Mucosal Immunity and HIV-1 Infection: Applications for Mucosal AIDS Vaccine Development

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Abstract Natural transmission of human immunodeficiency virus type 1 (HIV-1) occurs through gastrointestinal and vaginal mucosa. These mucosal tissues are major reservoirs for initial HIV replication and amplification, and the sites of rapid $CD4^+$ T cell depletion. In both HIV-infected humans and SIV-infected macaques, massive loss of $CD4^+$ $CCR5^+$ memory T cells occurs in the gut and vaginal mucosa within the first 10–14 days of infection. Induction of local HIV-specific immune responses by vaccines may facilitate effective control of HIV or SIV replication at these sites. Vaccines that induce mucosal responses, in particular $CD8^+$ cytotoxic T lymphocytes (CTL), have controlled viral replication at mucosal sites and curtailed systemic dissemination. Thus, there is strong justification for development of next generation vaccines that induce mucosal immune effectors against HIV-1 including $CD8^+$ CTL, $CD4^+$ T helper cells and secretory IgA. In addition, further understanding of local innate mechanisms that impact early viral replication will greatly inform future vaccine development. In this review, we examine the current knowledge concerning mucosal AIDS vaccine development. Moreover, we propose immunization strategies that may be able to elicit an effective immune response that can protect against AIDS as well as other mucosal infections.

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Current Topics in Microbiology and Immunology (2012) 354: 157–179
DOI: 10.1007/82_2010_119

Published Online: 4 January 2011

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1 Introduction

Gastrointestinal (GI) and vaginal mucosal tissues are major sites of HIV entry and initial infection (Veazey et al. 1998; Berzofsky et al. 2001; Belyakov and Berzofsky 2004; Neutra and Kozlowski 2006; Wilkinson and Cunningham 2006; Morrow et al. 2007; Belyakov and Ahlers 2008; Ahlers and Belyakov 2009a; Belyakov and Ahlers 2009b). An early sign of immunodeficiency virus infection is depletion of $CD4^+$ $CCR5^+$ memory T-cells in the mucosa (Brenchley et al. 2004; Li et al. 2005; Mehandru et al. 2004; Veazey et al. 1998, 2003). Dysfunction of the mucosal immune system during the early stages of AIDS leads to major structural abnormalities in the gut of infected individuals and to the development of opportunistic infections (Clayton et al. 2001; Heise et al. 1994; Kotler et al. 1984; Sharpstone et al. 1999). Subsequent systemic immune activation is considered a hallmark of the disease. Thus, AIDS can be considered primarily as a disease of the mucosal immune system (Belyakov and Berzofsky 2004). There is substantial evidence indicating that virus replication is rapid in the mucosa, eliminating $CD4^+$ target cells before dissemination into blood 2–7 days later (Spira et al. 1996; Zhang et al. 1999). Thus, the induction of $CD8^+$ CTL and $CD4^+$ T helper cells in concert with protective antibodies will be important criteria for an effective HIV vaccine (Kozlowski et al. 1997; Murphey-Corb et al. 1999; Baba et al. 2000; Barouch et al. 2000; Amara et al. 2001; Shiver et al. 2002; Kozlowski and Neutra 2003; McMichael 2006; Manrique et al. 2009; Sui et al. 2010).

2 Role of Mucosal $CD8^+$ CTL in Protection Against Local Viral Infection

Initially, studies pertaining to the mucosal immunology of HIV infection focused on induction of envelope specific antibody responses and the role of secretory IgA (S-IgA) and IgG in viral control (Funkhouser et al. 1993; Mazzoli et al. 1997). Failure to detect an early neutralizing antibody response in infected individuals,

and the possibility that infection was primarily amplified by cell-to-cell spread, suggested that an effective antibody response was subverted by HIV infection. Furthermore, S-IgA antibodies were infrequently detected in mucosal secretions of HIV-infected individuals (Mestecky and Jackson 1994). In contrast, a major role for $CD8^+$ CTL in initial virus control was suggested in seminal studies showing that the depletion of peripheral $CD8^+$ cells in SIV-infected macaques significantly increased virus loads (Castro et al. 1992; Jin et al. 1999; Schmitz et al. 1999). However, evidence of a role for mucosal $CD8^+$ CTL in control of immunodeficiency virus infection was limited (Gallichan and Rosenthal 1996; Porgador et al. 1997; Belyakov et al. 1998a, b; Berzofsky et al. 1999; Berzofsky et al. 2004; Belyakov and Ahlers 2008; Ahlers and Belyakov 2009a).

Early studies in our laboratory attempted to understand the role of mucosal $CD8^+$ T cells in reducing mucosal viral loads and delaying the appearance of virus in the blood. We asked whether immunization with CD4Th-CD8 epitope peptide constructs through different mucosal routes (e.g., intrarectal, intragastric, intranasal) could elicit HIV-specific $CD8^+$ CTL in small intestinal Peyer's patches (PP) or lamina propria (Belyakov et al. 1998b). Mice were immunized with 4 doses of the synthetic HIV-1 Th-CTL envelope peptide construct, PCLUS3-18IIIB, on day 0, 7, 14 and 21 in combination with cholera toxin (CT) mucosal adjuvant. In a comparison of the three mucosal routes of immunization, only intrarectal (I.R.) immunization induced long-lasting, antigen-specific $CD8^+$ CTL memory in both the inductive PP and lamina propria effector sites as well as in the spleen. The CTL responses in spleen after I.R. immunization were similar to those induced by subcutaneous (S.C.) immunization (Belyakov et al. 1998b). In contrast, S.C. immunization with PCLUS3-18IIIB induced systemic $CD8^+$ CTL responses with little evidence of mucosal $CD8^+$ T cell responses (Belyakov et al. 1998b).

Strong long-lasting mucosal $CD8^+$ CTL responses can also be generated by mucosal immunization with recombinant vaccinia virus vectors (Belyakov et al. 1998d, 1999; Wyatt et al. 2008). In a follow up study, we demonstrated the mucosal immunogenicity of replication-defective modified vaccinia Ankara (MVA) virus expressing the HIV89.6 gp160 envelope protein in mice (Belyakov et al. 1998d). A single I.R. immunization with MVA89.6 generated antigen-specific $CD8^+$ CTL in both PP and intestinal lamina propria, at least as efficiently as a replication-competent recombinant vaccinia virus expressing 89.6 gp160 (Belyakov et al. 1998d). Furthermore, $CD8^+$ CTL responses were detected in PP up to 6 months after I.R. immunization with MVA89.6 and were slightly higher than those after immunization with WR89.6 virus (Belyakov et al. 1998d). In contrast, intraperitoneal (I.P.) immunization with MVA89.6 induced CTL in the PP but not in the intestinal lamina propria. The magnitude of the response in PP of I.P. immunized animals was modest compared to the spleen, and this result was reproducible in three independent experiments (Belyakov et al. 1998d).

Transcutaneous immunization has also been shown to induce immune responses in the GI tract (Glenn et al. 1998; Scharton-Kersten et al. 2000). The application of antigen and adjuvant directly onto the skin has induced robust IgG and S-IgA responses, as well as $CD8^+$ CTL in PP and lamina propria (Glenn et al.

2000; Gockel et al. 2000; Belyakov et al. 2004b). In addition, studies have demonstrated protection against mucosal challenge with toxin or live virus following transcutaneous immunization (Glenn et al. 1998; Gockel et al. 2000; Scharton-Kersten et al. 2000; Belyakov et al. 2004b). Transcutaneous vaccination targets antigen to bone marrow-derived Langerhan's dendritic cells (DC) resident in the outer epidermal layers of skin. In a recent study, we demonstrated that activated DC carrying skin-derived antigen migrate from the skin to PP and present antigen directly to resident lymphocytes (Belyakov et al. 2004). By using an in vivo pulsed antigen-presenting cell (APC)/T cell co-culture model for tracking migrating APC by flow cytometry, we demonstrated that $CD11c^+$ DC carrying skin-derived antigens can be isolated from PP inductive sites in intestinal mucosa within 24 h following transcutaneous immunization with HIV peptide vaccine and CT mucosal adjuvant (Belyakov et al. 2004). The ex vivo treatment of bone marrow-derived $CD11c^+$ DC with vitamin D3, CT, or forskolin has been shown to increase the ability of DC to migrate to inductive mucosal sites and to induce mucosal immune responses (Enioutina et al. 2000).

Direct evidence supporting the ability of local $CD8^+$ CTL to mediate protection against mucosal viral transmission has been difficult to obtain (Belyakov et al. 1998a). Furthermore, previous studies demonstrating protection against mucosal viral challenge had not elucidated immune mechanisms involved in protection (Marx et al. 1993). A number of studies have shown a role for $CD8^+$ CTL in protection against mucosal infections, such as influenza (Gao et al. 1991; Ulmer et al. 1993). However, understanding the role of $CD8^+$ CTL at mucosal sites of infection in control and resolution of infection where antibody plays a prominent role in protection is complex (Eichelberger et al. 1991; Lukacher et al. 1984; Taylor and Askonas 1986). In an early study, we were able to demonstrate CTL-mediated protection against mucosal viral challenge and showed that $CD8^+$ CTLs present at the mucosal site of challenge were required for protection (Belyakov et al. 1998a). Using a novel model mucosal viral challenge system with recombinant vaccinia virus that expresses HIV-1 gp160 in infected cells but not in the virus particle (in order to eliminate the contribution of antibody-mediated responses), we found that I.R. immunization with the synthetic HIV envelope peptide vaccine, PCLUS3-18IIIB, induced a mucosal $CD8^+$ CTL response that protected mice against vaccinia-gp160 challenge up to 6 months after mucosal immunization (Belyakov et al. 1998a). Protection was attributed to a specific T cell response against gp160 since mice were not protected against challenge with vaccinia virus expressing an unrelated protein (Belyakov et al. 1998a). Importantly, protection against I.R. challenge with vaccinia-gp160 was dependent on $CD8^+$ CTL as it was abrogated by treatment of I.R. immunized mice with anti-CD8 antibody (Belyakov et al. 1998a). Because S.C. HIV peptide immunization, which elicited a similar level of $CD8^+$ CTL in the spleen but not in the mucosa, did not protect, we concluded that protection against mucosal challenge requires local $CD8^+$ CTL. Local mucosal (but not systemic) delivery of IL-12 with CT in the vaccine formulation significantly increased mucosal and systemic HIV-specific CTL activity as well as the level of protection (Belyakov et al. 1998a).

Furthermore, we showed that the effect of IL-12 was dependent on induction of IFN-γ, as no effect of IL-12 in enhanced protection was seen in IFN-γ knock-out mice. This was the first study to demonstrate that mucosal $CD8^+$ CTL can mediate protection following local virus challenge in the mucosa (Belyakov et al. 1998a).

It is important to note that mucosal T cell responses and partial protection can be achieved with systemic immunization routes (Kaufman et al. 2008; Lin et al. 2007; Pal et al. 2006; Tatsis et al. 2007). However, it is the authors' opinion that optimal mucosal immune responses and protective immunity are achieved through oral, nasal, rectal, or vaginal mucosal immunization routes (Ahlers and Belyakov 2009a; Belyakov and Ahlers 2008, 2009). Also, it is important to state that mucosal DC are the main target for mucosal vaccination and systemic immunization may have a limited effect on these DC. DC precursors that are recruited to mesenteric lymph nodes (MLN) during inflammation are fully capable of secreting IL-12 and are potent inducers of Th1 IFN-γ responses. A recent study identified a population of $CD11c^{hi}$ $CD11b^{hi}$ lamina propria DC that express toll-like receptor (TLR)-5 and produce proinflammatory cytokines such as IL-6 and IL-12, but not IL-23 or IL-10, in response to flagellin (Uematsu et al. 2008). Understanding the unique properties of mucosal DC and the mucosal milieu in regulating local T and B cell responses and "mucosal memory" will be important for the delivery of vaccines that can provide protection against mucosal infections. Although the magnitude, quality of response, and tissue residency of cells that migrate to mucosal sites following systemic immunization needs further investigation, the targeting of mucosal DC for induction of local immune responses by mucosal vaccination has proven more effective for containing mucosal infections. Thus, next generation HIV-1 vaccines and vaccines against other mucosal pathogens will require formulations and delivery strategies which can effectively induce frontline mucosal immune responses and memory (Belyakov and Ahlers 2009a).

3 Cytokine and Adjuvants for Enhancing Mucosal CTL Responses

Cytokines and mucosal adjuvants are two major factors that can significantly augment mucosal $CD8^+$ CTL responses and protective efficacy of mucosal vaccines (Table 1) (Beagley and Elson 1992; Belyakov et al. 1999a; Belyakov et al. 2000; Staats et al. 2001; O'Neill et al. 2002; Ahlers et al. 2003; Belyakov et al. 2004a; Zhu et al. 2008; Ahlers and Belyakov 2009b; Zhu et al. 2010). Identification of cytokines, chemokines, and immunomodulatory molecules that augment mucosal CTL responses and resistance to mucosal viral challenge has largely been empirical (Belyakov et al. 1998c; Belyakov et al. 2004c; Belyakov et al. 2006a, b). We asked whether the combination of GM-CSF, which recruits DC to inductive sites, and IL-12, which drives $CD4^+$ Th1 function and $CD8^+$ CTL responses, could enhance mucosal immunogenicity and protective efficacy of an HIV-1 peptide vaccine given with mucosal adjuvants CT or LT(R192G). CT, one of the most

Table 1 Strategies for optimizing HIV vaccines

Strategy	Mechanism of activity	References
Mucosal route of immunization for induction of CTL in the mucosa	Protection against mucosal viral transmission was accomplished by establishing $CD8^+$ CTL in the mucosal tissue prior to exposure. Generation of functional $CD8^+$ CTL and compartmentalized immunity by mucosal vaccination was associated with the preservation of $CD4^+$ T cells in the colonic lamina propria after mucosal challenge with pathogenic virus	Barnett et al. (2008), Belyakov et al. (1998a, b, d, 1999, 2000, 2001a, 2006b, 2007a), Bruhl et al. (1998), Caputo et al. (2008), Egan et al. (2004), Kaneko et al. (2000), Li et al. (2008), Manrique et al. (2009), Mercier et al. (2007), Pinczewski et al. (2005), Ranasinghe et al. (2006), Sharpe et al. (2003), Shata et al. (2001), Vajdy et al. (2001)
Heterologous mucosal prime/boost	High quality $CD8^+$ CTL responses were generated in the intestinal mucosa after mucosal priming with HIV gp160 envelope DNA vaccine and mucosal boosting with recombinant viral vector expressing the same envelope gene. A single systemic immunization with rMVA was sufficient for induction of high-avidity $CD8^+$ CTL in systemic lymphoid organs, whereas a single mucosal immunization with rMVA was not able to elicit high-avidity $CD8^+$ CTL in the mucosa. A heterologous mucosal DNA prime-viral vector mucosal boost strategy was needed to induce functional HIV-1-specific $CD8^+$ CTL in intestinal mucosa	Allen et al. (2000), Amara et al. (2001), Belyakov et al. (2008), Eo et al. (2001), Evans et al. (2003), Gherardi et al. (2003), Gherardi et al. (2004), Hanke et al. (1998), Masopust et al. (2006), Neeson et al. (2006), Peacock et al. (2004), Ranasinghe et al. (2006), Ranasinghe et al. (2007), Sharpe et al. (2003), Zhou et al. (2007), Huang et al. (2007)

(continued)

Table 1 (continued)

Strategy	Mechanism of activity	References
Generation of high avidity CTL	High-avidity CTL are readily activated by low concentrations of peptide/MHC presented on target cells, while low avidity CTL require higher concentrations of peptide to become fully activated and exert effector function. Strong costimulation skew CTL toward higher avidity cells. High-avidity CTL exert selective pressure on HIV during the acute phase of infection, resulting in the emergence of escape variants. Vaccines that induce high-avidity mucosal CTL reduce dissemination of virus from the mucosa to the blood	Alexander-Miller et al. (1996), Belyakov et al. (2006b, 2007a, b, 2008), Bennett et al. (2007), Dzutsev et al. (2007), Estcourt et al. (2002), O'Connor et al. (2002), Oh et al. (2003), Ranasinghe et al. (2007), Sedlik et al. (2000), Snyder et al. (2003), Yoshizawa et al. (2003)
Inclusion of cytokines, chemokines, costimulatory molecules, and TLR ligands that enhance vaccine efficacy	Cytokine and chemokine combinations and TLR-triggering can help recruit monocytes, macrophages and neutrophils to local lymph nodes. TLR fusion proteins may help target antigen to the appropriate APC, stimulate maturation of DC, steer cellular immune responses toward Th1-type, and enhance mucosal S-IgA and IgG antibodies and isotype balance. Synergistic combinations of cytokines and immunomodulating molecules may be required for protection against mucosal challenge with virus. Mucosal adjuvant LT(R192G) alone was as effective as CT plus IL-12. GM-CSF synergized with LT(R192G). A triple cytokine combination of GM-CSF, IL-12, and TNF-α was synergistic for induction of $CD8^+$ CTL and for antiviral protection. Choice of adjuvants affects the interplay of cytokines and chemokines in regulation of mucosal CTL	Ahlers et al. (2001a, b, 2003), Belyakov et al. (1998c, 2000), Biragyn et al. (2002), Lena et al. (2002), O'Neill et al. (2002), Staats et al. (2001), Staats and Ennis 1999), Trumpfheller et al. (2008)

(continued)

Table 1 (continued)

Strategy	Mechanism of activity	References
Counteracting Treg mechanisms that dampen immune responses	Depletion of Treg with anti-CD25 antibody significantly enhanced $CD8^+$ T cell immunodominant responses in both the acute and memory phases of the immune response. The depletion of $CD4^+$ T cells enhanced long-lasting CD8-mediated protective immunity upon protein vaccination. In vivo inactivation experiments attributed enhancement primarily to MHC class II-restricted $CD4^+$ Treg cells which suppress the differentiation process towards effector memory $CD8^+$ T cells. Controlling suppressor effects at the time of vaccination may produce more effective long-term immunity	Denning et al. (2007), Heit et al. (2008), Shevach 2002), and Suvas et al. (2003)
Push–pull approach to maximize vaccine efficacy	A synergistic enhancement of vaccine mediated $CD8^+$ CTL generation and antiviral protection by GM-CSF and the costimulatory molecule CD40L, combined with the relief of suppression mediated by $CD4^+$ Treg cells, including $CD4^+$ NKT cells may provide optimum induction of $CD8^+$ CTL. Cytokines, costimulatory molecule agonists, and TLR synergies in combination with antibodies that block IL-10 or IL-13 produced by Type II NKT cells may provide optimum long term memory and protection	Ahlers et al. (2002) and Sutmuller et al. (2001)

commonly used mucosal adjuvants in experimental animals, is unsuitable for humans because of potent toxicity associated with a massive luminal secretory response (Xu-Amano et al. 1993; Marinaro et al. 1995; Elson 1996; Braun et al. 1999). A number of studies have introduced mutations into CT in an attempt to eliminate the toxicity associated with the ADP-ribosyltransferase activity of the A subunit and induction of cAMP in cells. These efforts have only been partially successful since induction of cAMP has been shown to be necessary for adjuvant activity. Dickinson and Clements constructed the LT(R192G) attenuated derivative of *Escherichia coli* heat-labile enterotoxin (LT) using site-directed mutagenesis (Dickinson and Clements 1995). They found that a single amino acid substitution of Glycine for Arginine at position 192 within the disulfide-subtended region of the LT A subunit separating A1 from A2 was effective for reducing toxicity while retaining significant adjuvant properties (Dickinson and Clements 1995; Dickinson and Clements 1996; Morris et al. 2000).

Using either CT or LT(R192G) and the HIV PCLUS3-18IIIB envelope peptide construct, we were able to demonstrate a synergistic effect between these mucosal adjuvants and the combination of GM-CSF and IL-12 for generating mucosal $CD8^+$ CTL in I.R. immunized mice (Belyakov et al. 2000). Furthermore, I.R. immunization with the HIV peptide plus LT(R192G) proved to be as effective for induction of HIV-specific $CD8^+$ CTL in PP and intestinal lamina propria as native LT or CT (Belyakov et al. 2000). After just two mucosal immunizations, the combination of the two cytokines synergistically enhanced the $CD8^+$ CTL response to the HIV-1 peptide vaccine (Belyakov et al. 2000). Co-administration of GM-CSF and IL-12 with peptide also markedly enhanced protection against mucosal challenge with vaccinia-gp160 when compared to animals immunized with each cytokine alone (Belyakov et al. 2000). However, supplementation of peptide vaccine with LT(R192G) and both cytokines afforded the greatest protection. The LT(R192G) adjuvant may produce a more favorable cytokine profile since, in contrast to CT, it did not inhibit IL-12 production (Belyakov et al. 2000). Also, much less IL-4 was induced by LT(R192G) than CT. Thus, the selection of mucosal adjuvant may be critical for influencing the cytokine environment and the induction of mucosal T cell responses that prevent viral transmission. It is important to note that different strategies from those used to elicit optimal cellular responses may be required for induction of humoral responses at mucosal sites. New HIV vaccine design incorporating $CD4^+$ and $CD8^+$ T cell epitopes with appropriate envelope immunogen structures and TLR agonists and the utilization of mucosal delivery strategies that target mucosal DC may induce both high-avidity $CD8^+$ CTL and local IgA and IgG neutralizing antibodies.

In a subsequent study, we demonstrated that GM-CSF, IL-12 and TNF-α also act synergistically in the induction of CD8+ CTL following systemic immunization (Ahlers et al. 2001a). The combination of IL-12 and TNF-α was essential for the optimal development of Th1 responses to provide help for $CD8^+$ CTL induction in vivo, while GM-CSF increased the number and activity of antigen-presenting DC in draining lymph nodes where the immune response was initiated (Ahlers et al. 2001a). Most importantly, significant improvement in protection against viral

challenge was achieved when the triple combination of cytokines (GM-CSF, IL-12 and TNF-α) was co-administered with peptide vaccine (Ahlers et al. 2001a).

The increased magnitude in CTL responses and protection against viral infection afforded by synergistic combinations of cytokines could be further improved using a "push–pull" approach to counteract natural negative regulatory mechanisms which dampen Th1-type immune responses (Table 1) (Sutmuller et al. 2001; Ahlers et al. 2002). In a recent study, we showed that both T regulatory (Treg) and Natural Killer-T (NKT) cells suppress vaccine-induced immune responses (Ahlers et al. 2002). We found that relief of suppression through in vivo depletion of regulatory $CD4^+$ cells, including $CD4^+$ NKT cells, or blockade of IL-13 with an IL-13 receptor competitive inhibitor significantly improved vaccine-mediated $CD8^+$ T cell responses and protection against surrogate viral challenge. These results were confirmed in CD1-deficient animals that lack NKT cells (Ahlers et al. 2002). We reasoned that in mice in which $CD4^+$ T cells were depleted by antibody, the combination of GM-CSF and CD40L might substitute for $CD4^+$ T cell help. We deduced that GM-CSF would recruit more professional APC to the draining lymph nodes where soluble CD40L would provide maturation and activation signals (Ahlers et al. 2002). Indeed, GM-CSF and CD40L given with HIV peptide vaccine did act synergistically to enhance CTL responses in CD4-depleted mice. The improved CTL responses achieved by this push–pull strategy translated to significant protection against vaccinia-gp160 challenge (Ahlers et al. 2002). Thus, mucosal vaccination strategies that utilize both synergistic combinations of Th1-promoting cytokines and approaches that inhibit negative regulatory mechanisms could significantly enhance protective immunity against mucosal HIV transmission.

4 Mucosal Vaccination for Induction of Protective CTL in the Mucosa

Immune correlates of protection against HIV-1 are still not very well understood (Acierno et al. 2006; Ahlers, 2009 #8052; McMichael 2006; Neutra and Kozlowski 2006; Belyakov et al. 2008). However, accumulating experimental evidence suggests that mucosal immune responses (including S-IgA, IgG and $CD8^+$ CTL) in combination with circulating HIV neutralizing antibodies, $CD8^+$ CTL and $CD4^+$ T helper cells, and innate immune responses can exert varying degrees of control of HIV or SIV replication (Baba et al. 2000; Mascola et al. 2000; Bafica et al. 2004; Acierno et al. 2006; McMichael 2006). Disappointing results of the Merck vaccine trial underscore the importance of eliciting frontline mucosal immune responses that can significantly reduce virus load in the intestinal mucosa and subsequent viral dissemination to blood and peripheral lymphoid tissues.

We next asked whether mucosal $CD8^+$ CTL induced by mucosal immunization of rhesus macaques could impact the course of mucosal pathogenic retroviral infection similar to our studies in the surrogate viral challenge model in mice (Belyakov et al. 2001a). We found that SIV-specific CTL could be induced in the

colon and mesenteric lymph nodes by I.R. immunization of monkeys with a synthetic HIV envelope/SIV gag, pol peptide vaccine and LT(R192G) adjuvant (Belyakov et al. 2001a). The SIV-specific intestinal $CD8^+$ CTL were able to traffic to systemic lymphoid tissues. Interestingly, S.C. immunized monkeys also developed significant $CD8^+$ CTL in the mesenteric lymph nodes. However, after rectal infection with chimeric simian–human immunodeficiency virus (SHIV)-ku2, the monkeys immunized by the I.R. route demonstrated a more rapid decline in blood and intestinal virus loads and a significantly lower viral load set point in blood when compared to S.C. immunized animals or adjuvant only controls. We speculated that more $CD8^+$ CTL induced by I.R. immunization were in the right place at the time of I.R. challenge with SHIVku2 (Belyakov et al. 2001a). The numbers of $CD4^+$ and $CD8^+$ T cells in I.R. immunized animals were also better preserved after SHIV challenge when compared to S.C. immunized animals. Thus, $CD8^+$ CTL induced at sites of mucosal challenge can significantly reduce immunodeficiency virus infection in primates, and mucosal immunization may be optimal to parenteral immunization for generating these cells. Rhesus macaques immunized by the intranasal route with a T cell-inducing SIV DNA/MVA vaccine have similarly demonstrated better control of rectal SIVmac251 infection when compared to macaques given the same vaccine by the I.M. route (Manrique et al. 2009). These studies provide strong rationale for the development of mucosal vaccines that generate HIV-specific CTL and T helper cells at sites of HIV exposure in humans.

5 Functional $CD8^+$ CTL for Preventing Immunodeficiency Virus Infection

5.1 Prime-Boost Strategies for Generating High-Avidity CTL

$CD8^+$ T cells that can be activated after recognition of peptide/MHC class I at low peptide concentration are defined as high-avidity $CD8^+$ CTL, whereas those that require high peptide concentrations are termed low-avidity $CD8^+$ CTL (Alexander-Miller et al. 1996; Snyder et al. 2003; Belyakov et al. 2006b; Belyakov et al. 2007b; Ahlers and Belyakov 2010c). It is well known today that high-avidity $CD8^+$ CTL are more effective for preventing viral infections (Alexander-Miller et al. 1996; Belyakov et al. 2006b, 2007a; Estcourt et al. 2002; Gallimore et al. 1998) and eliminating tumors (Yee et al. 1999; Zeh et al. 1999). Also, a functional impairment of HIV-specific $CD8^+$ CTL has been associated with clinical AIDS progression (Acierno et al. 2006; Ahlers and Belyakov 2010a; Appay et al. 2000; Hel et al. 2001; McKay et al. 2002). Thus, immunization strategies that generate high-avidity $CD8^+$ T cells in mucosal and systemic lymphoid tissues could significantly impact initial virus infection and progression of disease. Different combinations of heterologous prime-boost immunization protocols are currently being investigated in multiple experimental and clinical trials for HIV-1, other infectious diseases and

cancer (Belshe et al. 1998; Allen et al. 2000; Barouch et al. 2000; Amara et al. 2000; Shiver et al. 2002; Gherardi et al. 2003; Dale et al. 2006) (Table 1).

In our studies, we employed a prime-boost immunization strategy consisting of a DNA prime and a rMVA or recombinant adenovirus (rAd) virus boost, all encoding an HIV-1 envelope protein, to evaluate immunization routes for ability to induce mucosal as well as systemic HIV-specific $CD8^+$ CTL in mice (Belyakov et al. 2008). A systemic I.M. prime-boost approach induced a strong CTL response in the spleen specific for the immunodominant CTL envelope epitope P18-I10 (measured by ^{51}Cr-release assay, 7 days after in vitro stimulation with P18-I10-peptide) and high-avidity CTL (determined by IFN-γ ELISPOT assay using titrated concentrations of P18-I10 peptide). However, the HIV-specific CTL responses in Peyer's patches were very low after I.M. DNA-MVA prime-boost immunizations (Belyakov et al. 2008). When the prime and boost routes were distinct, the delivery site of the boost had a greater impact than the site of DNA priming. For example, I.M. DNA prime and I.R. MVA boost was more effective than I.R. DNA prime and I.M. MVA boost for eliciting high-avidity $CD8^+$ CTL in the intestine. The optimal CTL response in the gut was observed after I.R. priming with HIV DNA vaccine and I.R. boosting with MVA (Belyakov et al. 2008). The I.R. prime-boost strategy also induced a very strong systemic P18-I10-specific CTL response. A single I.M. immunization with MVA was sufficient to elicit high-avidity $CD8^+$ CTL in systemic lymphoid organs (Belyakov et al. 2008). However, a single I.R. immunization with MVA was not able to elicit high-avidity $CD8^+$ CTL in the mucosa. These results indicate that a mucosal prime-mucosal boost strategy might be crucial to induce optimal cellular immunity in the mucosa. The requirement of a booster vaccination for induction of functionally active CTL in mucosal tissues using mucosal immunization routes may also be more stringent than that for generating high-avidity CTL in systemic tissues using systemic immunization routes (Belyakov et al. 2008).

5.2 *Vaccine-Induced Mucosal High-Avidity $CD8^+$ CTL Preventing Virus Dissemination from Mucosa*

It is a strongly debated subject whether mucosal or systemic $CD8^+$ CTL are necessary to prevent or reduce virus dissemination from the initial mucosal infection site to systemic tissues (Belyakov et al. 2004a; Belyakov and Berzofsky 2004; Belyakov et al. 2006b; Neutra and Kozlowski 2006; Belyakov and Ahlers 2008; Kaufman et al. 2008; Ahlers and Belyakov 2010b; Ahlers and Belyakov 2010c). Also, the role of CTL avidity in control of mucosal AIDS virus transmission is unknown. To address these questions, we used rhesus macaques to compare a peptide-based vaccine, a viral vector-based vaccine, and a combination peptide-prime/viral vector boost regimen (Belyakov et al. 2006b). We chose a prime-boost strategy with replication-incompetent recombinant NYVAC poxvirus expressing HIV envelope and SIV gag, pol proteins because similar systemic

prime-viral vector boost strategies have been shown to elicit strong systemic $CD8^+$ CTL responses (Amara et al. 2001; Hanke et al. 1998; Hel et al. 2002; Shiver et al. 2002). The peptide vaccine contained a mixture of HIV and SIV CTL epitopes presented by Mamu-A*01, the class I antigen expressed by the macaques selected for this study (Belyakov et al. 2006b). All vaccines were delivered I.R. with a combination of GM-CSF, IL-12 and CpG oligodeoxynucleotides (ODN) as adjuvants. Four weeks after the last immunization, all macaques were challenged I.R. with SHIVku2 and monitored for plasma viral loads and $CD8^+$ CTL responses. Two weeks after the last immunization, we analyzed avidity of mucosal $CD8^+$ CTL in the MLN. To examine avidity, the T cell responses were evaluated by plotting specific lysis versus epitope concentration on peptide-coated target cells (Belyakov et al. 2001b; Belyakov et al. 2006b). We found that both vaccination regimens which included the peptide vaccine, GM-CSF, IL-12, and CpG ODN led to similar avidity, whereas I.R. NYVAC immunization alone produced CTL responses of lower magnitude and avidity. Thus, the peptide immunization and a combination of cytokines and CpG ODN improved CTL avidity and functionality, whereas boosting with NYVAC improved the magnitude of the $CD8^+$ CTL responses but not the quality. The prime-boost regimen was necessary to obtain responses with both the highest magnitude and avidity.

Next, we performed I.R. challenge and measured acute-phase peak viremia in blood as an indicator of systemic dissemination (Belyakov and Ahlers 2008; Belyakov et al. 2006b). We found that macaques given the peptide prime-poxvirus boost exhibited a significant (2.5 week) delay in peak viremia compared to macaques immunized with peptide or poxvirus alone. We interpreted this delay in the peak viremia to most likely reflect a temporary local mucosal control of initial virus replication by high-avidity $CD8^+$ CTL preventing rapid dissemination of virus from the intestinal mucosa into the bloodstream. At day 17 after challenge, when viral loads were near their peak, we found a strong inverse correlation between viremia and the numbers of antigen-specific $CD8^+$ T cells in the colon but not those in the blood. In addition, the animals that had $CD8^+$ CTL with the highest avidity in MLN were those that demonstrated the best viral control (Belyakov et al. 2006b). Thus, we demonstrated for the first time that a peptide-prime and poxvirus-boost vaccine that induced high levels of high-avidity mucosal $CD8^+$ CTL can delay dissemination of I.R. administered pathogenic SHIVku2 in macaques, and that such protection correlates better with mucosal rather than systemic $CD8^+$ CTL (Belyakov et al. 2006b).

5.3 Localization of High Quality $CD8^+$ CTL at Sites of Vaccine Delivery

A number of recent studies have demonstrated that antigen-specific $CD8^+$ CTL and partial protection against mucosal challenge with pathogenic SHIV can be achieved after systemic vaccination (Horner et al. 2001; Pal et al. 2006; Stevceva et al. 2002;

Vogel et al. 2003). However, as we and others have shown, mucosal vaccination can be even more effective for the clearance of virus from the major site of replication in the mucosa (Belyakov et al. 2006b, 2007a). The mechanism of protection generated by mucosal vaccination is not well understood. For example, the localization of $CD8^+$ effector and memory T cells after mucosal vaccination has not been well characterized. However, for many mucosal intracellular pathogens (including HIV and Herpes), an effective vaccine strategy will require induction and long-term maintenance of antigen-specific B cells, $CD4^+$ Th cells and CTL at the site of viral transmission. In one study, we performed mucosal versus systemic immunization and compared $CD8^+$ CTL avidity in lymphoid tissues proximal and distal to the site of immunization (Belyakov et al. 2007a). We observed a novel compartmentalization of functional HIV-specific $CD8^+$ CTL in tissue most proximal to the site of immunization (Belyakov et al. 2007a). In this study, mice were immunized with MVA by the S.C. or I.R. immunization routes. To determine the extent of compartmentalization, we measured vaccinia B8R peptide-specific $CD8^+$ T cells by tetramer staining (Tscharke et al. 2005) and by IFN-γ production using ELISPOT with cells from the spleen, small intestinal epithelium, and lamina propria (Belyakov et al. 2007a). We found that both systemic and mucosal routes of immunization generated vaccinia-specific $CD8^+$ T cells in both systemic and mucosal compartments, when measured as total numbers of B8R tetramer-positive $CD8^+$ T cells. However, when we characterized the functional activity of the cells by IFN-γ production, the cell distribution was asymmetric (Belyakov et al. 2007a). The S.C. vaccination with MVA induced a significant number of IFN-γ-producing cells in the spleen, but not in the gut, while I.R. immunization generated greater numbers of IFN-γ secreting $CD8^+$ T cells in the intestinal epithelium and lamina propria. Thus, mucosal immunization produced a much higher ratio of IFN-γ-secreting cells to the total B8R-tetramer positive cells in the gut when compared to systemic immunization (Belyakov et al. 2007a). We also found that I.R. immunization induced more IL-12-producing DC in the colon, while S.C. vaccination induced more IL-12-producing DC in axillary lymph nodes (ALN). Thus, differences in local DC activation could account for the differences in functional T cells in proximal versus distal tissues (Belyakov et al. 2007a) (Fig. 1).

We also characterized the function and avidity of CTL in mucosal MLN and systemic ALN of Mamu-A*01+ rhesus macaques after I.R. and S.C. vaccination with an HIV–SIV Th-CTL peptide vaccine by using a functional ^{51}Cr release assay with different concentrations of peptide (Kuroda et al. 1998)-coated target cells and ELISPOT assay for IFN-γ secretion (Belyakov et al. 2007a). We observed that after I.R. immunization, specific lysis by MLN cells was very high against both low and high concentrations of CTL peptide on target cells, indicating a large proportion of high-avidity $CD8^+$ CTL. In contrast, S.C. vaccination induced greater levels of high-avidity $CD8^+$ CTL in ALN and was less effective for induction of functional $CD8^+$ CTL in MLN. Moreover, we found a strong inverse correlation between the number of high-avidity $CD8^+$ CTL in the mucosal compartment and viral load in the colon 200 days after I.R. challenge with SHIVku-2 (Belyakov et al. 2007a). There was also a strong positive correlation between the percentage of $CD4^+$ T cells

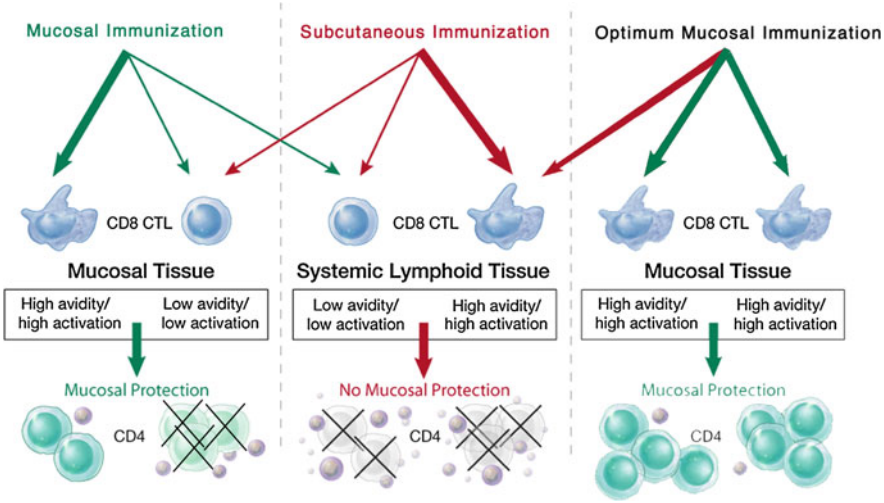

Fig. 1 Avidity of CTL in relation to vaccination route and protection. High-avidity $CD8^+$ CTL are optimal to low-avidity CTL for containing immunodeficiency virus infection. Suboptimal mucosal immunization (e.g., with vaccine lacking adjuvants) can induce high-avidity CTL at the vaccination site but it also produces low-avidity $CD8^+$ CTL or T cell anergy, which results in limited mucosal protection. Systemic immunization produces high-avidity CTL in systemic tissues but not mucosal tissues, allowing rapid $CD4^+$ T cell depletion and viral spread to the periphery. Optimal mucosal immunization establishes high-avidity $CD8^+$ CTL in both mucosal and peripheral lymphoid tissues. Multiple variables influence the quality of the CTL response achieved by mucosal vaccination including: dose, frequency, the use of synergistic combinations of mucosal adjuvants, cytokines, chemokines, and TLR ligands to enhance vaccine efficacy. Furthermore, heterologous prime-boost strategies and push–pull approaches will be needed to maximize vaccine efficacy

in colonic lamina propria and the number of high-avidity antigen-specific $CD8^+$ CTL in the same location (Belyakov et al. 2007a). Thus, a mucosal AIDS vaccine reduced viral load and the depletion of $CD4^+$ T cells in the intestinal mucosa. Control of mucosally transmitted immunodeficiency virus infection can be generated by local mucosal immunization, and the mechanism for this control can be attributed to the focusing of high quality cellular responses at sites of viral exposure (Belyakov et al. 2007a). The additional induction of mucosal and systemic antibodies should improve protection even more significantly by working in concert with CTL to prevent viral entry and replication in mucosal tissues.

6 Conclusion

Local mucosal $CD8^+$ CTL and antibody may completely prevent HIV transmission at mucosal surfaces or potentially control virus replication within mucosal tissues prior to systemic dissemination. We believe that a number of approaches can be

employed to effectively elicit mucosal immune responses. We have demonstrated that local immunization with appropriate adjuvants and synergistic combinations of cytokines and TLR agonists are effective for induction of potent $CD8^+$ CTL responses in the intestine. Further vaccine improvements in combination with push–pull strategies that target and activate mucosal DC while relieving local suppression should additionally promote desired mucosal $CD4^+$ Th1 and $CD8^+$ CTL responses. The enhanced magnitude and quality of immune responses could lead to highly efficacious HIV vaccines. The next important step for HIV vaccine development will be to move these new design and delivery strategies for mucosal immunization into Phase 1 clinical trials. We are optimistic that moving in this direction will greatly accelerate the efforts of so many in bringing a protective AIDS vaccine to fruition in our lifetime.

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