Protection from Secondary Human Immunodeficiency Virus Type 1 Infection in Chimpanzees Suggests the Importance of Antigenic Boosting and a Possible Role for Cytotoxic T Cells


Department of Virology, Biomedical Primate Research Centre, Rijswijk, The Netherlands

Recent evidence suggests a much higher prevalence of human immunodeficiency virus type 1 (HIV-1) recombinants than previously anticipated. These recombinants arise from secondary HIV infections in individuals already infected with the virus. It remains unclear why some individuals acquire secondary HIV-1 infections and others do not. To address this question, a study was undertaken of a small cohort of chimpanzees with well-defined HIV-1 infection.

After exposure to an infectious dose of heterologous primary isolate, 4 of 8 HIV-1 seropositive chimpanzees resisted secondary infection, whereas 2 naive controls became readily infected. Only animals who were immunologically boosted were protected. Protection from heterologous secondary exposure appeared to be related to the repertoire of the cytolytic CD8+ T cell responses to HIV-1. Data suggested that immunologic boosting by HIV-1 antigens or exposure to subinfectious doses of virus may be important events in sustaining sufficient immunity to prevent secondary infections from occurring.

One of the major obstacles facing human immunodeficiency virus (HIV) type 1 vaccine development is virus variability. Not only does genetic drift result in a high degree of antigenic diversity, but it is also clear that recombination occurs between different strains, possibly resulting in major antigenic changes [1]. Accumulating data clearly reveal that HIV-1 infected persons become dually or multiply infected with different HIV strains more often than previously expected [1–3]. In Brazil, Thailand, and several African nations where multiple subtypes of HIV-1 are prevalent, individuals are documented to be infected with recombinants of several subtypes [4–7]. Recombinants may also have pathogenic consequences, as was observed in a chimpanzee who was infected with 2 strains of HIV and who developed AIDS after the emergence of a recombinant virus [8, 9].

Why certain individuals are susceptible to multiple HIV infections and others seem to resist such coinfections is unknown. A study of HIV-2 infected macaques suggested that a short window of susceptibility existed immediately after primary infection, during which the immune responses to the initial infection had not sufficiently matured [10]. In a similar setting, live attenuated infections (vaccines) have been demonstrated to afford the best protection from heterologous and mucosal exposure in several studies [11–13]. Despite the safety concerns of the use of live attenuated vaccines in humans, they have been pursued for proof-of-principle studies and as model vaccines, in an effort to elucidate the immune mechanisms of HIV vaccine protection [14]. Despite many studies, the nature of the protective immune responses elicited by preexisting lentiviral infections has not been defined clearly [15]. The compiled data from the many live attenuated vaccine studies suggest that very early immunity can be generated by the primary infection and that secondary infection can occur late after the primary infection because of subclinical immunodeficiency preceding the development of AIDS.

To investigate the possible role of cell-mediated immune (CMI) responses in protection from secondary infection, we designed a study of a cohort of 8 seropositive and 2 naive chimpanzees. The study included chimpanzees with a history of chronic asymptomatic HIV-1 infection for ≥5 years. Since boosting of immunologic responses recently was suggested to be important, as observed in the Nairobi commercial sex worker cohort [16], we elected to boost 6 of the seropositive animals with a candidate HIV-1 Env vaccine recently used in a human clinical trial [17]. To determine whether either chronic asymptomatic or immunologically boosted asymptomatic chimpanzees could be protected from secondary infection, we exposed animals to an infectious dose of a heterologous primary HIV-1 isolate.

Materials and Methods

Study design. A cohort of 2 naive chimpanzees and 8 HIV-1mnr-seropositive chimpanzees (Pan troglodytes verus) was used in
this study (table 1). The animals, who were 13–34 years old, were divided into 3 groups: group 1 consisted of 2 naive chimpanzees who served as controls for infection with the clinical isolate HIV-1_Han2; group 2 consisted of 2 chronically infected chimpanzees who did not receive booster immunizations [18], and group 3 consisted of 6 seropositive chimpanzees who, at 8 and 4 weeks (weeks −8 and −4) before challenge (week 0), were boosted intramuscularly with 200 μg of HIV-1_W6.1D Env formulated to a volume of 1 mL with AS2 adjuvant [19]. All 10 animals were challenged intravenously with the clinical isolate HIV-1_Han2 (table 1). The 6 seropositive animals from group 3 (Ch-La, Ch-Ze, Ch-Th, Ch-Er, Ch-Bi, and Ch-Os) had been used in a homologous prophylactic HIV-1 vaccine efficacy study 4 years earlier [20, 21]. In that study, they received a homologous challenge, and all except Ch-Os became chronically infected with HIV-1_Han2.

Detection of heterologous infection in vivo. Quantification of viral RNA in plasma and detection of proviral DNA in peripheral blood mononuclear cells (PBMC) were done as follows: RNA in plasma was measured using a polymerase chain reaction (PCR)–based kit (AmpliCor HIV-1 Monitor; Roche Diagnostic Systems). Proviral DNA in PBMC was determined quantitatively, as described by ten Haaf et al. [21], with some minor modifications. In brief, DNA was extracted by SDS–proteinase K digestion, followed by ethanol precipitation. DNA concentrations were standardized at 50 ng/μL. A nested HIV-GAG PCR and a nested HIV-ENV V3 PCR were done with primer sets described elsewhere [22], with detection limits being sensitive enough to detect 1 proviral HIV-1 copy in a background of 1 μg of genomic DNA (equivalent to 1.5 × 10^6 cells). The PCR products were analyzed by ethidium bromide–agarose gel electrophoresis. HIV-1_w6.1D and HIV-1_Han2 were discriminated by restriction fragment length polymorphism of the PCR products.

T helper (Th) responses. Two weeks before and 2 weeks after each immunization and every 2–4 weeks until 40 weeks after challenge, PBMC from each chimpanzee were assayed in vitro for Gag- and Env-specific T cell responses. An ELISPOT assay was used to enumerate antigen-specific cytokine (interleukin [IL]-2 and IL-4)–secreting cells, and an antigen-specific lymphoproliferative (LP) assay was performed elsewhere [23]. In brief, 1 μg of recombinant Gag of HIV-1_Han2 (P24, ARP-620; Medical Research Council AIDS Reagent Program [MRC]), 1 μg of Env of the clinical isolate HIV-1_W6.1D (Gp120, EVA-48; MRC), or 1 μg of the laboratory strain HIV-1_W6.1D (Gp120, EVA-607; MRC) was used as antigen; medium alone was used as a negative background control. Concanavalin A (5 μg/mL; Sigma) was used as mitogen stimulator (positive control), to test responsiveness of the cells. For the LP assay, PBMC were cultured for 5 days with antigen and then were pulsed with 3H-thymidine overnight before being harvested. LP responses were considered to be positive when they exceeded the negative control by 2-fold. HIV-1_w6.1D specific responses could not be measured, because purified antigens were not available.

Virus-specific cytotoxic T lymphocytes (CTL). CTL responses were assayed, as described elsewhere [24, 25], using Gag and Env overlapping 20-mer peptides. Peptides from HIV-1_W6.1D or HIV-1_Han2 were not available, so the assays were done with available overlapping peptides spanning the Gag of HIV-1_SC (ARP-740; MRC). They consisted of 22 20-mer peptides overlapping by 10 aa, spanning Gag amino acid residues 135–364. The responses against Gag were measured against pools A (peptides 1–8), B (peptides 9–16), and C (peptides 17–22). Overlapping peptides spanning

<table>
<thead>
<tr>
<th>Study group, animal</th>
<th>Provirus^a</th>
<th>Boosted with HIV-1_w6.1D Gp120^b</th>
<th>Secondary exposure dose, TCID_{50}</th>
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<tbody>
<tr>
<td>Naive</td>
<td></td>
<td>100</td>
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<tr>
<td>Ch-SuA</td>
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<td>Ch-On</td>
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<td>Nonboosted seropositive</td>
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<tr>
<td>Ch-Bu</td>
<td>+</td>
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<tr>
<td>Ch-Ma</td>
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<tr>
<td>Boosted seropositive</td>
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<tr>
<td>Ch-La</td>
<td>+</td>
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<td>Ch-Th</td>
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<td>Ch-Os</td>
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^a +, Provirus is present; -, provirus is absent.

^b +, Animal was boosted; -, animal was not boosted.

Env of HIV-1_Han2 (ARP-740; MRC) consisted of 47 20-mer peptides overlapping by 10 aa, spanning Env amino acid residues 28–457. Responses were tested against 2 peptide pools: pool 1 (peptides 1–23) and pool 2 (peptides 24–47).

In brief, the cytotoxicity assay was performed as follows: target cells were autologous Epstein-Barr virus immortalized B lymphoblastoid cell lines that were labeled with 150 μCl of Na^235CrO_4 (Amer sham International) for 1 h and then were pulsed with pools of peptides (25 μg/mL) for 1 h at 37°C, followed by a 16-h incubation with the same peptides at 2.5 μg/mL. Unpulsed target cells were used as controls. Target cells subsequently were washed and were plated at 5 × 10^3 cells/well in 96-well U-shaped plates (Costar) together with effector cells at 3 effector-to-target cell ratios (E:T). After a 5-h incubation at 37°C, supernatants were harvested and were counted in a Cobra 5 gamma-counter (Canberra Packard Benelux NV). Percentages of specific ^235Cr release were calculated as 100 × (experimental release – spontaneous release):maximum release – spontaneous release. All experimental values were determined in duplicate or triplicate, and maximum and spontaneous releases were done in quadruplicate. Specific responses of ≥10% above lysis of control unpulsed targets were scored as positive. Spontaneous release was 5%–30%.

Results

Naive animals. The 2 naive animals, Ch-SuA and Ch-On, readily became infected with the challenge strain, thus confirming the infectivity of the challenge stock of the clinical isolate HIV-1_Han2. Ch-SuA received 100 TCID_50, but as little as 10 TCID_50 was sufficient to infect Ch-On [26].

As shown in figure 1, LP responses to HIV-1_W6.1D Env remained negative at all times tested (figure 1I). In both chimpanzees, LP responses to HIV-1_Han2 Env were also negative (data not shown). However, positive LP responses to Env and Gag were seen in cells isolated from lymph nodes obtained at week 4 and later in both animals, confirming exposure to HIV (data not shown). The responses were comparable when cells were stimulated with HIV-1_HAN2 Env or HIV-1_W6.1D Env.
Antigen-specific IL-2 and IL-4 production was measured at the single cell level during this study, representing Th1 and Th2 type cellular immune responses, respectively. Antigen-specific IL-2 responses were not detected after secondary infection with HIV-1\textsubscript{Han2} (figure 1A). The number of HIV-1\textsubscript{W6.1D} Env-specific IL-4 producing cells also remained very low in these 2 chimpanzees.

In the 2 naive control animals, a specific CTL response to Gag peptides was detected after infection. In cells from Ch-SuA, the response was specific for pool A of the Gag peptides (figure 1B). Responses were first observed 6 weeks after infection. The response reached a peak after 10 weeks of infection and declined below detection limits after 12 weeks. In cells from Ch-On, the Gag-specific cytolytic response was directed against pool B of the Gag peptides (figure 1C). Env-specific CTL were not detected in these animals.

Nonboosted chronically infected animals. The 2 nonboosted chronically asymptomatic HIV-1\textsubscript{IIIB} infected animals who did not receive booster immunizations, Ch-Bu and Ch-Ma, both became infected after exposure to HIV-1\textsubscript{Han2}, which confirmed that coinfection occurred in chronically infected asymptomatic chimpanzees. Thus, prior HIV-1\textsubscript{IIIB} infection did not afford protection from secondary HIV-1\textsubscript{Han2} infection.

LP responses were not detected in PBMC from Ch-Bu and Ch-Ma either before exposure or during the 40-week follow-up after HIV-1\textsubscript{Han2} coinfection (figure 2A). Lympocyte from Ch-Bu and Ch-Ma did produce a low Env-specific IL-2 and IL-4 response after coinfection with HIV-1\textsubscript{Han2} (figure 2A). CTL responses were not detected at the time of challenge. However, 6 weeks after HIV-1\textsubscript{Han2} coinfection, cytolytic responses to the Gag peptides were detected, but not against Env. Cells from Ch-Bu responded to Gag pool A (figure 2B), whereas cells from Ch-Ma responded to Gag pools A and B (figure 2C).

Immune boosted animals. Of the 6 animals that received booster immunizations, 4 (Ch-Th, Ch-Os, Ch-Bi, and Ch-Er) were protected from secondary HIV-1\textsubscript{Han2} infection. Two animals (Ch-La and Ch-Ze) became dually infected. Plasma viral RNA loads are shown in figure 3. Ch-Er always maintained a high virus load with only a small increase after the period of heterologous exposure; however, it was found that this was only HIV-1\textsubscript{IIIB} and that he was protected against secondary HIV-1\textsubscript{Han2} infection. All the immune boosted animals developed an increase in virus load after the secondary exposure. This subsequently declined. Ch-Os was protected in the previous study against primary infection with HIV-1\textsubscript{IIIB}. In that study, a correlation was found with the neutralizing antibody titer and IL-2 production and protection. Ch-Os again was found to be protected against the heterologous HIV-1\textsubscript{Han2} challenge, but a correlation with heterologous neutralizing antibody titers was not found with this virus (authors’ unpublished data).

After an initial induction, the Env-specific LP responses de-
Cellular immune responses of 2 chronically human immunodeficiency virus type 1 (HIV-1) IIIB–infected chimpanzees, Ch-Bu and Ch-Ma, after secondary exposure without immune boosting. A, HIV-1W6.1D Env-specific lymphoproliferative responses (LPs) and enumeration of antigen-specific interleukin (IL)–2– and IL-4–secreting cells on the day of challenge (week 0) and 2 and 6 weeks thereafter. Antigen-specific LP remained negative, and the number of IL-2– and IL-4–producing T cells was low after HIV-1Han2 challenge. Positive cytotoxic T cell responses were detected against a peptide pool of Gag 1–8 with peripheral blood mononuclear cells (PBMC) of Ch-Bu at week 6 (B) and peptide pools of Gag 1–8 and Gag 9–16 with PBMC of Ch-Ma at week 6 (C). E:T ratio, effector-to-target ratio.

clined after the secondary infection with HIV-1Han2 in all animals except Ch-Bi (figure 4A). In Ch-Ze (coinfected) and Ch-Os (not infected), this initial induction was not observed. The LP responses were comparable in all animals when stimulated with either HIV-1IIIB Env or HIV-1W6.1D Env (data not shown). Gag-specific LP responses were detected but fluctuated between animals. Gag-specific LP responses were detected in Ch-Th, Ch-Ze, and Ch-Bi, with stimulation index (SI) values between 2 and 9 (data not shown). The LP responses of Ch-La, Ch-Er, and Ch-Os remained below the level of detection.

The 6 immune boosted animals had detectable Env-specific cytokine responses of 3–31 IL-2–producing cells per 2 × 10^5 PBMC after 2 immunizations (figure 4A). Comparable numbers of IL-2–producing cells were observed in PBMC cultures stimulated with HIV-1IIIB or HIV-1W6.1D Env (data not shown). The observed number of Env-specific IL-2–producing cells was 0–50 cells per 2 × 10^5 PBMC. Stimulation of PBMC with either HIV-1IIIB or HIV-1W6.1D Env generated comparable numbers of IL-2–producing cells.

Cytolytic T cell responses were detected by use of different peptide pools, which suggested the presence of several HIV-1 epitopes recognized by chimpanzee CTL. Before HIV-1W6.1D Env immunization, Gag-specific cytotoxicity was present in Ch-La. CTL were detected against a pool of Gag peptides 1–3 (figure 4B). Epitope mapping led to the identification of a 9-mer peptide recognized by the CTL of Ch-La when presented in the context of the Patr-B*02 molecule [25]. This strong monospecific response persisted up to at least 50 weeks after the second infection with HIV-1Han2.

At the same time, a Gag-specific CTL response was observed in Ch-Ze against a pool of peptides 11–14 (figure 4C). Epitope mapping led to the identification of a 9-mer peptide recognized by the CTL of Ch-Ze when presented in the context of the Patr-B*03 molecule [25]. This monospecific response also persisted up to 50 weeks after secondary infection. The CTL activity was directed against a Gag epitope different from that detected in Ch-La. Cytotoxic responses to Gag also were observed in Ch-Th after exposure to HIV-1Han2 for 20 weeks. The CTL activity was directed against ≥3 peptides in HIV-1 Gag in pools 1–4, 5–8, and 13–16 (figure 4D). Env-specific CTL were not detected in Ch-La, Ch-Ze, or Ch-Th by use of HIV-1IIIB peptides. In the uninfected Ch-Os, however, Env-specific CTL against pool 1 were detected in cultures during the sequential follow-up (figure 4E) after immunizations even before challenge, which indicates that these were vaccine induced (data not shown). An Env-specific response against pool 1 also was seen in Ch-Bi at week 8 (figure 4F), and Ch-Er had Env-specific CTL responses against peptide pools 1 and 2 at week 6 (figure 4G). All Env-specific CTL responses were detected at several time points but were found to be transient over time; however, the responses...
Figure 3. Plasma viral RNA loads in 6 human immunodeficiency virus type 1 (HIV-1)–immune chimpanzees over time after exposure to HIV-1\textsubscript{Han2} after antigenic boosting. Broken lines, 4 animals protected from secondary infection; solid lines, 2 animals that became dually infected (*) after heterologous exposure.

in Ch-Os were more persistent (positive on at least weeks -2, 2, and 6).

**Immune responses that correlate with protection from secondary HIV-1 infection.** The characteristics of the different cellular immune responses in the immunologically boosted animals are shown in Table 2. The 2 naive and the 2 persistently asymptomatic chimpanzees infected with HIV-1\textsubscript{IIIB} became infected with the secondary challenge isolate HIV-1\textsubscript{Han2}. Of the 6 animals that received booster immunizations, 2 (Ch-La and Ch-Ze) became HIV-1\textsubscript{Han2} coinfected, whereas the other 4 (Ch-Er, Ch-Bi, Ch-Os, and Ch-Th) resisted secondary infection. Two weeks before secondary exposure (week -2), the SI of LP responses ranged from 2 to 92. Ch-Os and Ch-Ze had the highest SI (∼90). Ch-Os was protected from secondary and primary infection, whereas Ch-Ze was infected with both viruses. Conversely, of the remaining 3 protected from secondary infection, Ch-Er had the lowest SI before exposure, and Ch-Er was protected, whereas Ch-La, who had a similar LP response, became coinfected. There clearly was no correlation evident with LP responses. Th1 responses were estimated by enumerating the number of antigen-specific IL-2 producing cells, whereas Th2 responses were measured by enumerating the number of antigen-specific IL-4 producing cells. However, there was also no correlation between these responses and secondary infection or protection (Table 2).

In the 6 boosted animals, CTL responses were detected to either Gag or Env peptides. Env-specific CTL responses were induced or boosted up to detectable levels by the exposure to HIV-1\textsubscript{Han2}. In the fourth protected animal (Ch-Th), there was no Env-specific cytolytic activity during the 20-week CTL follow-up after secondary infection; however, at multiple time points, Gag-specific CTL responses were measured and were found to be directed to ≥3 different peptides. This broad Gag-specific CTL activity is in contrast to the monospecific CTL response seen in Ch-La and Ch-Ze, both of whom became coinfected with HIV-1\textsubscript{Han2}. Hence, animals that became secondarily infected had only monospecific Gag-specific CTL responses, whereas chimpanzees that were protected from secondary exposure to the heterologous HIV-1\textsubscript{Han2} had either multiple Gag-specific CTL responses or an Env-specific CTL response.

**Discussion**

Current molecular epidemiologic data from the HIV pandemic have revealed that dual or multiple HIV-1 infections occur in individuals more frequently than previously suspected [1]. To determine whether specific CMI responses play a role in protecting certain infected individuals from secondary HIV infections, we undertook a study in chronic asymptomatic seropositive chimpanzees. Of the 10 animals studied, 2 served as naive controls, and 2 asymptomatic chronically HIV-1 infected animals with normal CD4\textsuperscript{+} T cell counts were used to determine whether healthy individuals could, as a result of their chronic infection, naturally resist a second HIV infection. The remaining 6 seropositive chimpanzees were boosted with a candidate HIV-1 vaccine, to determine whether additional immunologic exposure to HIV-1 antigen might be necessary for acquiring the ability to resist secondary infection. The results revealed the latter to be the case.

The 2 naive and 2 asymptomatic infected chimpanzees be-
Figure 4. Cellular immune responses of 6 animals who were antigenically boosted. A. Human immunodeficiency virus type 1 (HIV-1) Env-specific lymphoproliferative responses (LPs) and enumeration of antigen-specific interleukin (IL)–2– and IL-4–secreting cells after immunizations but before challenge (week 2) and at weeks 2 and 6 thereafter. Positive cytotoxic T cell responses were detected against peptide pools of Gag 1–3 with peripheral blood mononuclear cells (PBMC) of Ch-La at week 10 (B); Gag 11–14 with PBMC of Ch-Ze at week 10 (C); Gag 1–3, 4–6, and 11–14 with PBMC of Ch-Th at week 10 (D); Env 1-23 with PBMC of Ch-Os at week 6 (E); Env 1-23 with PBMC of Ch-Bi at week 8 (F); and Env 1-23 and 24-47 with PBMC of Ch-Er at week 6 (G). E:T ratio, effector-to-target ratio. *Animals who became dually infected after heterologous exposure.

came infected after exposure to the clinical isolate HIV-1_Han2. This strongly suggested that immunologic boosting might be required to generate sufficient immunity to protect from heterologous HIV-1 infection, since 4 of the 6 seropositive boosted animals resisted secondary infection. Preexposure virus loads did not have an effect on protection from secondary infection. Ch-Bi, Ch-Os, and Ch-Th all had plasma viral RNA loads below detection, as did Ch-La and Ch-Ze, although the latter 2 chimpanzees became dually infected. There also was no correlation with antibody titers or neutralizing antibodies and protection from secondary infection. In the light of these observations, we set out to investigate whether certain CMI responses in these chimpanzees correlated with protection.

The possible role of Th immune responses in secondary protection was investigated by monitoring lymphocyte proliferation and by enumerating the number of antigen-specific cells that secreted IL-2 (Th1) or IL-4 (Th2) cytokines in the presence of Gag or Env antigens. Clearly, neither the naive controls nor the asymptomatic chronically infected animals elicited strong Th responses. Some, but not all, of the boosted animals developed Th responses to Gag and Env, but there was no evident correlation with protection from secondary infection with Th responses.

The results of CTL analysis, however, suggested an interesting correlation with protection from secondary infection. The CTL responses to 2 HIV-1 proteins (Env and Gag) were studied. Env was studied because it was used for the vaccine boost, and
Gag was studied because CTL responses to conserved regions had been reported previously in HIV-infected chimpanzees [25]. Of the 6 animals that received booster immunizations, 2 became dually infected after secondary exposure. These animals were able to mount only a monospecific CTL response that was directed only to a single Gag epitope. In contrast, protected animals had CTL responses to Env peptides or, in the case of 1 animal, to multiple Gag epitopes. These results suggested that the repertoire of the CTL response might have had a role in protecting these animals from secondary HIV-1 infection. However, it should be noted that CTL responses against other viral proteins—such as Nef, Rev, Tat, or Pol—were not evaluated ever, it should be noted that CTL responses against other viral proteins—such as Nef, Rev, Tat, or Pol—were not evaluated but very likely may also have a role in preventing subsequent new HIV-1 infections after repeated exposure. Previous data have revealed that there is a protective effect of certain specific CTL responses during primary infection, which may persist to prevent or delay disease progression [27, 28, 38–41]. To our knowledge, this is the first report to demonstrate clearly the importance of immunologic boosting in protection from secondary infection. Furthermore, although limited to a few animals, studies of the immune responses of “protected vs. infected animals” point toward a possible role of the quality of specific CTL responses or the repertoire of CTL responses in protection from secondary HIV-1 infection.

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References


