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Intramuscular rather than oral administration of replication-defective adenoviral vaccine vector induces specific CD8⁺ T-cell responses in the gut

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Abstract

Gut-associated lymphoid tissue (GALT) is the primary replication site for HIV-1, resulting in a pronounced CD4⁺ T cell loss in this tissue during primary infection. A mucosal vaccine that generates HIV-specific CD8⁺ T cells in the gut could prevent the establishment of founder populations and broadcasting of virus. Here, we immunized mice orally and systemically with a chimpanzee derived adenoviral vector expressing HIV *gag* (AdC68gag) and measured frequencies of *gag*-specific interferon-gamma (IFN- γ) producing CD8⁺ T cells in the GALT. A single oral administration was inefficient at eliciting responses in the mesenteric lymph nodes and Peyer's Patches, while a single intramuscular administration elicited strong systemic and detectable mucosal responses. The *gag*-specific CD8⁺ T cell responses were present in both acute and memory phases following intramuscular administration.

Keywords

adenovirus; mucosal; gut; oral; intramuscular

Introduction

Following the rapid acute phase of infection with HIV-1, the virus causes persistent, systemic CD4⁺ T cell depletion[1]. Ideal conditions for explosive viral production and marked CD4⁺ T cell depletion exist in the largest lymphoid organ, the gut-associated lymphoid tissue (GALT). From there, the virus and infected cells are disseminated to draining lymph nodes and other lymphoid tissues through the circulation. Although immune responses are activated, they are delayed and of insufficient magnitude or quality to successfully eradicate the infection[2]. Vaccines that elicit strong and durable host immune responses in the GALT may prevent the broadcasting of virus from the gut.

Oral vaccines can induce both systemic and mucosal immunity. Some evidence suggests that the route of immunization influences the trafficking of T cells[3,4], and some routes including mucosal administration of viral vectors have been shown to be efficient at inducing mucosal immune responses which may sometimes be protective[3,5–8]. Mucosal T cells receive instructions to migrate to the intestines during priming by dendritic cells in the mesenteric

lymph nodes (MLN) and other inductive sites[9,10]. Thus, eliciting CD8⁺ T cell responses in the gut may require antigen encounter in the intestinal immune system[11]. This study aimed to test virus-specific CD8⁺ T cell responses in GALT elicited by oral and intramuscular vaccine regimens based on replication-defective adenoviruses.

In order to circumvent pre-existing immunity in many human populations to vectors derived from common human serotype adenoviruses, new vectors have been derived from chimpanzee adenoviruses[12]. Chimpanzee adenoviral vectors have been shown to elicit strong CD8⁺ T cell responses to transgene products[13]; however, it is unknown whether oral vaccination with these vectors induces mucosal CD8⁺ T cells including in animals with pre-existing immunity to a common human adenovirus serotype. Here we tested the intestinal mucosal responses to oral and intramuscular (i.m.) administration of an E1-deleted adenoviral vector of chimpanzee serotype C68 (also called C9)[12]. The vector was designed to carry the HIV-1 *gag* transgene. We also tested oral-prime, i.m.-boost strategies to induce HIV-*gag*-specific CD8⁺ T cell responses in the mesenteric lymph nodes (MLN) and Peyer's Patches (PP).

Materials and methods

Mice

Female 4- to 6-week-old BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Ace Animals, Inc (Boyertown, PA) and housed at the Animal Facility of The Wistar Institute (Philadelphia, PA). All experiments were performed according to institutionally approved protocols.

Vectors

The E1-deleted adenovirus recombinant human serotype 5 (AdHu5) expressing the rabies glycoprotein, AdHu5rab.gp, has been previously described [14]. Mice were immunized i.m. with 1×10^8 pfu AdHu5rab.gp to generate neutralizing titers to AdHu5. The AdHu5 and AdC68 recombinant viruses carrying the *gag* gene of HIV-1 clade B were obtained from the University of Pennsylvania Vector Core Facility (Philadelphia, PA). Doses of these vectors are noted in the text below. Vaccine vectors were diluted in sterile saline to a total volume of 100ul, and mice were immunized with a feeding tube or immunized by injection into the lower leg muscle.

Isolation of lymphocytes

Lymphocytes were isolated as previously described [15]. Briefly, blood was collected in heparin and red blood cells were lysed with ACK Lysing Buffer (Invitrogen, Carlsbad, CA). Spleens and popliteal lymph nodes were dissociated against metal screens and washed with Leibovitch's L-15 Mod. (Mediatech, Inc., Herndon, VA). Peritoneal lavage was performed with 1X Phosphate Buffered Saline (PBS) (Mediatech, Inc., Herndon, VA) and cells were washed with L-15. MLN and PP were dissociated against metal screens and washed with 10% FBS in 1X Hank's Balanced Salt Solution (HBSS) (Mediatech, Inc., Herndon, VA). Intraepithelial lymphocytes (IELs) were isolated from small intestines by first removing PP, and the intestines were cut longitudinally and then into 1-cm pieces. They were incubated in a HBSS/HEPES (Invitrogen, Carlsbad, CA) bicarbonate buffer containing 10% fetal bovine serum (FBS) (Gemini BioProducts, West Sacramento, CA) for 15 minutes with slow stirring, 4 times. Final preparations of IELs were purified on a 40%–70% Percoll (Sigma, St. Louis, MO) gradient (2000rpm at 10°C for 20 minutes).

Flow cytometry

To examine *gag*-specific interferon-gamma- (IFN- γ)-secreting CD8⁺ T-cell frequencies[16], lymphocytes were incubated (1×10^6 cells/sample) with a BALB/c-specific AMQMLKETI

peptide for 5 hours at 37°C with 5% CO₂. Control cells were stimulated with an irrelevant peptide, and during analysis background controls were subtracted from sample values before plotting. Cells were surface stained with anti-CD8 conjugated to Fluorescein Isothiocyanate (FITC), then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Jose, CA) for intracellular cytokine staining with anti-IFN- γ conjugated to Phycoerytherin (PE). Tetramer analysis was done[16] with Allophycocyanin (APC) conjugated *gag* AMQMLKETI BALB/c specific H-2K^d MHC class I restricted tetramers (NIAID Tetramer Facility, Atlanta, GA). Flow cytometry analysis of the cells was performed with the Beckman-Coulter XL (Beckman-Coulter, Fullerton, CA) and FACSCalibur (Becton-Dickinson, San Jose, CA) flow cytometers at The Wistar Institute Flow Cytometry Core Facility (Philadelphia, PA); data was analyzed with WinMDI 2.8 (Howard Scripps Institute, La Jolla, CA) and FlowJo 7.1.1 (Tree Star, Inc., Ashland, OR). Statistical analysis to calculate p-values was performed with Intercooled Stata 8.2 (StataCorp LP, College Station, TX). All antibodies were purchased from BD Pharmingen (San Jose, CA) unless otherwise noted.

ELISpot

IFN- γ capture enzyme-linked immunospot (ELISpot) was performed with lymphocytes as previously described[16,17]. Briefly, 96-well Millipore polyvinylidene difluoride (PVDF) plates (Millipore, Billerica, MA) were coated with mouse anti-IFN- γ capture antibody (BD Pharmingen, San Jose, CA) diluted in PBS and incubated overnight at 4°C. After washing with PBS, plates were blocked with complete RPMI-1640 (Mediatech, Inc., Herndon, VA) with 10% FBS for 2 hours at 37°C. Lymphocytes were added in triplicate and stimulated with the BALB/c specific 9 mer peptide AMQMLKETI and co-stimulatory molecules mouse anti-CD28 and mouse anti-CD49d (BD Pharmingen, San Jose, CA) for 18–20 hours at 37°C with 5% CO₂. Cells were removed and plates were washed with 0.01% Tween (Sigma, St. Louis, MO) in PBS and incubated with biotin-labeled secondary antibody (BD Pharmingen, San Jose, CA) in 5%FBS in 0.01% Tween/PBS for 2 hours at room temperature. Plates were washed and streptavidin alkaline phosphatase (MATBECH AB, Cincinnati, OH) was added at room temperature for 1 hour, and the spots were developed by adding BCIP/NBT developer (Pierce, Rockford, IL) to each well for 5 minutes at room temperature. Plates were washed in water and dried before counting the spots using the C.T.L. Series 3A Analyzer and ImmunoSpot 3.2 (Cellular Technology Ltd, Cleveland, OH). Data from unstimulated cells was used as background control and values were subtracted from sample values before plotting.

Results

We tested whether AdC68 could be used as an oral vaccine to induce a mucosal response, particularly in the gut. However, since many human populations have neutralizing antibody titers to AdHu5, we first determined whether using AdC68 orally could overcome this pre-existing immunity. Groups of 5 mice were immunized i.m. with 1x10⁸pfu AdHu5rab.gp to induce neutralizing antibody titers to AdHu5 of 1/160 to 1/320 at three weeks post exposure. These and naïve mice were then orally immunized with AdC68gag or AdHu5gag at the dose of 5x10¹⁰vp. Mice were sacrificed 10 days after oral immunization, and spleen lymphocytes were harvested for intracellular cytokine staining. While frequencies of gag-specific IFN- γ -producing CD8⁺ T cells in mice orally immunized with AdC68gag in the pre-exposed and naïve mice were not significantly different (p=0.55), frequencies in mice orally immunized with AdHu5gag in the pre-exposed mice were significantly reduced (p=0.04) compared to the naïve mice (Figure 1 shows representative data from one of three repeated experiments).

Although the chimpanzee-origin adenovirus vector AdC68 used as an oral vaccine in mice was able to overcome neutralizing antibodies to AdHu5, *gag*-specific IFN- γ -producing CD8⁺ T cell frequencies were low (0.1–0.5% at day 10) in the spleens of these mice. Furthermore,

frequencies were undetectable in the MLN and PP after a single oral dose of 5×10^{10} vp AdC68HIVgag. Therefore, we tested different approaches: a single i.m. administration and oral-prime, i.m.-boost. In contrast to single oral immunization, a single i.m. immunization with the same dose of virus elicited high frequencies in the spleen and blood and detectable frequencies in the MLN and PP (Figure 2) at day 10 and month 3 after immunization.

Considering that gag-specific CD8⁺ T cells could be found in both systemic and mucosal compartments after i.m. immunization, we then tested a heterologous oral-prime, i.m.-boost strategy to determine if frequencies could be increased. Groups of 5 mice were orally primed with 5×10^{10} vp AdC68gag and after 2 months, boosted i.m. with 1×10^9 vp AdHu5gag (Figure 3A). At day 10 and month 2 after the boost, mice were sacrificed and lymphocytes from spleen, MLN, and PP were harvested for IFN- γ capture ELISpot analysis (Figure 3B). Although responses in each compartment after the boost improved over the single oral dose, an oral prime does not subsequently increase the response after the i.m. boost when compared to a single i.m. immunization. To show that gag-specific memory CD8⁺ T cells were present in the spleen and MLN, cells were harvested at month 2 after the boost and stained with a gag tetramer (Figure 3C). Paralleling the ELISpot data, responses in both compartments after the boost improved over the single oral dose frequencies, but the response was did not increase beyond the single i.m. dose.

Sustained CD8⁺ T cell frequencies in the gut elicited by a vaccine may be paramount for preventing CD4⁺ T cell loss in HIV-1 infection. Since our data indicates that a single i.m. immunization elicited responses in the gut, we tested whether gag-specific IFN- γ -producing CD8⁺ T cell frequencies could be detected for prolonged times in mucosal tissues after i.m. immunization. Mice were immunized i.m. with a high dose of 5×10^{11} vp AdC68gag for these experiments. Animals were analyzed 10 days later to determine peak frequencies during the effector phase and a separate group 18 months later to assess late memory T cell responses. Lymphocytes were isolated from the spleen, blood, peritoneal cavity (PerLav), popliteal lymph nodes (LN), MLN, and PP for intracellular cytokine staining. Results (Figure 4) show that a high i.m. dose of the chimpanzee-derived adenoviral vector preferentially induced a systemic response early after immunization although some vaccine-induced CD8⁺ T cells could be detected in the PP. After 18 months frequencies in most compartments such as spleen, blood and peritoneal lavage declined while frequencies in lymph nodes including MLN increased. Frequencies in PP remained stable. These data indicate that i.m. vaccination not only elicits a systemic CD8⁺ T cell response but also strong responses in gut mucosal tissues. Therefore, further vaccine regimens focusing on responses in the gut may consider incorporation of these vectors in i.m. administration rather than oral administration.

Discussion

Previous studies using replication defective adenovirus as an oral vaccine have elicited strong transgene product-specific antibodies in serum and, in particular, mucosal secretions such as vaginal wash or saliva [4,18,19], even in the face of pre-existing immunity to the virus[20]. In our study, oral administration of AdC68gag can overcome pre-existing immunity to AdHu5, and it elicited a low gag-specific CD8⁺ T cell response in the spleen and blood at early time points but not in the GALT. While some oral vaccine vectors have shown efficacy in eliciting CD8⁺ in both systemic and regional immunity[21], similar adenoviral vectors tested orally in mice have also shown better systemic rather than intestinal mucosal immune responses[22].

Since HIV-1 depletes the gut reservoir of CD4⁺ T cells during primary infection[23] and recovery of CD4⁺ T cells in the gut is associated with long-term non-progressing cases[24], an effective vaccine that induces a response in the gut could eliminate infected cells and reduce viral spread[2]. The oral mode of delivery has the potential to confer long-term mucosal

immunity against pathogens such as HIV-1 if it can induce antigen specific cells to home to the gut as shown in previous studies [25–28], but efforts to stimulate immunity through this route may inadvertently induce oral tolerance instead[29]. Oral vaccine development using replication defective adenovirus, such as the vector we have tested, may be impeded by vector instability in the acidic environment of the stomach, through which it must pass to ensure antigen presentation and maximum exposure to and uptake by the antigen presenting cells of the intestine. Genomic DNA from adenovirus vector administered orally to mice is found in very limited amounts in the stomach, small intestines, and PP at the early time point of Day 4 (as compared to higher amounts found in the oral cavity) and is nearly not detectable after Day 10[19].

In our study, the adenovirus vaccine given orally or as an oral prime to an i.m. booster immunization protocol failed to induce significant CD8⁺ T cell responses in GALT. Instead a single intramuscular administration of a high dose of AdC68gag induces strong systemic and detectable mucosal responses during both the acute and the memory phases following vaccination.

Adenoviral vectors of human and, more recently, chimpanzee serotypes have been described as attractive vaccine vectors as they induce both innate and adaptive responses[30,31], and here we demonstrate that they can also induce responses in the lymphoid tissues of the intestinal mucosa. This implies that imprinting by mucosal dendritic cells is not an absolute requirement for primed T cells to migrate to the gut[32,33]. In fact, dendritic cells from the spleen or lymph node which do not induce gut-homing receptors can still prime T cells that migrate to the intestine and other non-lymphoid tissues in some experimental systems [34].

An alternative explanation for the induction of antigen-specific CD8⁺ T cells in the gut mucosa following i.m. immunization is that although anatomical sources of the antigen-presenting cells may be important for inducing site-specific CD8⁺ T cells [35], virus antigen may still be carried away and presented within the GALT and thus generate antigen-experienced CD8⁺ T cells that localize to the gut mucosa[33,36,37]. In fact, it is interesting that infection with a strictly respiratory virus such as the Sendai virus that infects locally can still generate antigen-specific CD8⁺ T cells in the gut mucosa or other non-lymphoid tissues [33]. In addition, it has been shown that the imprinting mechanism for gut-homing T cells is related to retinoic acid synthesis in dendritic cells [38], and it is possible that gut-homing T cells can be imprinted in regions other than the GALT [36,39–41]. Thus, an i.m. administration of our adenovirus vector could induce an inflammatory condition, causing retinoic acid synthesis in non-intestinal dendritic cells to initiate gut-specific imprinting on the T cells that they activate, or perhaps, there are other, non-retinoic acid-dependent intestinal imprinting mechanisms [42].

In conclusion, oral immunization with the adenoviral vectors is thus far suboptimal for stimulating the potent transgene product-specific CD8⁺ T cell responses elicited by systemic administration. Future studies may focus on using adenoviral vectors with alternate mucosal delivery routes such as intranasal, vaginal, and rectal immunization[43] or further characterization of the mucosal response upon systemic administration, particularly in the effector compartments of the lamina propria [44] or the intestinal epithelium. There have been heterologous prime-boost studies done with chimpanzee-derived adenoviral vectors[45] as well as other viruses and vectors[46] to drive an increase of effector memory CD8⁺ T cell population in the gut. Heterologous prime-boosting through the i.m. route has the potential to facilitate both an increase in systemic as well as mucosal immunity. Further studies will focus on whether repeated activation of T cells may initiate a differentiation pathway allowing for increased trafficking of systemically activated CD8⁺ T cells to mucosal sites.

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References

1. Veazey RS, Lackner AA. HIV swiftly guts the immune system. *Nat Med* 2005;11(5):469–70. [PubMed: 15875046]
2. Haase AT. Perils at mucosal front lines for HIV and SIV and their hosts. *Nat Rev Immunol* 2005;5(10):783–92. [PubMed: 16200081]
3. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med* 2005;11(4 Suppl):S45–53. [PubMed: 15812489]
4. Sharpe S, Fooks A, Lee J, Hayes K, Clegg C, Cranage M. Single oral immunization with replication deficient recombinant adenovirus elicits long-lived transgene-specific cellular and humoral immune responses. *Virology* 2002;293(2):210–6. [PubMed: 11886240]
5. Stevceva L, Strober W. Mucosal HIV vaccines: where are we now? *Curr HIV Res* 2004;2(1):1–10. [PubMed: 15053336]
6. Wang Y, Xiang Z, Pasquini S, Ertl HC. The use of an E1-deleted, replication-defective adenovirus recombinant expressing the rabies virus glycoprotein for early vaccination of mice against rabies virus. *J Virol* 1997;71(5):3677–83. [PubMed: 9094641]
7. de Souza AP, Haut LH, Silva R, Ferreira SI, Zanetti CR, Ertl HC, et al. Genital CD8(+) T cell response to HIV-1 gag in mice immunized by mucosal routes with a recombinant simian adenovirus. *Vaccine*. 2006
8. Xiang ZQ, Yang Y, Wilson JM, Ertl HC. A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* 1996;219(1):220–7. [PubMed: 8623532]
9. Cheroute H, Kronenberg M. Mucosal T lymphocytes-peacekeepers and warriors. *Springer Semin Immunopathol* 2005;27(2):147–65. [PubMed: 15931528]
10. Macpherson AJ. IgA adaptation to the presence of commensal bacteria in the intestine. *Curr Top Microbiol Immunol* 2006;308:117–36. [PubMed: 16922088]
11. Kantele A, Arvilommi H, Iikkanen K, Savilahti E, Makela HP, Herzog C, et al. Unique characteristics of the intestinal immune system as an inductive site after antigen reencounter. *J Infect Dis* 2005;191(2):312–7. [PubMed: 15609243]
12. Farina SF, Gao GP, Xiang ZQ, Rux JJ, Burnett RM, Alvira MR, et al. Replication-defective vector based on a chimpanzee adenovirus. *J Virol* 2001;75(23):11603–13. [PubMed: 11689642]
13. Fitzgerald JC, Gao GP, Reyes-Sandoval A, Pavlakis GN, Xiang ZQ, Wlazlo AP, et al. A simian replication-defective adenoviral recombinant vaccine to HIV-1 gag. *J Immunol* 2003;170(3):1416–22. [PubMed: 12538702]
14. Wiktor TJ, Dietzschold B, Leamnson RN, Koprowski H. Induction and biological properties of defective interfering particles of rabies virus. *J Virol* 1977;21(2):626–35. [PubMed: 833940]
15. Pinto AR, Fitzgerald JC, Gao GP, Wilson JM, Ertl HC. Induction of CD8+ T cells to an HIV-1 antigen upon oral immunization of mice with a simian E1-deleted adenoviral vector. *Vaccine* 2004;22(5–6):697–703. [PubMed: 14741162]
16. Sun Y, Iglesias E, Samri A, Kamkamidze G, Decoville T, Carcelain G, et al. A systematic comparison of methods to measure HIV-1 specific CD8 T cells. *J Immunol Methods* 2003;272(1–2):23–34. [PubMed: 12505709]
17. Ranasinghe C, Medveczky JC, Woltring D, Gao K, Thomson S, Coupar BE, et al. Evaluation of fowlpox-vaccinia virus prime-boost vaccine strategies for high-level mucosal and systemic immunity against HIV-1. *Vaccine* 2006;24(31–32):5881–95. [PubMed: 16759767]
18. Xiang Z, Li Y, Gao G, Wilson JM, Ertl HC. Mucosally delivered E1-deleted adenoviral vaccine carriers induce transgene product-specific antibody responses in neonatal mice. *J Immunol* 2003;171(8):4287–93. [PubMed: 14530353]

19. Zhou D, Cun A, Li Y, Xiang Z, Ertl HC. A Chimpanzee-Origin Adenovirus Vector Expressing the Rabies Virus Glycoprotein as an Oral Vaccine against Inhalation Infection with Rabies Virus. *Mol Ther*. 2006
20. Xiang ZQ, Gao GP, Reyes-Sandoval A, Li Y, Wilson JM, Ertl HC. Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier. *J Virol* 2003;77(20):10780–9. [PubMed: 14512528]
21. Xin KQ, Ooki T, Mizukami H, Hamajima K, Okudela K, Hashimoto K, et al. Oral administration of recombinant adeno-associated virus elicits human immunodeficiency virus-specific immune responses. *Hum Gene Ther* 2002;13(13):1571–81. [PubMed: 12228012]
22. 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(8):1045–6. [PubMed: 16203062]
23. Johnson RP. HIV pathogenesis and vaccine development. *Top HIV Med* 2005;13(1):9–15. [PubMed: 15849368]
24. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 2006;107(12):4781–9. [PubMed: 16467198]
25. Lundin BS, Johansson C, Svennerholm AM. Oral immunization with a *Salmonella enterica* serovar typhi vaccine induces specific circulating mucosa-homing CD4(+) and CD8(+) T cells in humans. *Infect Immun* 2002;70(10):5622–7. [PubMed: 12228290]
26. Kantele A, Zivny J, Hakkinen M, Elson CO, Mestecky J. Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans. *J Immunol* 1999;162(9):5173–7. [PubMed: 10227989]
27. Rojas OL, Gonzalez AM, Gonzalez R, Perez-Schael I, Greenberg HB, Franco MA, et al. Human rotavirus specific T cells: quantification by ELISPOT and expression of homing receptors on CD4+ T cells. *Virology* 2003;314(2):671–9. [PubMed: 14554094]
28. Rott LS, Rose JR, Bass D, Williams MB, Greenberg HB, Butcher EC. Expression of mucosal homing receptor alpha4beta7 by circulating CD4+ cells with memory for intestinal rotavirus. *J Clin Invest* 1997;100(5):1204–8. [PubMed: 9276738]
29. Shalaby WS. Development of oral vaccines to stimulate mucosal and systemic immunity: barriers and novel strategies. *Clin Immunol Immunopathol* 1995;74(2):127–34. [PubMed: 7828366]
30. Tatsis N, Ertl HC. Adenoviruses as vaccine vectors. *Mol Ther* 2004;10(4):616–29. [PubMed: 15451446]
31. Tatsis N, Tesema L, Robinson ER, Giles-Davis W, McCoy K, Gao GP, et al. Chimpanzee-origin adenovirus vectors as vaccine carriers. *Gene Ther* 2006;13(5):421–9. [PubMed: 16319951]
32. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 2001;291(5512):2413–7. [PubMed: 11264538]
33. 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(8):4875–82. [PubMed: 15067066]
34. Cheroutre H, Madakamutil L. Mucosal effector memory T cells: the other side of the coin. *Cell Mol Life Sci* 2005;62(23):2853–66. [PubMed: 16314932]
35. Calzascia T, Masson F, Di Bernardino-Besson W, Contassot E, Wilmotte R, Aurrand-Lions M, et al. Homing phenotypes of tumor-specific CD8 T cells are predetermined at the tumor site by crosspresenting APCs. *Immunity* 2005;22(2):175–84. [PubMed: 15723806]
36. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Roseblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 2003;424(6944):88–93. [PubMed: 12840763]
37. Westermann J, Bode U. Distribution of activated T cells migrating through the body: a matter of life and death. *Immunol Today* 1999;20(7):302–6. [PubMed: 10379047]
38. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 2004;21(4):527–38. [PubMed: 15485630]

39. Campbell DJ, Butcher EC. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med* 2002;195(1):135–41. [PubMed: 11781372]
40. Johansson-Lindbom B, Svensson M, Wurbel MA, Malissen B, Marquez G, Agace W. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. *J Exp Med* 2003;198(6):963–9. [PubMed: 12963696]
41. Svensson M, Marsal J, Ericsson A, Carramolino L, Broden T, Marquez G, et al. CCL25 mediates the localization of recently activated CD8alphabeta(+) lymphocytes to the small-intestinal mucosa. *J Clin Invest* 2002;110(8):1113–21. [PubMed: 12393847]
42. Mora JR, von Andrian UH. Retinoic acid: an educational "vitamin elixir" for gut-seeking T cells. *Immunity* 2004;21(4):458–60. [PubMed: 15485623]
43. 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(12):1320–6. [PubMed: 11726972]
44. Cheroutre H, Madakamutil L. Acquired and natural memory T cells join forces at the mucosal front line. *Nat Rev Immunol* 2004;4(4):290–300. [PubMed: 15057787]
45. Pinto AR, Fitzgerald JC, Giles-Davis W, Gao GP, Wilson JM, Ertl HC. Induction of CD8+ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol* 2003;171(12):6774–9. [PubMed: 14662882]
46. Masopust D, Ha SJ, Vezys V, Ahmed R. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J Immunol* 2006;177(2):831–9. [PubMed: 16818737]

Figure 1. Pre-existing immunity to AdH5 does not inhibit T-cell responses when using AdC68gag orally

BALB/c mice were immunized with 1×10^8 pfu AdHu5rab.gp to induce neutralizing antibodies to AdHu5. Then they were immunized orally with 5×10^{10} vp of either AdHu5gag or AdC68gag. Ten days later, splenocytes were collected and stimulated with a CD8⁺-specific *gag* epitope and frequencies of IFN- γ ⁺ cells were determined by intracellular cytokine staining. Frequencies shown are averages calculated from individual mice minus background (between 0.01–0.2%), and error bars shown here represent standard deviations.

Figure 2. Single oral administration of AdC68gag is inefficient at eliciting responses in the mesenteric lymph nodes and Peyer's Patches, while single intramuscular administration elicits strong systemic and weak mucosal responses

BALB/c mice were immunized orally and intramuscularly with 5×10^{10} vp AdC68gag, and lymphocytes from tissues were isolated 10 days and 3 months later and stimulated with a CD8⁺-specific *gag* epitope and frequencies of IFN- γ ⁺ cells were determined by intracellular cytokine staining. Lymphocytes from spleens were analyzed from individual mice, while blood, MLN, and PP were pooled from groups of 5 mice for this assay. Frequencies shown are averages (spleens) or values (blood, MLN, PP) with background frequencies (between 0.1–0.5%) subtracted, and error bars shown here represent standard deviations.

Figure 3. Priming orally does not raise gag-specific CD8⁺ T cell frequencies with i.m. boost
 Groups of 5 BALB/c mice were orally immunized with 5×10^{10} vp AdC68gag and i.m. immunized 2 months later with 1×10^9 vp AdHu5gag, as shown in the timeline (A). Lymphocytes from spleen, MLN, and PP were isolated from sacrificed animals at day 10 and month 2 after the boost, and were stimulated for gag-specific IFN- γ ELISpot (B). Data for spleen represent mean + SD of five individual mice, whereas data for MLN and PP represent mean + SD of pooled cells in triplicate wells. Unstimulated cells from each sample were used as background control, and the numbers of background spots, which were similar for each tissue (<100 SFU/1E6), was subtracted from each sample before plotting the data. (C) Spleen and MLN cells were harvested at the Month 2 timepoint post boost and stained with a gag

tetramer. Percentage of tetramer⁺ CD8⁺ cells are shown in the upper right corner of each dot plot. The plots for spleen are representative samples from individual mice, while the plots for MLN show pooled lymphocytes from 5 mice per group.

Figure 4. A high intramuscular dose of AdC68gag maintains specific CD8⁺ T cells in central and mucosal compartments during the late memory phase

BALB/c mice were immunized intramuscularly with 5×10^{11} vp AdC68gag. Lymphocytes from spleen, blood, popliteal lymph nodes (LN), peritoneal lavage (PerLav), MLN, and PP and were isolated after day 10 and month 18 and stimulated with a CD8⁺-specific *gag* epitope and frequencies of IFN- γ ⁺ cells were determined by intracellular cytokine staining. Background levels (between 0.1–0.8%) were subtracted from each sample before plotting the frequencies here. Spleens were analyzed from individual mice, while the other tissues were pooled from 5 mice per group.