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

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The presence of high-avidity CTLs in the right compartment can greatly affect clearance of a virus infection (for example, AIDS viral infection of and dissemination from mucosa). Comparing mucosal vs systemic immunization, we observed a novel compartmentalization of CTL avidity and proportion of functionally active Ag-specific CD8$^+$ T cells to tissues proximal to sites of immunization. Whereas both s.c. and intrarectal routes of immunization induced tetramer$^+$ cells in the spleen and gut, the mucosal vaccine induced a higher percentage of functioning IFN-γ^+ Ag-specific CD8$^+$ T cells in the gut mucosa in mice. Translating to the CD8$^+$ CTL avidity distribution in rhesus macaques, intrarectal vaccination induced more high-avidity mucosal CTL than s.c. vaccination and protection of mucosal CD4$^+$ T cells from AIDS viral depletion, whereas systemic immunization induced higher avidity IFN-γ-secreting cells in the draining lymph nodes but no protection of mucosal CD4$^+$ T cells, after mucosal challenge with pathogenic simian/human immunodeficiency virus. Mucosal CD4$^+$ T cell loss is an early critical step in AIDS pathogenesis. The preservation of CD4$^+$ T cells in colonic lamina propria and the reduction of virus in the intestine correlated better with high-avidity mucosal CTL induced by the mucosal AIDS vaccine. This preferential localization of high-avidity CTL may explain previous differences in vaccination results and may guide future vaccination strategy. *The Journal of Immunology,* **2007, 178: 7211–7221.**

Since the introduction of the first vaccines by Edward Jenner and Louis Pasteur, a controversy on the appropriate route of immunization for optimal vaccine efficacy has been one of the major issues of vaccine development. This question was addressed many times, but never was resolved (1, 2). However, multiple studies in vaccine development against respiratory (3, 4), gastrointestinal (5), polio (6), AIDS (7–9), and other infectious diseases predicted that mucosal vaccines may be even more effective than systemic vaccines against those infections. AIDS epidemics continue to spread worldwide and over 40 million people are infected with HIV (10). The gastrointestinal and vaginal mucosa are the major routes of natural HIV transmission and the gut mucosa is a major reservoir for HIV and SIV replication (11–16). Several groups (12–14, 17) have shown recently that intestinal CD4$^+$ T cells are selectively and rapidly depleted in the intestine of HIV-infected patients. They found that depletion of intestinal CD4$^+$ T cells occurs at all stages of infection regardless of highly active antiretroviral therapy (18). Other studies demonstrated that memory CD4$^+$ T cells are rapidly depleted from all systemic and mucosal tissues, but because the majority of memory CD4$^+$ are located in the intestinal mucosa, this results in massive CD4$^+$ T cell depletion in the gut (19–21). The majority of memory CD4$^+$ T cells coexpress CCR5, the major coreceptor expressed by mucosally transmitted strains of HIV and SIV (20–23). The study by Mattapallil (21) has shown that 30–60% of CD4$^+$ memory T cells throughout the body are infected by SIV at the peak of infection and the majority of these cells disappear within 4 days. Also, a recent study by Mattapallil et al. (24) showed that systemic vaccination reduces the massive destruction of CD4$^+$ memory T cells across all the tissue compartments during SIV Mac251 infection, leading to better survival and long-term outcome. However, the efficacy of mucosal vs systemic immunizations for prevention of CD4$^+$ memory T cell depletion is unknown. These studies opened a new round of discussion in the press on their implications for vaccine development. They particularly support the case for developing a vaccine that especially protects the gut. Thus, a mucosal immunity component (both CD8$^+$ CTL and mucosal Ab) is needed for a successful AIDS vaccine (10, 25–32). Indeed, our previous studies (7, 8) and studies of other groups (9, 33–36) demonstrated a strong rationale for development of a mucosal AIDS vaccine to generate sufficient mucosal immune responses (both mucosal Ab and CD8$^+$ CTL) to keep the virus under control locally in the gut mucosa and in the systemic circulation as well. We showed that in mice, a mucosal vaccine can be more effective than the same vaccine given s.c. and this protective effect is mediated by mucosal CD8$^+$ CTL (37–39); we also showed that in intrarectally (IR)[2] immunized macaques, the viral titers were eliminated more completely both in blood and intestine than in s.c.-immunized animals (7). Recently, we found that a mucosal prime-boost vaccine induced a high level of high-avidity mucosal CTL and

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[2] Abbreviations used in this paper: IR, intrarectal; SHIV, simian/human immunodeficiency virus; GALT, gut-associated lymphoid tissue; DC, dendritic cell; LP, lamina propria; MVA, modified vaccinia Ankara; NASBA, nucleic acid sequence-based amplification; IEL, intraepithelial lymphocyte; LPL, LP lymphocyte; MLN, mesenteric lymph node; ALN, axillary lymph node; LT, labile toxin.

reduced viral dissemination from the initial mucosal site of infection to the systemic circulation (8). This delay of AIDS viral dissemination from mucosa correlated better with the level of high-avidity mucosal CTL than with systemic CTLs (8).

Recent studies demonstrated that some Ag-specific CD8$^+$ CTL and partial protection against mucosal viral transmission after challenge with pathogenic simian/human immunodeficiency virus (SHIV) can be induced after systemic vaccination (40–44). However, the "major phenomenon" of mucosal vaccination is that mucosal HIV vaccines more effectively induce local mucosal immune responses in genital and gastrointestinal mucosa and more effective clearance of virus from main site of replication in the intestinal mucosa (7, 8). The mechanism of effective antiviral protection based on the distribution of functionally active CD8$^+$ CTL after mucosal vaccination was not previously studied. In our current study, we now establish proof of principle that a mucosal vaccine can protect macaques against massive AIDS viral depletion of mucosal CD4$^+$ T cells, whereas a systemic vaccine was not effective in this regard. In the current study, we re-examined this question in murine and rhesus macaque models, and found that indeed systemic immunization did induce tetramer-binding cells in the mucosa, but the functional activity (lytic activity and IFN-γ production) and CTL avidity correlated with proximity to the site of immunization. Subcutaneous immunization induced a higher CTL avidity in the regional lymph nodes, whereas the IR immunization generated a higher percent of a functionally active CD8$^+$ T cells and higher avidity CTL in the gut-associated lymphoid tissue (GALT). Our data suggested that this correlation with proximity to the site of immunization relates to differential dendritic cell (DC) activation at the proximal site. Generation of functionally active CD8$^+$ CTL by mucosal vaccination was associated with the preservation of CD4$^+$ T cells in the colonic lamina propria (LP) after IR challenge with SHIV. This pattern of the CTL avidity after IR immunization with an AIDS vaccine in rhesus macaques was inversely correlated with the viral load in the gut mucosa. This difference between Ag-binding cells and functionally active high-avidity T cells may resolve an apparent discrepancy in the literature regarding the induction of mucosal immunity through different routes of immunization.

Also, we are not convinced that we have the most optimal mucosal vaccine to prevent massive memory CD4$^+$ T cells depletion in the gut. Our current study is only the proof of principle that a mucosal vaccine can be more effective in protection of CD4$^+$ T cells in the intestinal mucosa. A mucosal vaccine that incorporates more epitopes and immunoenhancing molecules may be even more effective at controlling virus infection by reducing the viral reservoir in the gut and at protecting memory CD4$^+$ T cells.

Materials and Methods

Mice

Female BALB/c and C57BL/6 mice were purchased from the Frederick Cancer Research Center (Frederick, MD).

Macaques

Indian rhesus macaques (*Macaca mulatta*) were matched for genetic origin, source, and comparable age and weight, and maintained in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care-International and under the approval of the Applied BioScience Laboratory's Animal Center Review Committee. All were seronegative for SIV, simian retrovirus 1, 2, and 5, and simian T cell leukemia/lymphotropic virus type 1 before the study, and were Mamu-A*01$^+$ MHC type as determined by PCR sequence-specific primers and direct sequencing (45).

Vaccines

The HIV/SIV vaccine peptides each contain an HIV envelope helper epitope (46–48) and an SIV Gag (49) or Pol (50) CTL epitope. Each CTL epitope was previously tested for immunogenicity (7). The B8R peptide (TSYKFESV) poxvirus CTL epitope restricted by H-2K^b was used in murine experiments (51). C57BL/6 mice were immunized IR or s.c. with modified vaccinia Ankara (MVA) (10^7 PFU/mouse) as described previously (52, 53). BALB/c mice were immunized IR or s.c. with P18-I10 peptide (50 μg/per immunization) plus LT(R192G) (7 μg/per immunization) (54).

Immunization and challenge of macaques and processing of macaque tissues

All experimental animals received a mixture of all four HIV/SIV vaccine peptides with mutant *Escherichia coli* labile toxin (LT) LT(R192G) (50 μg per immunization). 1) PCLUS3-CL10 (HIV-env plus SIV-Gag-181) (KQ IINMWQEVGKAMYAPPISGQIRCTPYDINQML), 2) PCLUS6.1-CL10 (HIV-env plus SIV-gag-181) (DRVIEVVQGAYRAIRHIPRRIRQGLERC TPYDINQML), 3) PCLUS3-Pol143 (HIV-env plus SIV-Pol-143) (KQII NMWQEVGKAMYAPPISGQIRLGPHYTPKIV), 4) PCLUS3-Gag-372 (HIV-env plus SIV-Gag-372) (KQIINMWQEVGKAMYAPPISGQIRLA PVPIPFA). For IR immunization or challenge, macaques were sedated with ketamine hydrochloride (10 mg/kg IM) and placed in a biosafety cabinet in ventral recumbency with hindquarters elevated. The tail was elevated dorsally and a 3-cc slip-tip syringe was atraumatically inserted into the rectum. Vaccine or virus was administered and the tail was lowered to assure complete delivery. Each s.c. vaccine dose was administered in the upper arm with the same peptide mixture emulsified 1:1 with Montanide ISA 51 adjuvant (Septic). Control animals received IR LT(R192G) alone (50 μg/dose) (7). The SHIV-ku2 virus is a chimeric virus containing the envelope gene from HIV-1 IIIB strain (HXBc2) and *gag* and *pol* genes from SIVmac239 and has been found to be pathogenic in rhesus macaques. SHIV-ku2 stock was isolated from PBMC of SHIV-ku2-infected rhesus macaques and titered in vivo by IR administration of 10-fold serial dilutions of the virus stock. One milliliter of undiluted virus infected 100% of animals whereas a 1/10 diluted virus stock infected 50%. Therefore, the animals were challenged IR in the current study with undiluted virus containing ~10 animal infectious doses (AID_{50}) for IR administration.

Determination of viral load

SHIV mRNA in plasma was quantified by nucleic acid sequence-based amplification (NASBA). mRNA extracted from plasma or tissue homogenates was subjected to isothermal amplification with primers specific for $SIV_{mac}251$ genes in SHIV-ku2 and then quantified by electrochemiluminescence chemistry using a coextracted internal standard. The detection limit of this assay is 2×10^3 RNA copies/input volume. In the case of colon and jejunum, snap-frozen samples of 300–500 mg were homogenized and lysed in 1 ml of lysis buffer. Twenty-microliter samples were analyzed and the results were normalized to 6 mg of tissue (equivalent to 300 mg/ml original tissue).

Isolation of LP and intraepithelial lymphocytes (IEL) in mice

To prepare IEL in mice, intestines from sacrificed mice were removed, flushed with RPMI 1640, and visible Peyer's patches were cut out (55). The intestines were longitudinally opened and washed with cold RPMI 1640. The intestines were then cut into 1-cm pieces, placed into flask, and shaken with a magnetic stirrer in 20 ml of Dulbecco's PBS containing 4 μM EDTA (Phoenix Biotechnologies) and 1 μM DTT (Pierce Biotechnology) for 40 min at 37°C. The supernatant was filtered through a 70-μM cell strainer (Falcon; BD Biosciences). IELs were isolated on 10 ml of Lympholyte-M cell separation medium (Laboratories Limited) by centrifugation for 30 min at 2000 rpm. For isolation of LP, intestinal pieces were digested in 5 ml of RPMI 1640 (Mediatech) containing 10% FBS (Invitrogen Life Technologies), 100 μg/ml penicillin-streptomycin solution (Mediatech), 2 mM L-glutamine (Mediatech), 80 U/ml collagenase type VIII, and 200 μg/ml DNase I (Sigma-Aldrich) for 40 min at 37°C (37). Then, the procedure for isolation of LP lymphocytes (LPL) was performed similar as for IEL cells.

CTL, IFN-γ ELISPOT, tetramer assays, and intracellular cytokine staining

Immune cells were activated overnight at 0.5×10^6 cells/well in a 12-well plate in the presence of 0.02 μM synthetic CTL epitope peptides in complete T cell medium (56). Mamu-A*01$^+$ target cells were pulsed with peptide from 10 to 10^{-4} μM for 2 h during ^{51}Cr labeling and cultured with effectors at 37°C for 4 h. Specific release was calculated as described (7).

IFN-γ ELISPOT assay was performed on mouse splenocytes, IEL, and LPL

Lymphoid cells were cultured in the wells of a 96-well ELISPOT plate in a volume of 200 μl, at 1×10^5 lymphoid cells/well. Splenocytes from naive C57BL/6 mice were used as target cells (2×10^5/well), pulsed in complete medium for at least 1 h with $B8R_{20-27}$ peptide (TSYKFESV), washed with complete medium twice, gamma-irradiated, and then added to cultures containing lymphocytes from the spleen, IEL, and LP isolated from immunized mice. The plates were incubated for at least 18 h at 37°C in 5% CO_2 and air. After that, the plates were washed thoroughly with PBS-0.05% Tween 20 (Sigma-Aldrich) followed by incubation with rat anti-mouse IFN-γ biotinylated monoclonal R4-6A2 (2 μg/ml; Mabtech) in PBS-0.5% BSA for 3 h at 37°C. After incubation with secondary mAbs, plates were washed with PBS-0.05% Tween 20, and incubated with alkaline phosphatase substrate prepared from VECTASTAIN Elite ABC kit (Vector Laboratories) for 1 h at room temperature. At the last stage, plates were washed with PBS-0.05% Tween 20 followed by PBS and developed with an ELISPOT AEC Substrate Set (BD Pharmingen). Spots were counted on an AID ELISPOT Reader (Cell Technology).

An IFN-γ ELISPOT assay was also performed on cells from macaque mesenteric lymph nodes (MLN) and axillary lymph nodes (ALN) per instructions (Cell Sciences). A total of 1×10^5 cells/well cells were activated with 10, 0.1, and 0.001 μM concentration of SIV CL10 peptide for 12 h (8). Spots were counted on an ELISPOT Reader (Cell Technology). The background level was subtracted from the number of IFN-γ-producing cells in the experimental groups. Soluble tetrameric K^b/B8R complex was conjugated to PE-labeled streptavidin (made by the National Institutes of Health Tetramer Core Facility) and was used to stain $CD8^+$ cells and analyzed on FACScan (BD Biosciences). Intracellular cytokine staining of IL-12-producing cells was performed using a BD Biosciences Cytofix/Cytoperm Plus kit according to the manufacturer's instructions. Briefly, cells were permeabilized with Cytofix/Cytoperm before incubation with anti-IL-12p70/40 (C15.6) mAbs. To identify DCs, LP, MLN, and ALN cells were stained with anti-mouse MHC class II (M5/114) (BD Biosciences) and CD11c (HL3) (BD Biosciences). Samples were analyzed with LSR II (BD Biosciences) and FlowJo software (Tree Star).

Statistics

Statistica-6 (StatSoft) and SigmaPlot-2000 (SSPS) were used. Statistical comparisons were performed using the Mann-Whitney, Wilcoxon, Kruskal-Wallis, and unpaired Student's *t* tests (57). Values of $p < 0.05$ were considered significant.

Results

Mucosal immunization induced a higher number of functionally active tetramer$^+$CD8$^+$ CTL in the gut mucosa, while s.c. immunization produced a greater number of IFN-γ-secreting cells in systemic sites

To study the role of mucosal vs systemic immunization in the formation of functionally active $CD8^+$ CTL in different systemic and mucosal sites, we immunized C57BL/6 mice by s.c. or IR routes with replication incompetent MVA at a dose of 10^7 PFU. We measured B8R tetramer binding (in which B8R is a dominant vaccinia epitope (51) restricted by H-$2K^b$) and IFN-γ ELISPOT by $CD8^+$ T cells in the spleen, small intestinal epithelium, and LP (Fig. 1, *A–C*). IR immunization with MVA on day 7 induced a higher frequency of B8R tetramer$^+$ cells in the spleen (Fig. 1*A*, $p < 0.05$, by Student *t* test) and in the intestinal LP (Fig. 1*C*; $p < 0.05$) compared with s.c. immunization with the same Ag. However, the difference in B8R tetramer$^+$ cells in the intestinal epithelium between s.c. and IR immunization was not significant. Thus, both routes of immunizations (systemic and mucosal) induced Ag-specific $CD8^+$ CTL in both compartments, systemic (spleen) and mucosal (LP and intestinal epithelium), when measured as cells expressing a specific receptor. However, when we examined functional activity instead of just receptor$^+$ cells, the situation was more asymmetric. Subcutaneous immunization induced a significant number of IFN-γ-producing cells in the spleen, but not in the intestinal mucosa (Fig. 1*D*). However, IR immunization induced a significantly much higher number of IFN-γ-secreting $CD8^+$ T cells in the intestinal epithelium and in the LP as well as an equal number in the spleen (Fig. 1*D*). This may explain the apparent discrepancy between the "asymmetry" in the induction of $CD8^+$ CTL in the gut mucosa and in the spleen after systemic vs mucosal immunization observed in our previous studies (7, 37, 58) and those of others (59–61) and some contrasting studies showing induction of tetramer-binding T cells in the mucosa after systemic immunization (40–42).

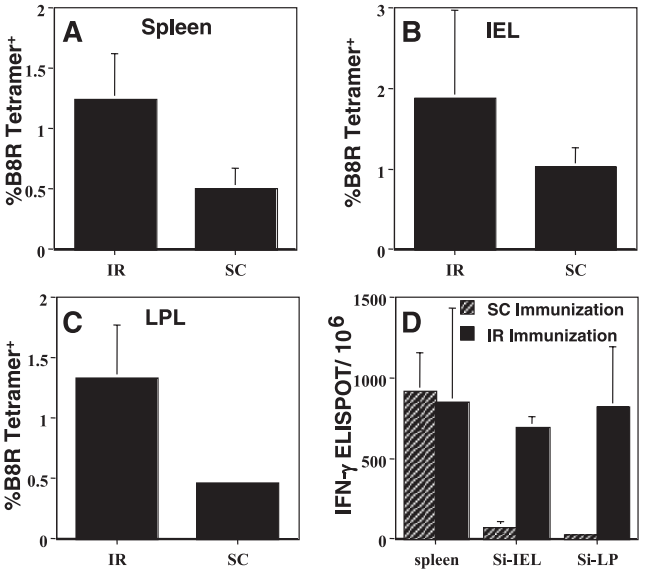

FIGURE 1. Mucosal immunization induced a higher frequency of H-$2K^b$/B8R tetramer$^+$ cells in the spleen, intestinal epithelium, and LP (*A–C*) and IFN-γ-secreting cells in the gut mucosa (*D*) compared with s.c. immunization. Groups of C57BL/6 mice (five per group) were immunized s.c. or IR with 10^7 PFU of MVA. Seven days after immunizations, we characterized the frequency of B8R-specific (TSYKFESV) $CD8^+$ CTL in the spleen (*A*), intestinal IEL (*B*), and LP (*C*) measured by H-$2K^b$/B8R tetramer staining, and the number of functional B8R-specific $CD8^+$ T cells in the spleen, the gut epithelium, and the LP by IFN-γ ELISPOT assay (*D*).

To determine whether routes of immunization play a significant role in generation of functionally active Ag-specific cells, we calculated the ratio of IFN-γ-secreting cells to the total B8R tetramer$^+$ cells in the spleen, in the intestinal epithelium, and intestinal LP (Fig. 2, *A–C*). A significantly greater percent of IFN-γ$^+$B8R tetramer$^+$ cells was found in the spleen after s.c. immunization (Fig. 2*A*, $p < 0.01$). IR administration generated a significantly lower percent of the functionally active B8R tetramer$^+$ cells in the spleen (Fig. 2*A*, $p < 0.01$) despite the fact that the number of Ag-specific T cells in the spleen after IR immunization was higher (Fig. 1*A*). Mice immunized IR generated a much higher ratio of B8R-specific IFN-γ$^+$ to B8R tetramer$^+$ cells in the gut epithelium and in the LP (Fig. 2, *B* and *C*). These data may explain why the generation of Ag-specific $CD8^+$ T cells in the mucosal sites after systemic immunization is not as effective for protective immunity against mucosal challenge as after mucosal immunization. We reasoned that the effectiveness of the mucosal protection is mediated by the generation of a higher number of functionally active Ag-specific $CD8^+$ CTL in the mucosal sites after mucosal immunization, rather than simply Ag-binding T cells.

It is well-known in murine and macaques studies that high-avidity $CD8^+$ CTLs are more effective than low-avidity CTLs in clearance of viral infections (8, 62–64). For this reason, we characterized the $CD8^+$ CTL avidity against B8R peptide (by using an IFN-γ ELISPOT assay) in the spleen, intestinal epithelium, and LP after systemic vs mucosal immunizations (Fig. 2*D*). We observed

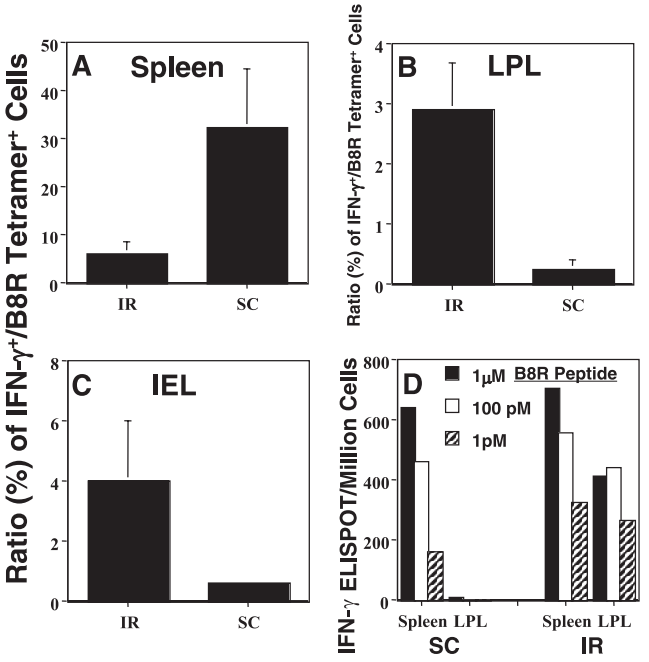

FIGURE 2. Subcutaneous immunization induced a higher ratio of IFN-γ-secreting Ag-specific cells to tetramer$^+$ cells in the spleen, while mucosal immunization produced a greater ratio of IFN-γ-producing to tetramer$^+$ cells in the intestine. The ratio of IFN-γ^+ responding to Ag functionally by ELISPOT to the number of H-2K^b/B8R tetramer$^+$ T cells was calculated in the spleen (*A*), the LP (*B*), and the intestinal epithelium (*C*). CD8$^+$ CTL avidity against B8R peptide was studied against different concentrations of B8R peptide (1 μM; 100 and 1 pM) in the spleen, and LP after s.c. or IR immunization in mice. C57BL/6 mice were s.c. or IR immunized with MVA (10^7 PFU). Seven days after the immunizations, we characterized the number of B8R-specific CD8$^+$ T cells against different concentrations of peptide (1 μM; 100 and 1 pM); presented data illustrate a representative individual mouse of 10 studied, with similar results (*D*).

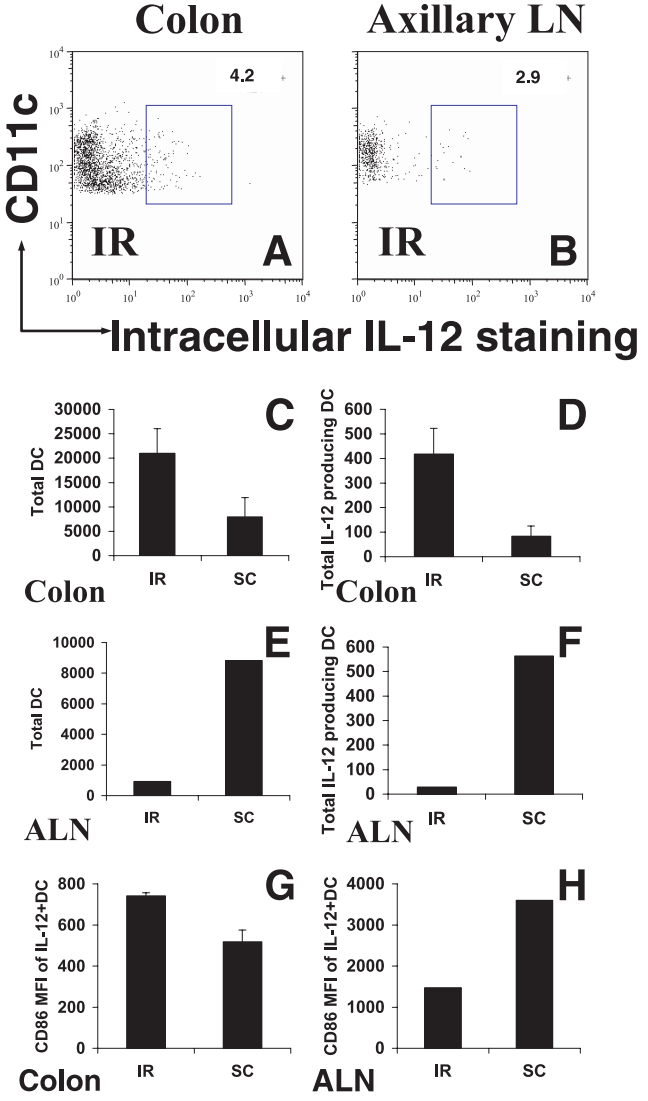

FIGURE 3. IR immunization induced a higher production of IL-12-producing DC in the colon, while s.c. immunization induced a higher production in the ALN. BALB/c mice (H-2D^d) were immunized IR or s.c. with peptide vaccine and LT(R192G) on days 0 and 1 and mice were sacrificed 1 day later (on day 2). Mononuclear cells from the colonic LP or ALNs were purified. DC cells were identified by flow cytometry with anti-mouse CD11c and anti-mouse MHC class II (M5/114) Abs and the gated population was assessed for IL-12 production by intracellular cytokine staining. The percentage of IL-12$^+$ DC in the colon (*A*) or in the ALN (*B*) after IR (*A* and *B*) or s.c. (data not shown) immunization of BALB/c mice (three mice per group) with peptide vaccine (50 μg per mouse per immunization) plus LT(R192G) (7 μg per mouse per immunization) was measured. The total DC (*C* and *E*) and the total IL-12-producing DC (*D* and *F*) were calculated in the colon (*C* and *D*) and ALN (*E* and *F*) after IR and s.c. immunization with peptide vaccine and LT(R192G). The mean of fluorescence index of CD86 on IL-12$^+$ DC in the colon (*G*) or ALN (*H*) after IR or s.c. immunization is shown in *G* and *H*. Presented is a representative experiment of two with similar results. Error bars (SD) in *E*, *F*, and *H* are too small to discern.

that the s.c. immunization induced high-avidity CD8$^+$ CTL (against 1 pM B8R peptide) in the spleen (Fig. 2*D*). However, IR immunization with MVA induced high-avidity T cells in the spleen as well (Fig. 2*D*). It was impossible to study the CTL avidity in LPL after s.c. immunization, because there was almost no measurable response (Fig. 2*D*). However, IR immunization generated the highest avidity CD8$^+$ CTL response against the B8R peptide in the gut LP, measured as the ratio of high-avidity T cells responding at 1 pM peptide to the total specific cells responding at 1 μM peptide (Fig. 2*D*).

The mechanisms of generation of high-avidity CTL in the local site after mucosal vs systemic immunization are not very well-understood. We reasoned that the local microenvironmental milieu (cytokines and costimulatory molecules) after the immunization with a vaccine plus an adjuvant might play a major effect in the generation of the functionally active CD8$^+$ T cells at the local sites. One of the major cytokine-producing cells in the intestinal mucosa is the DC. To determine whether peptide vaccine plus adjuvant LT(R192G) predominantly increased cytokine production locally (but not at the distant site), we studied IL-12 production by DC in the rectal mucosa and by DC in ALNs after the IR or s.c. immunization of BALB/c mice (H-2D^d) with P18-I10 HIV-1 CTL epitope (50 μg/per immunization) plus LT(R192G) (7 μg/per mouse/per immunization). We immunized mice IR (group 1) or s.c. (group 2) with the peptide vaccine plus LT(R192G) or with peptide vaccine alone without LT(R192G) given by IR (group 3) or by s.c. (group 4) immunization. Animals were immunized IR or s.c. twice (on days 0 and 1), and were sacrificed 1 day later (on day 2). DC from the colonic LP (Fig. 3 *A*, *C*, and *D*), the MLN (data not shown), and the ALN (Fig. 3, *B*, *E*, and *F*) were purified and the intracellular cytokine staining and FACS analyses were used to characterize the IL-12 production by DC from the local and the distant sites. The total number of CD11c$^+$ cells (Fig. 3, *C* and

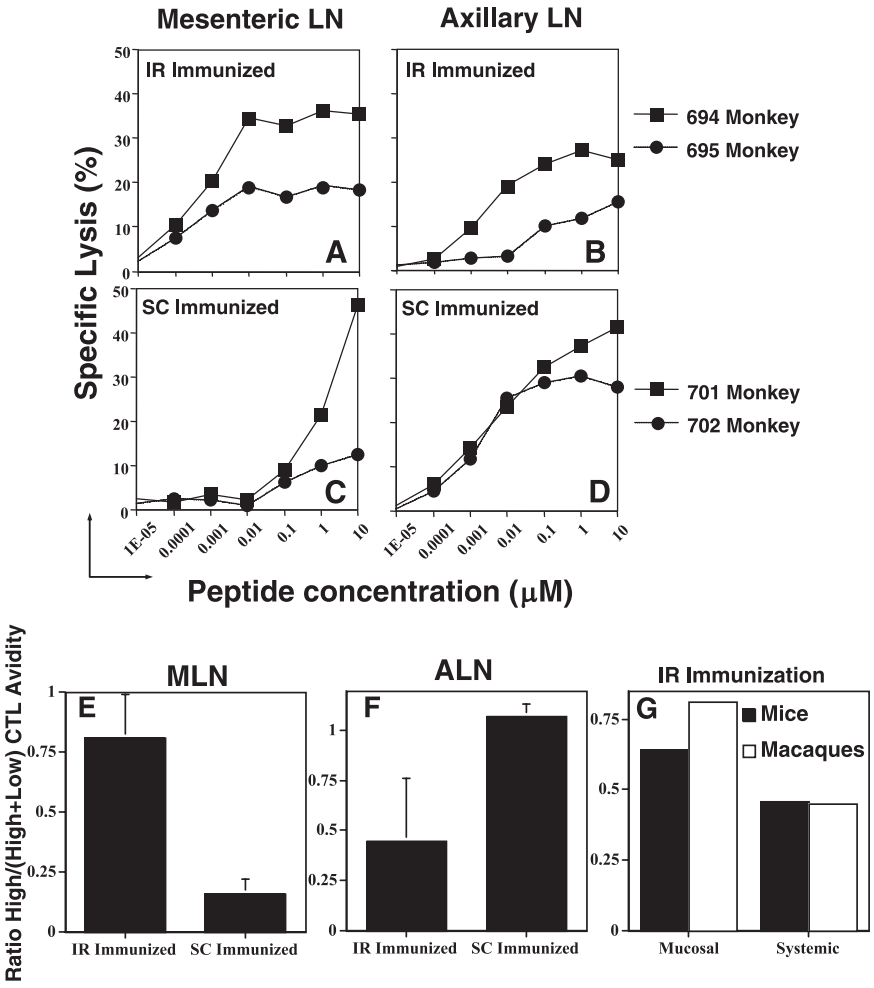

FIGURE 4. Mucosal AIDS peptide vaccine induces high-avidity $CD8^+$ CTL responses in the GALT, whereas systemic AIDS peptide vaccine induces high-avidity $CD8^+$ CTL responses in systemic lymphoid tissues of rhesus macaques. CTL activity as a function of peptide concentration for SIV Gag CL10 in MLN (*A* and *C*) or ALN (*B* and *D*) after mucosal (*A* and *B*) vs systemic (*C* and *D*) immunization with HIV/SIV peptide vaccines. Mamu-A*01^+ target cells were pulsed with different concentrations of peptides from 10 to 10^{-4} μM, as indicated, and lysis by MLN or ALN cells stimulated just overnight with 0.02 μM peptides (to avoid skewing the repertoire) was studied by ^{51}Cr release assay at a 100:1 E:T ratio. *E* and *F*, Ratio high/(high + low) CTL avidity. *E*, MLNs; *F*, ALNs. Ratio of percent specific lysis against 0.001 μM peptide (high-avidity CTLs) to percent-specific lysis against 10 μM peptide (total low- and high-avidity CTLs). *G*, Ratio of CTL activity as a function of peptide concentration in LP and spleen after IR immunization in mice vs CTL activity as a function of peptide concentration for CL10 in MLN and ALN after IR immunization in rhesus macaques.

E) and IL-12^+ DC (Fig. 3, *D* and *F*) in the colon after IR immunization with the peptide vaccine plus LT(R192G) was significantly higher compared with s.c. immunization with the same vaccine (Fig. 3, *C* and *D*) ($p < 0.05$). However, the total number of $CD11c^+$ cells and IL-12^+ DC in the ALNs after s.c. immunization was significantly higher compared with IR immunization (Fig. 3, *E* and *F*) ($p < 0.01$). The total number of IL-12^+ DCs in MLN after IR immunization was also higher than the IL-12^+ DCs in MLN after s.c. immunization (data not shown). IR or s.c. immunization with peptide alone was much less effective in production of IL-12 by DC (data not shown). The expression level (measured as mean of fluorescence intensity) of CD86 on IL-12^+ DC in the colon after IR immunization was significantly higher than after s.c. immunization (Fig. 3*G*) ($p < 0.05$). Conversely, s.c. immunization induced a higher level of CD86 expression on IL-12^+ DC in ALN (Fig. 3*H*) ($p < 0.01$). These data suggest that the different routes of immunization produced different levels of local cytokine secretion and costimulatory molecule expression by DC in the regional tissue proximal to the Ag application vs in the distal lymph nodes. This difference in DC activation locally could account for the difference in T cell function induced in the proximal vs distal sites.

Thus, our murine model demonstrated that mucosal immunization induced a higher number of functionally active tetramer$^+$ $CD8^+$ CTL in the gut mucosa, while the s.c. immunization produced a greater number of IFN-γ-secreting cells in the systemic site. Does this pattern apply to other species (for example, rhesus macaques, when we immunize rhesus macaques by s.c. or IR routes with an AIDS vaccine)? Does the generation of high quality of $CD8^+$ CTL responses correlate with protection against mucosal challenge with the pathogenic virus and protect macaques against $CD4^+$ T cell loss in the gut mucosa?

Preferential induction of high-avidity CTL in the mucosal site after IR immunization (not s.c. administration) in rhesus macaques

To examine the influence of mucosal vs systemic immunization on the development of CTL avidity in macaques, we compared IR synthetic HIV/SIV vaccine vs s.c. administration of the same AIDS vaccine in rhesus macaques that were described previously (7). In the previous published study, we compared whether a mucosal vaccination with HIV/SIV vaccine could induce local $CD8^+$ CTL and protect Mamu-A*01 macaques against IR challenge with SHIV more effectively than the same vaccine administered s.c. (7). In that study, we characterized $CD8^+$CTL in MLN, colon, PBMC, and ALN by ^{51}Cr release assay, $CD4^+$ Th response by [^{3}H]thymidine proliferation assay, and viral load in PBMC and mucosal tissues by NASBA test. In the current study, we used preserved samples from that previous preclinical trial in macaques to address new questions regarding the role of the $CD8^+$ T cell avidity by IFN-γ ELISPOT and ^{51}Cr release assay proximal and distal to the site of immunization in protection, and the preservation of $CD4^+$ T cells in colonic LP after IR challenge with pathogenic SHIV in rhesus macaques immunized with an HIV/SIV peptide vaccine by IR or s.c. routes. We used a peptide vaccine for the immunization just to avoid a live recombinant vector that might disseminate to

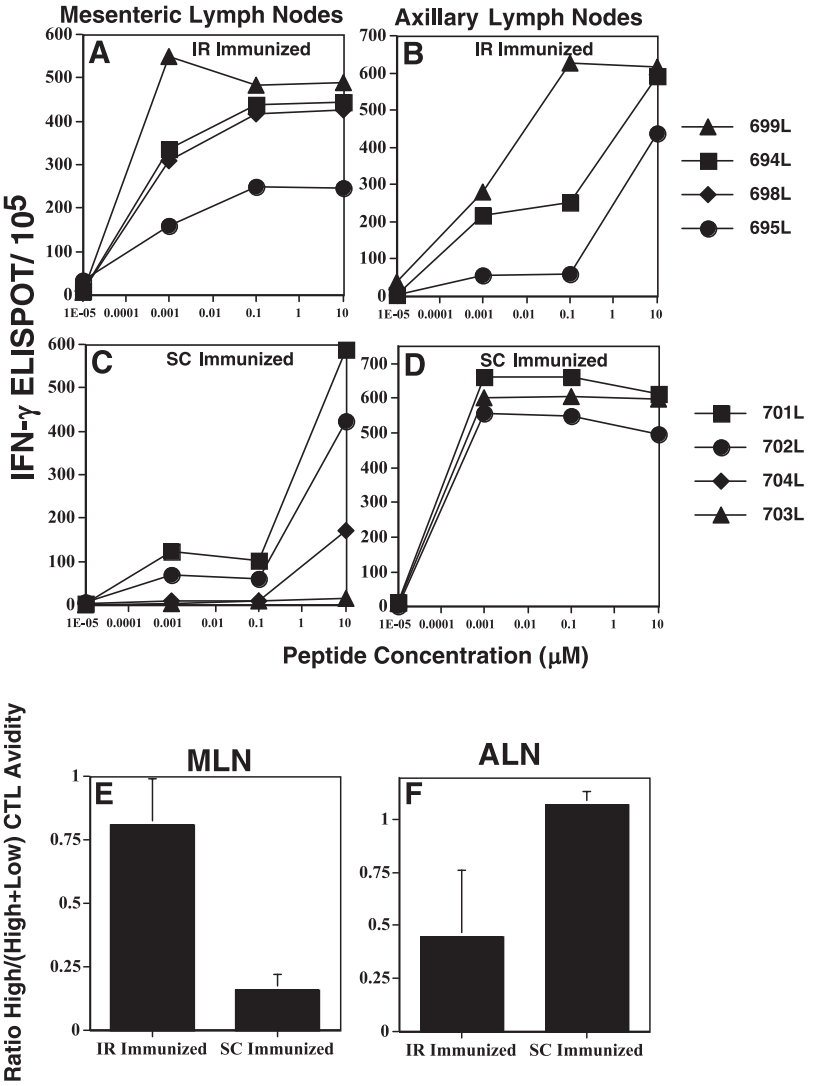

FIGURE 5. Mucosal immunization of rhesus macaques with HIV/SIV vaccine induces high-avidity CD8$^+$ IFN-γ-producing T cells in the GALT, but not in ALN, whereas systemic immunization induces predominantly high-avidity CD8$^+$ T cells in ALN, but not in MLN. *A*, CD8$^+$ T cell activity measured as IFN-γ-producing cells by ELISPOT as a function of peptide concentration for SIV Gag CL10 peptide in the MLN after IR immunization with HIV/SIV peptide vaccine with LT(R192G). T cells were activated with different concentration of CL10 peptides (10, 0.1, 0.001 μM and no peptide, as indicated). *B*, IFN-γ-producing cells as a function of peptide concentration for SIV Gag CL10 peptide in the ALN after IR immunization with HIV/SIV peptide vaccine with LT (R192G). *C*, IFN-γ-producing cells as a function of peptide concentration for SIV Gag CL10 peptide in the MLN after s.c. immunization with HIV/SIV peptide vaccine with Montanide ISA 51. *D*, CTL IFN-γ-producing cells as a function of peptide concentration for SIV Gag CL10 peptide in the ALN after s.c. immunization with HIV/SIV peptide vaccine with Montanide ISA 51. *E* and *F*, Ratio high/(high + low) CTL avidity. *E*, MLNs; *F*, ALNs. Ratio of the number of IFN- γ$^+$ cells against 0.001 μM peptide (high-avidity T cells) to the number of IFN-γ-producing cells against 10 μM peptide (total low- and high-avidity T cells).

other tissues and lymphoid organs, when comparing routes of immunization. Each peptide vaccine construct contained one Th epitope (which was broadly recognized by Th cells from mice, macaques, and humans) and one CD8$^+$ CTL epitope (presented by the rhesus MHC class I molecule, Mamu-A*01), and all rhesus macaques were preselected for the class I molecule Mamu-A*01 (7). All s.c.- and IR-immunized rhesus macaques received a mixture of all four AIDS vaccine constructs. Subcutaneous immunization with peptides was done in an emulsion with Montanide ISA 51 (7), whereas IR administration was performed with a mixture of all four peptides with a mutant *E. coli* LT(R192G), as a less toxic adjuvant with higher potency to induce a Th1 cytokine pattern, stronger CTL response, and protection (65).

The characterization of CD8$^+$ CTL avidity only by enumeration of IFN-γ-secreting cells responding to a different concentration of peptides may have some limitation, in that it reflects only one functional activity, and does not provide any comprehensive measure of all functions. Therefore, we also studied mucosal and systemic CTL avidity after IR (macaques 694 and 695) and s.c. (701 and 702) immunizations in macaques by using a functional ^{51}Cr release assay against different concentrations of SIV CL10 peptide on target cells (from 10 to 10^{-4} μM, as indicated in Fig. 4, *A–D*), as well as by IFN-γ production (Fig. 5). These two assays of cytokine production and lytic activity together may give a broader picture of the activity of these CD8$^+$ T cells. To characterize CTL avidity, these cytolytic responses were evaluated by plotting specific lysis vs epitope concentration. In these studies, we define "functional avidity" as the ability of a T cell to respond to low concentrations (high avidity) or high concentrations (low avidity) of Ag. This functional avidity may reflect a number of factors in T cell activation besides the intrinsic affinity of the TCR. CTL avidity in the MLN and ALN was studied 2 wk after completion of the last IR or s.c. immunization (week 17). After IR immunization, the percent-specific lysis in MLN of immunized macaques was very high against both low (0.001 μM) and high (10 μM) concentrations of SIV CL10 peptide (Fig. 4*A*), indicating a large proportion of high-avidity CTL. Our monkeys were outbred for MHC class II and therefore heterogeneous in their Th cell responses. This fact may explain the differences in the magnitude of CTL lytic activity in immunized animals. However, it is the relative activity at high vs low concentrations that defines the avidity, not the absolute magnitude of the response. Subcutaneous immunization induced specific lytic activity in the MLN predominantly against targets with a high concentration of SIV CL10 peptide (10 μM, indicative of predominantly low-avidity CTL) (Fig. 4*C*). In contrast, s.c. immunization induced a much higher level of specific lysis against the low concentration of SIV CL10 peptide in the ALN (Fig. 4*D*), whereas mucosal immunization was less effective in the generation of high-avidity CTL responses in the ALN (Fig. 4*B*). Thus, there was a reciprocal pattern in which the high-avidity

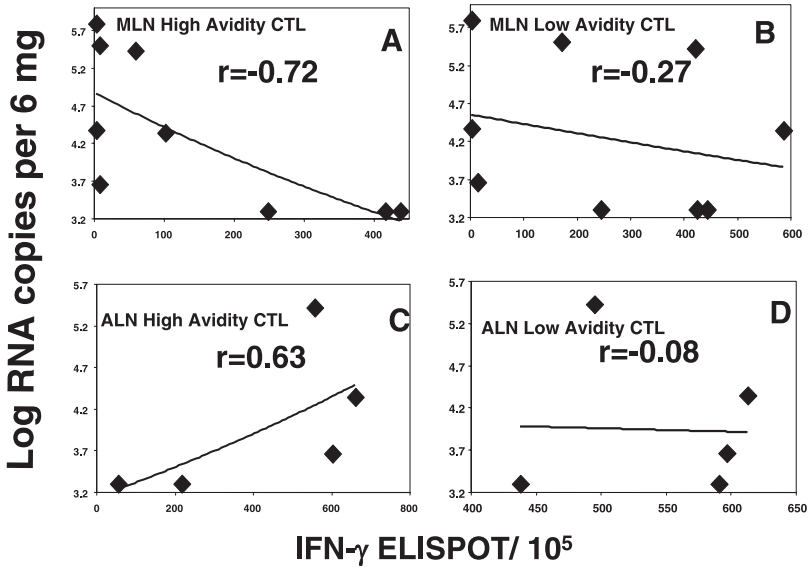

FIGURE 6. Viral load in colon inversely correlated with the level of high-avidity SHIV-specific $CD8^+$ T cells in the mucosal lymphoid tissue (*A*), but not low-avidity CTL in the MLN (*B*) before the challenge, nor with high- or low-avidity SHIV-specific $CD8^+$ T cells in the ALN (*C* and *D*). Viral load is expressed as log RNA copies per 6 mg of colonic tissue (by NASBA assay) vs number of IFN-γ-producing cells in MLN or ALN before challenge.

cells were predominantly found in the nodes proximal to the site of immunization.

To quantify this comparison, we calculated the ratio between the percent-specific lysis against a low (0.001 μM) peptide concentration (high-avidity $CD8^+$ T cells) and the percent-specific lysis against a high (10 μM) concentration (high- plus low-avidity $CD8^+$ T cells) for the CTL to SIV-CL10 peptide separately for the MLN and ALN after s.c. or IR immunizations with the peptide AIDS vaccine (Fig. 4, *E* and *F*). The higher values of this ratio indicate skewing of the CTL population toward high-avidity cells, whereas lower values indicate a smaller proportion of high-avidity cells. The avidity ratio was higher in MLN after IR immunization (Fig. 4*E*), while s.c. induced a slightly higher avidity ratio in the ALN (Fig. 4*F*). Thus, IR immunization induces higher CTL avidity in the local mucosal site (MLN) and the avidity tended to be higher at the site more proximal to the site of immunization. We calculated a similar ratio (high/high plus low) of CTL avidity against the B8R peptide in the spleen and LP after IR immunization in mice. This phenomena of "compartmentalization" of high-avidity T cells to the site proximal to the immunization was consistently demonstrated in both mice and macaques (Fig. 4*G*).

To complement and confirm the results of the lytic assays, we also characterized the $CD8^+$ T cell avidity against CL10 peptide to the AIDS peptide vaccine administered IR or s.c. by IFN-γ ELISPOT assay. We asked whether IR immunization with the AIDS peptide vaccine induced a higher proportion of high-avidity IFN-γ-secreting CTLs in the GALT of rhesus macaques that could be activated at low SIV-CL10 peptide concentrations compared with s.c.-immunized animals in the same compartment. A significant number of IFN-γ^+CD8^+ T cells in the MLN after IR immunization were detected to react with high (10 μM) and low (0.001 μM) concentrations of CL10 peptide (Fig. 5*A*). However, after s.c. immunizations of animals with AIDS peptide vaccine a comparable number of IFN-γ^+CD8^+ CTLs in MLN was observed against a high concentration of peptide (10 μM), but only a small proportion of cells responded to a lower concentration of CL10 peptide (0.001 μM) indicative of high avidity (Fig. 5*C*). The spectrum of $CD8^+$ CTL avidity in ALN of macaques after s.c. and IR immunizations was the reverse (Fig. 5, *B* and *D*). Subcutaneous immunization of rhesus macaques consistently induced a very high proportion of high-avidity $CD8^+$ T cells producing IFN-γ responding to low concentrations of CL10 peptide in the ALN (Fig. 5*D*), whereas IR immunization induced a consistently lower proportion of high-avidity $CD8^+$ T cells in the ALN (Fig. 5*B*). These data strongly suggest the compartmentalization of high functional CTL avidity proximal to the site of immunization, at the local mucosal site after IR immunization and in the draining lymph nodes after s.c. immunization.

Thus, we observed that the shape of the dose-response curves in MLNs was more convex up in the group of macaques immunized IR compared with those immunized s.c., indicating a high proportion of high-avidity CTL that could kill targets at low peptide concentration. The dose-response curves after s.c. immunization was more convex up in the ALN, but not in the MLN. To quantify this difference, we calculated the ratio between the number of IFN-γ-secreting cells against a low (0.001 μM) epitope concentration (reflecting high-avidity CTLs) and the number of IFN-γ-secreting cells against a high (10 μM) concentration (reflecting both high- and low-avidity CTL) for the CTL to the SIV-CL10 epitope (Fig. 5, *E* and *F*). Higher values of this ratio indicated the skewing of CTL toward high-avidity cells, whereas lower value of this ratio indicated skewing toward low-avidity cells. The avidity ratio in MLN was significantly higher after IR immunization ($p < 0.05$) (Fig. 5*E*), whereas s.c. immunization increased the avidity ratio in ALN ($p < 0.05$) (Fig. 5*F*).

To understand the protective role of mucosal vaccine-induced high-avidity CTL in the GALT, we investigated possible correlations between log of viral load in the colon at the time of necropsy, 200 days after challenge, and the numbers of the IFN-γ^+ cells in MLN against high and low concentrations of SIV CL10 peptide after IR immunization (before challenge) (Fig. 6, *A* and *B*). We found a strong inverse correlation ($r = -0.72$; $p < 0.01$) between the number of high-avidity CTL in MLNs and the level of viral load in the colon at the time of necropsy (Fig. 6*A*). In contrast, we found only a weak but nonsignificant trend ($r = -0.27$, $p > 0.05$) between the number of low-avidity CTL in the MLN and the level of viral load in the colon (Fig. 6*B*), and no significant inverse correlation between high- and low-avidity CTL in the ALN and viral load (Fig. 6, *C* and *D*). In fact, if anything, the weak trend (not significant) in the ALN was in the opposite direction (Fig. 6*C*). These data suggested the importance of local compartmentalization of high-avidity mucosal CTL (but not low-avidity CTL or CTL in distant peripheral lymph nodes) for clearance of virus in the major reservoir of AIDS virus replication in the gut mucosa.

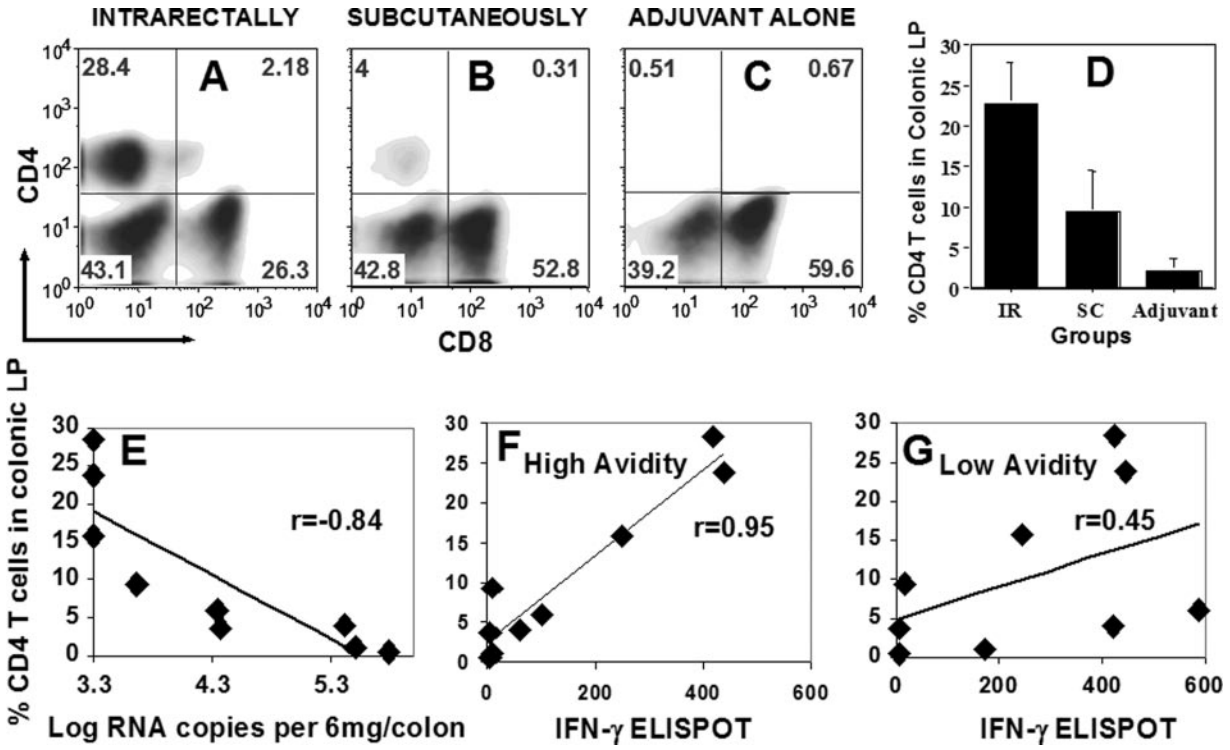

FIGURE 7. Mucosal immunization of rhesus macaques with HIV/SIV vaccine prevented massive $CD4^+$ T cell depletion in the mucosa, while a systemic vaccine or adjuvant alone was not effective for preservation of $CD4^+$ T cells in the LP. Percentage of $CD4^+$ and $CD8^+$ cells in colonic LP in rhesus macaques immunized with HIV/SIV vaccine IR, s.c., or with adjuvant alone (*A–D*). *A–C* are representative animals from each group; means and SD values are plotted for all the animals in the three groups in *D*. Percentage of $CD4^+$ cells in LP inversely correlated with viral load in the gut mucosa (*E*). Preservation of $CD4^+$ T cells in the gut was directly correlated with the presence of high-avidity $CD8^+$ T cells in mucosa (*F*), but not with low-avidity $CD8^+$ T cells (*G*).

To understand the role of mucosal vaccination and functionally active $CD8^+$ CTL at the local mucosal site for prevention of massive $CD4^+$ T cell depletion from the mucosa, we examined the percentage of $CD4^+$ T cells in colonic LP 180 days after IR challenge with pathogenic SHIV in rhesus macaques immunized IR, s.c. with Ag, or with adjuvant alone IR. Six months after IR challenge with pathogenic SHIV, we found a profound depletion of $CD4^+$ T cells in colonic LP in macaques immunized s.c. or with adjuvant alone (Fig. 7, *B–D*), while the number of $CD8^+$ cells in colonic LP were preserved in all groups (Fig. 7, *A–D*). However, in macaques immunized IR with the vaccine, there was a preservation $CD4^+$ T cells in the intestinal LP (Fig. 7, *A* and *D*). The $CD4^+$ cell loss was related to the SHIV infection in that there was a strong inverse correlation ($r = -0.84$) between the viral load in the colonic LP and the number of $CD4^+$ T cells in colon (Fig. 7*E*). A strong positive correlation ($r = 0.95$) between the percent of $CD4^+$ T cells in colonic LP and the number of high-avidity $CD8^+$ CTL in the same location was observed (Fig. 7*F*). However, there was no significant correlation ($r = 0.45$) between the percent of $CD4^+$ cells and low-avidity $CD8^+$ CTL (Fig. 7*G*). It is clear now that $CD4^+$ T cell depletion is observed much earlier in the gut mucosa than in systemic lymphoid tissue (12–14). Thus, the mucosal AIDS vaccine not only reduced viral load in the gut reservoir, but as result of this could prevent an early massive $CD4^+$ T cell depletion in the mucosa. This protection correlates with the level of functionally active $CD8^+$ CTL at the mucosal site of viral entry. This decrease of $CD4^+$ T cells in the gut is very stable and cannot be restored even after aggressive highly active antiretroviral therapy (12–14). This is why the generation of functionally active $CD8^+$ CTL at the mucosal site is so important. Such desirable immune responses can be generated by mucosal vaccination, because of the avidity compartmentalization observed here in mouse and macaque models.

Discussion

One of the major goals of a vaccine against HIV/AIDS is to protect against massive loss of $CD4^+$ T cells. It is clear today that the major loss of $CD4^+$ T cells in the early stage of AIDS infection occurs in the intestinal mucosa, which is the major site of HIV/AIDS viral replication (12–14). Our current study demonstrated that an IR AIDS vaccine was much more effective in preservation of $CD4^+$ T cells in the gut mucosa. Such mucosal protection correlates with the induction of functionally high-avidity $CD8^+$ CTL in the mucosa, which was better induced by the mucosal AIDS vaccine. This localization of high-avidity cells and protection supported the compartmentalization of high-avidity CTL responses we observed proximal to the immunization site in both mice and primates.

Functional impairment of virus-specific memory $CD8^+$ T lymphocytes has been associated with clinical disease progression following HIV, SIV, and SHIV infection (44, 66–68). It has been recently reported that dysfunction of IL-2 and TNF-α production might be observed even earlier than the loss of IFN-γ production by HIV-specific $CD8^+$ CTL (66). It was demonstrated that i.m. vaccination was associated with the preservation of Gag-specific central memory $CD8^+$ T cells in peripheral lymphoid tissue that were functionally capable of producing IFN-γ, and effector memory $CD8^+$ T cells that were capable of producing granzyme B after exposure to SIV (66, 68). However, the influence of route of immunization on the generation of functionally active $CD8^+$ T cells, especially high-avidity $CD8^+$ CTL, has not been well-appreciated. In these studies, we defined functional avidity of $CD8^+$ CTL by ex

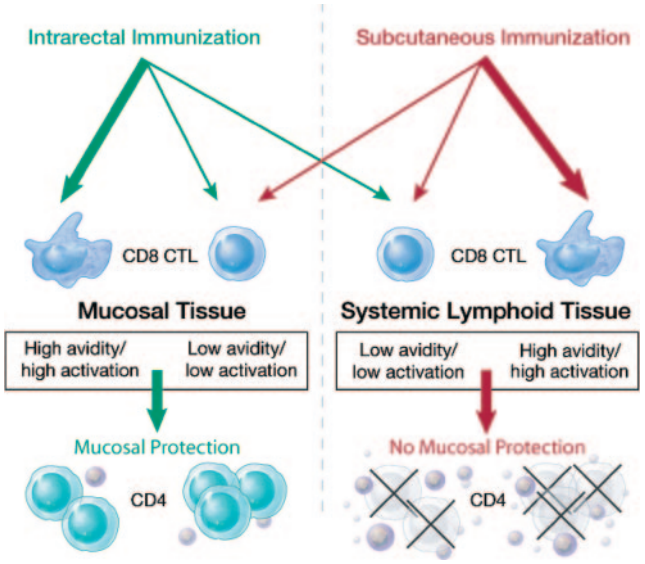

FIGURE 8. Scheme of functional CTL activity and avidity compartmentalization according to the site of immunization and prevention of massive $CD4^+$ T cell depletion.

vivo enumeration of Ag-specific $CD8^+$ CTL responding to different peptide concentrations by lytic activity or cytokine production (8, 62–64, 69, 70). The functional avidity of $CD8^+$ CTL is different from the intrinsic affinity of the TCR to MHC class I-peptide complexes, which is only one component of the overall avidity of the cell. However, the functional avidity is the parameter previously seen to correlate with ability to clear virus in mice (62).

In this study, we compared the effect of mucosal vs systemic immunization for generation of high-avidity $CD8^+$ CTL and functionally active $CD8^+$ T lymphocytes at the local sites of immunization vs distally in mice and rhesus macaques. We observed a novel phenomenon of compartmentalization of $CD8^+$ CTL avidity and proportion of functionally active Ag-specific $CD8^+$ CTL to the local sites after IR (to the GALT) or s.c. (to local draining lymph nodes) immunization (Fig. 8). Reduction of viral titer in the colon in macaques and preservation of $CD4^+$ T cells in intestinal LP, after IR challenge with pathogenic SHIV, were inversely correlated with the level of high avidity of $CD8^+$ T cells in the MLN induced by the vaccination. These were better induced by mucosal (IR) immunization. The compartmentalization phenomenon was reproduced in both species, mice and macaques. This is important because such reproducibility in mice and macaques is not always the fact for many other observations. Also, it is very possible that Ab affinity, neutralizing activity, or subclass of Ab could compartmentalize according to the site of immunization, but these were not studied here.

Several studies demonstrated that systemic immunization is capable of inducing Ag-specific $CD8^+$ T cells in the mucosa (40, 41). However, the majority of these studies used as a readout the number of $tetramer^+$ (Ag-specific) cells in the mucosal sites. In this study, we see that the comparison of Ag-specific cells by the nonfunctional tetramer-binding assay does not reveal the functional differences between compartments clearly. In contrast, a comparison of functionally active $CD8^+$ T cells and $CD8^+$ T cell avidity after different routes of immunizations with the same Ag reveals more striking differences and correlations of these parameters with protection against AIDS virus. These findings may explain apparent discrepancies in the literature about the need for a mucosal route of immunization to induce mucosal CTL, as the result depends on whether the readout is Ag-binding cells or functional activity and avidity.

We find that mucosal protection inversely correlates with higher $CD8^+$ CTL avidity in the local mucosal tissue after IR immunization. This novel phenomenon can be described as a predominant compartmentalization of the higher avidity $CD8^+$ T cells proximal to the site of vaccination (Fig. 8). We did not expect the difference in avidity was due to higher Ag concentrations proximal to the site of immunization, as these might select for lower avidity CTL. Therefore, we asked whether DC near the site of immunization were more effectively activated. In this study, we have seen a correlation between the site of vaccination and the generation of IL-12-producing DC with high levels of the costimulatory molecule CD86 in the proximal tissues that may in part account for the more efficient induction of high-avidity CTL. We believe that greater DC activation may explain the results because in previous studies, we have found that increasing costimulatory molecule expression on the cells presenting Ag (71), or increasing production of cytokines like IL-15 by these cells (72), can induce higher avidity CTL. Thus, the increased expression of the CD86 costimulatory molecule in the sites proximal to the immunization may in part account for the induction of higher avidity CTL locally. Also, although it is not yet possible to measure intracellular IL-15 as it is for IL-12, it is likely that DC that are activated to make IL-12 also make IL-15. Thus, we speculate that this mechanism also may be operative in the local sites near the immunization site but not at distal sites, accounting for the induction of higher avidity T cells proximal to the vaccination site. However, additional mechanisms beside DC activation may contribute to the mechanism of $CD8^+$ avidity compartmentalization. Thus, further study in this area will be needed.

Our previous study (73) and studies from other groups (74, 75) demonstrated that just a simple application of the Ag mucosally would not guarantee success of a mucosal vaccine. The generation of functionally active $CD8^+$ T cells in the local site after mucosal vaccination will depend on multiple factors including type of mucosal adjuvant, type of Ag, frequency of immunization, and others. However, the current study supports the conclusions that generation of high-avidity CTL in the GALT is greatly dependent on a mucosal route of vaccination and CTL in the GALT should be functionally active and of high avidity to protect against massive $CD4^+$ T cell depletion in the mucosa and to have an impact on AIDS viral dissemination from the intestinal mucosa.

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Disclosures

The authors have no financial conflict of interest.

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