccinated mice with an adenovirus vector that expressed the envelope gene from HIV and observed immune responses in the peripheral blood, spleen, liver, mesenteric lymph nodes, and Peyer's patches. We found that (1) the number of HIV-specific CD8 T cells was dramatically lower in GALT than in other tissues; (2) the programmed cell death protein-1 (PD-1) was expressed at high levels in HIV-specific CD8 T cells including memory T cells in GALT; and (3) high levels of HIV-specific CD8 T cell apoptosis were occurring in GALT. These results suggest that contributing to GALT becoming an HIV reservoir during infection is a combination of exhaustion and/or dysfunction of HIV-specific CTLs at that site. These results emphasize the importance of developing of an effective mucosal vaccine against HIV.

Keywords

Programmed cell death protein-1 (PD-1); Vaccine; Virus vector; Mouse

1. Introduction

Gut-associated lymphoid tissue (GALT) contains an abundance of activated effector memory CD4 T cells and is an early target for infection by and site of high replication of human immunodeficiency virus (HIV) [1–3]. Severe depletion of CD4 T cells occurs in GALT following primary HIV infection, a reduction that is not reflected in the peripheral blood

*Corresponding author at: Department of Molecular Biodefense Research, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. Tel.: +81 45 787 2602; fax: +81 45 787 2509. mshimada@yokohama-cu.ac.jp (M. Shimada).

until the chronic stage of disease [4–6]. Memory cell loss correlates with progression to AIDS (acquired immunodeficiency syndrome) and HIV-associated mortality [7,8]. Vaccination, in addition to giving rise to better viral suppression, can induce immune activation in the GALT, which can limit CD4 T cell loss [9]. Systemic vaccination using replication-deficient viral vectors elicits a systemic immune response as well as a much weaker mucosal immune response [10,11]; however, the details of this mechanism are unclear.

Adenovirus vectors have many advantages for vaccine development, including high viral titer and easy access to multiple cell types including professional antigen presenting cells (APCs), such as dendritic cells (DCs). DCs play a critical role in mediating Ag uptake, processing, expression, and transport to lymphocytes in primary lymphoid organs. These DC activated lymphocytes then initiate primary immune reactions that culminate in the generation of Ag-specific CTLs. Unfortunately, a recent phase IIb clinical trial using an adenovirus vector-based HIV vaccine failed to prevent HIV infection, suggesting that such a vaccine is more effective at inducing systemic immunity rather than protection at mucosal surfaces such as GALT [12]. Indeed, when replication-deficient viral vectors are administered systemically, they typically elicit strong systemic immune responses but relatively weak mucosal immunity [10,11].

Loss of T cell function occurs during persistent infection by a diverse range of viruses, including HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV) in humans, and lymphocytic chori-omeningitis virus (LCMV) in rodents [13]. This pattern is consistent with conserved mechanisms of immunosuppression that down-regulate T cell activity during persistent viral infection. Recent studies demonstrate that programmed death-1 (PD-1), a member of the CD28 gene family, plays a critical role in the suppression of CD8 T cell activity during chronic Ag exposure [14]. PD-1 is usually expressed by activated CD4 and CD8 T cells, natural killer cells (NK cells), B cells, and monocytes [15,16]. Other inhibitory T cell receptors (TCRs) including LAG-3, CD244, CD160, CTLA-4, and Tim-3 are also up-regulated on exhausted CD8 T cells during chronic viral infection [17,18]. These receptors act cooperatively to mediate a decline in CD8 T cell function. The PD-1 pathway is a more potent inhibitor of TCR-induced T cell activation than CTLA-4 because it blocks more membrane-proximal signals and exerts a wider inhibition of T cell activation [19–23]. The blockade of PD-1 as well as LAG-3 or Tim-3 during chronic viral infection in mice is more efficient in reversing CD8 T cell exhaustion and diminishing viral burden than the inhibition of either pathway alone [18]. To date, very little is known about the effects of vaccination on the expression/function of these inhibitory TCRs by CTLs, especially those in the GALT.

It is difficult to study cells from multiple lymphoid organs in humans and non-human primates, so most of the studies relied on samples derived from the peripheral blood. In this study, we investigated the variation of PD-1 expression, HIV-specific CTL response, and levels of apoptosis-related molecules in multiple lymphoid tissues (spleen, liver, mesenteric lymph node (MLN), and Payer's patch (PP)) as well as peripheral blood from BALB/c mice after vaccination with a high HIV gp160 expressing adenovirus vector (Ad-HIV). Compared to their frequency in the peripheral blood, few HIV-specific CTLs were detected in MLN or PP after vaccination, although vaccination did appear to induce CTL PD-1 expression in all

samples. Additional apoptosis-related molecules were also identified in HIV-specific CTLs and memory cells derived from MLN and PP. These results demonstrate the importance of developing vaccines that generate a strong CTL response in GALT.

2. Materials and methods

2.1. Ethics statement

All animal works have been conducted according to relevant Japan and international guidelines. All experimental procedures were carried out in accordance with the Administrative Panel on Laboratory Animal Care (APLAC) protocol and the institutional guidelines set by Yokohama City University. The protocols used in this study were proved by Institutional Animal Care and Use Committee (IACUC)/Ethics committee of Yokohama City University.

2.2. Vaccine

A chimeric virus vector expressing HIV-1_{III_B} rev and env gp160 proteins (Ad-HIV) was used as a vaccine, and a virus vector expressing green fluorescent protein (Ad-GFP) was used as a control [24–26]. The virus vector is an E1, E3-deletion, replication-defective recombinant adenovirus type 5 virus with adenovirus type 35 fiber. HIV genes were driven by a CAG promoter and inserted into the E1 region of the chimeric virus. The Ad-HIV was propagated in HEK293 cells and purified by two repetitions of the CsCl methods described elsewhere [27]. The total concentration of virions in each preparation was calculated from the optical density at 260 nm (OD₂₆₀), using the formula 1OD₂₆₀ = 1 × 10¹² viral particle (vp)/mL.

2.3. Animal immunization

Eight-week-old female BALB/c mice (H-2D^d) were purchased from Japan SLC, Inc. (Shizuoka, Shizuoka-ken, Japan). Five to ten mice per each group were received a single intramuscular (i.m.) injection with 7 × 10¹⁰ vp of the Ad-HIV vaccine in 100 µL of sterile PBS divided equally between both quadriceps muscles. Naïve mice and the mice received i.m. injection with 7 × 10¹⁰ vp of Ad-GFP were used as negative controls. The sampling and assays were performed 1 week after immunization. In some experiments, sampling and assays were performed on days 7, 10, 21, 28 and 35 after immunization (Fig. 2b).

2.4. Antibodies for flow cytometry

The following antibodies were purchased from eBioscience (San Diego, CA): anti-CD8a-FITC, anti-CD8a-PE, anti-CD4-FITC, anti-CD4-PE, anti-IFN-γ-PE, anti-IFN-γ-FITC, anti-TNF-α-APC, anti-PD-1 (CD279)-FITC, and anti-rat IgG-FITC. The anti-CD8a-APC-Cy7 and anti-PD-1 (CD279)-PE antibodies were purchased from BioLegend (San Diego, CA) and the anti-CD127-Alexa fluor 647 antibody was from Bio-Rad Laboratories (Hercules, CA). The annexin V-FITC apoptosis detection kit was purchased from BioVision, Inc. (Milpitas, CA).

2.5. Isolation of lymphocytes

Isolation of lymphocytes from PP, MLN, and liver was performed as previously described [28]. Briefly, PPs were excised from the intestinal wall and liver pieces were mechanically disrupted by passing them through a stainless steel mesh in RPMI1640 containing 5% FBS. The cells were dissociated using collagenase solution containing 0.5 mg/mL collagenase, 0.5 mg/mL DNase I (Roche, Indianapolis, IN), 2% FBS, 20 mM HEPES (pH 7.2), and penicillin/streptomycin in RPMI1640 at 37 °C for 20 min to obtain single-cell suspensions. The cell suspension was washed twice with RPMI-1640, and subjected to Percoll gradient separation to remove epithelial cells. Cells were collected by centrifugation and resuspended in RPMI-1640 containing 5% FBS.

2.6. Tetramer assay

For the tetramer assay, we used a PE-conjugated H-2D^d/p18-I10 tetramer (RGPGRFVIT_I), as previously described [24,29,30]. In brief, heparin-treated whole blood was collected from anesthetized mice. For staining of PBMCs, 100 µL samples of blood were stained with anti-mouse CD8 antibody, anti-mouse PD-1 antibody, anti-CD127 antibody, and tetramer reagent at room temperature for 30 min. An equal volume (100 µL) of Optilyse-lysing solution (Beckman Coulter, Brea, CA) was mixed with the Ab-stained cells at room temperature for 10 min, to fix the cells. A 5-fold volume of H₂O was then added to lyse the red blood cells. The cells were washed twice with staining buffer (3% FBS, 0.1% sodium azide (NaN₃) in PBS) and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ). For staining lymphocytes isolated from tissues, cells were incubated with PE-conjugated H-2D^d/p18-I10 tetramer and antibodies on ice for 1 h.

2.7. Detection of apoptotic cells

The assay was performed using the Annexin V apoptosis detection kit according to the manufacturer's instructions. Briefly, PBMCs were stained with Annexin V-FITC and propidium iodide (PI), anti-CD8 antibody, and tetramer reagent at room temperature for 30 min. Then, an equal volume (100 µL) of Optilyse-lysing solution (Beckman Coulter, Brea, CA) was mixed with the Ab-stained cells and incubated at room temperature for 10 min, followed by an addition of a 5-fold volume of H₂O to lyse the red blood cells. The cells were washed twice with staining buffer (3% FBS, 0.1% sodium azide (NaN₃) in PBS) followed by flow cytometric analysis (BD Biosciences, Franklin Lakes, NJ). For staining of lymphocytes isolated from tissues, cells were incubated with Annexin V-FITC, PI, and antibodies on ice for 1 h, followed by flow cytometric analysis.

2.8. Statistical analyses

All values were expressed as means ± standard error (S.E.). Statistical analysis of the experimental data and controls was conducted with one-way factorial analysis of variance. Significance was defined at 95% confidence ($p < 0.05$) in the statistical analysis.

3. Results

3.1. Ad-HIV vaccination induces cytotoxic T lymphocytes (CTLs)

Previous studies showed that an adenovirus vector (Ad) vaccine against HIV could induce a strong Ag-specific immune response in humans and animal models [12,25,26,31,32]. However the Ad vaccine failed to protect against HIV infection in STEP human clinical trials [12,32]. Analysis of these clinical trials, and studies of non-human primates, typically focuses on the activity of CTLs present in the peripheral blood. In this study, we compared the frequency of HIV-specific CTLs in PBMCs to those from other organs including the spleen, liver, MLN and PP. The analysis was conducted 1 week after intramuscular immunization with the Ad-HIV vaccine. Surprisingly, the liver contained the highest frequency of CTLs (Fig. 1a). Consistent with previous reports, the frequency of HIV-specific CTLs among PBMCs was almost two-fold higher than the frequency of such cells in the spleen [11,33]. Yet we did not confirm previous findings concerning the frequency of HIV-specific CTLs in the MLN and PP, noting them to be approximately 10 times lower than in PBMCs (Fig. 1a) (the percentage of H-2D^d/p18-I10 tetramer⁺ in CD8⁺ T cells derived from naïve mice and the mice received Ad-GFP administration was less than 0.1%). We also analyzed the frequency of HIV-specific memory CD8 T cells in these tissues and found the frequency of HIV-specific memory CD8 T cells was higher in the spleen and PP than in the PBMCs, liver, and MLN (Fig. 1b) (the percentage of CD127⁺ cells in H-2D^d/p18-I10 tetramer⁺CD8⁺ T cells derived from naïve mice and the mice received Ad-GFP administration was less than 0.1%).

3.2. PD-1 expression is upregulated on CD8 T cells post-vaccination

Chronic viral infections lead to persistent CD8 T cell activation and functional exhaustion. High levels of PD-1 expression are associated with decreasing CD8 T cell function during HIV infection [34–36]. We therefore examined the effect of Ad-HIV vaccination on PD-1 levels. Mice were immunized i.m. with the Ad-HIV vaccine. PD-1 expression on HIV-specific CD8 T cells was monitored weekly through day 35. As seen in Fig. 2a, administration of the Ad-HIV vaccine induced PD-1 expression on the surface of HIV-specific CD8 T cells on day 7. Although the level of PD-1 present at day 28 was lower than on day 7, they were still higher than those observed on day 0. The frequency of CD8 T cells expressing PD-1 that were H-2D^d/P18-I10 tetramer positive (e.g., specific for the vaccine antigen), as well as their mean fluorescence intensity (Fig. 2b) was highest on day 7 and fell gradually through day 35 (Fig. 2b). Significantly greater numbers of CD8 T cells in the liver, MLN, and PP of vaccinated mice expressed PD-1 when compared to those isolated from the PBMCs and spleen (7.8–9% vs. 1.4–3.8%, $p < 0.05$) (Fig. 3a). (The percentage of PD-1⁺ cells in CD8⁺ T cells derived from naïve was less than 1.0%.) When analysis was restricted to those CD8 T cells specific for HIV, those expressing PD-1 was significantly higher in the liver (22.5%), MLN (28.9%), and PP (73.6%) when compared to PBMC (5.8%) or spleen (3.3%) (Fig. 3b) (the percentage of PD-1⁺ cells in H-2D^d/p18-I10 tetramer⁺CD8⁺ T cells derived from naïve mice and the mice received Ad-GFP administration was less than 0.1%). Of particular interest, the expression of PD-1 by CD8 T cells that were both HIV-specific and of a memory phenotype was >99% and 67% in the PP and MLN, respectively (Fig. 3c) (the percentage of PD-1⁺ cells in H-2D^d/p18-I10 tetramer⁺CD8⁺CD127⁺ T cells derived

from naïve mice and the mice received Ad-GFP administration was less than 0.1%). These findings indicate that PD-1 is significantly up-regulated on HIV-specific CTL following vaccination, especially those with the memory phenotype residing in the PP and MLN.

3.3. Apoptosis of HIV-specific CD8 T cells in GALT

We examined the magnitude of apoptosis in HIV-specific CD8 T cells among vaccinated mice by staining with PI to detect dead cells and Annexin V to detect cells undergoing apoptosis. One week after vaccination, there was a significant increase in the frequency of HIV-specific Annexin-V⁺ CD8 T cells in the MLN and PP (79.00% and 77.28%, respectively) compared to PBMCs, spleen, and liver (less than 20%; Fig. 4, top panel). However only in the PP (24.54%) were the number of dead (PI⁺) HIV-specific CD8 T cells significantly increased (in all other tissues the frequency was <2%, Fig. 4, bottom panel) (the percentage of Annexin V⁺ cells or PI⁺ cells in H-2D^d/p18-I10 tetramer⁺CD8⁺ T cells derived from naïve mice and the mice received Ad-GFP administration was less than 0.1%). These data suggest that Ad-HIV vaccination results in a large amount of HIV-specific CD8 T cell death, mediated via apoptosis, in the GALT and particularly in the PP.

4. Discussion

Most primary isolates of HIV and SIV utilize both CD4 and the chemokine receptor CCR5 for attachment, fusion, and entry into host cells [37,38]. The observation that CD4 T cells in the gut are considerably more likely to express CCR5 than those in the peripheral blood may explain why T cells in the GALT but not PBMCs are rapidly depleted in early SIV infection [39]. Many studies confirm the preferential depletion of CD4 T cells during early HIV/SIV infection [4,6,40] and it is well established that the GALT is major reservoir for HIV [1,2,10]. Others and we found that CCR5-expressing CD4 T cells in the GALT could provide an important target of initial HIV/SIV infection via the mucosal route (data not shown) [6]. However, efforts to prevent infection by vaccination have failed to induce strong antigen-specific CTLs in GALT.

The initial site of infection influences where Ag exposure and subsequent immune responses develop [41,42]. Similar anatomical localization of primary and secondary responses have been observed following site-specific vaccination [43–47], although more global/systemic responses (including CD8 T cell responses) can be elicited by various pathogens and vaccination strategies [30,48,49]. In this study, we compared the nature and magnitude of the immune response generated in the PBMCs, spleen, liver, MLN, and PP after adenovirus-based vaccination via the intramuscular route. We found that antigen-specific immune responses induced by the vaccine were markedly lower in MLN and PP than in other tissues. We also detected HIV-specific CD8⁺ responses in other mucosal tissues such as nasal associated lymph tissues after vaccination. The responses were almost undetectable, and furthermore, fewer amounts of cells were isolated, which was limited for further analysis. So we did not include the analysis in this study (data not shown).

The distribution of naïve and activated immune cells through secondary lymphoid organs and other peripheral tissues is determined by their expression of homing receptors [50,51]. Kaufman et al. [11] found that i.m. administration of an adenovirus vector-based vaccine

induces potent, durable and functional mucosal immunity through the up-regulation of mucosal homing integrins and chemokine receptors by CD8 T cells. Similar with our results, they also found that i.m. vaccination induces lower antigen-specific CD8 effector T cells and high antigen-specific CD8 memory T cells in PP than in blood and spleen. Thus, it is not the systemic distribution of the adenovirus vector but rather the up-regulation of these homing receptors that is responsible for the rapid traffic-king of Ag-specific T cells to mucosal surfaces and other anatomic compartments. Isakov et al. [52] found that functional and compositional differences between the mucosal compartments during the effector and memory phases of the immune response, indicating limited crosstalk and microenvironmental differences between mucosal and systemic lymphocytes. Belyakov et al. [53] showed that the local microenvironmental factors (such as cytokines and costimulatory molecules) might play a major effect in the generation of the functionally active CD8+ T cells at the mucosal sites. The current work explores why, despite the migration of vaccine-activated cells, the level of immunity induced in the GALT is much lower than the systemic response.

During chronic viral infections, T cell “exhaustion” is commonly observed during chronic viral infection. This state is characterized by non-responsiveness of Ag-specific CD8 T cells and is associated with up-regulation of PD-1. We found that a remarkably high percentage of HIV-specific CD8 T cells, particularly those with a memory phenotype, T cells of PP, expressed PD-1 after Ad-HIV vaccination (Fig. 3). The reason for this enhanced expression is unclear. Kinter et al. reported that cytokines belonging to the common cytokine receptor γ -chain family, such as IL-2, IL-7, and IL-15, can induce the expression of PD-1 and PD-1 ligands (PD-L1 and PDL2) [15,54]. PD-L1 is expressed on many cell types, including T, B, endothelial, and epithelial cells, while PD-L2 is only expressed on professional APCs, such as DCs and macrophages. Each ligand binds to the PD-1 receptor with a different affinity [55,56] with PD-L2 being three times more potent than PD-L1 [19]. We plan to explore the expression of these cytokines and ligands in GALT compared to other tissues to elucidate the mechanism of enhanced cytokine secretion following vaccination.

PD-1 and PD-L1 are implicated in CD8 T cell apoptosis in hepatocellular carcinoma patients [57]. Thus, we examined PD-1 expression by CD8 T cells after Ad-HIV immunization. During the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner layer of the plasma membrane to the outer layer of the membrane and can be easily detected with Annexin V [58]. In contrast, PI is taken up by dead cells, and is used to detect the late stages of apoptosis [59]. We found little evidence of apoptosis in cells isolated from the PBMCs, spleen or liver yet most of the HIV-specific CD8 T cells in MLN and PP were positive for Annexin V (Fig. 4, top panel), while those in the PP also stained with PI (Fig. 4, bottom panel). The expression levels of Annexin V and PI on HIV-specific CD8 T cells were positively paralleled with the expression level of PD-1 (Figs. 3b and 4). These findings suggest that there are very high levels of apoptosis among Ag-specific CD8 T cells in the GALT, which may explain the difficulties encountered inducing mucosal immunity at that site.

To evaluate vaccine-induced response, Gag-based HIV/SIV vaccine has been well used in monkey model study [11,53]. Due to well known of HIV env epitope (p18-I10) in H-2D^d

mouse model, other groups and our group also use HIV env-based vaccine to evaluate the responses [26]. However, Gag-based vaccine may be more suitable for valuation of vaccine-induced immune response than Env-based vaccine, since we recently found that Gag-based vaccine induces higher responses than env-based one by IFN-gamma-ELISPOT assay and intracellular cytokine staining assay (data not shown) and antigen-specific response is more easily for further functional analysis.

Taken together, our data indicated that HIV-specific immune responses in the GALT are much lower than those generated in PBMCs, spleen, and liver after i.m. administration of an adenovirus-based vaccine. The magnitude of this immune response was inversely correlated with PD-1 expression in all tissues investigated. These results suggest that the weak immune response observed in the GALT after systemic immunization reflects dysfunction of Ag-specific CD8 T cells. This dysfunction includes the “exhaustion” (manifest by increased PD-1 expression) and apoptosis of HIV-specific T cells, helping to explain why mucosal surfaces remain susceptible to ongoing HIV infection.

Acknowledgements

We thank Dr. Akio Yamashita for critical reading of the manuscript. This work was partially supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 26462531). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study. The authors have declared that no competing interests exist.

References

- [1]. Dandekar S Pathogenesis of HIV in the gastrointestinal tract. *Curr HIV/AIDS Rep* 2007;4:10–5. [PubMed: 17338855]
- [2]. Ivanov II, Diehl GE, Littman DR. Lymphoid tissue inducer cells in intestinal immunity. *Curr Top Microbiol Immunol* 2006;308:59–82. [PubMed: 16922086]
- [3]. Veazey RS, DeMaria M, Chalifoux LV, Shvetz DE, Pauley DR, Knight HL, et al. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 1998;280:427–31. [PubMed: 9545219]
- [4]. Guadalupe M, Reay E, Sankaran S, Prindiville T, Flamm J, McNeil A, et al. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* 2003;77:11708–17. [PubMed: 14557656]
- [5]. Li Q, Duan L, Estes JD, Ma ZM, Rourke T, Wang Y, et al. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* 2005;434:1148–52. [PubMed: 15793562]
- [6]. Mehandru S, Poles MA, Tenner-Racz K, Manuelli V, Jean-Pierre P, Lopez P, et al. Mechanisms of gastrointestinal CD4+ T-cell depletion during acute and early human immunodeficiency virus type 1 infection. *J Virol* 2007;81:599–612. [PubMed: 17065209]
- [7]. Letvin NL, Mascola JR, Sun Y, Gorgone DA, Buzby AP, Xu L, et al. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science* 2006;312:1530–3. [PubMed: 16763152]
- [8]. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 2005;434:1093–7. [PubMed: 15793563]
- [9]. Mattapallil JJ, Douek DC, Buckler-White A, Montefiori D, Letvin NL, Nabel GJ, et al. Vaccination preserves CD4 memory T cells during acute simian immunodeficiency virus challenge. *J Exp Med* 2006;203:1533–41. [PubMed: 16735692]

- [10]. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med* 2009;15:293–9. [PubMed: 19219024]
- [11]. Kaufman DR, Liu J, Carville A, Mansfield KG, Havenga MJ, Goudsmit J, et al. Trafficking of antigen-specific CD8+ T lymphocytes to mucosal surfaces following intramuscular vaccination. *J Immunol* 2008;181:4188–98. [PubMed: 18768876]
- [12]. McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, Janes H, et al. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* 2008;372:1894–905. [PubMed: 19012957]
- [13]. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006;439:682–7. [PubMed: 16382236]
- [14]. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. *Cell* 2009;138:30–50. [PubMed: 19596234]
- [15]. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* 2005;23:515–48. [PubMed: 15771580]
- [16]. Chen L Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol* 2004;4:336–47. [PubMed: 15122199]
- [17]. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 2007;27:670–84. [PubMed: 17950003]
- [18]. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 2009;10:29–37. [PubMed: 19043418]
- [19]. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* 2010;236:219–42. [PubMed: 20636820]
- [20]. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 2007;8:239–45. [PubMed: 17304234]
- [21]. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci U S A* 2001;98:13866–71. [PubMed: 11698646]
- [22]. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 2004;173:945–54. [PubMed: 15240681]
- [23]. Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett* 2004;574:37–41. [PubMed: 15358536]
- [24]. Xin KQ, Jounai N, Someya K, Honma K, Mizuguchi H, Naganawa S, et al. Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV. *Gene Ther* 2005;12:1769–77. [PubMed: 16079886]
- [25]. Wang HB, Kondo A, Yoshida A, Yoshizaki S, Abe S, Bao LL, et al. Partial protection against SIV challenge by vaccination of adenovirus and MVA vectors in rhesus monkeys. *Gene Ther* 2010;17:4–13. [PubMed: 19759567]
- [26]. Shimada M, Wang HB, Kondo A, Xu XP, Yoshida A, Shinoda K, et al. Effect of therapeutic immunization using Ad5/35 and MVA vectors on SIV infection of rhesus monkeys undergoing antiretroviral therapy. *Gene Ther* 2009;16:218–28. [PubMed: 18923453]
- [27]. Lieber A, He CY, Kirillova I, Kay MA. Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. *J Virol* 1996;70:8944–60. [PubMed: 8971024]

- [28]. Belyakov IM, Hammond SA, Ahlers JD, Glenn GM, Berzofsky JA. Transcutaneous immunization induces mucosal CTLs and protective immunity by migration of primed skin dendritic cells. *J Clin Invest* 2004;113:998–1007. [PubMed: 15057306]
- [29]. Jounai N, Okuda K, Kojima Y, Toda Y, Hamajima K, Ohba K, et al. Contribution of the rev gene to the immunogenicity of DNA vaccines targeting the envelope glycoprotein of HIV. *J Gene Med* 2003;5:609–17. [PubMed: 12825200]
- [30]. Oomura K, Xin KQ, Takakura M, Shinoda K, Jounai N, Okuda K. Oral administration of the adenovirus vector induces systemic immunity rather than intestinal mucosal immunity. *Vaccine* 2006;24:1045–6. [PubMed: 16203062]
- [31]. Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002;415:331–5. [PubMed: 11797011]
- [32]. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 2008;372:1881–93. [PubMed: 19012954]
- [33]. Xin KQ, Ohta S, Jounai N, Takeshita F, Okuda K. Comparison of antigen-specific cellular immune responses between individual PBMCs and splenocytes. *Vaccine* 2005;23:2954–5. [PubMed: 15811639]
- [34]. Blattman JN, Wherry EJ, Ha SJ, van der Most RG, Ahmed R. Impact of epitope escape on PD-1 expression and CD8 T-cell exhaustion during chronic infection. *J Virol* 2009;83:4386–94. [PubMed: 19211743]
- [35]. Peretz Y, He Z, Shi Y, Yassine-Diab B, Goulet JP, Bordi R, et al. CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. *PLoS Pathog* 2012;8:e1002840. [PubMed: 22916009]
- [36]. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006;443:350–4. [PubMed: 16921384]
- [37]. Marx PA, Chen Z. The function of simian chemokine receptors in the replication of SIV. *Semin Immunol* 1998;10:215–23. [PubMed: 9653048]
- [38]. Berger EA, Doms RW, Fenyo EM, Korber BT, Littman DR, Moore JP, et al. A new classification for HIV-1. *Nature* 1998;391:240. [PubMed: 9440686]
- [39]. Veazey RS, Mansfield KG, Tham IC, Carville AC, Shvetz DE, Forand AE, et al. Dynamics of CCR5 expression by CD4(+) T cells in lymphoid tissues during simian immunodeficiency virus infection. *J Virol* 2000;74:11001–7. [PubMed: 11069995]
- [40]. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 2004;200:749–59. [PubMed: 15365096]
- [41]. Offit PA, Cunningham SL, Dudzik KI. Memory and distribution of virus-specific cytotoxic T lymphocytes (CTLs) and CTL precursors after rotavirus infection. *J Virol* 1991;65:1318–24. [PubMed: 1847457]
- [42]. Koelle DM, Liu Z, McClurkan CM, Topp MS, Riddell SR, Pamer EG, et al. Expression of cutaneous lymphocyte-associated antigen by CD8(+) T cells specific for a skin-tropic virus. *J Clin Invest* 2002;110:537–48. [PubMed: 12189248]
- [43]. Bukawa H, Sekigawa K, Hamajima K, Fukushima J, Yamada Y, Kiyono H, et al. Neutralization of HIV-1 by secretory IgA induced by oral immunization with a new macromolecular multicomponent peptide vaccine candidate. *Nat Med* 1995;1:681–5. [PubMed: 7585151]
- [44]. Gallichan WS, Rosenthal KL. Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. *J Exp Med* 1996;184:1879–90. [PubMed: 8920875]
- [45]. Belyakov IM, Derby MA, Ahlers JD, Kelsall BL, Earl P, Moss B, et al. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. *Proc Natl Acad Sci U S A* 1998;95:1709–14. [PubMed: 9465081]

- [46]. Belyakov IM, Hel Z, Kelsall B, Kuznetsov VA, Ahlers JD, Nacsa J, et al. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. *Nat Med* 2001;7:1320–6. [PubMed: 11726972]
- [47]. Santosuosso M, Zhang X, McCormick S, Wang J, Hitt M, Xing Z. Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. *J Immunol* 2005;174:7986–94. [PubMed: 15944305]
- [48]. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 2001;291:2413–7. [PubMed: 11264538]
- [49]. Masopust D, Vezys V, Usherwood EJ, Cauley LS, Olson S, Marzo AL, et al. Activated primary and memory CD8 T cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. *J Immunol* 2004;172: 4875–82. [PubMed: 15067066]
- [50]. Brinkman CC, Rouhani SJ, Srinivasan N, Engelhard VH. Peripheral tissue homing receptors enable T cell entry into lymph nodes and affect the anatomical distribution of memory cells. *J Immunol* 2013;191:2412–25. [PubMed: 23926324]
- [51]. Warnock RA, Askari S, Butcher EC, von Andrian UH. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J Exp Med* 1998;187: 205–16. [PubMed: 9432978]
- [52]. Isakov D, Dzutsev A, Belyakov IM, Berzofsky JA. Non-equilibrium and differential function between intraepithelial and lamina propria virus-specific TCR α beta(+) CD8 α beta(+) T cells in the small intestinal mucosa. *Mucosal Immunol* 2009;2:450–61. [PubMed: 19571797]
- [53]. Belyakov IM, Isakov D, Zhu Q, Dzutsev A, Berzofsky JA. A novel functional CTL avidity/activity compartmentalization to the site of mucosal immunization contributes to protection of macaques against simian/human immunodeficiency viral depletion of mucosal CD4+ T cells. *J Immunol* 2007;178: 7211–21. [PubMed: 17513770]
- [54]. Kinter AL, Godbout EJ, McNally JP, Sereti I, Roby GA, O’Shea MA, et al. The common gamma-chain cytokines IL-2, IL-7 IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. *J Immunol* 2008;181:6738–46. [PubMed: 18981091]
- [55]. Zhang X, Schwartz JC, Guo X, Bhatia S, Cao E, Lorenz M, et al. Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity* 2004;20:337–47. [PubMed: 15030777]
- [56]. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;192:1027–34. [PubMed: 11015443]
- [57]. Shi F, Shi M, Zeng Z, Qi RZ, Liu ZW, Zhang JY, et al. PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. *Int J Cancer* 2011;128:887–96. [PubMed: 20473887]
- [58]. Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995;182:1545–56. [PubMed: 7595224]
- [59]. Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc* 2006;1:1458–61. [PubMed: 17406435]

Fig. 1. Induction of HIV-specific CD8 T cells by intramuscular immunization with the Ad-HIV vaccine. Cells were prepared 1 week after vaccination from the PBMCs, spleen, liver, mesenteric lymph node (MLN), and Peyer's patches (PP) of vaccinated mice. (a) HIV-specific CD8 T cells and (b) HIV-specific memory CD8 T cells were detected by flow cytometry, using a HIV-specific tetramer. The assay was performed 1 week after immunization. The mean $\pm$ SE of five mice is shown.

Fig. 2.

Up-regulation of PD-1 in PBMCs after vaccination with Ad-HIV. (a) PD-1-expressing HIV-specific CD8 T cells were detected in PBMCs from immunized mice on days 0, 7, and 28 after vaccination using flow cytometry. (b) The frequency of HIV-specific CD8 T cells (top panel) and of HIV-specific CD8 T cells expressing PD-1 (middle panel), and mean fluorescence intensity (MFI) of PD-1 expression by HIV-specific CD8 T cells in PBMCs isolated from vaccinated mice at the indicated time points. The mean $\pm$ SE of 10 mice is shown.

Fig. 3.

Up-regulation of PD-1 expression by HIV-specific CD8 T cells. Cells were prepared from PBMC, spleen, liver, MLN, and PP of immunized mice 1 week after vaccination. Values indicate the percentage of CD8⁺ T cells that express PD-1 as determined by FACS. (a) Total CD8 T cells, (b) HIV-specific CD8 T cells, and (c) HIV-specific CD8 T cells with the memory phenotype expressing PD-1. The mean $\pm$ SE of 10 mice is shown.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4.

Ad-HIV vaccination enhances the apoptosis of CD8 T cells. Mice were immunized as described in Fig. 1. Cells from various organs were isolated 1 week later, and stained with an HIV-specific tetramer plus anti-CD8 Ab. Apoptosis was evaluated by staining cells with annexin-V and PI. The mean $\pm$ SE of five mice is shown.